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# Pseudomonas Aeruginosa Prophages and Engineered Temperate Phages

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# *PSEUDOMONAS AERUGINOSA* PROPHAGES AND ENGINEERED TEMPERATE PHAGES

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ABSTRACT

The rising rate of antibiotic-resistant bacteria is a global health concern, and *Pseudomonas aeruginosa* is estimated to be a leader in serious threats. Bacteriophages, or viruses that infect bacteria, have gained renewed interest in Western Medicine as an alternative to antibiotics. Phage therapy of *P. aeruginosa* infections has primarily focused on using obligately lytic phages. However, prophages, or phages in the lysogenic life cycle, are estimated to far outnumber obligately lytic phages. In theory, these prophages can be genetically modified to be obligately lytic and thus ideal candidates for phage therapy. To date there has not been a comprehensive analysis on the diversity of *P. aeruginosa*-infecting prophages. Furthermore, genetic engineering of *P. aeruginosa*-infecting prophages has yet to be explored.

Here I present a 2-fold study including a comprehensive analysis of the prevalence and diversity of prophages within all publicly available *P. aeruginosa* genomes paired with the engineering of 2 *P. aeruginosa*-infecting temperate phages. 6,852 high confidence prophage sequences were identified from 5,383 genomes of *P. aeruginosa* with 68% of the genomes containing at least 1 high confidence prophage. While 57% of these predicted prophages displayed sequence similarity to publicly available phage genomes, novel prophages were discovered. There was extensive diversity observed among the prophages, including diversity within the widely conserved integrase and C repressor coding regions – the two genes responsible for prophage entering and persisting through the lysogenic life cycle. Two *P. aeruginosa* temperate phages, Dobby and D3112, were engineered to become obligately lytic

phages. This engineering included removal of Dobby's integrase and D3112's C repressor genes producing phage strains eDobby and eD3112, respectively. To our knowledge, this is the first instance of a *P. aeruginosa*-infecting temperate phage being engineered to remove the C repressor for production of an obligately lytic phage. Both the ancestral and engineered strains were capable of infecting clinical *P. aeruginosa* isolates. Furthermore, both eDobby and eD3112 were able to kill and debilitate *P. aeruginosa* ATCC 15692 at MOIs of 1 and 10.

This study has identified numerous additional prophage candidates, which can be engineered using the approach presented here, for phage therapy of *P. aeruginosa* infections.

# CHAPTER ONE

# INTRODUCTION

## *Pseudomonas aeruginosa*

*Pseudomonas aeruginosa* is a Gram-negative, rod-shaped, and opportunistic pathogen that, when not found in infections, can be commonly isolated from environments closely linked with human activity, e.g., soil and water (Crone et al., 2020). *P. aeruginosa* is the sixth most common cause of nosocomial infections with highest prevalence in catheter-associated urinary tract infections, ventilator-associated pneumonia, and surgical-site infections (Weiner et al., 2016). The CDC considers *P. aeruginosa* to be a serious threat to the United States population due to its multi-drug resistance (CDC, 2021). The low permeability of the outer membranes of *P. aeruginosa* cells make it naturally resistant to chemotherapies (Nicas & Hancock, 1983). The bacteria's ability to form biofilms also creates a barrier that is difficult for antibiotics, or even the host's own immune responses, to cross (Drenkard, 2003). Production of antibiotic-inactivating enzymes, such as β-lactamase, and expression of efflux pumps are 2 additional intrinsic resistance systems of *P. aeruginosa* (Li et al., 2000). The bacterial cells can also frequently acquire drug resistance through mutational changes or horizontal gene transfer of resistance genes (Breidenstein et al., 2011). With such high rates of antibiotic resistance and difficulty in treating infections, new treatments have been explored to combat *P. aeruginosa*, including the use of bacteriophage (phage) therapy.

## Phages and Phage Therapy

Phages are viruses that infect bacteria. While phages are the most abundant biological entity on the planet with an estimated $10^{31}$ phages in existence, the majority of our knowledge about phages comes from marine environments where they have an estimated rate of $10^{23}$ infections/second (Suttle, 2005, 2007). Phages are in fact ubiquitous; they are prevalent in many environments, including natural bodies of water, soil, sewer systems, and all examined human body sites (Dion et al., 2020; Navarro & Muniesa, 2017). The genome sizes of phages range anywhere from 2,000 bp to over 300,000 bp, but the average phage genomes are 30,000 to 50,000 bp in size (Hatfull, 2008). While most isolated phages are tailed dsDNA phages (order: *Caudovirales*), there are also ssDNA phages that do not have tails (e.g., family: *Microviridae*) or are filamentous (e.g., family: *Inoviridae*), as well as dsRNA and ssRNA phages (Ackermann, 2007).

Phages are major drivers of microbial ecology and evolution (Klimenko et al., 2016). The majority of phages have a host range limited to 1 bacterial species and many are even specific to only certain strains or serotypes of a singular species (Drullis-Kawa, 2017). The particular host range of a phage is dependent on the host cell surface receptors as well as on host-range-determining regions of the phage's tail fiber protein (Bertozzi Silva et al., 2016; Yehl et al., 2019). This selectivity is in direct contrast with antibiotics, which often affect both pathogenic bacteria as well as non-pathogenic or commensal species, contributing to the development of antibiotic resistance (Yehl et al., 2019). As phages are typically unaffected by the antibiotic-resistance mechanisms of bacteria, they could be used to treat even antibiotic-resistant bacterial infections (Międzybrodzki et al., 2012). Felix d'Herelle was the first to use phages against

bacteria therapeutically and treated 4 cases of bacterial dysentery with phages in 1919 (Chanishvili, 2012). With the discovery of antibiotics, phage therapies were quickly abandoned by Western medicine.

Phage therapy has gained renewed interest in Western medicine due to the increasing rates of antibiotic resistance. This is especially true for infections due to *P. aeruginosa* (Kakasis & Panitsa, 2019). Animal models have been instrumental in ascertaining the efficacy of phage therapy of *P. aeruginosa* infections. Injection of phages into mice with *Pseudomonas* bacteremia was able to rescue mice survival even with delayed administration (Wang et al., 2006). In another study, phages administered through eye-drops were able to resolve a *P. aeruginosa* infection of *Pseudomonas* keratitis in murine models (Fukuda et al., 2012). Phage treatments were also found to not only dismantle *P. aeruginosa* infections of gut-derived sepsis in mice, but also improve the survival of the mice (Watanabe et al., 2007). Mice infected with *P. aeruginosa* in their lungs were able to be treated with phages that significantly slowed the spread of infection (Pabary et al., 2016). In a 2019 study, *P. aeruginosa* infections in zebrafish with cystic fibrosis were resolved using phage treatments, an optimistic result for human patients plagued with cystic fibrosis (Cafora et al., 2019).

With such promising results from animal trials, clinicians in the US and Europe have recently turned to using phage therapy to treat patients in extenuating circumstances. In 2017, the first instance of successful intravenous phage therapy against *P. aeruginosa* septicemia in humans was reported (Jennes et al., 2017). In another case, a single application of phage treatment was successfully able resolve a *P. aeruginosa* infection of an aortic graft (Chan et al., 2018). Additionally, phages have been used to resolve *P. aeruginosa* infections in other

individuals (Aslam, Courtwright, et al., 2019; Dedrick et al., 2019; Jault et al., 2019; Kortright et al., 2019; LaVergne et al., 2018; Łusiak-Szelachowska et al., 2014; Nir-Paz et al., 2019; Petrovic Fabijan et al., 2020; Reardon, 2014; Rhoads et al., 2009; Rose et al., 2014; Schooley et al., 2017; Armata Pharmaceuticals, Inc., 2016, 2019b, 2019a, 2019d, 2019g, p. 2, 2019h, p. 2, 2019c, p. 2, 2019g, 2019f, 2019e, 2021; BiomX, Inc., 2021, p. 002; Bristol-Myers Squibb, 2011; Leitner et al., 2017; Locus Biosciences, 2021; National Cancer Institute (NCI), 2015, p. 5; University Hospital, Montpellier, 2013; Weir, 2019, 2020; Wolcott, 2011; Armata Pharmaceuticals, Inc., 2016; MicroPhage, Inc., 2010; National Institute of Allergy and Infectious Diseases (NIAID), 2008; Aslam, Pretorius, et al., 2019; Cano et al., 2020; *Case Studies – Adaptive Phage Therapeutics*, n.d.; Doub et al., 2020; C. Duplessis et al., 2018; C. A. Duplessis et al., 2019; Gainey et al., 2020).

## Phage Life Cycles

Phages lack the ability to replicate on their own, thus requiring the use of their bacterial host's machinery to produce progeny (Dennehy & Abedon, 2021). While there are several life cycles of phages, most phages tend toward 2 common circuitions, the lysogenic and the lytic life cycles, as displayed in **Figure 1** (Dennehy & Abedon, 2021; Hobbs & Abedon, 2016). In the lytic life cycle, the phage DNA is inserted into the host cell whereupon the bacterial machinery is used for replication followed by fatal host lysis and the release of the phage progeny (Dennehy & Abedon, 2021). In the lysogenic life cycle, after the phage DNA is inserted into the host cell, the DNA integrates into the host cell's genome or persists as an extrachromosomal plasmid. The phage's genome is thus replicated as the bacteria reproduces (Dennehy & Abedon, 2021). Lysogenic phages are referred to as prophages. While some phages persist solely through a

single life cycle, e.g., obligately lytic phages continue only in the lytic life cycle, temperate phages are able to switch between the lysogenic and the lytic life cycles. Integrase and C repressor genes can be indicative of a phage persisting as a temperate phage through both life cycles (Reyes et al., 2010).

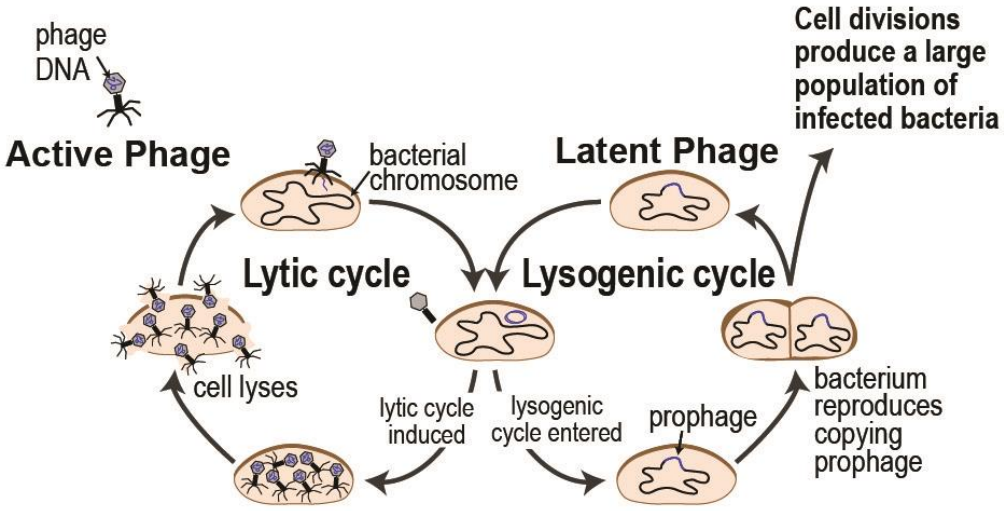


Figure 1. Lytic and lysogenic life cycles of phages. A phage begins infection by attaching to a host cell and inserting its DNA into the cell. In the lytic cycle, the phage replicates using the host cell machinery and its mature progeny burst the host cell, killing the bacteria. In the lysogenic cycle, the inserted phage DNA is integrated into the bacterial genome, and it replicates as the bacteria itself replicates. Temperate phages switch between the two life cycles.

Integrases are site-specific recombinase enzymes that directly mediate the integration of phages into the bacterial genome during a temperate phage's life cycle (Groth & Calos, 2004). As illustrated in **Figure 2**, the integrase recognizes the phage attachment site *attP* and the bacterial attachment site *attB* (Groth & Calos, 2004). The enzyme then recombines the two attachment sites to integrate the phage DNA into the bacterial genome with the phage DNA being flanked on both sides by hybrid sites, *attL* and *attR*, consisting of half *attP* and half *attB* sequences (Groth & Calos, 2004). When the temperate phage is ready to exit the lysogenic life cycle and enter the lytic life cycle, the excisionase, paired with the integrase, will use the *attL*

and *attR* sites to excise the phage DNA from the bacterial genome (Groth & Calos, 2004). Integrases can be classified as either tyrosine recombinases or serine recombinases (Fogg et al., 2014). The two families of site-specific recombinases are not related in their protein sequence and they have distinct mechanisms of recombination (Grindley et al., 2006). Tyrosine integrases recombine DNA through a tyrosine active site that both cleaves and then reseals the DNA by forming a 3'-phosphotyrosine intermediate (Landy, 2015). The serine integrases use a serine nucleophile to cleave the DNA for recombination and members of the serine family share a conserved catalytic domain (Stark, 2014). Without an integrase gene, temperate phages would be unable to integrate into a bacterial genome, limiting the lysogenic portion of their life cycles.

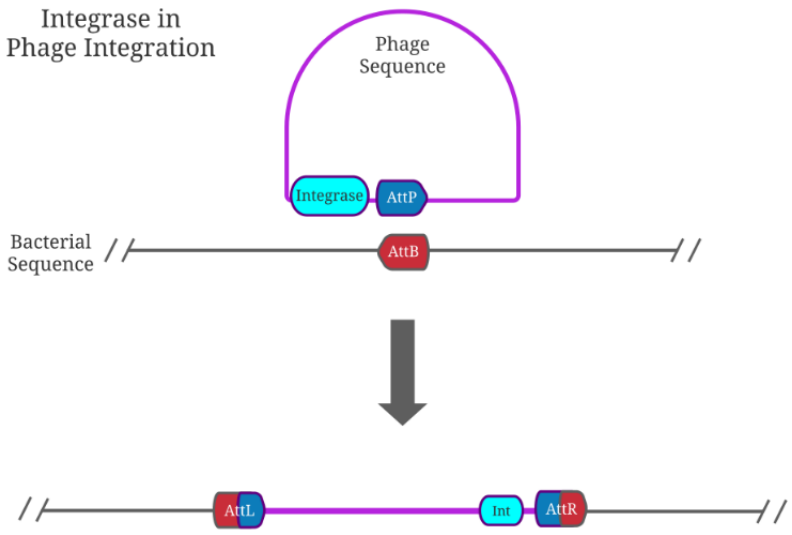


Figure 2. Role of the integrase during phage integration. The integrase assists in integration of the phage into the bacterial host genome by recognizing the phage attachment site, *attP*, and the bacterial attachment site, *attB*. It then recombines the 2 attachment sites to integrate the phage DNA with *attL* and *attR* as the new attachment sites.

The C repressor, also known as the CI regulator in the model phage λ, is a repressor and activator of transcription that plays a role in both maintaining the lysogenic life cycle and the

induction switch to the lytic life cycle (Blakely, 2004). **Figure 3** illustrates this process. Briefly, the C repressor maintains the lysogenic state by repressing the transcription of the pL and pR promoters responsible for the expression of lytic genes (Oppenheim et al., 2005). One protein transcribed from the pR promoter is the Cro protein which is considered the counterpart to the C repressor (Folkmanis et al., 1977). The C repressor stunts transcription of pR which promotes transcription of Cro, and once Cro is transcribed it represses the transcription of the C repressor by inhibiting the pRM promoter (Svenningsen et al., 2005). The pRM promoter begins transcription of the C repressor in an already established lysogen to maintain the lysogenic life cycle, while the pRE promoter stimulates transcription of the C repressor following phage infection for integration (Oppenheim et al., 2005). Cro also regulates the production of CII, a lysogenic regulator that activates the pRE promoter to synthesize the C repressor (Oppenheim et al., 2005). Without the C repressor, temperate phages would be unable to maintain the lysogenic status in the bacterial genome.

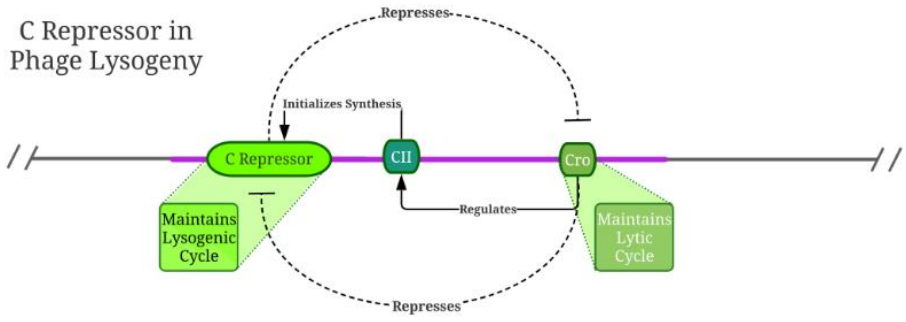


Figure 3. Role of C repressor in maintaining the lysogenic life cycle. The C repressor assists in maintaining the lytic cycle by repressing Cro through promoter inhibition. Cro is responsible for maintaining the lytic cycle and it in turn represses the C repressor through promoter inhibition. Cro also regulates CII, a lysogenic regulator that initializes the synthesis of the C repressor.

Temperate phages, which can switch between the lytic and lysogenic life cycles, contribute greatly to the wide genomic diversity of bacteriophages (Dion et al., 2020). The mosaic nature of phages is due to horizontal gene transfer and recombination between phage ancestors (Dion et al., 2020). Because temperate phages are able to integrate and excise their genomes into and out of bacterial hosts, they can pick up an extra gene from another prophage or the host or they can leave a gene behind for other temperate phages to acquire (Dion et al., 2020). The phage terminase gene is a useful gene marker for the diversity and evolutionary relationships of phages as well as identifying recombination events (Adriaenssens & Cowan, 2014; Sørensen et al., 2020). Terminases are involved in the packaging of the phage DNA and are conserved among all tailed phages (order: *Caudovirales*) (Casjens & Thuman-Commike, 2011).

## Scope of Thesis

As of May 2021, 21,367 complete phage genomes are publicly available in the NCBI Nucleotide database (*NCBI Virus*, n.d.-a). Of these, only 452 entries are phages that infect *P. aeruginosa* (*NCBI Virus*, n.d.-b). These 452 phages mainly represent obligately lytic phages or temperate phages that have been isolated and characterized in the lab through their propagation in the lytic life cycle. As it is known that lysogenic phages outnumber obligately lytic phages in human microbiome (Manrique et al., 2017), it can be assumed that there are many unknown prophage sequences inhabiting *P. aeruginosa* genomes. Temperate phages that reside in bacterial genomes as prophages can also be induced to display lytic activity against their host species (Dion et al., 2020). Thus, the catalogue of temperate phages represents an unexplored collection of phages with potential use as phage therapies.

Here I present a study of *P. aeruginosa*-infecting temperate phages, integrating bioinformatic and synthetic biology approaches. Chapter 2 details the first comprehensive catalogue of *P. aeruginosa* lysogenic phages. Over 5,000 publicly available *P. aeruginosa* genomes were examined for prophage sequences. The identified prophage sequences were further investigated for the presence of integrase and C repressor genes. I next explored the viability of temperate phages for phage therapy of *P. aeruginosa* infection (Chapter 3). A concern for using temperate phages is their propensity for integrating rather than lysing (clearing) of the infectious bacterium. Thus, I engineered two temperate *P. aeruginosa*-infecting phages, Dobby and D3112, by excising their integrase and C repressor genes, respectively. These engineered, now obligately lytic phages, were then evaluated for their efficacy against clinical *P. aeruginosa* isolates. Chapter 4 offers conclusions and future directions of this thesis work.

# CHAPTER TWO

# *PSEUDOMONAS AERUGINOSA* PROPHAGE ANALYSIS

## Introduction

Prophages are lysogenic phages that have integrated into their bacterial host's genome and they can account for up to 20% of the host's genome (Casjens & Hendrix, 2004). Nearly half of all sequenced bacterial genomes contained at least 1 prophage and those with larger genomes are more likely to contain prophages than bacteria with smaller genomes (Touchon et al., 2016). The presence of a prophage within the bacterial genome can give the host a selective advantage (Little, 2005). Lysogenic conversion is the expression of genes from the prophage genome that can change the phenotype of the bacterial host (Little, 2005). This can include extracellular toxins, adhesions to assist in bacterial attachment, effector proteins to assist in bacterial invasion, and virulence enzymes (Brüssow et al., 2004). Prophages can also protect the bacterial cell from environmental stressors and confer antibiotic resistance to the bacterial host (X. Wang et al., 2010). Thus, prophages can contribute to both the virulence and the genetic diversity of their bacterial hosts.

Several computational tools have been developed to predict the presence of prophages within the genomes of bacterial species, e.g., VirSorter, VirFinder, PHAST, and PHASTER (Arndt et al., 2016; Camarillo-Guerrero et al., 2021; Roux et al., 2015; Zhou et al., 2011). VirSorter is able to detect and identify viral genome sequences from both complete and fragmented microbial genomes as well as metagenomes (Roux et al., 2015). It uses primary and

secondary metrics based on sequence similarity to known viruses and similarity to viral-like genome structures, respectively (Roux et al., 2015). The tool then builds probabilistic models to indicate whether regions of the input sequences contain more viral-like genes than would be found by random chance (Roux et al., 2015). VirSorter achieves comparable results to similar phage prediction tools but outperforms the other tools in analyses with larger-scale and fragmented microbial data sets (Roux et al., 2015).

A study to investigate the diversity of prophages in the bacterial genomes of the urinary microbiome used VirSorter to predict viral sequences (Miller-Ensminger et al., 2018). 86% of the urinary bacterial genomes were found to contain at least 1 prophage sequence, displaying the prevalence of phages within the urinary tract system (Miller-Ensminger et al., 2018). Another study mined through 2,898 cultured gut bacterial genomes and 28,060 gut metagenomes using VirSorter paired with VirFinder (Camarillo-Guerrero et al., 2021). The authors of this work identified 142,809 phage sequences and created the Gut Phage Database to explore phage diversity (Camarillo-Guerrero et al., 2021). VirSorter has also been applied across data sets from a variety of environmental niches. In a study published in 2015, VirSorter was used to mine through almost 15,000 publicly available bacterial and archaeal genomic data sets to identify over 12,000 prophage sequences, expanding the known viral sequences at the time by 10-fold (Roux et al., 2015). In a subsequent study, VirSorter was used to identify over 125,000 partial viral genomes from approximately 3,000 geographically diverse metagenomic samples (Paez-Espino et al., 2016).

The availability of bioinformatic tools for prophage prediction have enabled investigations of the diversity of prophages within specific bacterial genera and species. A study

on the diversity of 71 published *Staphylococcus aureus* temperate phages was conducted to determine the prevalence of phages from 386 different bacterial isolates, concluding that the distribution of phages from the strains differed greatly based on bacterial lineages (Goerke et al., 2009). In 2013, 500 prophages were identified from 21 *Salmonella* and 48 *Escherichia* genomes using Phage Finder, PHAST, and Prophinder prediction tools (Bobay et al., 2013; Fouts, 2006; Lima-Mendez et al., 2008). The 500 predicted prophages were then compared to all publicly available enterobacteria phage genomes, finding that temperate phage genomes and lytic phage genomes form distinct clusters without overlap of the 2 groups (Bobay et al., 2013). A 2015 study isolated and sequenced 627 *Mycobacterium smegmatis* phages to investigate their genomic similarities (Pope et al., 2015). They determined that, while a majority of the phage genomes clustered into 28 distinct groups, there was still the clear genetic mosaicism characteristic of bacteriophages (Pope et al., 2015). A study of 20 sequenced *Klebsiella pneumoniae* isolates harboring prophages identified 20 prophage genomes and found that only 2 conserved regions, *int* and *umuCD*, existed between the 20 prophages (F. Wang et al., 2019). In 2017, 442 Pneumococcal genomes were analyzed using PHAST and prophage DNA existed in every genome (Brueggemann et al., 2017). The full-length phage predictions clustered into 4 different genetic groups that had very few genes shared between the groups, displaying the diversity of Pneumococcal prophages (Brueggemann et al., 2017). In yet another study, 49 *Salmonella enterica* isolates were screened for prophages both by PCR and PHASTER to compare the number of integrase genes amplified and annotated integrases, respectively. The PCR assays detected 147 integrase regions while PHASTER only identified 75 integrase genes from 102 predicted prophages (Colavecchio et al., 2017). The results demonstrate the possibility of using

the integrase gene to identify prophages in bacterial genomes that some phage prediction tools may miss (Colavecchio et al., 2017). A subsequent study into *S. enterica* prophages identified 154 different prophage sequences via PHASTER from 1,760 *S. enterica* genomes; it was found that the prophage sequences from the same serovar tended to share conserved regions, but there was little overlap between the serovars (Mottawea et al., 2018). The use of phage sequence prediction tools throughout these studies shows the high prevalence of prophages within a multitude of different bacterial genera. Furthermore, these studies reveal that while there are several conserved gene regions, e.g., integrases and terminases, between phages that infect the same bacterial species, there is also distinct mosaicism that results in broad phage diversity.

Prophages of *P. aeruginosa* have also been studied, primarily with respect to their association with increased bacterial pathogenicity and fitness. The Liverpool Epidemic Strain (LES) of *P. aeruginosa*, which has been associated with infections in cystic fibrosis patients, harbors 5 prophages, LESϕ2, LESϕ3, LESϕ4, LESϕ5, and LESϕ6 (Davies et al., 2016). Not only do the prophages improve the invasiveness of LES, but they also assist in the displacement of other *P. aeruginosa* strains by LES, allowing LES full competitive advantage in lung infections (Davies et al., 2016). Another. *P. aeruginosa* prophage that has been investigated is the *P. aeruginosa* phage FIZ15, which causes lysogenic conversion in *P. aeruginosa* PAO1 leading to PAO1's increased resistance to phagocytosis, increased resistance to normal human serum, and increased adhesion to human epithelial cells (Vaca-Pacheco et al., 1999). The *Pseudomonas* φCTX phage, isolated from a cytotoxin-producing strain of *P. aeruginosa*, was able to induce cytotoxin production in non-cytotoxin-producing strains of *P. aeruginosa* when integrated into their genomes (Hayashi et al., 1990). *Inoviridae* prophages, such as the Pf family of prophages,

can promote biofilm formations in the *P. aeruginosa* that they infect (Ambroa et al., 2020). As these four examples clearly demonstrate, *P. aeruginosa* prophages can have a profound effect on bacterial phenotype, competitiveness, and pathogenicity (Tsao et al., 2018).

In comparison to other bacterial taxa, there has not been any comprehensive studies of the diversity or prevalence of *P. aeruginosa* phages, a likely factor of the vast observed differences in genetic content and genome sizes of known lytic *Pseudomonas* phages (Xu et al., 2020). The prevalence of Pf1-like prophages in *P. aeruginosa* was studied in 2015 (Knezevic et al., 2015). 241 strains of *P. aeruginosa* were screened with primers for both universal Pf1-like genes and genes specific to Pf1, Pf4, and Pf5 (Knezevic et al., 2015). Approximately 60% of the *P. aeruginosa* strains screened were found to have at least 1 Pf1-like genetic element and 56% of the strains contained universal elements of Pf1-like phages, displaying the prevalence of Pf1-like prophages in *P. aeruginosa* genomes (Knezevic et al., 2015). The diversity of lytic *P. aeruginosa* phages was investigated in 2012 using a collection of 68 isolated phages (Sepúlveda-Robles et al., 2012). The 68 phages were classified into 12 different phage species using phage-phage homology by DNA hybridization (Sepúlveda-Robles et al., 2012). 6 species were novel identifications at the time (Sepúlveda-Robles et al., 2012). In 2019, the *P. aeruginosa* phage diversity was expanded to include the 149 sequenced phages containing lytic proteins from the UnitProt database (Valero-Rello, 2019). While this study focused on investigating the lytic proteins of these phages, they were able to display the effect of phage lifestyle on protein variability within the phages (Valero-Rello, 2019).

While there have been a few studies focused on the diversity of specific types of *P. aeruginosa* phages, there has yet to be a comprehensive study on the presence and diversity of

prophages in all publicly available genomes of *P. aeruginosa*, which now exceeds 5383 genomes. The diversity and putative roles that prophages play in *P. aeruginosa* fitness and pathogenicity has yet to be catalogued. Furthermore, these prophages represent a reservoir of potential candidates for use in phage therapy to resolve *P. aeruginosa* infections.

## Methods

### Cataloguing *P. aeruginosa* Prophages.

Through the Genome Information by Organism section of the NCBI Genome database, a 5383 *P. aeruginosa* bacterial genomic assemblies were identified (September 2020). The assembly files were retrieved from the GenBank database using the FTP links for each genome. All of the 5383 genome assemblies were then entered into VirSorter v.1, a bioinformatic tool to predict phage sequences found inside of bacterial genomes (Roux et al., 2015). VirSorter v.1 detects viral signals using both reference-based homology as well as reference-independent methods. It then predicts phage sequences with confidence levels ranging from 1 to 3 for extrachromosomal phages, e.g., lytic or plasmidial phages, and 4 to 6 for prophages, where 1 and 4 are the highest confidence predictions and 3 and 6 are the lowest confidence predictions. For the entirety of Section I, only category 1 and category 4 predicted phages were retained for analysis.

### Taxonomic Classification of *P. aeruginosa* Prophages.

All category 1 and 4 sequences were compared to previously characterized phage genomes in an effort to determine their likely taxonomic family. Each sequence was queried against all complete and partial genome sequences in GenBank (Organism: “Virus” and Division: “PHG”) using local BLAST (Camacho et al., 2009). This database includes 26,381

sequences and the BLASTn algorithm was used. Homologous results with a query coverage greater than 50% and a percent identity over 70% were considered to be acceptable and the taxonomies of the resulting similar phages were used to predict the taxonomy of the query phages.

**Evaluating Genetic Diversity of *P. aeruginosa* Prophages.**

Predicted phage sequences were then examined using Anvi'o v.6.2 to find the number of shared genes between each predicted phage (Eren et al., 2015). Anvi'o was used to identify homologs given the following parameters: MCL inflation value of 2 during cluster identification and a minbit heuristic score of 0.35 to remove weak gene matches. A python script was used to parse the Anvi'o output to identify homologous genes shared between genomes and produce an edgelist file, listing each pair of genomes (nodes) and the number of genes shared (edge weight). The edgelist file was then pruned using a python script to generate an undirected graph edgelist representation, eliminating self-loop edges or duplicate edges. Phage gene similarity was visualized using Cytoscape v.3.8.2 (Shannon et al., 2003). Different thresholds of edge weights (number of genes in common between phage genomes) were considered.

**Diversity of *P. aeruginosa* Prophage Integrase, C Repressor, and Terminase Coding Sequences.**

After analyzing the diversity of the category 1 and 4 predicted *P. aeruginosa* phages as a whole, the diversity of integrase and C repressor genes was then investigated. The predicted phages were annotated using PATRIC v.3.6.9 with the Bacteriophage Domain, the Genetic Code for Bacteria and Archaea, and the Bacteriophage Annotation Recipe (Brettin et al., 2015; Davis et al., 2020). The resulting annotations were parsed to identify coding regions identified as an

integrase using the words “integrase” and “Integrase”. The same process was repeated to identify C repressor coding regions using the word “repressor” in combination with the words “cI”, “CI”, “c1”, or “C1”. Finally, the terminase coding regions were identified using the words “terminase” and “Terminase”. The annotated integrase, C repressor, and terminase coding regions were then compared for similarity to all other annotated coding regions from the predicted phages. A local BLASTp with a maximum of 1 target sequence was used with the non-integrase coding regions as the database and the integrase coding regions as the query sequences. Any gene that had a percent identity greater than 70% and a query coverage greater than 70% was then added to the respective list of integrases and the same process was repeated for the C repressors and terminases. USEARCH v.11.0.667 (Edgar, 2010), a tool that clusters sequences based on sequence similarity, was used to create clusters of the integrase coding regions, clusters of the C repressor coding regions, and clusters of terminase coding regions with percent identities greater than or equal to 70%. The clusters for the 3 genes were then uploaded to Geneious Prime® 2020.1.2 to visualize the quality of the clusters. The top 5 largest clusters for each gene were aligned using MAFFT v.7.450 (Katoh & Standley, 2013) multiple alignment with the automatic algorithm option with its default parameters. Using FastTree v.2.1.11 (Price et al., 2010) with its default parameters, Newick trees were constructed and then visualized in iTOL v.6.1 (Letunic & Bork, 2007). For easier visibility, clades with an average branch length less than 0.00056 were collapsed.

The phage memberships in the integrase, C repressor, and terminase clusters output from USEARCH were examined. A python script using Biopython SeqIO, NumPy, and pandas

packages counted the number of phages shared between each of the 3 sets of coding region clusters (Cock et al., 2009; Harris et al., 2020; Jeff Reback et al., 2021).

**Predicting Antibiotic Resistance and Virulence Factors Encoded by Prophages.**

ResFinder 4.1 (Bortolaia et al., 2020), an antibiotic resistance prediction tool that uses k-mer based alignment against a curated database to locate possible genes associated with antibiotic resistance, and its databases were installed and downloaded from the tool's bitbucket page (https://bitbucket.org/genomicepidemiology/resfinder/src/master/). All of the predicted prophages were separately run through the tool's pipeline from the command-line in a Linux environment using the authors' suggestions of an 80% threshold and 60% minimum coverage for acquired antibiotic resistance genes. The virulence factors of the phages were then predicted using VFDB, a curated database of pathogenic bacterial virulence factors (Chen et al., 2005). The full data set that encompasses all virulence factor genes, both predicted and experimentally known, was downloaded from the VFDB website (http://www.mgc.ac.cn/cgi-bin/VFs/v5/main.cgi?func=VFanalyzer) and used as the BLAST database in a local BLASTn. Each predicted phage was compared for sequence similarity to the virulence factor database entries with a maximum target of 1 sequence.

## Results

**Prophages of *P. aeruginosa*.**

VirSorter identified 49,102 prophages in the 5,383 publicly available *P. aeruginosa* genomes examined here (**Table 1**). Of the total predicted prophages, 6,852 prophages were of category 1 and category 4, the highest confidence categories for unintegrated and integrated prophages, respectively. Given that these high confidence predictions likely represent viable

temperate phages, further analysis was restricted to category 1 and 4 prophage sequences only. 3,672 out of 5,383 *P. aeruginosa* genomes encoded for prophage sequences identified as either category 1 or 4. An average of 1.879 prophages were predicted per bacterial strain, with a maximum of 15 category 1 or 4 prophages predicted for 1 strain, *P. aeruginosa* XDR-PA (GCA_900707735.1). The 5,383 *P. aeruginosa* genomes were isolated from body sites, the environment, and industrial settings. Of the isolation sites, the urinary tract/urine, blood, the ear, feces, and hospitals had the highest average predictions per strain, ranging from 10 to 12 predicted prophages per strain of *P. aeruginosa* from that isolation site category. A One-Way ANOVA determined the isolation sites were significantly associated with the numbers of predicted prophages for the strains of *P. aeruginosa* after normalization ($p<2.2e-16$).

| | Category 1 | Category 2 | Category 3 | Category 4 | Category 5 | Category 6 |
|---|---|---|---|---|---|---|
| Number of Prophages Predicted | 2668 | 12579 | 5666 | 4184 | 17367 | 6638 |

Table 1. Summary statistics of VirSorter prophage prediction results. Categories 1 and 4 are the highest confidence predictions. Categories 3 and 6 are the lowest confidence predictions e.g., typically partial phages or phage-like genes.

**Genetic Diversity of *P. aeruginosa* Prophages.**

The taxonomy of each prophage was determined by querying each predicted prophage sequence to all publicly available characterized, sequenced phages. Of the 6,852 predicted prophages, 11 were classified as Inoviridae, 106 as Microviridae, 672 as Myoviridae, 105 as Podoviridae and 2,744 as Siphoviridae. The remaining 3,214 prophages were not homologous to any characterized, sequenced phages and thus a taxonomic classification could not be made.

The diversity of *P. aeruginosa* prophages was visualized through a network consisting of the prophages as nodes and the connecting edges representative of the number of genes shared between each prophage. The network is composed of 4 connected components (data not shown). The largest component contains 6,472 predicted tailed prophages (Myoviridae, Podoviridae, and Siphoviridae), Inoviridae prophages, and taxonomically uncharacterized ("Unknown") prophages. The second connected component contains all 106 of the predicted Microviridae prophages. The final 2 connected components consist of 2 pairs of Myoviridae prophages. Both pairs were predicted from the same bacterial strain, *P. aeruginosa* ENVO278 (GCA_006704765.1), which also contained another 7 predicted Myoviridae prophages (placed in the largest connected component). Following a BLAST analysis, 10 of the 11 Myoviridae prophages from *P. aeruginosa* ENVO278 were found to be highly similar (greater than 97% identity) to the characterized *Pseudomonas* phage EL (NC_007623.1).

Further investigation into the category 1 and 4 prophage sequences revealed high confidence predictions of sequences too small to be a viable phage. While the average length of the predicted prophages was ~27Kbp, the smallest prophage sequence predicted with high confidence by VirSorter was 606 bp; this is far smaller than any documented prophage or phage for *P. aeruginosa*. In an effort to focus our investigation on complete prophages, we introduced a threshold for further investigation of the *P. aeruginosa* prophage network. Only edges representative of 5 or more shared genes between prophages (nodes) were retained; nodes that were not connected to any other node were removed from further consideration. After this thresholding, 6,676 of the originally predicted 6,852 (97.43%) prophages remained (**Figure 4**). The network contained a total of 3,814,212 edges representing the genes shared between the

prophages. It contains 2 Inoviridae prophages, 105 Microviridae prophages, 635 Myoviridae prophages, 99 Podoviridae prophages, 2,662 Siphoviridae prophages, and 3,173 prophages assigned to the Unknown taxonomic class.

The prophages cluster within 5 connected components with the majority (6,513 prophages; 97.56%) belonging to 1 large, connected component. This largest connected component includes prophages characterized as tailed prophages (Myoviridae, Podoviridae, and Siphoviridae), the Inoviridae prophages, and unclassified ("Unknown") prophages. The 105 Microviridae prophages (green nodes in **Figure 4**) belong to their own connected component, sharing genes only among other Microviridae prophages. The third connected component in **Figure 4** contains 21 Siphoviridae prophages, which are distinctly different from other Siphoviridae prophage sequences within the largest connected component. These prophage sequences were identified from 18 different *P. aeruginosa* genomes. Further investigation of these sequences revealed genetic homology to strains of *Escherichia coli* as well as to the *E. coli* Lambda phage.

The other 2 separated clusters contained prophages classified as Unknown (orange nodes in **Figure 4**). The first of the unknown clusters consisted of 27 prophages predicted from 27 different genomes of *P. aeruginosa*. While BLAST queries of these prophage sequences revealed homology to a pyocin gene fragment (L06240.1), BAGEL4 (van Heel et al., 2018), a bacteriocin prediction tool, did not predict these sequences to be bacteriocins. The other cluster classified as Unknown contained 10 prophages predicted from 7 different *P. aeruginosa* genomes. The prophages were queried via BLAST and found to all have high similarity (over 75%) to at least 1 of 2 uncultured Caudovirales phages (clone 3S_15: MF417945.1 and clone 3S_19:

MF417971.1), both identified through a skin metavirome project. Neither these 10 prophage sequences nor the 2 uncultured Caudovirales phage sequences exhibit significant sequence homology to any phage genome and thus may be representative of a new family of phages yet to be characterized.

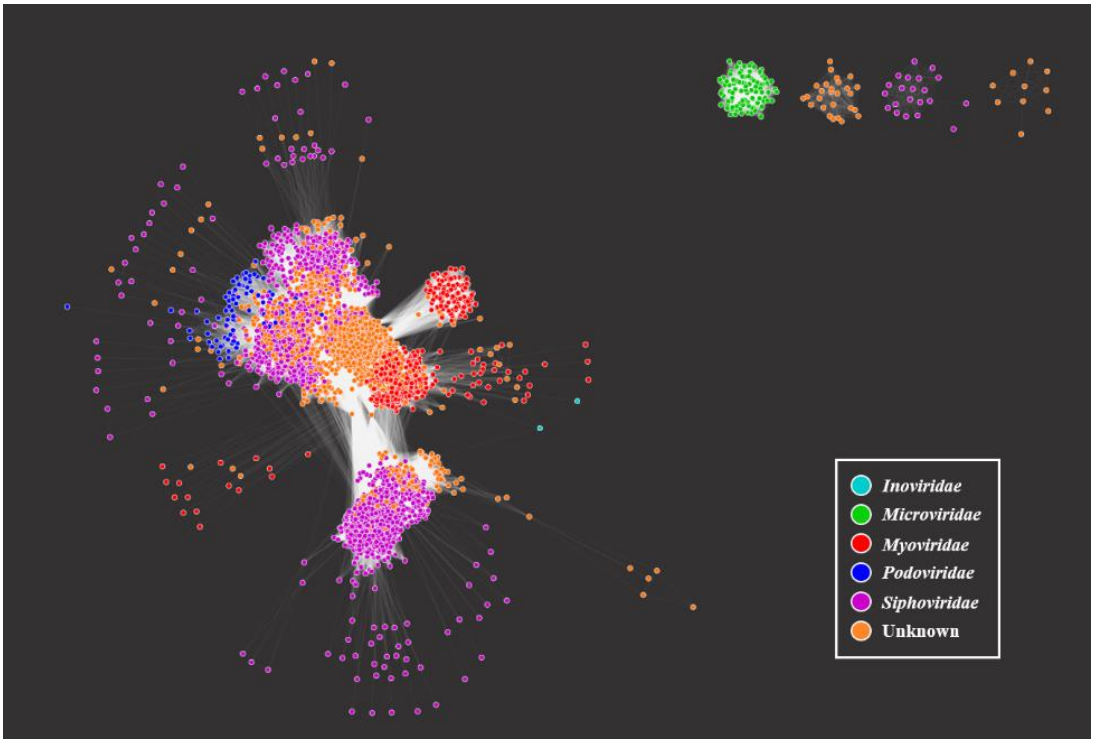


Figure 4. Network of *P. aeruginosa* predicted prophages. Prophages are the nodes color-coded by taxonomy: Inoviridae, Microviridae, Myoviridae, Podoviridae, Siphoviridae, or Unknown. Prophages are connected by the number of shared genes between each prophage.

**Diversity of *P. aeruginosa* Prophage Integrases, C Repressors, and Terminase Coding Sequences.**

PATRIC identified 283,493 coding regions within the 6,852 predicted prophage sequences. These annotations included 1,358 integrase coding regions, with 54 specified as tyrosine integrases and 1 as a serine integrase, 2,344 C repressor coding regions, and 4,546 terminase coding regions. To ensure that all integrase, C repressor, and terminase genes within

these prophage sequences were included in our analysis, we conducted additional BLAST homology searches for representatives of these 3 genes. These queries identified an additional 12, 17, and 24 integrase, C repressor, and terminase coding regions, respectively. Thus, a total of 1,370 integrases, 2,361 C repressors, and 4,546 terminases are encoded within the predicted *P. aeruginosa* prophage sequences.

To investigate the diversity of integrase sequences within the *P. aeruginosa* prophages, a phylogenetic tree was derived (**Figure 5**). The 5 red circles in **Figure 5** indicate clades, inclusive of 154 sequences, that were identified as tyrosine integrases. The 1 orange circle in **Figure 5** represents the 1 integrase coding region identified as a serine integrase. As the integrases identified as tyrosine integrases are not closely related to each other, there is no correlation between integrase type (serine or tyrosine) on the tree in **Figure 5**. As the phylogenetic tree shows, there are distinct lineages of integrase genes among the prophages. We next performed clustering of the integrase coding regions (n=1370). In total, 72 different clusters of integrases were identified, with the largest containing 110 integrase coding regions. The integrase sequences within this cluster exhibit little sequence variation (average pairwise identity 98.3%) (**Figure 6**).

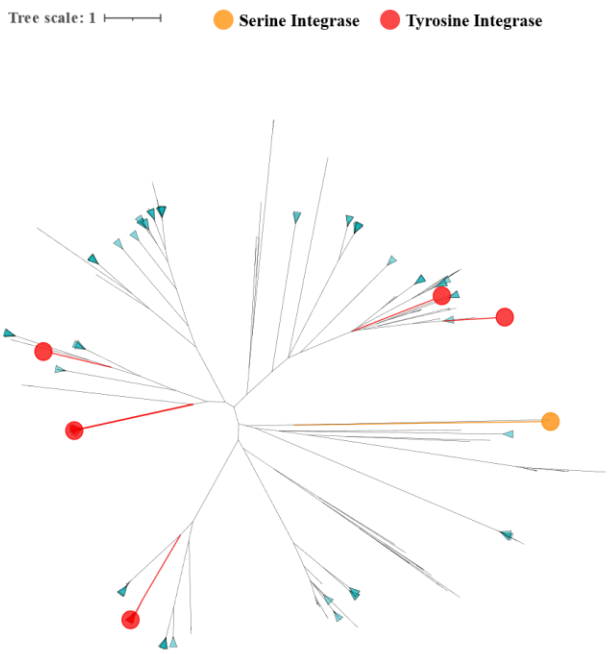


Figure 5. Phylogenetic tree of all annotated integrase coding regions. Average amino acid pairwise identity of 16.1% (n=1,370). Red circles indicate integrase clades identified as tyrosine integrases. The orange circle indicates the integrase cluster identified as a serine integrase.

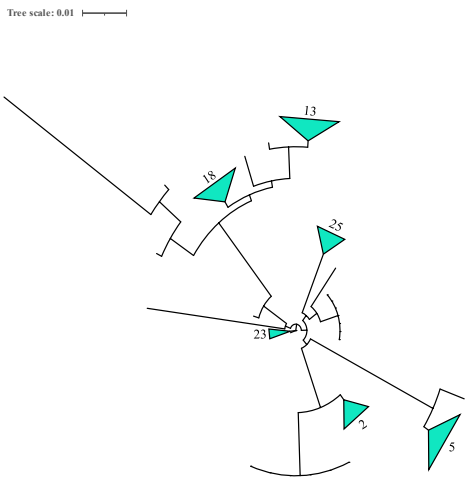


Figure 6. Phylogenetic tree of largest homologous cluster of integrase coding regions. Average amino acid pairwise identity of 98.3% (n=110).

The integrase from the temperate *P. aeruginosa* phage Dobby (NC_048109) was added into the list of all 1,370 integrases to identify coding regions similar to Dobby. After performing clustering on the coding regions, the integrase from the Dobby phage was not similar to any of the 1,370 integrase coding regions from the predicted prophages. A BLAST analysis was then used to compare the full Dobby genome against the 6,852 predicted prophage sequences. The Dobby phage was highly similar (≥70% identity) to 250 of the predicted prophages. Less than half (48.8%) of these predicted prophages contained an integrase coding region. The integrase coding regions from those that did have one were not similar to the integrase coding region from the Dobby phage (≤30% identity).

We also examined the sequence diversity of the 2,361 C repressor coding sequences within the predicted *P. aeruginosa* prophages (**Figure 7**). The C repressor sequences were next clustered based upon sequence similarity resulting in 44 clusters. Most of the C repressor sequences (n=1604; 67.94%) clustered together. This largest cluster of 1,604 C repressor coding regions contains an average pairwise identity of 99.7% and its sequence diversity is shown in **Figure 8**.

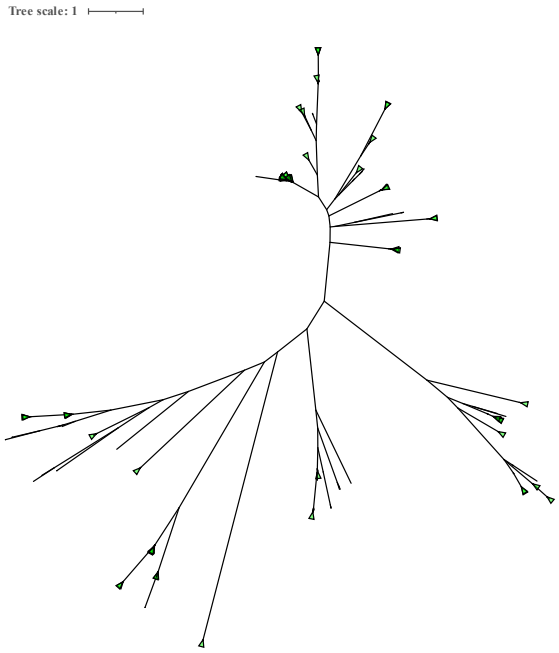


Figure 7. Phylogenetic tree of all annotated C repressor coding regions. Average amino acid pairwise identity of 58.0% (n=2,361).

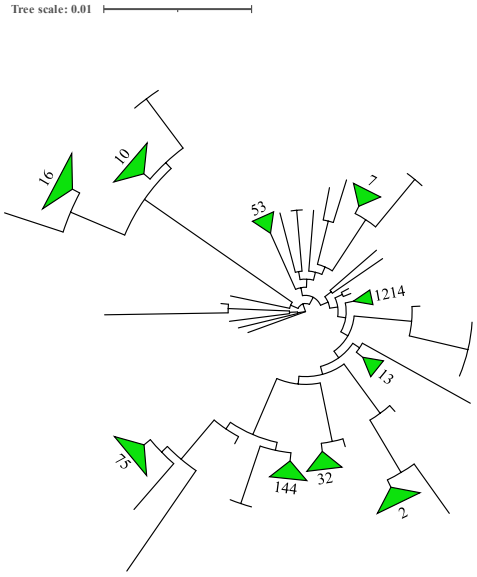


Figure 8. Phylogenetic tree of largest homologous cluster of C repressor coding regions. Average amino acid pairwise identity of 99.7% (n=1,604).

The C repressor gene from the temperate *P. aeruginosa* phage D3112 (NC_005178) was added into the list of all 2,361 C repressors to identify similar coding regions. After clustering was performed on the C repressor coding regions, the C repressor from D3112 was not found to be similar to any of the 2,361 C repressor coding regions from the predicted prophages. A BLAST analysis compared the full D3112 genome against all 6,852 predicted prophages and D3112 was found to be highly similar (≥70% identity) to 314 of the predicted prophages. Out of these similar prophages, none contained a C repressor coding region.

To investigate the homology of the terminases (n=4,570) within the prophages, the terminase coding regions were clustered into 81 homologous clusters of terminases. The largest of the terminase clusters contained 424 terminase coding regions with an average pairwise identity of 89.6%.

**Phage Membership Comparisons of Integrases and C Repressors to Terminases.**

Terminases are found in the genomes of all tailed phages and, therefore, is often used as a marker gene for phage diversity (Grose & Casjens, 2014). The associations between prophages that share homologous terminases and also share homologous integrases or C repressors were considered to investigate the genetic reassortment between prophage genomes. All 6,852 prophages were inspected for the presence of a terminase, integrase, and C repressor coding region. 3,028 of the prophages contained terminase coding regions, 1,115 contained integrase coding regions, and 2,318 contained C repressor coding regions. 850 prophages encoded for both a terminase and an integrase, 391 prophages encoded for both a terminase and a C repressor, and 713 prophages contained coding regions for all 3 of these genes.

Each prophage was next associated with the integrase, C repressor, and terminase clusters identified in the previous section. Between all 72 integrase clusters and all 81 terminase clusters, 40 integrase clusters shared all of their prophages with individual terminase clusters and 3 terminase clusters shared all of their prophages with individual integrase clusters. **Figure 9** displays the distribution of shared prophages between the integrase clusters and the terminase clusters with the highest numbers of co-occurring prophages existing between the largest integrase clusters and the largest terminase clusters.

Of the 44 C repressor clusters and the 81 terminase clusters, 17 C repressor clusters shared all of their prophages with individual terminase clusters and 5 terminase clusters shared all of their prophages with individual C repressor clusters. The C repressor clusters and the terminase clusters in **Figure 10** display a similar deduction to **Figure 9**, wherein the most shared prophages are between the largest C repressor clusters and the largest terminase clusters.

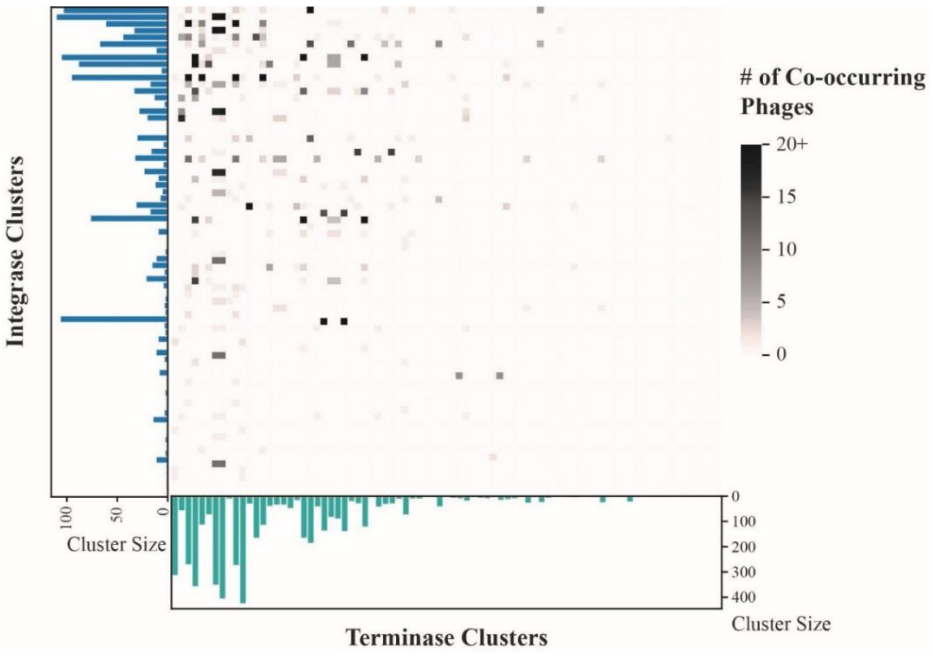


Figure 9. Prophage membership shared between integrase clusters and terminase clusters. The largest terminase clusters tend to share the most prophages with the largest integrase clusters.

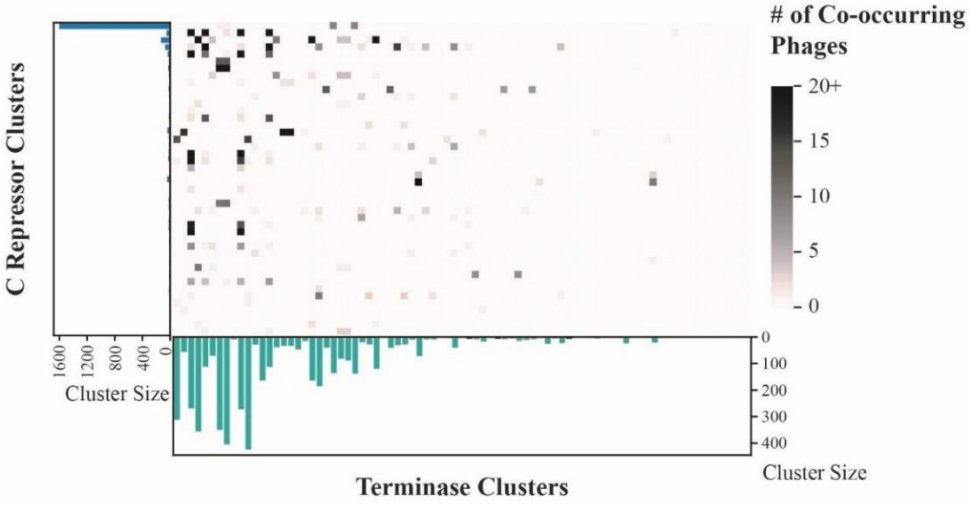


Figure 10. Prophage membership shared between C repressor clusters and terminase clusters. The largest terminase clusters tend to share the most prophages with the largest C repressor clusters.

To look for possible associations between terminase clusters and integrase or C repressor clusters, a phylogenetic tree of all terminase coding regions was constructed with color-coded bands to represent whether the prophage that possessed the terminase also contained an integrase, a C repressor, both, or neither of the coding regions (**Figure 11**). Using a One Sample t-Test, it was found that the integrase cluster membership of a phage is not associated with the terminase cluster membership of the phage; phages within terminase clusters contain integrases from significantly more than 1 integrase cluster type (p=8.275e-06). A similar finding was determined using a One Sample t-Test for the C repressor. The C repressor cluster member of a phage is not associated with the terminase cluster membership of the phage; phages within terminase clusters contain C repressor sequences from significantly more than 1 C repressor cluster (p=8.079e-04).

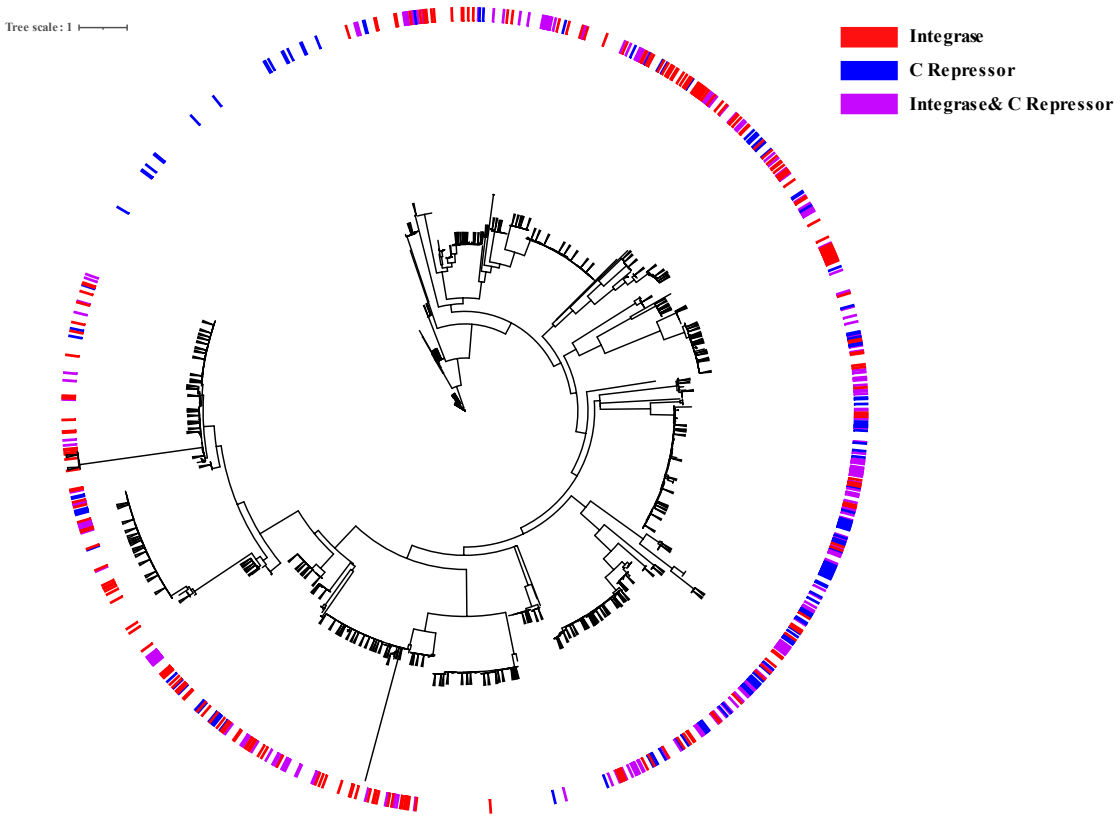


Figure 11. Phylogenetic tree of all annotated terminase coding regions. Average pairwise identity of 8.9% (n=4,570). Terminases are color-coded by shared phage membership with integrase and/or C repressor homologous clusters.

**Antibiotic Resistance and Virulence Factors Encoded by Prophages.**

To analyze the possibility of prophages conferring antibiotic resistance to their bacterial host, all category 1 and 4 predicted prophage sequences were examined using ResFinder 4.1 (Bortolaia et al., 2020). The antibiotic resistance prediction tool identified 71 predicted antibiotic resistance genes from 42 different prophage genomes, just 0.6% of the prophage sequences screened. The antibiotic resistance genes exhibited high percent identity (greater than 94%) and high query coverage (greater than 97%) to gene sequences within the ResFinder database. Of the 71 results, 26 genes are associated with resistance to three classes of antibiotics: aminopenicillins

(such as amoxicillin), antipseudomonal penicillins, and 2nd and 3rd generation cephalosporin antibiotics. Aminopenicillins are typically used to treat Gram-positive aerobic bacteria and Gram-negative rod bacteria (*Overview of Antibiotic Therapy*, n.d.). Antipseudomonal penicillins also target Gram-negative rod bacteria, especially *Pseudomonas* species (*Overview of Antibiotic Therapy*, n.d.). 2nd and 3rd generation cephalosporins are used to treat most Gram-negative bacteria as well as some Gram-positive bacteria (*Overview of Antibiotic Therapy*, n.d.). 24 genes are predicted to confer resistance to Chloramphenicol, a broad-spectrum antibiotic that is now rarely used and is typically reserved for severe infections due to its association with causing aplastic anemia (PubChem, n.d.-a). Additionally, 20 genes are associated with resistance to Kanamycin and Neomycin, broad-spectrum aminoglycoside antibiotics that are used to treat a variety of bacterial infections (PubChem, n.d.-b, n.d.-c). The final antibiotic resistance gene identified by ResFinder was a beta-lactam gene. These results display that the majority of the predicted prophages do not carry antibiotic resistance genes.

Next, the possibility of prophages conferring increased pathogenicity to their bacterial host by encoding genes associated with virulence was determined. Prophage sequences were screened against the Virulence Factors Database (VFDB) (Chen et al., 2005). Of the 6,852 prophage sequences, 502 prophages possessed a total of 515 predicted virulence factor sequences. The 515 predicted sequences were composed of 72 unique virulence factor sequences for 63 unique virulence genes. Some of the more frequent virulence factors found included Type IV pili related genes, GacS/GacA system related genes, alginate regulation genes, Type II/III/VI secretion system related genes, and flagella related genes. 41 prophages contained both predicted

virulence factors and predicted antibiotic resistance genes. These results suggest that these 41 prophages may increase their host's pathogenicity.

## Discussion

This is the first known report of the prevalence and diversity of prophages among *P. aeruginosa* strains. We limited our analysis to only the highest category prophage predictions from VirSorter, categories 1 and 4. Other investigations of phages in genomic and metagenomic data sets have taken a less conservative approach, including category 1, 2, 4 and 5 predictions (Camarillo-Guerrero et al., 2021; Miller-Ensminger et al., 2018; Paez-Espino et al., 2016). While others have noted that the lower confidence categories (3 and 6) tend to include only partial prophage genomes (Camarillo-Guerrero et al., 2021; Miller-Ensminger et al., 2018; Paez-Espino et al., 2016), our own prior work predicting prophages in the urinary microbiome found that most (∼71%) of the phage sequences in the category 2 and 5 predictions are not complete prophage sequences (Miller-Ensminger et al., 2018). Even with our stringent threshold, we identified 6,852 prophages in 5,383 *P. aeruginosa* genomes. 68.21% of the genomes examined contained at least one category 1 or 4 prophage prediction. This falls in line with, and even surpasses, previous work estimating nearly half of all sequenced bacteria to contain prophages (Touchon et al., 2016).

### Diversity of *P. aeruginosa* prophages.

57% of the predicted prophages exhibited sequence similarity to publicly available phage genomes. These similarities were most frequent to siphoviruses (n=2,744). The remaining predicted prophage sequences (n=3,214) shared no significant homology to characterized phages, thus representative of novel phages infectious of *P. aeruginosa*. It is important to note that even

though a conservative approach was taken when selecting VirSorter predictions, there are likely false positives and prophage artifacts (nonfunctional prophages) included in these higher confidence predictions. For instance, *Pseudomonas* phages have a wide range of genome sizes, the smallest being the PRR1 phage (NC_0082941) at 3,573 bp and the largest being the 201phi2-1 phage (NC_010821) at 316,674 bp in length. While the majority of our *P. aeruginosa* predicted prophage sequence lengths fall within this range, there are obvious false positives, e.g., our smallest predicted prophage is just 606 bp long. In fact, 332 prophage predictions were less than 3,000 bp. These smaller predicted prophage sequences may also be the result of fragmented bacterial assemblies, as many of the genomes examined here draft assemblies.

To minimize the impact of false positive predictions on our analysis, we implemented a threshold: a given predicted prophage must include 5 genes with another predicted prophage in order to be included in our data set. While this thresholding excluded these fragmented predictions, it also removed 9 of the 11 predicted inoviruses, which typically consist of only 8 genes. Inoviruses are filamentous phages. While there are very few characterized inoviruses, recent research predicts that they are far more diverse and pervasive than previously thought (Roux et al., 2019). Extant prophage prediction tools, including VirSorter, frequently miss inoviruses (Paez-Espino et al., 2016, 2017; Roux et al., 2015). As such new tools have been developed to explicitly identify inoviruses, e.g., Inovirus Detector (https://bitbucket.org/srouxjgi/inovirus/src/master/).

Our analysis did identify the inovirus Pf1. A previous study in 2015 studied the prevalence of Pf1-like phages in *P. aeruginosa* (Knezevic et al., 2015). The results displayed approximately 60% of the 241 strains screened via PCR contained at least 1 Pf1-like genetic

element (Knezevic et al., 2015). Of the 5,383 strains of *P. aeruginosa* analyzed in this work, only 11 were identified as strains of Pf1 via BLAST analysis. Pf1-like genetic elements were identified in an additional 93 predicted prophages. The prophage sequences had a high sequence similarity (>75% identity), but identities were only identified between one or a few genes (query coverage averaged 20%). These Pf1-like genetic elements were identified in prophage sequences classified as siphoviruses, myoviruses, or Unknown. It is these shared genes that "connect" the inoviruses to other taxa in the large, connected component of **Figure 4**. Our findings, however, are not consistent with the prior study. We find only ~2% of the *P. aeruginosa* genomes contain a Pf1-like genetic element. This inconsistency could be due to non-specific PCR primers used in the aforementioned study, e.g., they are amplifying non-Pf1 phages, or these genes are included in the Category 2, 3, 5, and/or 6 prophage predictions not examined here.

Of the 672 predicted myoviruses, 11 were predicted from a single genome assembly of *P. aeruginosa* ENVO278 (GCA_006704765). 10 of these predicted prophage sequences were highly similar (>97% identity) to the genome of Phage EL (NC_007623.1), a giant Phi KZ-like *Pseudomonas* phage with a genome size of 211,215 bp. The 10 predicted prophages are significantly shorter than Phage EL; combined their length is just 88 Kbp, ~42% of the Phage EL complete genome. However, they can be perfectly and uniquely mapped to the Phage EL genome (average query coverage of 99.1%). It is possible that Phage EL, or a similar phage, may have previously infected this specific strain of *P. aeruginosa* and left behind gene fragments that were identified by VirSorter. Alternatively, Phage EL (or a relative) could have been infecting this strain when it was sequenced but was not able to be assembled into a single contig. The 10 predicted prophages are in fact 10 separate contigs suggesting that they may not be integrated

within the *P. aeruginosa* ENVO278 genome. Without experimental investigation of this strain, we can only hypothesize whether or not VirSorter identified only small segments of Phage EL while it was infecting the host.

While the majority of the predicted prophages were taxonomically classified using homologous phages, 3,214 were unable to be associated with any known, characterized phage. The network analysis performed here, however, provides a means to predict the putative taxonomic classification of many of these phages. The taxonomies of the unknown prophages could be assumed according to the classified prophages nearby in **Figure 4** . After the threshold of 5 shared genes was added, the majority of the unknown prophages (n=3,136) clustered near the siphoviruses or near the myoviruses. However, the taxonomies of the 2 clusters that were separate connected components only contained phages of unknown classification. Thus, their taxonomies could not be deduced.

Of the unknown phages, 1 of the separated clusters contained 27 related prophages and was found to have similarity to a single pyocin gene fragment (L06240.1). It is known that bacteriophage tails can resemble the structures and sequences of R-type and F-type pyocins. While BAGEL4 did not identify any bacteriocins within the 27 predicted prophages, it is possible that small fragments of the pyocin gene from the bacterial genomes were included in the prophage predictions without including the full functional pyocin.

The second separated unknown cluster contained 10 prophages that were found to have high similarity (>76% identity) to at least 1 of 2 uncultured Caudovirales phages (MF417971 and MF417945). 6 of the prophages were also similar (>73% identity) to *Halomonas* phage phiHAP-1 (EU399241) and 3 to both *Ralstonia* phage RSY1 (AB981169) and *Ralstonia* phage RSA1

(AB276040). The *Halomonas* phage and the *Ralstonia* phages are all classified as myoviruses, so it is possible that the 10 unknown prophages are also myoviruses.

**Evolution of *P. aeruginosa* Prophages.**

Following annotation of the high confidence predicted prophages, several temperate phage marker genes were identified for analysis: terminases, integrases, and C repressors. Of the 6,852 predicted prophages, only 3,028 prophages encoded a terminase, a conserved gene among tailed phages. As expected, the 117 predicted microviruses and inoviruses do not contain terminase genes. That leaves 3,707 predicted prophages that should have contained a terminase gene and did not. The majority (n=2,127) of these prophages lacking terminases were classified as unknown taxonomy. Within the network of the predicted prophages, 2 separate connected components of 27 and 10 unknown prophages displayed homology to a pyocin gene fragment and to uncultured Caudovirales phages, respectively. This first group may not actually be phages, rather relatives of bacteriocins. Some of the remaining unknown prophages lacking a terminase shared significant sequence identity with characterized siphoviruses and myoviruses and thus are likely members of these taxonomic groups. The lack of the terminase may signify that they are nonfunctional or their VirSorter prediction is incomplete, i.e., the predicted sequence does not contain all phage coding regions. Those unknown prophage sequences that only exhibit modest sequence similarity with characterized tailed phages, may in fact be tailless and thus should not have a terminase. As our prior analysis of Pf1-like genes showed, there are gene homologs between inoviruses and caudoviruses.

Along with predicted prophages missing terminase coding regions, many phages did not encode integrase or C repressor genes which are typically conserved genes of temperate phages.

Of the 4,570 prophages that included a terminase coding region, 2,616 prophages lacked an integrase or C repressor coding regions. While again microviruses and inoviruses lack these genes, their absence in other predicted prophages echoes the earlier challenge in distinguishing between complete and incomplete prophage sequences the methods of prediction. It is possible that many of the "high confidence" predicted prophages are not complete and are rather small segments of phage-like genes residing within the bacterial sequences. These genes may be evidence of previous phage infections of the bacteria, or they may be nonfunctional phages that no longer have the ability to excise from the bacterial genomes. Of the predicted prophages that lacked a terminase, 1,311 were category 1 predictions and 2,576 were category 4 predictions. The category 1 prophages are predicted to be "free" phages that are not integrated into a host genome. It would be expected that the prophages that lacked an integrase or a C repressor would more likely be category 1 predictions. However, the category 4 prophages greatly outnumbered the category 1 prophages by almost double with 4184 and 2668 predictions, respectively (**Table 1**).

The integrase and C repressor coding regions that were identified in the predicted prophages exhibited extensive diversity. The integrase coding regions shared an average amino acid pairwise identity of only 16.1%. Once clustered based on sequence similarity, the integrases formed 72 distinct clusters with very high sequence similarity (>95% average pairwise identity) in the largest cluster (n=110). The C repressor coding regions were more similar to each other with an average pairwise identity of 58.0%. After being clustered, the C repressors formed 44 separate clusters with high sequence similarity (>86% average pairwise identity) within the

largest cluster (n=1604). While the integrase gene and the C repressor gene are widely conserved in temperate phages, they display substantial diversity between their clusters.

The terminase coding regions also had broad diversity with an average pairwise identity of only 8.9%. As terminase genes are widely conserved among phages alongside integrases and C repressors (Catalano, 2000), it was unexpected to find no association between prophages that share homologous terminases with prophages that share homologous integrases or C repressors. The most phages that shared similar terminases with similar integrases were clustered in the largest of the homologous gene clusters, and a similar result was found for the C repressors. Phages that contain a terminase belonging to 1 cluster do not necessarily contain integrases of only one integrase cluster. On average, phages with a terminase also contain integrases from approximately 3 different integrase clusters. The same applies to C repressors where on average phages with a terminase also contain C repressors from approximately 2 different C repressor clusters. There is no distinct association between phages with terminases of one homologous group also containing integrases or C repressors from only one cluster.

The presence of antibiotic resistance genes and virulence factors was also investigated within the predicted prophages. Only 71 antibiotic resistance genes were predicted from 42 different prophage sequences, just 0.6% of the sequences screened. While *P. aeruginosa* is known to be pathogenic and commonly resistant to antibiotics (Azam & Khan, 2019) and prophages have been identified in 68.21% of the bacterial strains, it does not seem there is any association between the presence of prophages with the presence of antibiotic resistance genes. However, 502 of the predicted prophages did contain virulence factors that could assist in the pathogenicity and fitness of *P. aeruginosa*. Future studies should look towards the association of

*P. aeruginosa* prophage presence with biofilm formations of the bacteria as prophages can be known to increase biofilm growth in *P. aeruginosa* strains (Rice et al., 2009).

# CHAPTER THREE

# ENGINEERING *PSEUDOMONAS AERUGINOSA* TEMPERATE PHAGES

## Introduction

While phage therapy is slowly being integrated as a treatment option in Western medicine, the therapeutic phages used to resolve bacterial infections in both animals and humans tend to be obligately lytic phages. They are predominantly used either alone or in a cocktail with other obligately lytic phages (Jault et al., 2019; Łusiak-Szelachowska et al., 2014; Rhoads et al., 2009; Rose et al., 2014; Wright et al., 2009). Obligately lytic phages continue only in the lytic life cycle and as such are attractive candidates for killing bacterial cells in therapeutic settings (Chan et al., 2013). However, temperate phages are far more abundant than obligately lytic phages, as nearly half of all sequenced bacteria contain prophages (Touchon et al., 2016). Because phages often have a very narrow host-range, i.e., they can only infect a particular bacterial species or even specific strains, expanding our candidate set of phages to include both obligately lytic phages and the more abundant temperate phages would likely increase our ability to identify phages capable of lysing infectious strains of bacteria.

There are a few issues with the use of temperate phages which are why treatments have focused on obligately lytic phages. While temperate phages may begin lysing bacterial cells when first administered, it is possible for the phage to switch to the lysogenic life cycle, and thus be ineffective in killing the bacteria. Furthermore, integration could introduce new virulence or antibiotic resistance genes to the bacterial genome through transduction (Harrison & Brockhurst,

2017; Monteiro et al., 2019; Oliveira et al., 2015). This can turn a non-virulent bacterial strain into a virulent one, a process known as lysogenic conversion (Davies et al., 2016; Harrison & Brockhurst, 2017). Integration of the temperate phage into the bacterial genome could also result in superinfection immunity, in which the prophage enables the bacteria to be resistant to infection by other phages, e.g., other phages used in a cocktail therapy or subsequent phage treatments (Dedrick et al., 2017).

Despite these concerns, temperate phages can be effective agents for killing bacteria. Temperate phages naturally reduce bacterial growth, and the integration of the phages can even lead to prevention of bacterial toxin release (Chung et al., 2012; Meader et al., 2013). Temperate phages are also effective treatments when used in combination with other temperate phages in a cocktail form. A 2015 study displayed successful results of temperate phage cocktails against strains of *P. aeruginosa*, as well as against *Clostridium difficile* strains (Burkal'tseva et al., 2011; Nale et al., 2016, 2018).

To eliminate the possibility of prophage integration into a targeted bacterial host, an alternative approach has recently been explored. Temperate phages can be genetically engineered to block entry into the lysogenic life cycle, a process known as virulent conversion (Kilcher et al., 2018). The integrase gene and the C repressor gene are responsible for the integration and maintenance of the prophage in the lysogenic life cycle, respectively (Monteiro et al., 2019). The engineering of prophages to remove or inactivate these genes creates obligately lytic phages (Monteiro et al., 2019). A 2013 study observed the natural recombination of a temperate *Enterococcus faecalis* phage with a prophage within the host bacteria to create a genetically modified, obligately lytic phage (Zhang et al., 2013). In 2018, an *in vitro* method was developed

for targeted modification of the integrase and C repressor for temperate phages to make obligately lytic variants (Kilcher et al., 2018). A 2019 study conducted the first treatment of a human mycobacterial infection with genetically engineered phages (Dedrick et al., 2019). A therapeutic cocktail consisting of 3 phages was used to treat the infection. 2 of the 3 phages were genetically engineered to either remove or mutate the C repressor gene, forcing the phages to become obligately lytic and improve their ability to lyse the bacteria (Dedrick et al., 2019). The patient did not have any adverse reactions to the cocktail of engineered phages and there was significant clinical improvement after treatment (Dedrick et al., 2019). With the rising rates of antibiotic resistance and our limited catalogue of obligately lytic phages, it is important to identify possible temperate phage candidates that could be engineered to be obligately lytic.

While there have been several examples of temperate phages being engineered to become obligately lytic, there has only been one example of this process being performed on *P. aeruginosa* phages to date (Mageeney et al., 2020). 5 prophages induced from 2 strains of *P. aeruginosa* were engineered to remove the integrase from each of the phages, resulting in 5 obligately lytic phages that killed *P. aeruginosa* PAO1 *in vitro* and *in vivo* (Mageeney et al., 2020). While this prior study removed the integrase genes from the *P. aeruginosa* temperate phages to render them obligately lytic, there has yet to be a study on removing the C repressor gene from *P. aeruginosa* temperate phages to produce obligately lytic phages.

To further display that *P. aeruginosa* phages can be engineered to become obligately lytic through removal of the integrase or the C repressor, I identified 2 temperate phages as potential candidates, D3112 and Dobby. Both phages were isolated from and infect strains of *P. aeruginosa*. D3112 was isolated from *P. aeruginosa* strain PAS429 and is known to infect many

different strains of *P. aeruginosa* (Roncero et al., 1990; Wang et al., 2004). The phage has a genome size of 37,611 bp and contains a C repressor gene that maintains the lysogenic state of the temperate phage when it is integrated into the host genome (Salmon et al., 2000). D3112 is closely related to the Mu-1 phage which has an infection latent period of 60 minutes with a burst size of 200 phages per bacterial cell (Campbell, A. M., n.d.). The Dobby phage was isolated from *P. aeruginosa* strain UMB2738 and is able to infect several strains of *P. aeruginosa* from the urinary tract (Johnson et al., 2019). Dobby has a genome size of 37,152 bp and it contains an integrase gene that is responsible for integrating the phage DNA into the bacterial host genome (Johnson et al., 2019). Dobby is closely related to φCTX, a member of the P2-like phages which have a typical infection latent period of 30 minutes and a burst size of 120 phages per bacterial cell (Campbell, A. M., n.d.; Nakayama et al., 1999). D3112 and Dobby would make promising candidates for virulent conversion by removing the C repressor or the integrase coding regions, respectively. Here I present the genetic engineering of these two phages and an evaluation of their ability to infect clinical isolates of *P. aeruginosa*.

## Methods

### Bacteriophage and Bacterial Isolates.

Two phages, Dobby and D3112, were obtained for experimentation. The Dobby phage (NC_048109.1) was spontaneously induced and isolated from *P. aeruginosa* strain UMB2738 (IRB: Indiana University 1803959731) that was cultured from a kidney stone (Johnson et al., 2019). The D3112 phage (NC_005178.1) was obtained from the Félix d'Hérelle Center for Bacterial Viruses. The strain of *P. aeruginosa* used in the experiments of this study, *P. aeruginosa* ATCC 15692 was obtained from ATCC.

**Bacteriophage DNA Isolation.**

Dobby and D3112 were grown to high titer using a diluted overnight culture of the laboratory strain *P. aeruginosa* ATCC 15692. 25mL of LB was inoculated with a single colony of *P. aeruginosa* ATCC 15692, then incubated with shaking at 37 degrees Celsius overnight. The culture was diluted the next day by 250uL culture in 25mL of LB incubated with shaking at 37 degrees Celsius for 1.5 hours. After 1.5 hours, 250uL of either Dobby phage lysate or D3112 phage lysate that had been stored at 4 degrees Celsius was added to the *P. aeruginosa* culture and placed in a shaking incubator 37 degrees Celsius overnight. The next day, 1mL of each of the inoculated cultures were removed and placed into 1.5mL microcentrifuge tubes and centrifuged at 13,000xg for 5 minutes. The supernatant was removed and filtered using 0.22um cellulose acetate membrane syringe filters and DNAse I, RNase-free (1U/uL) (Thermo Fisher Scientific, Thermo Scientific ™) was used following the recommended protocol to remove leftover bacterial DNA. Next, the Zymo *Quick*-DNA Viral Kit (ZymoResearch, CA, USA) was used to extract phage DNA following the manufacturer's protocol. DNA was quantified using a Qubit fluorometer.

Because the host used to amplify Dobby and D3112 also contains a prophage, PCR was used to confirm the source of the DNA. PCR primers were created using Primer3 (Untergasser et al., 2012, p. 3) and the genome sequence for each of these phages (Dobby: CCACCACTCACGGACCTC and AGCCATGACTGCGCTACC; D3112: CCGACCGACGTCTATTCC and CGCGCCTGACTGTTGTAG). Primers were checked for self-dimerization using the ThermoFisher Multiple Primer Analyzer (Thermo Fisher Scientific, Thermo Scientific ™) and synthesized by Eurofins Genomics LLC (Louisville, KY). Using 10uL

of Go-Taq Master Mix (Promega, Madison, WI), 7uL of nuclease-free $H_2O$, 1uL of each primer (10mM), and 1uL of phage DNA were combined for a 20uL reaction. PCR amplification was verified using a 1.2% agarose gel.

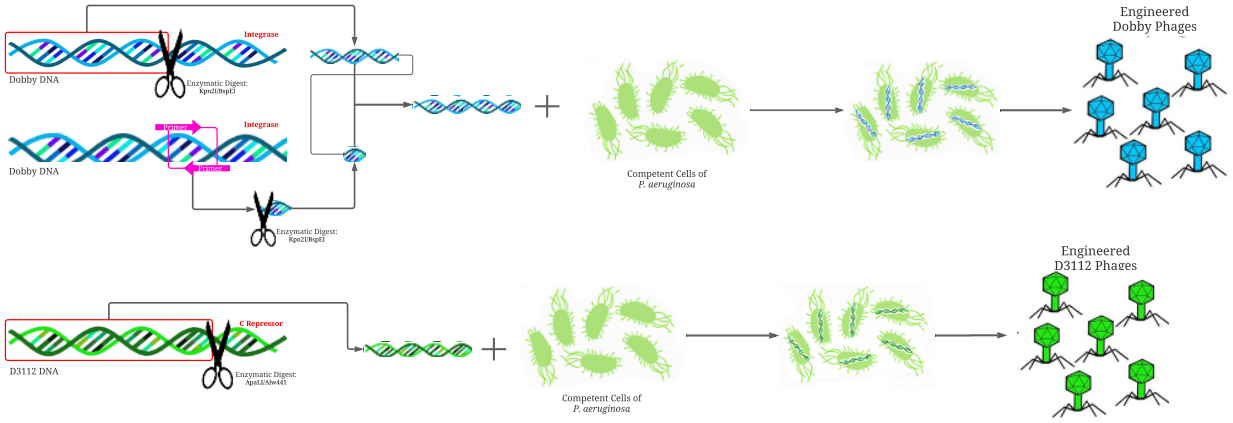


Figure 12. Workflow of engineering process for Dobby and D3112. Dobby is the top blue genome and D3112 is the bottom green genome. The enzymatic digestion begins on the left, the digested (and ligated in Dobby's case) genomes are transformed within *P. aeruginosa* competent cells to produce viable engineered eDobby and eD3112 phages.

**Digestion of Bacteriophage DNA.**

The Dobby phage DNA and the D3112 phage DNA were then engineered using enzymatic digestion to remove the integrase gene from Dobby and the C repressor gene from D3112. **Figure 12** displays the workflow of the entire engineering process for Dobby and D3112. NEBCutter v2.0 was used to identify restriction enzymes closest to the integrase/C repressor with single cut sites within the phage genome sequence (Vincze et al., 2003). The restriction enzyme Alw44I (Thermo Fisher Scientific, Thermo Scientific ™) was used to digest the DNA of D3112 with the recommended protocol. The restriction enzyme Kpn2I (Thermo Fisher Scientific, Thermo Scientific ™) was used with the recommended protocol to digest the Dobby DNA. 10uL of the digested DNA of each of the two phages with 3uL loading dye and

10uL of each of the undigested DNA with 3uL loading dye for comparison were then loaded into a 1.2% agarose gel with 4uL ethidium bromide, the gel electrophoresis was run, and the gel was visualized in a UV light box to examine band sizes of the digested DNA and the undigested DNA of the two phages. Once verified, the largest band of each of the phages' digested DNA was cut out of the gel and extracted using the Omega Bio-tek E.Z.N.A. Gel Extraction Kit (Omega Bio-tek, Inc., GA, USA) and its recommended protocol.

The digested DNA for each phage was established to be lacking the integrase or C repressor genes using PCR primers constructed with Primer3 (Untergasser et al., 2012) to amplify a section of the phages' sequences that included the cut sites and several hundred base pairs following the cut site (**Table 2**). These primers were then used on both the two digested phages as well as the undigested original two phages to show that the original phages still contained the cutsites and the successive base pairs and that the digested phages did not contain nucleotides following the cutsite. The amplicons of the PCR reactions were visualized through gel electrophoresis using a 1.2% agarose gel. The digested Dobby DNA and the digested D3112 DNA were stored at -20 degrees Celsius for further downstream analysis.

| Primers | Forward Sequence | Reverse Sequence | Expected Amplicon Size |
|---|---|---|---|
| D3112 Digestion Site | ACATAGCCACCATCCCGAAA | GTTGTCCTCGTCAATCCAGC | 230 |
| Dobby Digestion Site | CATTCGACCGGCAGCCTG | TCAAGCGACTGGATGATGCA | 181 |
| Dobby Missing Genes | CACTACGGCGTGATGG | CCTCATAGGATTCTGCTGTC | 2061 |

Table 2. Primer sequences for verification of Dobby and D3112 engineering process.

**Ligation of Dobby DNA.**

Due to the location of the Kpn2I cut position within the Dobby genome, 4 additional genes were removed in addition to the integrase. These coding regions were recovered for the engineered phage. The cut site of the genome was at 33,840bp and the integrase gene began at 35,866bp until the end of the genome. PCR primers were created using Primer3 (Untergasser et al., 2012) that replicated and amplified the genes of the original Dobby that were missing from the digested Dobby DNA (**Table 2**). The primers began at 33,810bp and the amplicon extended to 35,871bp, amplifying the 4 missing genes and the 5' end of the integrase. After running the PCR protocol using the new primers on the original Dobby DNA, the resulting amplicon was then digested using Kpn2I and its recommended digestion protocol. After the enzymatic digestion was completed, 10uL of the digested amplicon was loaded into a 1.2% agarose gel with 4uL ethidium bromide and the gel electrophoresis was run. The gel was then visualized in a UV light box to identify and cut out the digested amplicon band. The digested amplicon band was then extracted using the Omega Bio-tek E.Z.N.A. Gel Extraction Kit (Omega Bio-tek, Inc., GA, USA) and its recommended protocol. The digested Dobby DNA and the digested amplicon were then ligated together using ThermoFisher T4 DNA Ligase (5U/uL) (Thermo Fisher Scientific, Thermo Scientific ™). 6uL of the digested amplicon, 11.8uL of the digested Dobby DNA, 2uL of the 10x T4 DNA Ligase Buffer, and 0.45uL of the T4 Ligase were combined and incubated at 22 degrees Celsius for 10 minutes.

**Creation of Competent *P. aeruginosa* ATCC 15692 Cells.**

Competent *P. aeruginosa* cells were created using the laboratory strain of *P. aeruginosa* ATCC 15692 (Chuanchuen et al., 2002). To begin, 25mL of LB were inoculated with a single

colony of *P. aeruginosa* ATCC 15692 and incubated at 37 degrees Celsius overnight with shaking. The next day the following protocol was completed while keeping all tubes on ice throughout the entirety of the steps. 1.5mL microcentrifuge tubes were chilled on ice for 5 minutes before use, then 1mL of the overnight *P. aeruginosa* culture was aliquoted into each tube. The tubes were then centrifuged at 13,000xg for 30 seconds and the supernatant was poured off. The cell pellets were then resuspended in 1mL of cold (4 degrees Celsius) 0.1M $MgCl_2$ and again centrifuged at 13,000xg for 30 seconds. The supernatant was poured off of the tubes and the cell pellets were resuspended in 1mL of cold (4 degrees Celsius) TG salts (75 mM CaCl2, 6 mM $MgCl_2$, 15% glycerol) solution and kept on ice for 10 minutes (Chuanchuen et al., 2002). After a final centrifugation of the tubes at 13,000xg for 30 seconds, the supernatant was poured off and the cell pellets were resuspended in 200uL of cold (4 degrees Celsius) TG Salt Solution. The final *P. aeruginosa* competent cells were then stored at -80 degrees Celsius until future use.

**Transformation of Bacteriophage DNA.**

The digested D3112 phage DNA and ligated Dobby DNA was transformed into the *P. aeruginosa* competent cells. 60uL of competent cells and 10uL of the phage DNA were combined in a 1.5mL microcentrifuge tube. The tube was then incubated on ice for 90 minutes. After the ice incubation, 1mL of LB warmed to 25 degrees Celsius was added to the tube and the mixture was incubated and shaken overnight at 37 degrees Celsius. The next day, 10uL spots of the transformed lysate were placed onto a lawn of 3mL LB soft agar (0.7% agarose) and 500uL of overnight culture of *P. aeruginosa* ATCC 15692 on an LB agar plate (1.7% agarose) and incubated overnight at 37 degrees Celsius. The next day, the plaques from the lysate spots were

harvested from the plate and placed into 1.5mL microcentrifuge tubes and 1mL of LB was added. The phage harvest was then disrupted for 10 minutes, centrifuged at 13,000xg for 2 minutes, and filtered into new microcentrifuge tubes using 0.22um cellulose acetate membrane syringe filters. The filtered lysates were again spot-plated onto a lawn of *P. aeruginosa* ATCC 15692 and incubated overnight. The remaining filtered lysates were stored at 4 degrees Celsius until future use.

**Verification of Engineered Phages.**

To validate that the phage harvested were from the digested/ligated phage DNA, the DNA was extracted from 300uL of the transformed digested phage lysate using the Zymo *Quick*-DNA Viral Kit (ZymoResearch, CA, USA). PCR protocols with the primers for identity verification of the ancestral Dobby and ancestral D3112 were run to establish the respective identities of the engineered Dobby and engineered D3112. The digest primers used to verify the initial digestion of the engineered Dobby and engineered D3112 were also used in PCR protocols to establish the difference between the ancestral phages and the engineered phages. The 2 validated engineered phages are hence referred to as eDobby and eD3112.

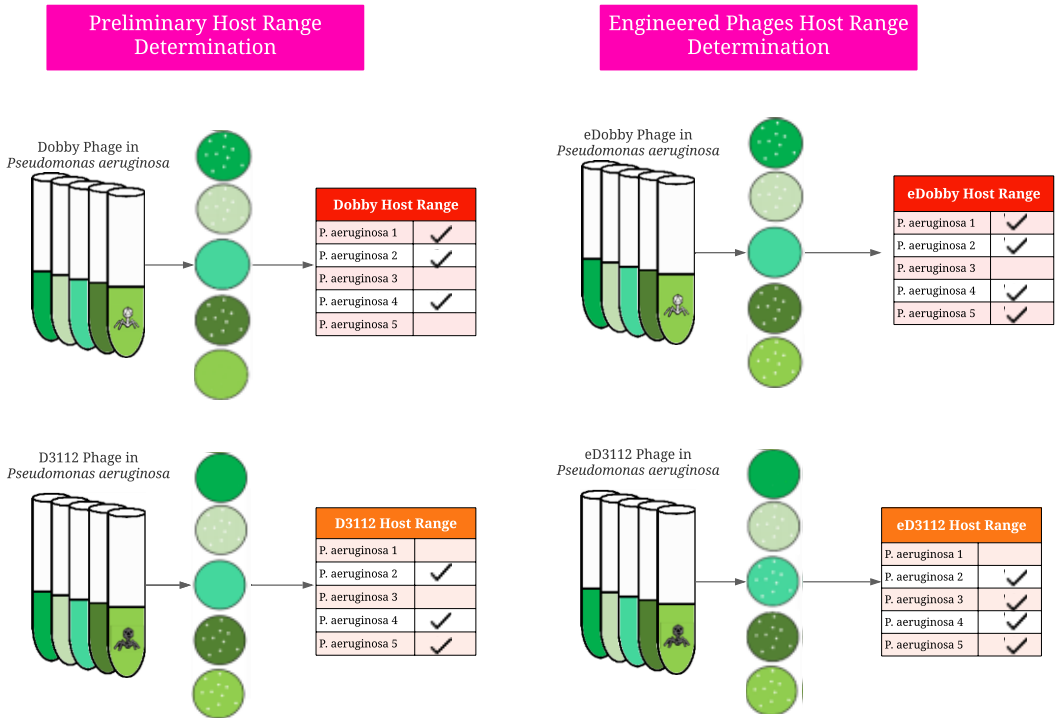


Figure 13. Display of host range testing process for ancestral and engineered phages. Phage lysates were added to bacterial lawns of 18 clinical isolates and 1 laboratory strain of *P. aeruginosa*. Strains that a phage displayed lytic activity in were considered the host range of the phage.

**Host Range Testing of Ancestral and Engineered Bacteriophages.**

The host ranges of the phages were determined using bacterial lawns of 19 different strains of *P. aeruginosa* and spots of phage lysate for the ancestral D3112, eD3112, ancestral Dobby, and eDobby. **Figure 13** displays the host range testing for the 4 different phages. The phage lysates had all been stored at 4 degrees Celsius. The 19 *P. aeruginosa* strains are listed as follows: laboratory strain *P. aeruginosa* ATCC 15692, UMB151, UMB0501, UMB0740, UMB0801, UMB0802, UMB1046, UMB1204, UMB2231, UMB2253, UMB2261, UMB2738, UMB4740, UMB4836, UMB5686, UMB6995, UMB7567, UMB7777, and UMB8183. The UMB strains were obtained from the Wolfe Lab at Loyola University Chicago's Stritch School

of Medicine, isolated as part of prior IRB-approved studies (IRB: LUC 203986; Indiana University 1803959731; LUC 204195; LUC 207152; LU 209983; LU 207906; LUC 207102; LUC 206129) (Hilt et al., 2014; Pearce et al., 2014, 2015; K. J. Thomas-White et al., 2016; Bajic et al., 2018; Price et al., 2016; K. Thomas-White et al., 2018). For each *P. aeruginosa* strain, a combination of 3mL LB soft agar (0.7% agarose) and 500uL bacterial culture were poured onto an LB agar plate (1.7% agarose). Once solidified, 10uL spots of each of the four phage lysates were pipetted onto the plate in replicates of 4. The plates were then incubated overnight at 37 degrees Celsius, and plaques were visualized the next day.

**Biofilm Growth and Testing of Phage Infection.**

Two strains of *P. aeruginosa*, ATCC 15692 and UMB8183, were evolved, selecting for biofilm formation, with duplicates to yield 4 *P. aeruginosa* biofilms. 1 mL of overnight cultures of each strain were placed into 6-well plates and 5 mL of LB were added to the cultures and the well-plates were incubated at 37 degrees Celsius. Every day for several days, the excess liquid was pipetted off the tops of the wells and 5 mL of LB was added each day. On the 7th day, the lab was officially shut down due to the COVID-19 pandemic. The well-plates were placed into an anaerobic chamber kept at room temperature for three months. The biofilms were occasionally replenished with LB about every 3 weeks and returned to the room temperature anaerobic chamber. After three months, the 4 biofilms were removed from the well-plates and transferred to 4 flasks. The biofilms continued to grow at room temperature with bi-monthly replenishment of either 5 mL or 10 mL of LB each time.

After over a year of growth, 1mL of each of the biofilms was pipette into a 1.5mL microcentrifuge tube. The 4 samples were then disrupted for 10 minutes and transferred to

QIAshredder cell-lysate homogenizers (Qiagen) and centrifuged at 16,000xg for 2 minutes. The 4 sets of homogenized bacterial cells were then each grown overnight in 10mL of LB media at 37 degrees Celsius shaken. The following day, 500uL of the overnight cultures were added to 3mL of LB soft agar (0.7% agarose) and poured onto LB agar plates (1.7% agarose). Duplicate 10uL spots of Dobby, eDobby, D3112, and eD3112 were pipetted onto the bacterial lawns, left to dry, and incubated overnight at 37 degrees Celsius.

**Dobby Integration Analysis.**

*P. aeruginosa* strains UMB151, UMB0801, UMB0802, UMB2231, UMB2253, and UMB2261 were each grown from a single colony in 5mL of LB media overnight at 37 degrees Celsius with shaking. The next day, the 6 bacteria were then subcultured with 500uL overnight culture in 10mL of fresh LB media and incubated at 37 degrees Celsius with shaking for 3 hours. 5uL of Dobby lysate that had been stored at 4 degrees Celsius was added to each of the 6 subcultures. An additional 6 subcultures were created without added phage lysate to serve as controls. The cultures were all incubated overnight at 37 degrees Celsius with shaking.

The next day, 1mL was taken from each of the overnight cultures and centrifuged at 13,000xg for 5 minutes. The lysates were completely removed from the tubes so only the bacterial cell pellets remained. The pellets were then washed with 500uL of PBS solution to remove phages from the cell pellet surface. The wash step was repeated 3 times. The cell pellets were then resuspended in 1mL LB media and vortexed to disperse the bacterial cells. Plastic loops were used to streak the resuspended cultures onto LB agar plates (1.7% agarose) that were incubated overnight at 37 degrees Celsius. The same process was repeated for the control cultures.

3 single colonies were picked from each of the 6 plates of bacteria treated with phage and each of the 6 control plates. Colony PCRs were run using primers specific to the Dobby phage (CCACCACTCACGGACCTC and AGCCATGACTGCGCTACC). The 50uL reaction consisted of 23uL of nuclease-free $H_2O$, 25uL of Go-Taq Master Mix (Promega, Madison, WI), 1uL of each primer (10mM), and 1 colony for a 50uL reaction setup. An additional reaction with 1uL of Dobby DNA instead of a bacterial colony was run as a positive control and a negative control (no bacterial colony or DNA) was run as well. PCR amplification was verified using a 1.2% agarose gel.

**Titer Quantification of Phages and Laboratory *P. aeruginosa*.**

Before testing the rate of infection for the ancestral D3112 and ancestral Dobby phages versus eD3112 and eDobby phages on the laboratory strain of *P. aeruginosa* ATCC 15692, the titer of each phage and of the bacterial strain needed to be determined. The phages had all been stored at 4 degrees Celsius for future use. Each of the four phages was diluted in a 10-fold serial dilution from $10^0$ to $10^{-9}$. Bacterial lawns comprised of 3mL soft agar (0.7% agarose) and 500uL *P. aeruginosa* ATCC 15692 overnight culture grown from a single colony were poured onto LB agar plates (1.7% agarose) for the dilution series. For each of the four phages, 10uL of each dilution series was spotted onto the bacterial lawn and incubated overnight at 37 degrees Celsius. The next day, the number of plaques at the lowest dilution that still contained visible plaques were counted and used to calculate the titer of each phage lysate.

For the laboratory strain of *P. aeruginosa* ATCC 15692, the titer of the bacteria was determined using dilutions as well. 200mL of LB was inoculated with a single colony of the *P. aeruginosa* ATCC 15692 and grown overnight shaken at 37 degrees Celsius. The next day 1mL

of culture was aliquoted and diluted 10-fold up to $10^{-6}$. 100uL of the $10^{-6}$ diluted culture was pipetted onto an LB agar plate (1.7% agarose) and a cell spreader was used to spread the culture across the entirety of the plate. The plate was incubated overnight at 37 degrees Celsius, and the number of visible colonies were counted the next day to determine the titer of the *P. aeruginosa* strain ATCC 15692.

**Phage Effect on *P. aeruginosa* Growth.**

The rate of infection for each of the 4 phages (ancestral D3112, eD3112, ancestral Dobby, and eDobby) was measured every hour for 8 hours using a spectrophotometer. The spectrophotometer measured the bacterial culture density at 600nm. Two multiplicities of infections (MOI), or the ratio of phage particles to bacterial cells, were tested: 1 and 10. Each MOI was tested in triplicate for each phage strain with a single control sample of *P. aeruginosa*. The protocol began by inoculating 25mL of LB with a single colony of *P. aeruginosa* ATCC 15692 that was grown overnight with shaking at 37 degrees Celsius. Due to the titer of the *P. aeruginosa* ATCC 15692 being similar to the phages' titers, the bacterial culture needed to be diluted 1:100 in LB media to be able to achieve an MOI of 10 for the phages. The next day, the bacterial culture was diluted as 3mL culture into 297mL LB to adjust bacterial concentrations to achieve the two MOIs to be tested.

The 300mL of diluted culture was then divided into 9 1mL microcentrifuge tubes, to serve as the bacterial control without phage, and 8 30mL flasks. Each of the 8 flasks included the addition of the appropriate amount of one of the 4 phages at one of the 2 MOIs. The ancestral D3112 phage required 0.1uL lysate per 1mL diluted bacterial culture to maintain an MOI of 1 and 1uL lysate per 1mL culture for an MOI of 10. eD3112 used 1uL lysate per 1mL diluted *P.*

*aeruginosa* for an MOI of 1 and 10uL lysate per 1mL bacterial culture to achieve an MOI of 10. Both ancestral Dobby and eDobby needed 0.1uL of phage lysates per 1mL of culture for MOIs of 1 and 1uL of lysate per 1mL culture to maintain MOIs of 10. Each flask was then divided into 27 1mL microcentrifuge tubes and incubated with shaking at 37 degrees Celsius. Three samples per phage/MOI and one sample of the control were immediately removed, transferred to 1mL cuvettes, and run through the spectrophotometer at 600nm. Every 60 minutes thereafter, 3 samples per phage/MOI and 1 sample of the control were similarly transferred to 1mL cuvettes and run through the spectrophotometer.

Along with the spectrophotometric analysis, all samples, including the control samples, from 0 hours, 1 hour, and 8 hours were saved for further testing. Each was centrifuged at 13,000xg for 5 minutes and the lysate was separated from the cell pellet in the microcentrifuge. For the 0 hour samples, the removed lysates were filtered through 0.22um cellulose acetate membrane syringe filters and stored at 4 degrees Celsius for plating. The 1 hour samples' cell pellets were resuspended in 1mL LB, vortexed for 30 seconds, and stored at 4 degrees Celsius for plating. The 8 hour samples had both their lysates filtered through 0.22um cellulose acetate membrane syringe filters, as well as their cell pellets resuspended in 1mL LB and vortexed for 30 seconds. Both the lysates and the resuspended cell pellets of the 8 hour samples were stored at 4 degrees Celsius for future plating. The 0 hour lysates and the 8 hour lysates were all diluted in 10-fold serial dilutions from $10^{0}$ to $10^{-7}$. Each dilution series was 10uL spot-plated onto lawns of 500uL *P. aeruginosa* ATCC 15692 and 3mL LB soft agar (0.7% agarose) on LB agar plates (1.7% agarose) that were then incubated overnight at 37 degrees Celsius. The 1 hour resuspended cell pellets and the 8 hour resuspended cell pellets were plated for bacterial colony counts. The

control samples without phage were required to be diluted in order to count the individual colonies, so they were diluted to both $10^{-4}$ and $10^{-5}$. 100uL of each of the dilutions were plated onto LB agar plates (1.7% agarose) and incubated overnight at 37 degrees Celsius. The triplicate samples that were inoculated with phage were not required to be diluted. 100uL of each of the samples were plated onto LB agar plates (1.7% agarose) and incubated overnight at 37 degrees Celsius.

## Results

### Verification of Ancestral and Engineered Phages.

The Dobby phage and the D3112 phage were successfully engineered to their counterparts, eDobby and eD3112. The genome of D3112 was enzymatically digested to remove the C repressor gene from the beginning of the sequence. The genome was cut at 1,207 bp, which removed the C repressor gene as well as part of a helix-turn-helix domain-containing protein. The digested DNA was then transformed in *P. aeruginosa* ATCC 15692 competent cells to yield the engineered eD3112 phage. Primers were created to amplify the cut site of the enzymatic digestion (**Table 2**). **Figure 14** displays that the ancestral D3112 phage retained the amplified cut site, but eD3112 did not retain that region.

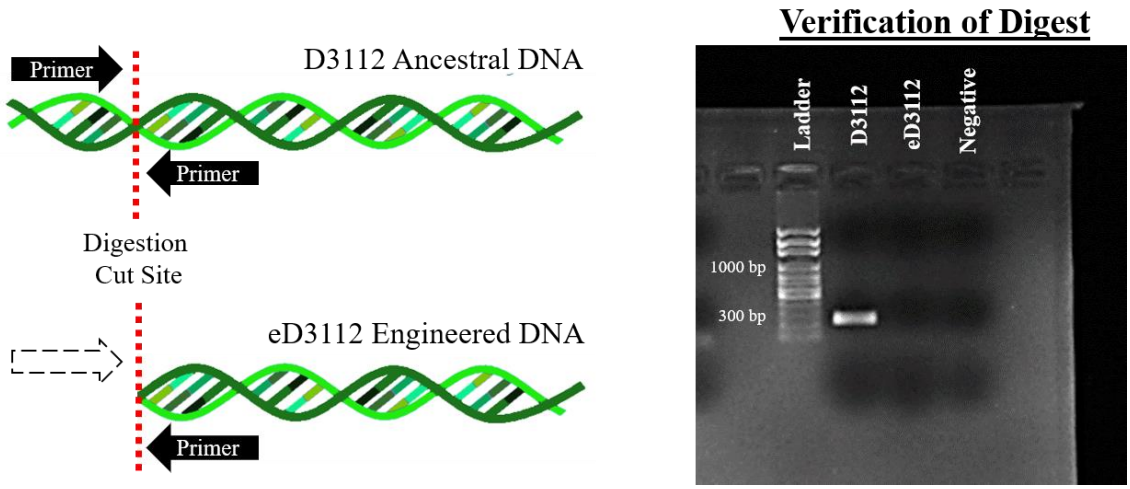


Figure 14. Verification of enzymatic digest for eD3112. Primers amplify the region around the cutsite from the digestive enzyme. The ancestral D3112 displays a band because it was not digested. The same region was not amplified from eD3112 because it was digested.

The genome of Dobby was also cut using an enzymatic digest to remove the integrase gene. The resulting DNA was then confirmed to have been digested using primers that amplify over the cut site of the enzymatic digestion (**Table 2**). **Figure 15** confirms that the digested DNA was cleaved at the restriction recognition site. The digestion cutsite at 33,840 bp excised the integrase gene along with 4 additional coding regions: a phosphoadenosine phosphosulfate reductase, a DUF4224 domain-containing protein, a hypothetical protein, and part of a DNA cytosine methyltransferase. The digested Dobby DNA could not be transformed into a viable phage without these 4 missing genes. Thus, primers were created to amplify these 4 coding regions from the Dobby genome (**Table 2**). The amplicon was digested and then ligated to the enzymatically digested genome. The product was transformed in *P. aeruginosa* ATCC 15692 competent cells to yield the engineered eDobby phage. **Figure 16** displays the presence of the 4 missing genes in both the ancestral Dobby phage and eDobby. **Figure 17** shows that the missing

genes were ligated at the digestion cut site as both Dobby and eDobby display amplicons from the Dobby digestion primers (**Table 2**).

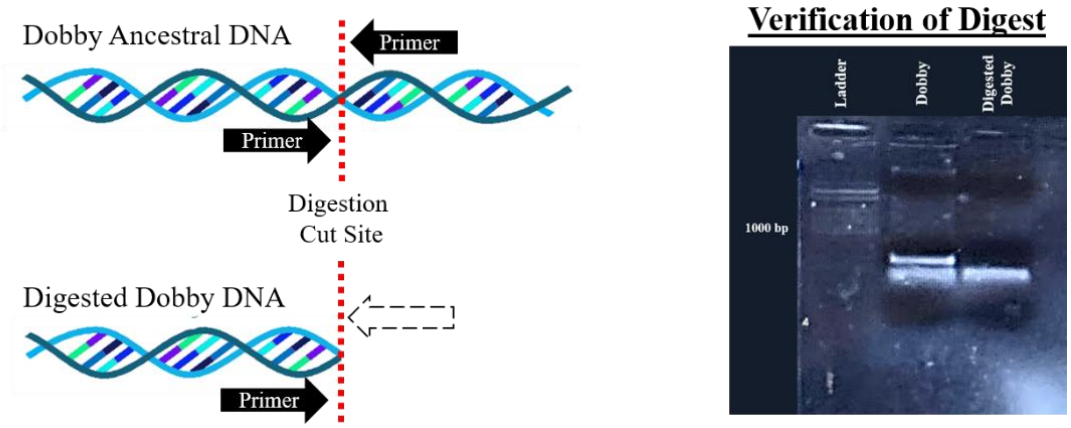


Figure 15. Verification of enzymatic digest for Dobby. Primers amplify the region surrounding the cutsite from the digestive enzyme. The ancestral Dobby displays a band because it was not digested. The digested Dobby did not have the region amplified because it was digested.

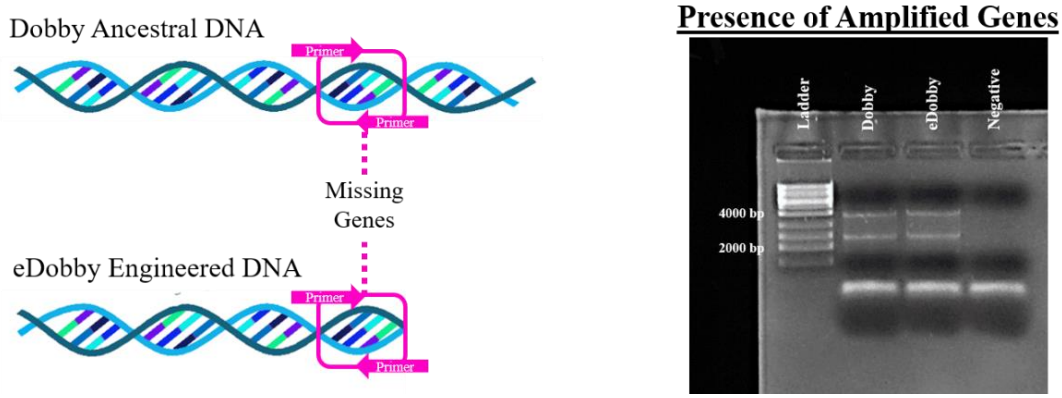


Figure 16. Presence of missing genes ligated onto end of eDobby. The missing genes were amplified from the ancestral Dobby using primers specific to those genes. Both Dobby and eDobby display amplified bands because the missing genes were ligated onto the digested Dobby DNA to produce eDobby.

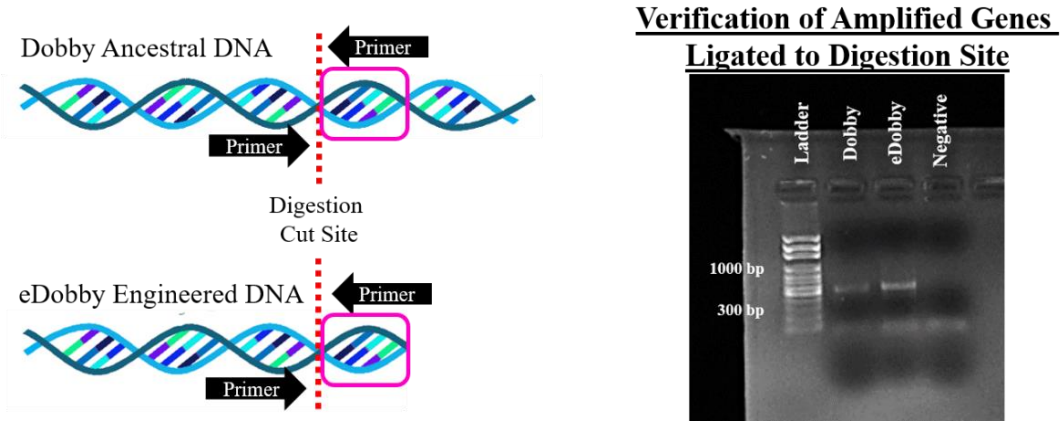


Figure 17. Verification of amplified genes ligated to digestion site. The same primers used to verify the digestion of the Dobby DNA were used to display the ligation of the missing genes to the end of the digested Dobby. After ligating the missing genes to the cutsite, eDobby displays an amplified band for the region surrounding the digestive cutsite.

**Host Ranges of Ancestral and Engineered Bacteriophages.**

The host range of the ancestral and engineered phages was determined using 19 *P. aeruginosa* strains - 18 clinical urinary isolates and 1 laboratory strain, *P. aeruginosa* ATCC 15692. The host range assays show that removing the integrase or C repressor could change the ability of the phages to lyse different strains of *P. aeruginosa*. The ancestral Dobby phage was able to lyse 9 of the 19 total strains of *P. aeruginosa* and eDobby was able to lyse 14 of the strains (**Figure 18**). After removing the integrase gene, eDobby lost the ability to lyse 1 strain of *P. aeruginosa* but gained the ability to lyse 6 more strains of *P. aeruginosa* than the ancestral Dobby (**Figure 18**). The host ranges of the ancestral and engineered D3112 phages were also compared. The ancestral D3112 phage was able to lyse 12 of the 19 *P. aeruginosa* strains and eD3112 was able to lyse 10 of those 12 strains (**Figure 19**); eD3112 was not able to lyse any additional *P. aeruginosa* strains. The differences in host ranges between the ancestral phages and their corresponding engineered phages confirms the hypothesis that removal of either the integrase gene or the C repressor gene can affect the phage's ability to lyse a bacterial strain.

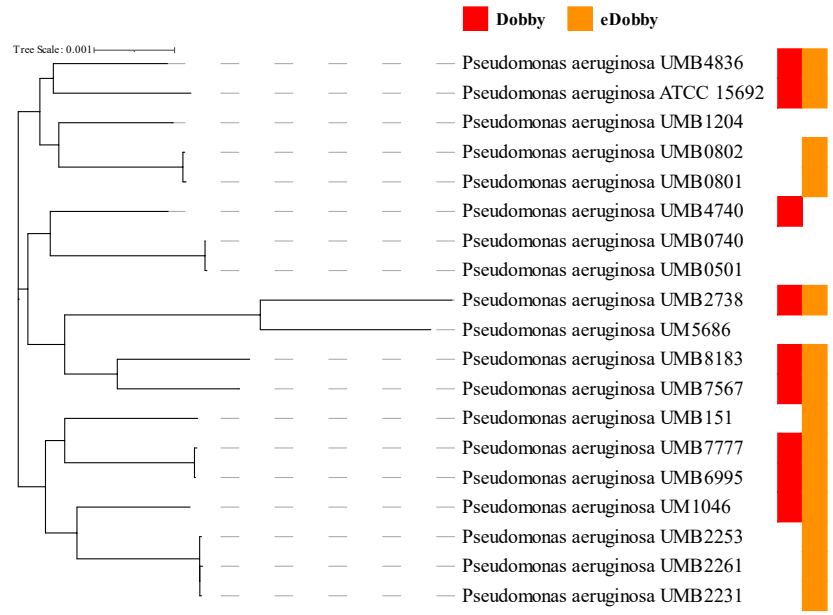


Figure 18. Host range of eDobby versus Dobby. The lytic activity of the 2 phages was observed on 18 clinical isolates and 1 laboratory strain of *P. aeruginosa*. eDobby could not lyse UMB4740 that Dobby could, but eDobby was able to lyse 6 more strains than Dobby, UM151, UMB0801, UMB0802, UMB2231, UMB2253, and UMB2261.

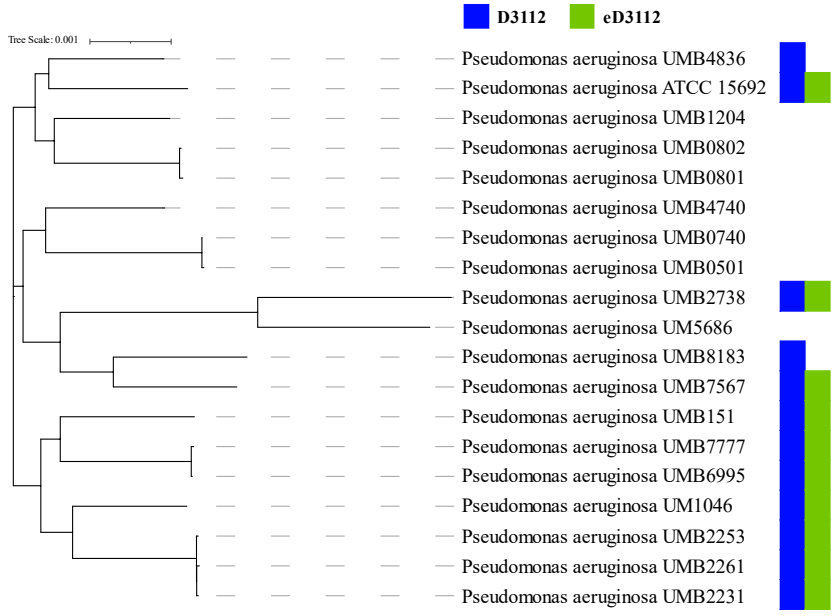


Figure 19. Host range of eD3112 versus D3112. The lytic activity of the 2 phages was determined on 18 clinical isolates and 1 laboratory strain of *P. aeruginosa*. eD3112 could not lyse 2 strains that D3112 was able to, UMB4836 and UMB8183.

The ancestral and engineered phages were also tested on four biofilm-forming strains. The 4 biofilming strains were evolved from two strains of *P. aeruginosa*, the laboratory strain tested in the prior host-range assays (ATCC 15692) and UMB8183; these strains were evolved with minimal nutrients and oxygen for over 1 year, producing dense biofilms (**Figure 20**). Samples were taken from each of the 4 biofilms, disrupted and homogenized to release the bacteria within the biofilms, and grown to produce bacterial cultures. The 4 phages were spotted in duplicate onto bacterial lawns of these 4 strains. All 4 phages shared similar lytic activities across the 4 biofilm-forming hosts. While the 4 phages were able to produce plaques on one of the strains evolved from UMB8183, they were all unable to produce lytic activity in the second UMB8183 lawn. None of the phages were capable of producing plaques on the lawns of the ATCC 15692 evolved strains. In fact, addition of phage increased the growth of the bacteria at the area of spotting, resulting in thicker bacterial formations.

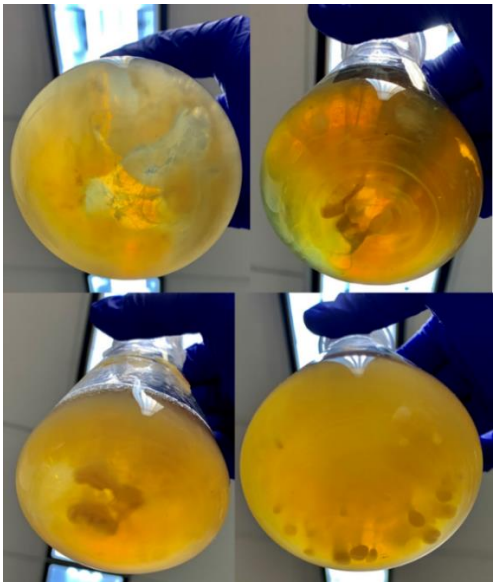

Figure 20. Four biofilms evolved for over a year from 2 strains of *P. aeruginosa*. 2 biofilms from each of *P. aeruginosa* ATCC 15692 (left) and *P. aeruginosa* UMB8183 (right) were grown with minimal nutrients and minimal oxygen for several months. After over 1 year of growth, they formed thick, distinct biofilms in flasks.

**Dobby Integration Analysis.**

eDobby gained the ability to lyse 6 strains of *P. aeruginosa* that the ancestral Dobby was not able to lyse: UMB151, UMB0801, UMB0802, UMB2231, UMB2253, and UMB2261. The possibility of the ancestral Dobby integrating into these strains rather than lysing them was investigated using colony PCRs with primers specific to the Dobby phage. The 6 bacterial strains exposed to Dobby lysate were compared to the same 6 strains without any additional phage and the reactions were run in triplicate. UMB151 displayed amplified bands in both the samples exposed to Dobby lysate and the control samples (**Figure 21**). After running a BLAST of the UMB151 assembly against Dobby's genome, it was found that UMB151 contains a prophage that is highly similar to Dobby.

UMB0802 displayed the presence of Dobby in the phage-exposed colony but not in the control colony reactions (**Figure 21**). After repeating the PCR for UMB0802 and obtaining the same results, it was confirmed that Dobby could be infecting and integrating into the genome of UMB0802 without showing any lytic activity against the bacteria **(Figure 22**). UMB0801, UMB2231, UMB2253, and UMB2261 did not display any evidence of Dobby integrating into the bacterial genomes (**Figure 21**).

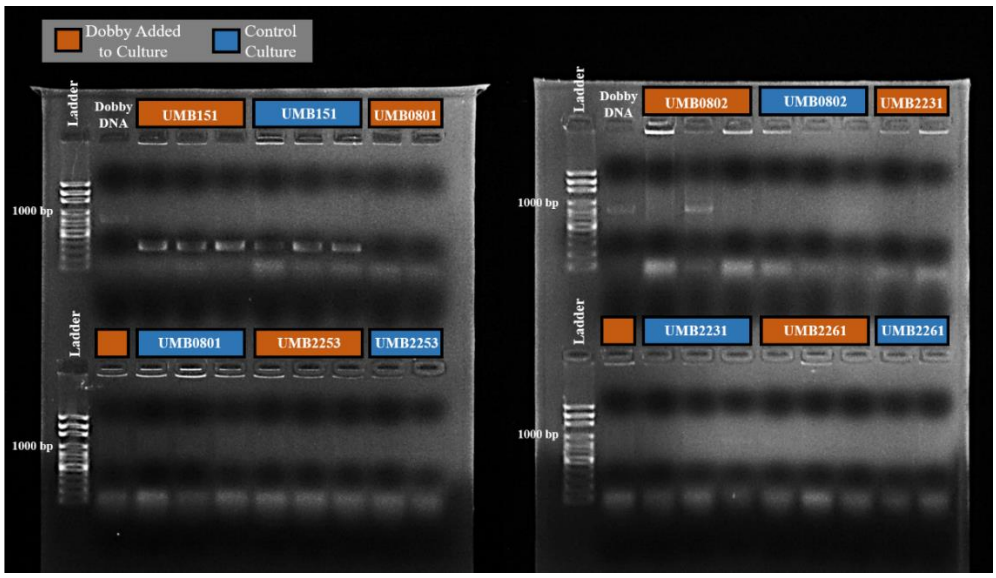


Figure 21. Integration analysis for *P. aeruginosa* strains lysed by eDobby but not Dobby. The 6 strains of *P. aeruginosa* that eDobby was able to lyse but Dobby could not lyse were evaluated for possible integration activity of Dobby. Dobby lysate was added to the 6 strains and the bacterial pellets were washed thoroughly to remove any free-floating phage. Colony PCR using Dobby primers was used to visualize signs of Dobby integrating into the host cells. UMB151 contained a prophage similar to Dobby which is why there are bands of different lengths amplified in both the cultures with added lysate and the control cultures. UMB0802 was the only strain to show integration activity of Dobby in the cultures with added lysate but not the control cultures.

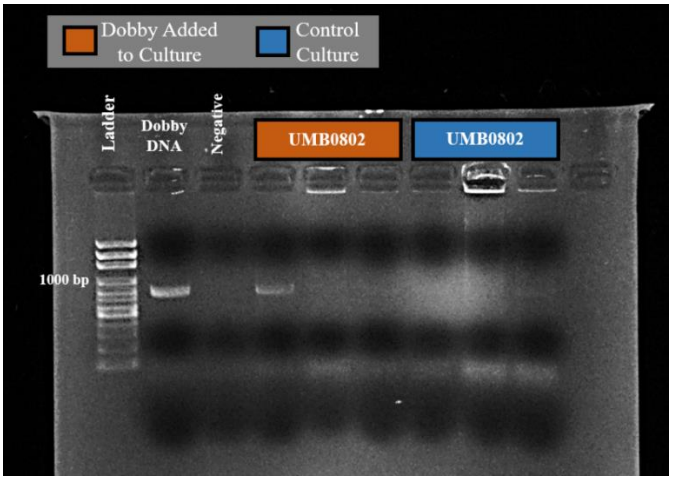


Figure 22. Repeated integration analysis of UMB0802 to verify results. The analysis was repeated for UMB0802 using several colonies to verify the results from Figure 21. The band in the culture with added Dobby lysate and no bands in the control cultures verifies that Dobby displayed lysogenic activity.

**Phage Effect on *P. aeruginosa* Growth.**

To compare the infection rate of the ancestral and engineered phages, one host was selected, the laboratory strain of *P. aeruginosa* ATCC 15692. Each phage was tested at two titers, one at a multiplicity of infection (MOI) of 1 and once at an MOI of 10, and each phage and MOI was performed in triplicate. Cultures were measured over time using optical density to quantify microbial growth over time in response to phage treatment.

**Figure 23** displays the bacterial growth in the presence of D3112 and eD3112 at both MOIs of 1 and 10 in comparison to the control *P. aeruginosa* ATCC 15692, grown without phage treatment. It can be seen that both phages inhibit bacterial growth over the course of 8 hours at both MOIs. While eD3112 performs slightly better than the ancestral D3112, it is not statistically significant. After 1 hour with eD3112 treatment, the bacteria no longer grew, and the optical density remained below 0.005 for the duration of the analysis. **Figure 24** shows the bacterial growth in the presence of Dobby and eDobby at both MOI 1 and MOI 10 in comparison to untreated *P. aeruginosa* ATCC 15692. Similar to that observed for D3112 treatment, both MOIs of Dobby and eDobby continued to inhibit bacterial growth over the 8 hours. Both phages at MOI of 10 performed slightly better than the phages at MOI 1, but all maintained the culture optical density below 0.01 for the entirety of the experiment after 1 hour.

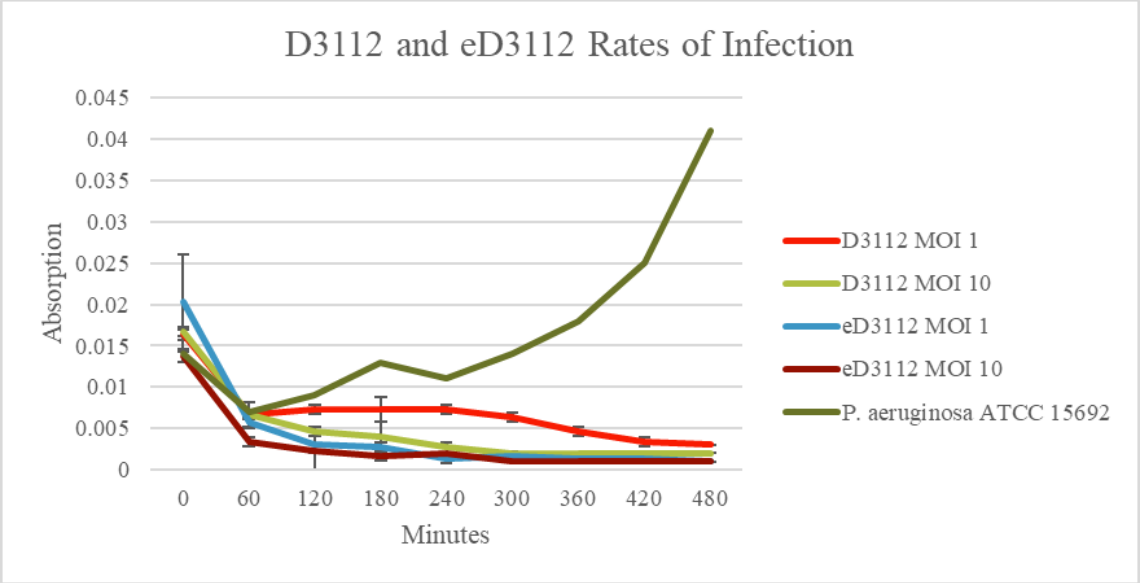


Figure 23. eD3112 and D3112 effect on *P. aeruginosa* growth. The growth of *P. aeruginosa* ATCC 15692 was analyzed over 8 hours using spectrophotometric data. Either eD3112 or D3112 was added to the bacterial cultures at an MOI of 1 or 10. The growth of the bacteria was measured and compared to the control culture without any added phage. Both phages at both MOIs severely stunted the growth of the *P. aeruginosa* strain throughout the 8 hours.

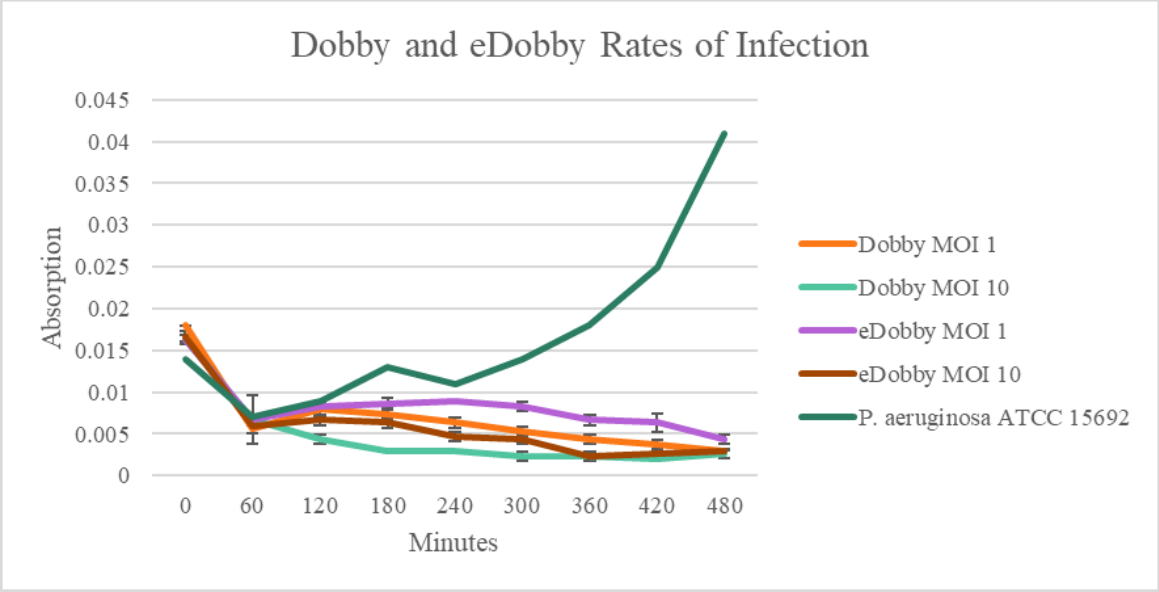


Figure 24. eDobby and Dobby effect on *P. aeruginosa* growth. The growth of *P. aeruginosa* ATCC 15692 was analyzed over 8 hours using spectrophotometric data. Either eDobby or Dobby was added to the bacterial cultures at an MOI of 1 or 10. The growth of the bacteria was measured and compared to the control culture without any added phage. Both phages at both MOIs severely stunted the growth of the *P. aeruginosa* strain throughout the 8 hours.

To complement, the spectrophotometric data collected, *P. aeruginosa* was also plated for colony counts. The resuspended cell pellets of the control *P. aeruginosa* strain ATCC 15962 and the cultures that had been treated with the 4 phages at both MOI 1 and MOI 10 from 1 hour and 8 hours were retained. **Figure 25** and **Figure 26** display the titers of all 1 hour and 8 hour bacterial cultures, both treated with phage and untreated. All 4 phages at both MOIs greatly reduced the number of colonies produced by the bacteria in comparison to the control culture. The control *P. aeruginosa* culture had a titer of $6.00x10^5$ cfu/mL at 1 hour and continued to grow throughout the experiment to $5.00x10^6$ cfu/mL at 8 hours.

The triplicate titers of the bacteria treated with the 4 phages remained consistently lower than the control *P. aeruginosa*. The cultures treated with ancestral D3112 at MOI of 1 had an average titer of $4.66x10^2$ cfu/mL at 1 hour that declined to an average of $3.97x10^1$ cfu/mL at 8 hours. The bacteria treated with D3112 at MOI of 10 had an average titer of $6.10x10^1$ cfu/mL at 1 hour and retained a similar titer of $8.53x10^1$ cfu/mL at 8 hours. The cultures treated with eD3112 at MOI of 1 were found to have an average titer of $6.13x10^1$ cfu/mL at 1 hour and increased slightly to $1.25x10^1$ cfu/mL at the 8 hour mark. The cultures treated with eD3112 at MOI of 10 had a very low average titer of $4.00x10^0$ cfu/mL at 1 hour and $2.60x10^1$ cfu/mL at 8 hours.

The titers of the bacteria treated with the ancestral and engineered Dobby phages also retained low titers in comparison to the control *P. aeruginosa*. The bacteria treated with the ancestral Dobby phage at MOI of 1 were found to have an average titer of $1.81x10^2$ cfu/mL at 1 hour that decreased to $5.87x10^1$ cfu/mL at 8 hours. When the MOI of Dobby was increased to 10, the bacteria had an average titer of $3.10x10^1$ cfu/mL at 1 hour and $2.19x10^2$ cfu/mL at 8 hours.

The cultures treated with eDobby at MOI of 1 maintained an average titer of $5.70x10^2$ cfu/mL at 1 hour and $5.37x10^1$ cfu/mL at 8 hours. At an MOI of 10, the bacteria infected with eDobby had an average titer of $1.28x10^2$ cfu/mL at 1 hour and $1.20x10^2$ cfu/mL at 8 hours. Overall, all *P. aeruginosa* bacterial cultures treated with the 4 phages at both MOIs maintained significantly lower titers than the control *P. aeruginosa* displaying the effectiveness of phage infection on bacterial growth.

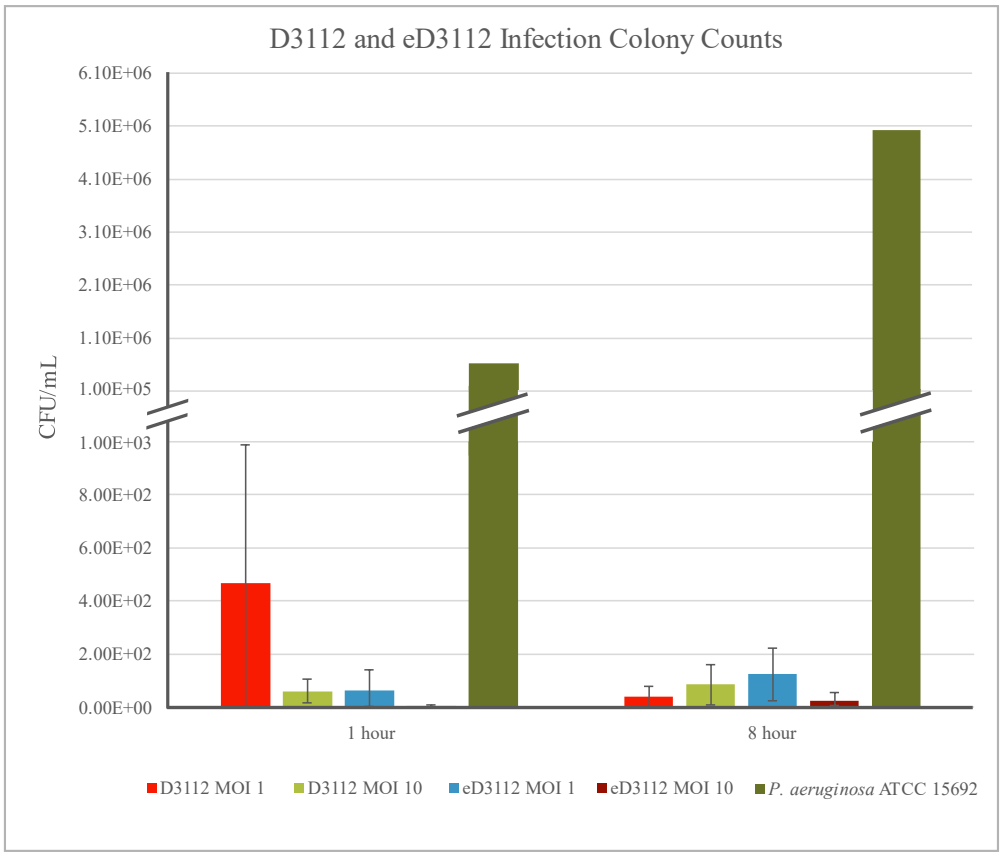


Figure 25. Growth of *P. aeruginosa* at 1 hour and 8 hours after eD3112 and D3112 added. Colony counts were taken from *P. aeruginosa* ATCC 15692 at 1 hour and 8 hours after being treated with either eD3112 or D3112 at MOIs of 1 or 10. The results are compared to the control bacteria without added phage. At 1 hour the growth of the bacteria was depleted and the trend continued through the 8 hours.

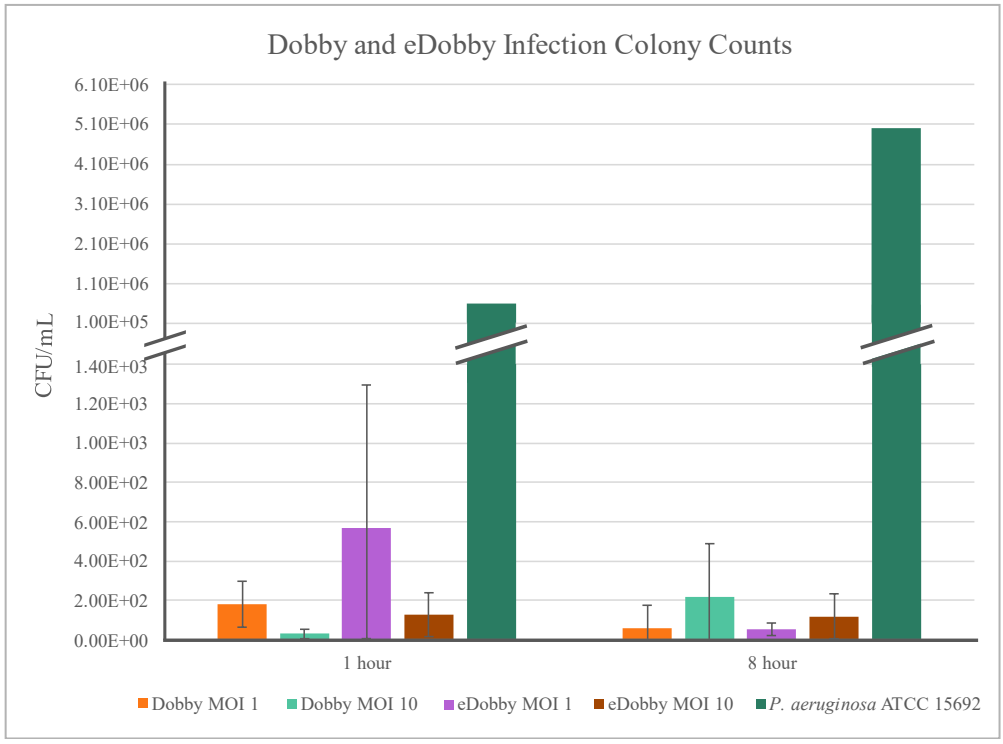


Figure 26. Growth of *P. aeruginosa* at 1 hour and 8 hours after eDobby and Dobby added. Colony counts were taken from *P. aeruginosa* ATCC 15692 at 1 hour and 8 hours after being treated with either eDobby or Dobby at MOIs of 1 or 10. The results are compared to the control bacteria without added phage. At 1 hour the growth of the bacteria was depleted and the trend continued through the 8 hours.

**Efficient Lysis by Ancestral and Engineered Phages.**

The filtered lysates of D3112, eD3112, Dobby, and eDobby at both MOIs from 0 hours and 8 hours were saved from the analysis to identify the titers of the phages during infection. **Figure 27** and **Figure 28** display the titers of all 4 phages at 0 hours and at 8 hours. As the experiment was performed in triplicate, the averages of the phage titers were graphed with standard deviation gridlines. All phages gained significantly higher titers at the 8 hour mark as they had ample time to replicate in the host bacterial cells.

D3112 MOI 1 had an average titer of $4.23 \times 10^8$ pfu/mL at 0 hours but increased to an average of $3.50 \times 10^{10}$ pfu/mL at 8 hours. A similar outcome was found in the phage at an MOI of

10 with an average titer of $4.57x10^8$ pfu/mL at 0 hours and $2.70x10^{10}$ pfu/mL at 8 hours. eD3112 MOI 1 had an average titer of $5.87x10^8$ pfu/mL at 0 hours which increased to $2.63x10^{10}$ pfu/mL at 8 hours. The phage at an MOI of 10 had an average titer of $2.21x10^9$ pfu/mL at 0 hours which was similar to the titer at 8 hours of $6.67x10^9$ pfu/mL.

Dobby MOI 1 displayed $5.03x10^8$ pfu/mL as its average titer at 0 hours and $2.63x10^{10}$ pfu/mL at 8 hours. At an MOI of 10, Dobby exhibited a similar shift in titers with an average of $7.46x10^8$ pfu/mL at 0 hours and $1.70x10^{10}$ pfu/mL at 8 hours. eDobby performed moderately better than its ancestor at both an MOI of 1 and an MOI of 10. eDobby with an MOI of 1 had an average titer of $4.27x10^8$ pfu/mL at 0 hours and $3.27x10^{10}$ pfu/mL at 8 hours. At MOI 10, eDobby had $4.37x10^8$ pfu/mL as its average titer at 0 hours and $2.00x10^{10}$ pfu/mL at 8 hours. All four phages at both MOI 1 and MOI 10 showed significant replication from the beginning to the end of the 8 hour experiment, displaying the ability of the phages to continually infect *P. aeruginosa* during phage treatment.

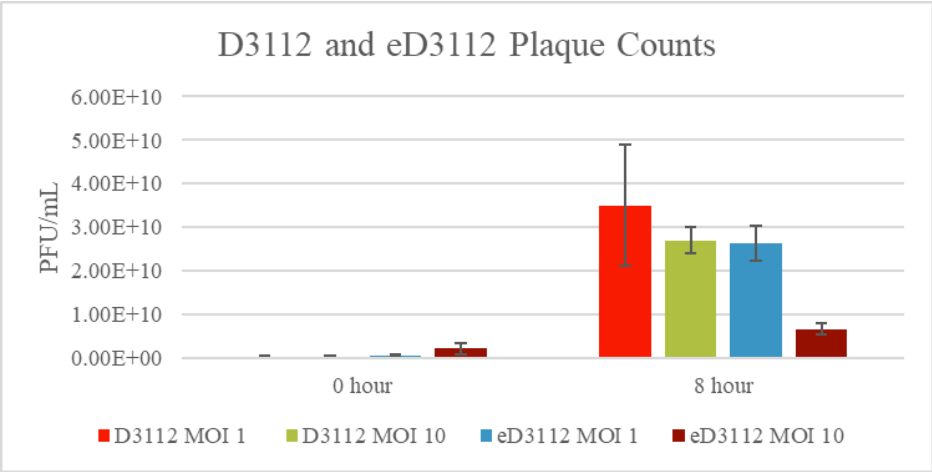


Figure 27. Plaque counts of eD3112 and D3112 at 0 and 8 hours post-inoculation of culture. After either eD3112 or D3112 were added to *P. aeruginosa* ATCC 15692 cultures at MOIs of 1 or 10, phage lysates were filtered from the cultures at 0 hours and 8 hours. Phage lysates were plated on bacterial lawns in dilution series. Both phages at both MOIs had large reproduction activity while maintaining lytic activity of the bacteria.

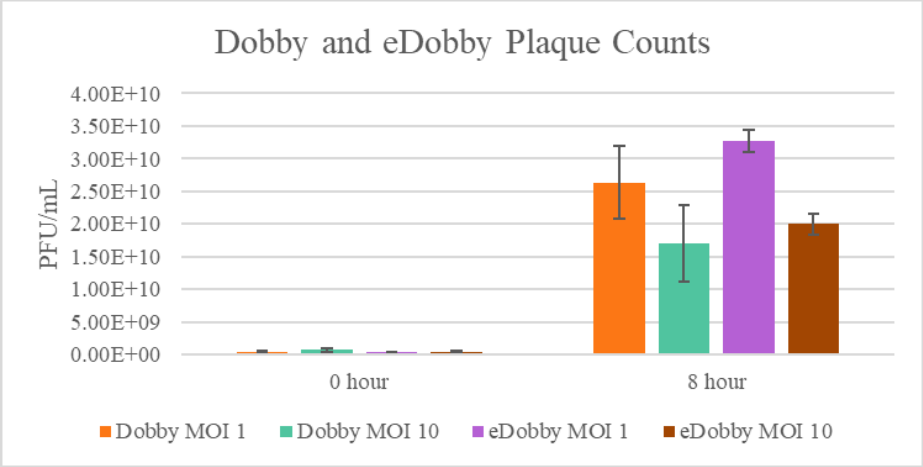


Figure 28. Plaque counts of eDobby and Dobby at 0 and 8 hours post-inoculation of culture. After either eDobby or Dobby were added to *P. aeruginosa* ATCC 15692 cultures at MOIs of 1 or 10, phage lysates were filtered from the cultures at 0 hours and 8 hours. Phage lysates were plated on bacterial lawns in dilution series. Both phages at both MOIs had large reproduction activity while maintaining lytic activity of the bacteria.

## Discussion

A broad spectrum of phages are needed to combat the rising rates of antibiotic-resistant infections in humans. The CDC considers antibiotic resistance to be one of the biggest health challenges in our society with approximately 2.8 million people contracting an antibiotic-resistant infection each year (CDC, 2020). As more strains of bacteria become resistant to antibiotic treatments, it is important to expand our phage therapy candidates to include not only obligately lytic phages, but also engineered temperate phages. Temperate phages that have been engineered to knock out the integrase or the C repressor have been proven successful at killing bacteria (Dedrick et al., 2019; Kilcher et al., 2018; Zhang et al., 2013), including *P. aeruginosa* (Mageeney et al., 2020).

The D3112 and Dobby phages isolated from *P. aeruginosa* are perfect examples of temperate phages to engineer for phage therapy against *P. aeruginosa* infections. Here, I have demonstrated the genetic engineering of D3112 and Dobby and their ability to infect clinical

isolates of *P. aeruginosa*. The C repressor of D3112 and the integrase of Dobby were removed via enzymatic digest to create obligately lytic variants of the phages that infect and kill *P. aeruginosa*. The integrase gene and the C repressor gene were located at the ends of the respective phages, making their removal ideal for enzymatic digestion. After the initial enzymatic digest to remove the integrase from the genome of Dobby, an extra 4 genes that were critical to the viability of the phage were also removed: a DUF4224 domain-containing protein, a phosphoadenosine phosphosulfate reductase, a hypothetical protein, and part of a DNA cytosine methyltransferase. Once the 4 coding regions were restored, we were able to produce viable engineered progeny.

The functionality of the DUF4224 domain-containing protein is unknown. It is commonly found within phage genomes, yet this particular protein from Dobby does not have any similarity to other DUF4224 proteins within phages (Schuster-Böckler et al., 2004). The protein is only 70 nucleotides long and while it is found in both bacterial and viral genomes, it is assumed to be of phage origin (Schuster-Böckler et al., 2004). The phosphoadenosine phosphosulfate reductase is involved in the reduction of activated sulfate into sulfite and is commonly found in *P. aeruginosa* genomes (Krone et al., 1990). As this protein is not characterized to have specific functionality in phage genomes, the protein was most likely acquired by the phage during integration and excision. The hypothetical protein encoded in the genome of Dobby is only 62 nucleotides long. The protein is highly similar to other hypothetical proteins found within *P. aeruginosa* genomes, but only shows similarity to the φCTX phage (Accession no.: NC_003278), a close relative of Dobby. DNA cytosine methyltransferases are frequently found in bacterial genomes, but the presence and functionality of the proteins in

phages has also been studied (Murphy et al., 2013). Lytic and lysogenic phages alike can encode methyltransferases and they assist in protecting the phages from the bacterial host's restriction endonucleases (Murphy et al., 2013). Future studies could explore which of these 4 genes are essential for Dobby's successful replication in and lysis of *P. aeruginosa*.

After successful creation of the engineered eD3112 and eDobby phages, both displayed changes to the bacterial isolates they were able to infect. eD3112 lost the ability to lyse 2 strains (UMB4836 and UMB8183) that were susceptible to the ancestral D3112 phage. After the removal of the integrase gene, eDobby could not lyse 1 strain (UMB4740) of *P. aeruginosa* that its ancestor could lyse. It is possible that there was interference with the phage receptors to recognize the bacterial host once the C repressor or integrase was removed. Alternatively, it could be that the lysis observed with the ancestral phages was the result of a native prophage in the UMB strain being induced. However, UMB4836 contained 1 high confidence prophage prediction within its genome.

While eD3112 was not able to lyse any additional strains of *P. aeruginosa* in comparison to its ancestral phage, eDobby was able to lyse 6 additional strains of *P. aeruginosa* that the ancestral Dobby could not. We hypothesized that the Dobby phage was integrating into the genomes of these 6 strains because it still contained the integrase gene. It was determined that of the 6 strains, Dobby was integrating into the genome of 1 strain, UMB0802. While we have ways to test for attachment of phages to bacterial cells, it is difficult to determine if a phage is really infecting a bacterial cell without extensive sequencing techniques. Engineering temperate phages to become obligately lytic displays a novel way to determine if a temperate phage is just unable to infect a bacterial strain or if it is actually integrating into the host cell's genome.

Along with being able to lyse the majority of the tested clinical isolates of *P. aeruginosa*, both engineered phages were also able to infect and kill the host cells within 1 of the 4 tested biofilms formed by *P. aeruginosa*. Most interesting was the fact that none of the phages, neither the ancestral nor the engineered strains, were capable of lysing the evolved biofilms of the laboratory *P. aeruginosa* ATCC 15692 strain, the same strain used to characterize the fitness of these phages. In fact, we observed increased growth of the evolved *P. aeruginosa* ATCC 15692 strain in the area of the phage spot. Our study also found that only one of the evolved *P. aeruginosa* UMB8183 was lysed by the phages, while the other evolved strain was not. This suggests different adaptations occurred in the two strains leading one and not the other to be resistant to phage lysis. As these methods only tested the phages on the bacterial cells from the biofilms, a future step should be to test the efficacy of the engineered phages on the biofilm formations themselves. Antibiotics are often unable to penetrate biofilms, making the infections difficult to resolve (Singh et al., 2016). The use of engineered phages to penetrate and lyse the bacterial cells within a biofilm would greatly expand the ability of physicians to treat complicated *P. aeruginosa* infections.

The 8 hour infection analysis of the engineered phages displayed their abilities to kill *P. aeruginosa* cultures and consistently reproduce throughout the 8 hours, which were comparable and even superior to the ancestral phages. Previous phage therapy studies have displayed resolution of bacterial infection with phage concentrations ranging from $10^5$ pfu/mL to $10^9$ pfu/mL per dose (Dedrick et al., 2019; Jault et al., 2019; Jennes et al., 2017; Rose et al., 2014; Wright et al., 2009). The pfu/mL for each of the engineered phages fell within the range of prior phage therapy studies required for therapeutic treatments. eD3112 displayed $5.87 \times 10^8$ pfu/mL at

the beginning of the *in vitro* testing against *P. aeruginosa* that quickly grew to $2.63x10^{10}$ pfu/mL after 8 hours. eDobby showed similar results with $4.27x10^{8}$ pfu/mL at the start of the analysis that grew to $3.27x10^{10}$ pfu/mL at 8 hours post-inoculation.

Other phage therapy studies noted MOI values rather than pfu/mL values for the phages. One study focusing on the phage treatment of an aortic graft noted only an MOI of 0.00001 was required to display significant reduction of *P. aeruginosa* biofilms which is much below the typical requirement for treatments (Chan et al., 2018). Another study using phages to treat a *P. aeruginosa* burn wound required an MOI of 10 to treat the infection (Dedrick et al., 2019; Rose et al., 2014). Similar results were seen in the engineered eD3112 and eDobby phages. Both an MOI of 1 and an MOI of 10 were tested against *P. aeruginosa*. Significant cell death and stunted growth of the bacteria was displayed at both MOIs. Further testing would be needed to find the minimum MOI needed to sufficiently treat a *P. aeruginosa* infection. With such high titers and their efficacy in killing *P. aeruginosa* cells, the engineered eD3112 and eDobby make noteworthy candidates for phage therapy against *P. aeruginosa* infections.

# CHAPTER FOUR

# CONCLUSIONS

This study produces the first known comprehensive catalogue of predicted prophages from all publicly available genomes of *P. aeruginosa*. High confidence predictions of prophages are prevalent in *P. aeruginosa,* and they are taxonomically diverse, including representatives of the order *Caudovirales* as well as to phages of the *Inoviridae* and *Myoviridae* families. Homologous genes are common across these taxonomic groups, as our network analysis identified connections between phages of *Caudovirales* and inoviruses. While many of the predicted prophage sequences resembled characterized phages and thus could be taxonomically classified, nearly half of the predictions are novel and thus could not be classified. Future analyses need to be done to determine the potential taxonomic families of these Unknown predicted prophages.

The integrase and C repressor coding regions, typically conserved among temperate phages, were found to be very diverse among the predicted prophages, especially the integrases. It was expected that phages with similar terminases, a conserved gene among tailed phages, would also share similar integrases or C repressors. However, this hypothesis was not observed among the predicted prophages, signifying substantial genetic exchange between *P. aeruginosa* prophages.

While there was no association found between the predicted prophages and antibiotic resistance genes in *P. aeruginosa*, there may be potential associations with virulence factors.

Prophages may also have an effect on the formation of biofilms by *P. aeruginosa*. Future studies are needed to examine the relationship between prophage presence and biofilms, as well as prophage effect on other pathogenic facets of *P. aeruginosa*.

This study also presented the successful engineering of temperate phages to obligately lytic phages, expanding the potential candidates for phage therapy. The C repressor was removed from the phage D3112 to create an obligately lytic eD3112 phage, the first known use of this technique for *P. aeruginosa* phages. The engineering produced a viable phage with similar, if not better, lytic activity against *P. aeruginosa* clinical isolates compared to the ancestral D3112 phage. There were changes to the host range after engineering that resulted in eD3112 not being able to lyse 2 clinical isolates that its temperate ancestor D3112 could not.

The obligately lytic eDobby phage was produced from Dobby by removing the integrase and replacing 4 additionally removed genes required for viability. eDobby displayed similar, if not better, lytic activity against clinical isolates of *P. aeruginosa* compared to the ancestral Dobby phage. Changes to the host range were observed after engineering, resulting in eDobby not being able to lyse 1 strain but being able to lyse 6 additional clinical isolates compared to Dobby. It was determined that Dobby was integrating into 1 of the 6 clinical isolates that eDobby was able to lyse after the integrase was removed. Engineering temperate phages could be used in future studies as an assay to determine whether the temperate phages are integrating into bacterial host genomes or if they are unable to infect cells when lytic activity is not observed.

Both engineered phages displayed lytic activity against the bacterial cells from 1 of the 4 tested *P. aeruginosa* biofilming lines, making the phages potential candidates for phage therapy against *P. aeruginosa* infections that include biofilms. However, future studies are needed to test

the phages on biofilms rather than only the bacterial cells from within the biofilms. *P. aeruginosa* biofilms in the lungs of cystic fibrosis patients can be lethal. As such, phages capable of disrupting these biofilms and clearing the infection are desperately needed. The next critical step in assessing the efficacy of engineered phages such as eD3112 and eDobby, or similar engineered temperate phages, is animal testing before the phages can be fully considered as candidates for phage therapy.

The ability to successfully engineer temperate phages to become obligately lytic greatly increases the catalogue of phages available to treat bacterial infections. With growing rates of antibiotic resistance, new treatment options are in high demand. The abundance of temperate phages in the environment and the human body cannot be ignored any longer. Engineering temperate phages to become obligately lytic alleviates the fears associated with possible integration of the phages meant to treat infections. As such, engineered temperate phages are ideal candidates to treat bacterial infections. While we have focused here on engineered temperate phages to combat *P. aeruginosa*, the same strategy can be employed for temperate phages infectious of other bacterial species.

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# VITA

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