Leveraging Temperate Phages To Enhance Antibiotic Effectiveness

LEVERAGING TEMPERATE PHAGES TO ENHANCE ANTIBIOTIC EFFECTIVENESS

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Lay Abstract

Bacteriophages (phages) are bacterial viruses that present a promising solution to the antibiotic resistance crisis. They can kill bacteria even when antibiotics fail, alone or in combination with them. Most work to date focuses on phages that immediately lyse the bacteria. However, phages that can go dormant within the host are far more abundant but largely ignored in therapy. Once integrated, these can awaken to switch into lytic replication by external triggers that stress the bacterial host, including antibiotics. Supported by this idea, in this thesis I show that these kinds of phages can synergistically interact with antibiotics by biasing the phage away from dormancy. This phenomenon is generalizable across host, phages, and antibiotics and shows effectiveness in an animal model.

Abstract

With a decline in antibiotic effectiveness, there is a renewed interest in using bacterial-specific viruses (bacteriophages or phages) to reduce bacterial loads, alone or with antibiotics. Most phages are therapeutically unsuitable because they are "temperate" and can integrate into the host genome, protecting the host from subsequent phage infections. However, dormant phages can be awakened by stressors such as antibiotics. Here we investigated whether antibiotics can uniquely interact with temperate phages to bias the phage away from dormancy. Model *E. coli* temperate phage and ciprofloxacin, a DNA-damaging antibiotic, exhibit a potent synergy, resulting in bacterial eradication at sublethal antibiotic concentrations, despite poor killing by the phage alone. Mechanistically, this synergy depletes survivors by awakening dormant phages. To broaden our findings, screening in the multi-drug-resistant pathogen *P. aeruginosa*, we identified phages that can synergize with four antibiotic classes, despite their widely differing targets - however, these are highly phage, antibiotic, and host-specific. Interestingly, ciprofloxacin also synergized with multiple phages, even in a ciprofloxacin-resistant clinical strain, functionally re-sensitizing the bacterium to the antibiotic. While some of these interactions operated through a mechanism independent of the temperate nature of the phages, ciprofloxacin and piperacillin, a cell wall synthesis inhibitor, specifically reduced the frequency of phage dormancy events. Finally, in a *Caenorhabditis elegans* infection model, temperate phage-ciprofloxacin pairing increased the lifespan of drug-resistant *P. aeruginosa* infected worms compared to the uninfected control. Similar rescue was also observed for the phage-carrying strain treated with the antibiotic, supporting that the phage even in its dormant

form can enhance antibiotic effectiveness. Overall, we show that temperate phages uniquely synergize with antibiotics at the level of biasing the phage away from dormancy. This is generalizable across phages, antibiotics, and hosts, and shows efficacy *in vivo*, thereby drastically expanding their therapeutic potential.

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List of Abbreviations and Symbols

Φ: Bacteriophage (phage)

AMR: Antimicrobial resistance

CFU: Colony forming units

DNase: Deoxyribonuclease

LB: Lysogeny broth

LYS: Lysogen

MIC: Minimum inhibitory concentration

M.O.I.: Multiplicity of infection

NGM: Nematode growth medium

OD600: Optical density at 600 nm

PAS: Phage-antibiotic synergy

PCR: Polymerase chain reaction

PFU: Plaque forming units

qPCR: Quantitative polymerase chain reaction

RNase: Ribonuclease

SDS: Sodium dodecyl sulfate

SKM: Slow killing medium

tPAS: temperate phage-antibiotic synergy

WT: Wild type

Declaration of Academic Achievement

Bacteriophage HK97 and its host *Escherichia coli* were obtained from the Félix d'Hérelle Reference Centre for Bacterial Viruses under the accession HER 382 and HER 1382, respectively. The *E. coli* Δ*recA* mutant as obtained from the Dharmacon KEIO collection through Horizon Discovery (Cambridge, UK). *Pseudomonas aeruginosa* PA14 was kindly gifted to us by Dr. Lori Burrows. The *P. aeruginosa* clinical strain collection was kindly provided by Dr. Gerry Wright. All other phages and strains were generated as part of this study. All sequencing was performed by the McMaster Genomics Facility. All experiments, analysis, interpretation, and writing were performed by Rabia Fatima, unless otherwise stated in each chapter preface.

Chapter 1 - Introduction

1.1 Antimicrobial Resistance

Since the discovery of penicillin in 1928, antibiotics have been extensively used to treat bacterial infections in humans, animals, and crops (Ventola, 2015). The widespread use of these agents has selected for resistance, resulting in a decline in their effectiveness and a rise in untreatable infections. In a recent systematic analysis published covering the global burden of antimicrobial resistance (AMR) crisis from 1990-2021, it was estimated that in 2021 alone, 1.41 million deaths worldwide could be directly attributed to AMR, with an additional 4.71 million associated deaths (Naghavi et al., 2024). This number is expected to rise to 1.91 million attributed and 8.22 million associated deaths by 2050. While the burden is disproportionately borne by developing countries, Canada is not insulated with 5400 lives lost because of AMR in 2018 and an expected increase to 13,700 by 2050 (Council of Canadian Academies 2019).

The priority bacterial pathogens with the highest risk to human health include *Acinetobacter baumannii, Escherichia coli, Klebsiella pneumonia, Pseudomonas aeruginosa, Staphylococcus aureus,* and *Streptococcus pneumonia* (Naghavi et al., 2024). They are well known for their extensive arsenal of tools which limit the action of antibiotics. These include low outer membrane permeability and constitutively expressed drug efflux pumps that contribute to intrinsic resistance, as well as acquired resistance such as plasmid encoding beta-lactamases, and adaptive mechanisms that involve changes in gene expression to survive environmental stress (Brown et al., 2014; Langendonk et al., 2021; Pragasam et al., 2018; Venter et al., 2017). The rise in resistance drastically narrows the panel of drugs that can be used against them in clinic. As a result, there has been a

renewed interest in new treatments with unique mode of actions and revisiting older therapies such as bacteriophages (phages).

1.2 Bacteriophages

Even prior to the discovery of the first antibiotic, researchers identified other naturally occurring biological entities that were capable of lysing bacteria all the while co-existing along with them. Bacteriophages (phages) were independently reported by Frederick William Twort in 1915 (Twort & Lond, 1915) and by Félix d'Hérelle in 1917 (D'Hérelle, 1917). Phages are viruses that infect bacteria and are the most abundant biological entities on the planet with an estimated population of $\geq 10^{31}$ (Brüssow et al., 2004). They are diverse in size, morphology, and genomic structure (Hatfull & Hendrix, 2011). However, at their core all phages are composed of nucleic acid surrounded by a protein capsid that protects the genome and mediates delivery into the host bacterial cell. Phages are highly host specific and usually infect a single bacterial species or strain (Kasman & Porter, 2022) .

1.3 Phage Replication Cycles

Phages have several known replication cycles, but the most well-characterized are the lytic and lysogenic cycle (Figure 1). The mode of infection of phages is dependent on several core steps. Phages attach to the host cell through binding to complementary receptors on the host cell surface in a process referred to as adsorption (Guttman et al., 2004). Bacterial surface components (e.g. LPS, outer membrane proteins) and pili are two of the major types of phage receptors (Coetzee et al., 1985; Rakhuba et al., 2010). Upon successful binding, the phage genome is injected into the host cytoplasm where it is able

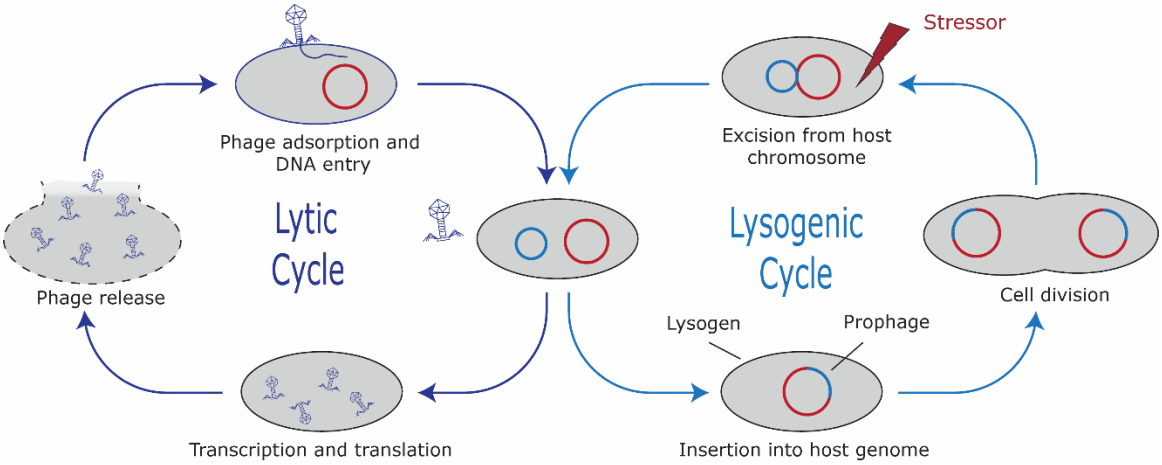


Figure 1. Lytic and lysogenic cycle of phage replication.

to either commit to a lytic or lysogenic cycle (Guttman et al., 2004). During the lytic cycle, the host machinery is redirected to synthesize new phage DNA which are assembled into new phage particles (Drulis-Kawa et al., 2012). This cycle results in host cell lysis and the release of new phage progeny that can go on to infect neighboring cells.

Lysogeny in contrast is a latent state in which the phage can integrate its genome into that of the bacterial host either by inserting itself into the host chromosome or existing as an autonomous plasmid (Czyz et al., 2001; Howard-Varona et al., 2017; Touchon et al., 2016). The phage and the bacterium are now referred to as a prophage and a lysogen, respectively. The prophage can awaken and switch from the lysogenic to lytic replication in response to external stressors from the environment in a process called induction. While virulent or strictly lytic phages can only undergo the lytic cycle, temperate phages can undergo both replication cycles.

1.4 Phage Resistance

Bacteria have evolved various resistance mechanisms that can target every step of the phage replication cycle from DNA entry, replication, or even by altruistic bacterial

death through abortive infection (Charbit et al., 1988; Hampton et al., 2020; Labrie et al., 2010). However, two the most commonly occurring, at least *in vitro* - and the earliest discovered - are surface receptor mutations and immunity to superinfection. Alteration of the receptor that the phage uses for recognition and attachment completely blocks infection at the very first step. For example, several mutations in *E. coli* LamB, the surface receptor for the phage Lambda, have been reported that confer host resistance to phage infection (Charbit et al., 1988). In contrast, in the case of successful infection, phage-carrying bacteria can become resistant to subsequent infections by the same phage or other closely related phage through phage-generated immunity (Mavrich & Hatfull, 2019). Superinfection exclusion is a result of phage-encoded proteins that prevent adsorption or DNA entry of an incoming phage. Examples of these include the *E. coli* lytic phage T4 Imm and Sp proteins (Lu & Henning, 1994) and *E. coli* temperate phage HK97 gp15 protein (Cumby et al., 2012). In contrast, superinfection immunity allows for DNA entry, but the resident prophage can silence the genome of incoming phages through a phage encoded repressor (Mavrich & Hatfull, 2019; Sun et al., 2006; Zinder, 1958). Superinfection immunity is only associated with lysogeny and is the primary concern in the therapeutic use of temperate phages.

1.5 Lambda and Lambdoid Phage HK97

The *E. coli* phage Lambda is the best studied temperate phage due to its role as a model organism and useful tool for microbial and molecular genetics. Since its discovery in 1951 by Esther Lederberg (Lederberg, 1951), work done with Lambda has been critical

for our current understanding of DNA viruses, gene regulation, and the genetic nature of lysogeny.

The establishment of a lysogenic state for Lambda requires two critical genes; *cI* and *int* (Bednarz et al., 2014; Griffiths et al., 2000). The *cI* gene has two promoters; PE is required for establishing lysogeny and PM for maintaining lysogeny through activation by the cI repressor itself. The cI repressor keeps the phage in its quiescent prophage state by effectively repressing the lytic activator Cro. It is also involved in providing Lambda lysogens with superinfection immunity by silencing the genomes of closely related phages in subsequent infections. The *int* gene encodes an integrase which works with bacterial integration host factor to mediate recombination between phage attachment site, *attP*, and bacterial attachment site, *attB*, flanked by the *E. coli* galactose and biotin operon (Cho et al., 2002; Tropp, 2012).

When external stressors comprise the host, the phage can exit lysogeny. The cI levels fall below the threshold, allowing transcription of Cro, initiating the lytic cycle (Bednarz et al., 2014). The excision of the phage genome from the bacterial host is mediated by the product of the *xis* gene, produced on the same mRNA as *int* (Davies, 1980; Schindler & Echols, 1981). The digestion of the mRNA transcript by host RNAse III results in higher concentration of Xis than Int, favouring excision (Schmeissner et al., 1984).

Since the discovery of Lambda, many Lamba-like (Lambdoid) phages have been characterized that infect distinct hosts but possess similar core proteins to Lambda, especially related to the establishment and maintenance of lysogeny (Biswas et al., 2014; Dhillon et al., 1980; Evseev et al., 2021). Of these, perhaps the most closely related is the

E. coli temperate phage HK97 which was first isolated from pig dung (Dhillon et al., 1980). It possesses the same host range as Lambda and has the same integration site. In addition, temperature dependent experiments using double Lambda and HK97 lysogenic bacteria revealed that the repressor protein produced by one prophage can suppress the lytic function of both prophages (Dhillon et al., 1980).

1.6 The Bacterial SOS Response System and Phage Induction

The lysogenic to lytic switch has been shown to heavily rely on the bacterial SOS response. The SOS response, first discovered in *E. coli* by Miroslav Radman in 1975, is an inducible bacterial DNA damage response system that works to synthesize DNA repair enzymes (Baharoglu & Mazel, 2014; Michel, 2005; Tippin et al., 2004). The SOS response operon is regulated by two key proteins; LexA, a repressor, and RecA, an inducer. Under normal condition, LexA binds to the SOS box in the operator region to prevent transcription and inhibit the SOS operon. Upon DNA damage, RecA/ssDNA nucleoprotein filament initiate the autocatalytic activity of LexA, which allows for the transcription of the SOS genes involved in homologous recombination, nucleotide excision repair, translesion synthesis, and cell division arrest.

Active RecA binding also leads to the cleavage of other LexA homologues. These include several phage repressor proteins such as Lambda cI repressor, φ80 (*E. coli* phage) cI repressor, and P22 (*Salmonella* phage) repressor (Eguchi et al., 1988; Sauer et al., 1982). Self cleavage of Lambda cI repressor occurs between Ala111 and Gly112 which separates the N-terminal DNA binding domain from the C-terminal oligomerization domain (Sauer et al., 1982). The cleavage of the repressor allows for prophage induction.

1.7 Lysis-Lysogeny Decision

While the genetic switch is encoded in the genome of many phages, the frequency with which a phage opts for lysogeny can be influenced by external factors. In a laboratory setting, Lambda lysogenizes fewer than 0.1% of cells at multiplicity of infection (M.O.I. or phage to host ratio) of 0.1 (Knoll, 1979), which can increase to ~90% as M.O.I. increases (Knoll, 1979; Kourilsky, 1973). This is further supported by fluorescent visualization of Lambda single phage infecting a single host cell where lysogeny is favoured at higher M.O.I.s (Trinh et al., 2017; L. Zeng et al., 2010). While the rate varies by M.O.I., it will still result in overgrowth of lysogen survivors. This rate of lysogeny however can clearly change in response to the environment. St-Pierre and Endy (2008) reported that the probability of Lambda lysogenization decreases by 4-5 fold as volume of the cell increases approximately 2-fold, which could be explained by the diluting of the CI and Cro. The host nutritional status can also influence the replication decision made by temperate phages at the time of infection. Starving *E. coli* in $MgSO_4$ before infection by Lambda promoted lysogeny 50-100-fold (Herskowitz & Hagen, 1980; Kourilsky, 1973; Kourilsky & Knapp, 1974).

1.7.1 SOS-dependent

It comes perhaps as no surprise that any threat to the host bacterium's DNA would be a threat to the prophage, and a relevant cue for lysis-lysogeny switch. Leveraging the knowledge gained from the genetic switch of Lambda, inducing the bacterial SOS response has been a practical approach for isolating phages. One of the most widely employed

methods is using mitomycin C, an anticancer drug that inhibits DNA replication (Fortier & Moineau, 2007; Martin et al., 1995; Motlagh et al., 2015; Quinones et al., 2005).

Another class of antibiotic that compromises the integrity of host DNA is the fluoroquinolones which inhibit DNA gyrase and DNA topoisomerase resulting in DNA damage (Blondeau, 2004). Subinhibitory concentrations of ciprofloxacin, a commonly prescribed fluoroquinolone for bacterial infections such urinary and respiratory infections (Kwong & Lindsay Grayson, 2017), was shown to cause prophage induction and increase the release of phage related virulence factors in *S. aureus* (Goerke et al., 2006). This has also been observed in multidrug resistant *Salmonella*, enterohemorrhagic *E. coli*, and *S. pneumonia* (Bearson & Brunelle, 2015; López et al., 2014; Walterspiel et al., 1992).

While it makes sense that antibiotics such as ciprofloxacin and mitomycin C, which compromise the host DNA, result in prophage induction, induction has also been observed with the β-lactam class of antibiotics (Maiques et al., 2006). β-lactams inhibit cell wall synthesis by binding to penicillin binding proteins, preventing peptidoglycan crosslinking, resulting in cell lysis (X. Zeng & Lin, 2013). The exact mechanism through which β-lactams potentially induce the SOS response is unclear and prophage induction was not tested further in a SOS deficient background (Maiques et al., 2006). In *E. coli*, Christine et al., (2004) showed that ampicillin induces the SOS response through a two-component signalling DpiBA system to survive the lethal effects of the antibiotic. Around the same time, Tatiana et al. (2005) reported that ceftazidime increased expression of *dinB*, a downstream SOS gene encoding DNA polymerase V, independently of LexA and RecA. In *S. aureus*, only β-lactam antibiotics targeting penicillin binding protein 1 such as

oxacillin and imipenem induce *recA* expression (Plata et al. 2013). In more recently reported work performed in *S. aureus*, an oxacillin-triggered SOS response was less pronounced under anaerobic conditions, suggesting that double stranded breaks caused by reactive oxygen species may play a role (Clarke et al., 2021).

Apart from antibiotics, other SOS dependent phage inducers include oxidative stress from hydrogen peroxide (Łoś et al., 2010; Tang et al., 2017) and a small genotoxin produced by members of the human gut (Silpe et al., 2022). While these directly operate through the SOS pathway, other stressors such as fructose or microbiota derived short chain fatty acids can result in prophage induction through indirect activation of this pathway (Oh et al., 2019).

Given the conservation of this stress response, while most studies do not directly measure whether induction is SOS-dependent, it is reasonable to assume that it is involved for most inducers. These include environmental pollutants such as copper (II) sulfate (Lee et al., 2006), pesticide mixture (Cochran et al., 1998), sunscreen (Danovaro & Corinaldesi, 2003), and solar oil (Danovaro & Corinaldesi, 2003). Similarly, many commonly consumed compounds such as oral medications (non-steroidal anti-inflammatory, chemotherapy, mild analgesic, cardiac, and antibiotic) (Sutcliffe et al., 2021) and dietary compounds (stevia, cloves, grapefruit seed extract) (Boling et al., 2020) have been shown to cause phage induction in gut isolates.

1.7.2 SOS-independent

The ability to factor in other signals of their host's well-being, or gauge the suitability of extracellular environment, would clearly be an immense value to the phage.

The earliest evidence of SOS-independent phage induction reported that overexpression of capsular polysaccharide synthesis results in Lambda phage induction in a *recA* independent manner (Rozanov et al., 1998). SOS-independent prophage induction can also result from chemical stressors such as citrate and EDTA, chelating agents that trigger induction of Stx2 phage (Imamovic & Muniesa, 2012). In addition, molecules in the environment released by neighbouring cells as in the case of acyl-homoserine lactones, a well-known signaling molecules of quorum sensing released by *P. aeruginosa* PA01, can trigger Lambda phage induction in *E. coli* through a SOS-independent mechanism (Ghosh et al., 2009). Interestingly, phages can also directly encode proteins to monitor host quorum sensing to facilitate the lysis-lysogeny switch (Silpe & Bassler, 2019). For example, a vibriophage encode a protein that can bind to the *Vibrio cholerae* quorum sensing autoinducer. This binding initiates the lysis program by increasing expression of another phage gene whose product sequesters the phage repressor of lysis. As a result, prophage induction is initiated when cell density is high and autoinducer concentration increases.

Independent of the host, phages can communicate with each other through a system termed “arbitrium”, discovered in *Bacillus* phages (Erez et al., 2017; Stokar-Avihail et al., 2019). This system consists of three phage genes that encode a communication peptide, an intracellular peptide receptor, and a non-coding RNA that negatively regulates lysogeny. During infection, each phage produces a hexapeptide that is released into the growth medium and taken up by neighboring cells. In subsequent infections, phage progeny enter lysogeny when they sense a high concentration of this peptide - indicative of many recent/past infections, and availability of nearby host. Arbitrium also plays a role several

generations after the initial infection at the level of prophage induction. Bruce et al. (2021) reported that lysogen formation only significantly increased at high concentrations when *Bacillus* cells were infected with non signal peptide producing phage in the presence of synthetic signal (Bruce et al., 2021). However, when non-signal producing lysogens were grown in the presence of synthetic signal, there was a significant decrease in prophage induction suggesting a favouring of lysogeny in the presence of the arbitrium. These examples provide clear evidence of biasing the lysis-lysogeny switch in the absence of a DNA damage response and that phages integrate a diverse range of inputs in the lysis-lysogeny equilibrium.

1.8 Transposable temperate phages

Much of our understanding of entry into and exit out of lysogeny comes from work done with Lambda, while non-Lambdoid phages remain relatively unexplored. This is potentially due to the inherent challenges that come with studying these phages as they can lack a specific integration site. Unlike Lambdoid phages, transposable temperate phages randomly integrate into the host genome and replicate during the lytic infection using transposition (Harshey, 2014; Hulo et al., 2015; O'Brien et al., 2019; Taylor, 1963). The earliest, and perhaps the most well-studied of these is the *E. coli* phage Mu – named for its mutagenic properties - discovered in the 1960s by Austin L. Taylor (Taylor, 1963). It uses a non-replicative transposition to carry out the initial integration, then uses a recombinase and replicative transposition to generate multiple copies in the host genome during its lytic cycle (Hulo et al., 2015). Early during the infection, RepC and Ner repressors are transcribed where the ratio of the two determines the phage infection cycle (Hulo et al.,

2015). RepC represses early promoters to establish lysogeny. In comparison, Ner represses RepC to allow transcription of the lytic genes. Several phages that possess Mu like genome organization have been isolated and these can infect diverse hosts such as *Burkholderia*, *Vibrio*, *Rhizobium*, *Haemophilus*, *Rhodobacter*, and appear especially prevalent in *Pseudomonas* (Hulo et al., 2015). While these appear to encode similar genetic components as Lambda, there are few studies exploring the induction of Mu-like phages.

1.9 History of Phage Therapy

Reference to phages as possible therapeutic agents dates to 1896 when Ernest Hankin detected antibacterial activity against *V. cholera* in unboiled water from the Ganges and Jumna rivers in India (Abedon, Thomas-Abedon, et al., 2011). However, their intentional use dates to their discovery in bacteria-free filtrate from stool sample of dysentery patients by Félix d'Hérelle in 1917 (D'Hérelle, 1917). He anticipated a role in recovery through their antibacterial properties and proposed the usage of laboratory prepared phages as therapy for bacterial infections. With evidence of therapeutic effectiveness, d'Hérelle carried out the first human trial of Shiga-bacteriophage for treatment of bacillary dysentery in 1919 (Summers, 1999). He later went on to also treat four cases of bubonic plague through phage administration (Summers, 2001). Phage therapy was commonly used during the Winter War for treating Soviet soldiers with streptococcal or staphylococcal infections using a phage cocktail, preventing amputation. In the United States, several pharmaceutical companies became interested in producing phage preparations in the 1930s (Straub & Applebaum, 1933). For example, Eli Lilly made

filtered lysates and a water-soluble jelly base for the treatment of respiratory infections, abscess, and mastoiditis.

After the second World War, phage therapy was mostly forgotten in the west. While major changes in attitude can be attributed to the discovery of antibiotics, an easier to produce broad-spectrum stable antibacterial agent, it is not the whole story. There was no standard for phage preparation or evaluating purity or potency. It also did not help that during this period, Eaton and Bayne-Jones (1934) published a report that questioned the successes of phage therapy cases and whether they could be attributed to phage-independent factors, as well as arguing that that phages were inanimate and could possibly be enzymes (Eaton & Bayne-Jones, 1934). Seven years later with further development in the phage field, Albert Krueger and Jane Scribner published a sequel to the original report, concluding that phages are not superior to any other preparations, such as bacterial vaccines, on the market (Krueger & Scribner, 1941), effectively halting interest in phage therapy in many countries (Sulakvelidze et al., 2001; Wu et al., 2013).

With the crisis of AMR, phage therapy has re-presented itself as a promising alternative. In 2024, the Belgium phage consortium presented outcomes of the first 100 consecutive cases of personalized phage therapy, reporting clinical improvement in 77.2% of cases and pathogen eradication in 61.3% cases (Pirnay et al., 2024). There have been several successful experimental phage therapy cases reported in the media such as that of Tom Patterson and his fight against resistant *A. baumannii* (Steffanie Strathdee, 2019) and Nicole Stringer born with cystic fibrosis and infected with resistant *P. aeruginosa* (Favaro & Philip, 2019) . The renewed interest in phage therapy has prompted the development of

phage therapy centers around the world, with three opening in the United States; The Center for Phage Technology in Texas, Center for Innovative Phage Applications and Therapeutics in San Diego, and Center for Phage Biology and Therapy at Yale.

Specifically, within Canada, recently phages were used to treat a 72-year individual with a life-threatening urinary tract infection caused by drug resistant *E. coli* (Favaro, 2023) and another patient suffering from *S. epidermidis* chronic joint infection, with signs of early sepsis (Favaro, 2024). While phage therapy shows promise, much work is required not only on the regulatory framework, with Canada largely modelling itself after the United States on this front, but also validated controlled clinical trials which include considerations on dosage, method of application, and combination treatments.

1.10 Traditional Phage Antibiotic Synergy

With antibiotics being the current standard of care, phages must be proven to work alongside or in place of them. There was a lot of concern that since antibiotics target many bacterial components that are also required for successful phage infections that phage-antibiotic combination treatments would be ineffective (Abedon, 2019; Matsui et al., 2017). Certain protein synthesis inhibitors have been shown to antagonize phages, with kanamycin interfering with the replication of *E. coli* lytic phage T3 (P. Zuo et al., 2021). Kanamycin and apramycin also reduced the efficiency of plaquing of temperate phage Lambda by up to 1000-fold (Kever et al., 2022). Protein synthesis inhibitors were also reported to antagonize *P. aeruginosa, S. aureus,* and *Enterococcus faecium* phages (Coyne et al., 2024), however, this was dependent on the type and amount of antibiotic being used.

Given that antibiotics interfere with host machinery needed for phage replication, it is then surprising that lytic phages can be combined with sublethal concentrations of antibiotics to effectively reduce bacterial load and manage resistance - a phenomenon known as phage-antibiotic synergy (PAS) (Chaudhry et al., 2017; Comeau et al., 2007; Kim et al., 2018). The concept originated with a 2007 study by Comeau et al. reporting that the plaques of phage φMFP, which infects *E. coli* MFP, were significantly larger in zones around sublethal concentration of β-lactam antibiotics aztreonam and cefixime (Comeau et al., 2007). They also showed that phage production increases in the presence of antibiotic. The PAS effects extended to other phages such as distantly related phages T4 and T7. The β-lactam class of antibiotics inhibits cell division and can potentially trigger the SOS response system. By carrying out experiments using *E. coli* SOS *lexA* mutant and mutants with altered cell filamentation responses, they were able to conclude that PAS is SOS-independent and a result of altered cell filamentation phenotype that allows for increased phage production.

This was also confirmed in another study in 2018 which showed that PAS occurs in both Gram-positive and Gram-negative bacteria and in the presence of other classes of antibiotics such as fluoroquinolones (Kim et al., 2018). Using *E. coli* Δ*recA* mutants, they also confirmed that PAS was SOS-independent and that while antibiotics do not affect phage adsorption, cellular filamentation and delayed lysis allow for increased phage progeny.

PAS been demonstrated against a number of hosts, including biofilm producing (Akturk et al., 2019; Chaudhry et al., 2017; Henriksen et al., 2019; Liu et al., 2020) and

antibiotic resistant strains (Engeman et al., 2021; Holger et al., 2022; Kamal & Dennis, 2015; Kebriaei et al., 2020; Liu et al., 2020; Rodriguez-Gonzalez et al., 2020), and has shown efficacy in infection-like conditions including serum and urine (Liu et al., 2020), as well as in clinical trials with patients suffering from multi-drug resistant *P. aeruginosa, S. aureus, Mycobacterium abscesses,* and pan-drug resistant *Achromobacter spp* (Aslam et al., 2020; Chan et al., 2018; Gainey et al., 2020). In fact, the 100 consecutive clinical cases from Belgium reports that the eradication of the pathogen was 70% less likely if no antibiotics were co-prescribed (Pirnay et al., 2024), highlighting that phages can work alongside antibiotics and that combination therapy is realistic approach to treating AMR infections.

1.11 Temperate Phages in Therapy

Current recommendations for phage therapy advise the use of strictly lytic phages (Pirnay et al., 2015; Suh et al., 2022). There are numerous concerns about the therapeutic application of temperate phages. Firstly, bacteria carrying prophages generally do not die. Lysogens exhibit superinfection immunity that protects them from subsequent phage infections. There are also concerns about potential phage mediated horizontal gene transfer which could facilitate the spread of antimicrobial resistance genes between neighbouring cells (Touchon, Bernheim, and Rocha 2016). However, the transfer of genetic material by phages has also been observed with virulent phages (Fineran et al., 2009; Wilson et al., 1979; Yasbin & Young, 1974). In addition, temperate phages can facilitate lysogenic conversion; the conversion of a nonvirulent strain to a virulent strain because of phage encoded toxins such as in cholera or enterohemorrhagic *E. coli* (Waldor & Mekalanos,

1996). However, phage toxins are also screened against when trying to identify virulent phages for therapy. Lastly, temperate phages can provide a source of genetic variation through repurposing of phage genes and as a product of their insertion into (and movement within) the bacterial genome, which can introduce novel mutations (Harrison & Brockhurst, 2017).

With recent renewed interest in phage therapy, isolating a strictly lytic phage for many pathogens presents a major challenge such as in the case of *Clostridioides* (previously *Clostridium*) *difficle* (Hargreaves & Clokie, 2014). Despites their drawbacks, it is important to note that the use of temperate phages could be highly beneficial. A study done in 2016 showed that nearly half of the sequenced bacterial genomes contain at least one dormant phage (Touchon et al., 2016). With advancements in bioinformatics tools, this estimate has increased to 75% (López-Leal et al., 2022). Temperate phages are far more abundant in nature and can be easily isolated using induction. They are also stably maintained long term in complex settings like the human gut (Shkoporov et al., 2019) suggesting they are probably active *in vivo*.

Studies exploring the potential usage of temperate phages in therapy in their natural state have been limited. Temperate phages isolated from clinical strains of *P. aeruginosa* were shown to decrease twitching motility, important for virulence, in lysogens (Chung et al., 2012). In addition, administration of temperate phage reduced bacterial counts and toxin production in an *in vitro* human colon model of *C. difficle* infection (Meader et al., 2013). Unfortunately, this was accompanied with an increase in spore formation which may increase the risk of subsequent infection. To limit emergence of resistances, several studies

have proposed the use of temperate phage cocktails for *C. difficle* (Nale et al., 2016, 2018). More recently, temperate phage of *A. baumannii* was observed to reduce mortality in a zebrafish model (Mardiana et al., 2023). The isolation and use of virulent mutants of temperate phages that can infect a lysogenic host has also been proposed for *P. aeruginosa* infections (Bourkal'tseva et al., 2011).

Researchers have experimented with genetically engineering temperate phages to remove lysogeny associated genes or use them as vehicle for delivering cargo that directly targets the bacteria. A group in 2018 designed synthetic genomes of *Listeria monocytogenes* temperate phages that lacked either the repressor of lysis or the entire lysogenic control region, including the integrase (Kilcher et al., 2018). The genetically engineered phages had a dramatically improved killing efficacy compared to wild type. In addition, knocking out the cI repressor and integrase from a *C. difficle* phage reduced fecal bacterial burden in mice by 2-logs relative to vehicle or wild type phage administration (Selle et al., 2020). The same phage could also be engineered to deliver a bacterial genome-targeting CRISPR RNA with improved *C. difficle* killing *in vitro* and *in vivo*. In 2019, a three-phage cocktail containing two engineered lytic derivatives of temperate phages was used for the treatment of a 15-year-old cystic fibrosis patient with a *M. abscessus* infection (Dedrick et al., 2019). The two engineered phages were also included in a bigger trial with 20 patients reporting favourable clinical outcomes (Dedrick et al., 2023). Despite the promising outcomes of temperate phage engineering, this approach is time consuming and not practical for large scale therapeutic use.

1.12 Research Aim

We have known for some time now that lysogens can be induced when exposed to stressors, many of which are known SOS response-inducing antibiotics. Supported by this idea, we sought to investigate if co-administering temperate phages with a known inducer such as an antibiotic can sufficiently bias the lysis-lysogeny equilibrium to select against lysogen survivors (chapter 2). In addition, would the resulting synergy be mechanistically distinct from PAS observed with lytic phages (chapter 2)? If so, is this a broadly generalizable phenomenon expanding across antibiotics, phages, and other hosts (chapter 3), and could it have applications *in vivo* (chapter 4)? This would be a scalable approach for the use of previously discarded temperate phages, in combination with antibiotics, for the treatment of antimicrobial resistant infections.

Chapter 2 - Temperate phage-antibiotic synergy eradicates bacteria through depletion of lysogens

2.1 Preface

Given antibiotics ability to induce prophages in several hosts (Goerke et al. 2006; Maiques, Ubeda, et al. 2006, Bearson and Brunelle 2015; López et al. 2014; Walterspiel et al. 1992), we sought to investigate if temperate phages can act as adjuvants to increase effectiveness of ciprofloxacin, a commonly used antibiotic for treating *E. coli* infections (Kwong & Lindsay Grayson, 2017). This chapter aims to investigate if a synergistic interaction exists between model *E. coli* Lambdoid temperate phage HK97 and sub-lethal ciprofloxacin (tPAS) and whether this interaction is mechanistically distinct from virulent PAS, specifically acting on the phage lysis-lysis equilibrium, biasing towards a lytic infection.

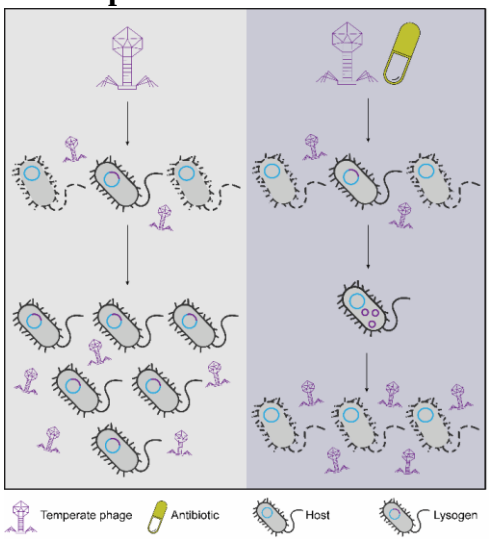
The work presented in this chapter has been previously published in the following paper (Reprint permission for this paper is granted by journal publisher. See Appendix A for approval).

Al-Anany, A. M.*, **Fatima, R.*,** & Hynes, A. P. (2021). Temperate phage-antibiotic synergy eradicates bacteria through depletion of lysogens. Cell Reports, 35(8).
*Equal contribution

2.2 Author contributions

A.M.A. performed all of the assays in Figures 2, 3, 5A, and 6, including a complete re-design for the overnight assays (Figure 3) and performing all assays in Figures S1 and S2. R.F. performed all of the assays in Figures 4 and 5B, including the design of all qPCR assays, and performing all of the assays in Figure S3. A.P.H. conceived of the study and performed pilot studies for Figure 2A. All of the authors contributed to the writing of the manuscript.

2.3 Graphical abstract



2.4 In Brief

Most bacterial viruses (phages) cannot be used in therapy as they are temperate and go dormant in their hosts. Al-Anany et al. eradicate *Escherichia coli* through the synergistic effects of a temperate phage and signals that awaken it. This targets conserved pathways and may be broadly applicable for phage therapy.

2.5 Summary

There is renewed interest in bacterial viruses (phages) as alternatives to antibiotics. All phage treatments to date have used virulent phages rather than temperate ones, as these can integrate into the genome of the bacterial host and lie dormant. However, temperate phages are abundant and easier to isolate. To make use of these entities, we leverage stressors known to awaken these dormant, integrated phages. Co-administration of the temperate phage HK97 with sub-inhibitory concentrations of the antibiotic ciprofloxacin results in bacterial eradication (≥8 log reduction) *in vitro*. This synergy is mechanistically distinct from phage-antibiotic-synergy described for virulent phages. Instead, the antibiotic

specifically selects against bacteria in which the phage has integrated. As the interaction between temperate phages and stressors such as ciprofloxacin are known to be widespread, this approach may be broadly applicable and enable the use of temperate phages to combat bacterial infections.

2.6 Introduction

Antimicrobial resistance (AMR) is a widespread health challenge (World Health Organization, 2014) that has led to a renewed interest in bacteriophage therapy as a promising alternative to antibiotics (Cooper et al., 2016). Bacteriophages (bacterial viruses, phages) are obligate parasites that exclusively infect bacteria (D'Hérelle, 1917). Upon infection, a phage will canonically end up in one of two life cycles: lytic or lysogenic (Oppenheim et al., 2005). In the case of virulent phages, the phage is strictly lytic. It quickly redirects the host machinery for its own DNA replication and protein synthesis, forming new phage particles (Howard-Varona et al., 2017). This cycle ends in host cell lysis and release of new virions that can go on to infect neighboring cells (Howard-Varona et al., 2017). In contrast, temperate phages may also choose to undergo a lysogenic life cycle, often integrating their genome into the bacterial chromosome (Czyz et al., 2001; Howard-Varona et al., 2017). As the host cell divides, a copy of the largely dormant phage (prophage) is vertically inherited. The bacterium carrying a prophage is referred to as a lysogen. In a process known as induction, upon exposure to external stressors (Czyz et al., 2001; Drulis-Kawa et al., 2012), the prophage is stimulated to awaken and switch to a lytic cycle (Howard-Varona et al., 2017).

Phage therapy in the clinic makes use only of virulent phages, as they do not risk a lysogenic cycle (Abedon, Kuhl, et al., 2011; Dedrick et al., 2019). Even regulatory agencies that have proceeded the furthest in approving phage therapy only allow for the use of virulent phages (Abedon, Kuhl, et al., 2011). The lysogenic life cycle is an enormous hurdle for the use of temperate phages in therapy for several reasons. First, if the phage integrates its genome into the host, then it will not have an immediate bactericidal effect (Fortier & Sekulovic, 2013). Second, the lysogenic cycle can affect host fitness and may leave the host with more virulence traits via the integrated prophage or via transduction by horizontal gene transfer (Fortier & Sekulovic, 2013). However, virulence factors are already screened for in phages used in therapy, and as transduction is also very common in virulent phages (Drexler, 1970; Ely & Johnson, 1977; Fineran et al., 2009), it cannot be avoided (Monteiro et al., 2019). A recent study has even highlighted that virulent phages may be more problematic because they can transduce a wider variety of genes across larger phylogenetic distances (Moura de Sousa et al., 2021). Furthermore, the lines between virulent and temperate phages are increasingly blurred (Song et al., 2019). In contrast, temperate phages with *cos* sites, high packaging specificity, and a known integration site near "harmless" genes, such as phages Lambda and HK97 (Tal et al., 2014), pose little threat from this perspective. Critically, however, the lysogenic life cycle will typically protect the host cell from further phage infections (Sun et al., 2006) through mechanisms such as superinfection immunity (Zinder, 1958).

Superinfection immunity is a property acquired by the bacterial host during lysogeny (Zinder, 1958). While the prophage regulates its lytic gene expression to maintain

a lysogenic life cycle, it also grants a fitness advantage to its host, making it resistant to subsequent phage infections, known as superinfections (Mavrich and Hatfull 2019; Sun et al. 2006). The well-studied model temperate phage lambda expresses the repressor cI to maintain a lysogenic life cycle. The excess repressor molecules produced inside the lysogen bind to operators in the genome of invading phages, blocking transcription and preventing a secondary infection (Fogg et al., 2011; Ptashne, 2004). The next phage can enter neither the lytic nor the lysogenic cycle. Prophages can also offer protection through superinfection exclusion, whereby the prophage encodes a protein that prevents the injection of DNA by other phages, as is the case in the lambda-like phage HK97 (Cumby et al., 2012; Uc-Mass et al., 2004). *In vitro*, a bacterial culture challenged with a temperate phage will regrow as a result of this phage-generated resistance, in addition to any other phage-selected resistance, which are a concern for virulent and temperate phages alike (He et al., 2016; Oechslin, 2018).

Despite these drawbacks, it is important to note that temperate phages have enormous unexploited potential as therapeutic agents. Phage therapy has been constrained by the difficulty in isolating virulent phages for many pathogens such as *Clostridioides* (formerly *Clostridium) difficile* (Hargreaves & Clokie, 2014; T. Zuo et al., 2018). In contrast, temperate phages are easier to find in nature; nearly half of the sequenced bacterial genomes contain at least one prophage (Touchon et al., 2016). Metagenomic analysis of fecal viral samples from healthy adults showed that these phages are stably maintained in settings such as the human gut over long periods of time, suggesting that they are active *in vivo* (Shkoporov et al., 2019).

Illustrating the therapeutic need for temperate phages is the drive to "make" them virulent. This includes isolating natural virulent mutants that can infect a lysogenic host or making use of genetic engineering approaches to remove components required for successful lysogenic events (Bourkal'tseva et al., 2011; Dedrick et al., 2019; Kilcher et al., 2018). In 2019, a phage cocktail that included an engineered virulent variant of a temperate phage was used for the successful treatment of a cystic fibrosis patient with a *Mycobacterium abscessus* infection (Dedrick et al., 2019). While engineering approaches show promise, they are time-consuming and not practical for large-scale application of temperate phages for therapy.

Given the challenges inherent to these approaches, there have been few studies examining the therapeutic potential of temperate phages without modification. In an *in vitro* human colon model of *C. difficile* infection, temperate phage administration reduced bacterial load and prevented toxin production (Meader et al., 2013). However, this was accompanied with an increase in spore formation, which may increase the risk of the re-emergence of infection. Several studies have also proposed the use of temperate phage cocktails for *C. difficile* (Nale et al., 2016, 2018). In addition, twitching motility, important for virulence, decreased in *Pseudomonas aeruginosa* strains lysogenized with temperate phages isolated from clinical strains (Chung et al., 2012). Phage administration also reduced bacterial load in mice and *Drosophila* models of *Pseudomonas* infection (Heo et al., 2009). Despite this promise, the stance of regulatory bodies on lysogeny mean these strategies are unlikely to make their way to the clinic in the short term.

One method of increasing the effectiveness of phages in general is through phage-antibiotic synergy (PAS) (Berryhill et al., 2021; Chaudhry et al., 2017; Comeau et al., 2007; Kamal & Dennis, 2015; Kebriaei et al., 2020; Nilsson, 2014; Rodriguez-Gonzalez et al., 2020; Tagliaferri et al., 2019). This phenomenon is characterized as the use of phages, in combination with antibiotics, to effectively reduce bacterial load and manage both phage and antibiotic resistance (Comeau et al., 2007). PAS has been studied since 2007 in virulent phages with antibiotics such as fluoroquinolones and β-lactams (Chaudhry et al., 2017; Comeau et al., 2007; Kamal & Dennis, 2015; Kim et al., 2018; Nilsson, 2014; Tagliaferri et al., 2019). Traditional PAS is proposed to be a result of antibiotic-induced cellular filamentation and resulting changes in phage replication, which help to increase phage production (Comeau et al., 2007; Kamal & Dennis, 2015; Kim et al., 2018). These antibiotics are known to trigger the bacterial DNA repair pathway known as the SOS response, but curiously that pathway does not appear to be necessary for synergy (Comeau et al., 2007; Kim et al., 2018). This is of particular interest in the context of temperate phages given that this stress response canonically results in phage induction in many model lysogens (DeMarini & Lawrence, 1992; Goerke et al., 2006; Maiques et al., 2006).

Here, we challenge bacteria with subinhibitory concentrations of antibiotics and temperate phages to bias the lysis/lysogeny decision in favor of a lytic life cycle. This proposed synergy, unique to temperate phages, would be temperate PAS (tPAS). Administered antibiotics may suffice on their own in uncomplicated and non-resistant infections. However, in case they are not, at inevitable sub-minimal inhibitory concentration (sub-MIC), they will stress the cells enough that any temperate phage within

will not be able to lysogenize (He et al., 2016). While any SOS response-inducing agent would suffice to achieve this, we choose to use antibiotics because phages must prove themselves to work along with antibiotics, the current standard of care in clinic.

2.7 Results and Discussion

2.7.1 Temperate phages and antibiotic synergistically inhibit bacterial growth

Our model phage, HK97, is closely related to lambda, the well-studied model for lysogeny and induction (Casjens & Hendrix, 2015; Dhillon et al., 1980; Heinemann & Howard, 1964; Weigle, 1953). Used alone, it is ineffective at reducing bacterial populations; a subset of phage-generated resistance events will rapidly overtake the population and result in their rapid growth both in solid (Figure 2A, II) and in liquid media (Figure 2B). This clearly illustrates the largest challenge facing the use of temperate phages in therapeutic applications.

Challenging our bacterial host with ciprofloxacin at sublethal ½ MIC together with HK97 at a multiplicity of infection (MOI) of ≥10 resulted in a synergistic effect (Figure 2A, IV). This synergy is revealed in the inhibition of bacterial growth with phage and antibiotic treatment compared to a bacterial lawn when challenged with just one of the two agents alone.

To more thoroughly investigate the dynamics of this synergy, we repeated our challenge assay in broth (Figure 2B), following the results over time. While the phage treatment alone almost resulted in a bacterial eradication after 2 h, the host was able to overcome this challenge and regrow. However, challenging the bacteria with both phage and antibiotic resulted in no detectable re-growth. To establish the range of antibiotic and

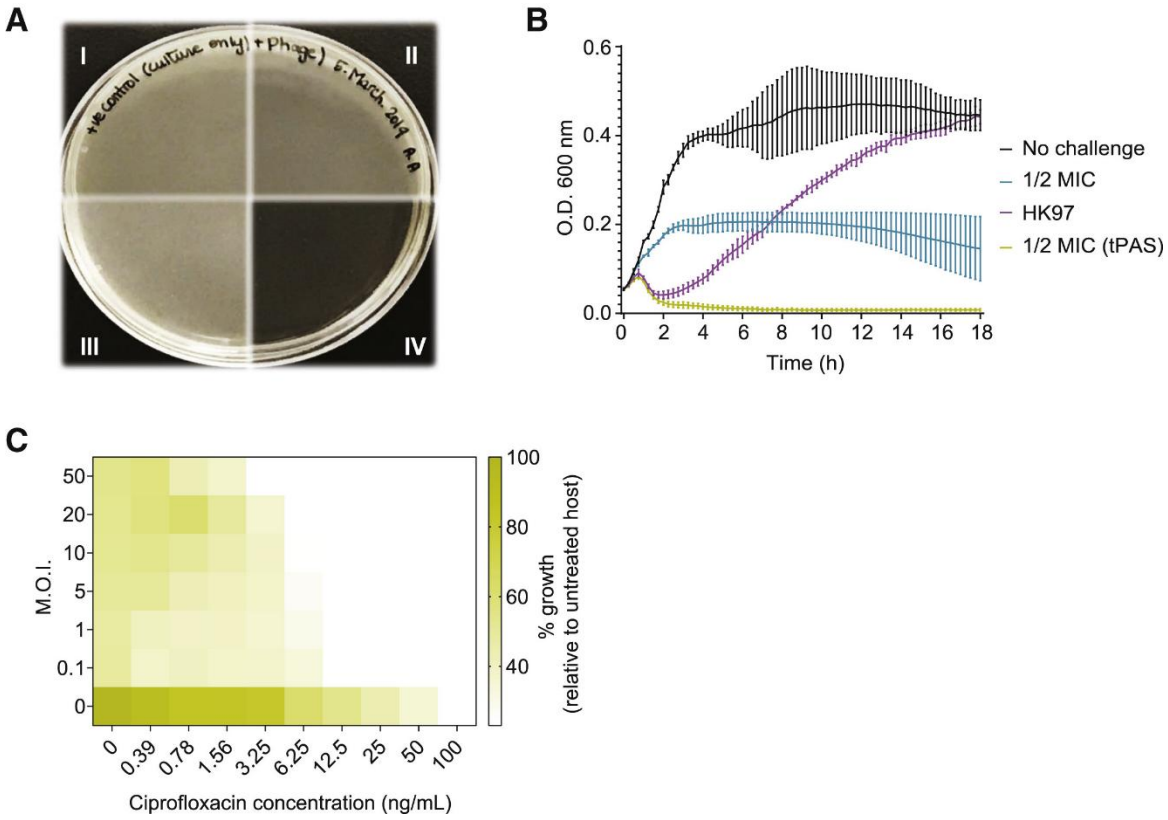


Figure 2. Infection of *E. coli* K-12 with the temperate phage HK97 in the presence of ciprofloxacin leads to a synergistic growth inhibition. **(A)** Illustrative images from 3 replicates of HK97 ciprofloxacin synergy in solid media. (I) Unchallenged *E. coli* K-12 resulting in a lawn of bacterial growth that is similar to (II) *E. coli* K-12 challenged with HK97 phage (MOI = 10) and (III) *E. coli* K-12 challenged with ciprofloxacin at a concentration ½ MIC. (IV) *E. coli* K-12 challenged with both HK97 (MOI = 10) and ciprofloxacin (½ MIC), resulting in no growth of a bacterial lawn, and only a few surviving colonies. **(B)** Growth curves in liquid culture for wild-type *E. coli* K-12 among 3 biological replicates ± standard deviation (SD), each of 2 technical replicates. Growth was tracked for no challenge, as well as challenges with phage at MOI = 10, antibiotics at ½ MIC, and with both phage and antibiotic combined. **(C)** Checkerboard assay. Endpoint growth relative to untreated bacterial control, averaged among 3 biological replicates, plotted as a heatmap.

phage concentrations that could produce this effect, we used a checkerboard assay (Martinez-Irujo et al., 1996). Infecting the bacterial host with temperate phages and ciprofloxacin reduced the antibiotic MIC 8-fold at a MOI of 0.1–10, 16-fold at MOI ≥ 20, and 32-fold at a MOI ≥ 50 (Figure 2C). These findings demonstrate that even at low doses (MOI = 0.1), the phage contributes to this synergy. While previous studies have

demonstrated that higher MOIs favor lysogeny (Trinh et al., 2017; L. Zeng et al., 2010), with tPAS, higher phage doses reduced antibiotic MICs, despite the lack of any effect on growth inhibition in the absence of the antibiotic (Figure 2C, leftmost column). Our assays established a clear synergistic inhibition of growth, but do not confirm a bactericidal effect, nor do they offer enough resolution to quantify the synergy.

2.7.2 Bactericidal effect of tPAS using quantification assay

To better quantify the effect of this synergy over a range of antibiotic concentrations, we exposed bacteria to phages and antibiotic for either 1 h, 1.5 h (Figure S1), or overnight (Figure 3A), then counted the number of survivors (colony-forming units [CFUs]). Our results demonstrated a mere 10-fold reduction in endpoint CFU for the phage alone condition regardless of exposure time, and a more pronounced exposure-dependent effect, rising from 10-fold (1 h, Figure S1) to 180-fold reduction (overnight) for the antibiotic at MIC. This inhibitory effect decreased with decreasing concentration of antibiotic (Figure 3A), regardless of exposure time (Figure S1). These recapitulate the poor efficacy of temperate phages alone, and that while the antibiotic affects the bacteria at these concentrations, it is far from sufficient to eradicate a bacterial population, even upon prolonged exposure.

In contrast, challenging our host with phage HK97 in the presence of ciprofloxacin at either MIC or at ½ MIC resulted in bacterial eradication ($\geq 2.71 \times 10^8$-fold reduction in colonies at endpoint relative to the control) with the overnight incubation. To calculate fold reduction arising from antibiotic concentrations at MIC and ½ MIC tPAS, the actual number of survivors (0) was substituted by the limit of detection (LOD) of 10 CFU/mL.

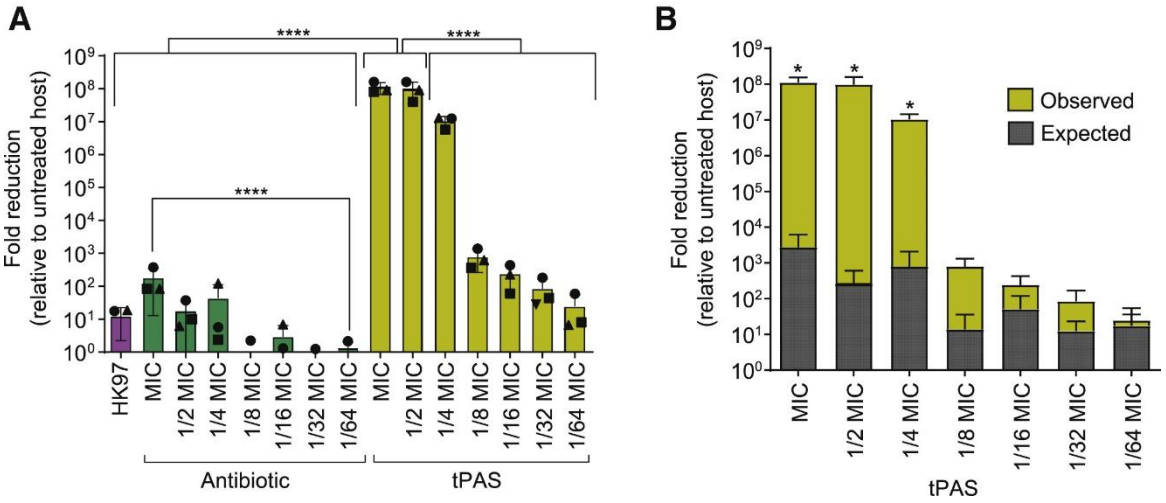


Figure 3. Overnight HK97 temperate phage ciprofloxacin synergy quantification. (A) Bars show average number of survivors relative to untreated cultures in 3 biological replicates, each of 3 technical replicates. Each biological replicate is represented by its own shape: circle, square, or triangle. LOD (10 CFU/mL) is represented at all points of MIC and ½ MIC tPAS data, as no counts were obtained, except "square" at ½ MIC. Error bars depict the SD, while **** $p \leq 0.0001$, from a 1-way ANOVA and Tukey post hoc test. **(B)** Bars show observed effect (yellow) versus expected (gray) effect. Average from the 3 biological replicates for each observed tPAS data from Figure 3A was compared to the calculated expected effect at the corresponding antibiotic concentration using paired t test, with $^{*}p \leq 0.05$. Assays depicting the effects of tPAS after only 1 or 1.5 h are in Figure S1.

This is particularly impressive, as the 18 h incubation would allow any survivors to regrow to a high density, should they emerge. However, the longer incubation also allows the untreated control to continue growing, which increases the perceived magnitude of the synergy slightly: a comparison of 18 h tPAS to the untreated control at 1 h (rather than at 18 h) would instead reveal a $\geq 1.04 \times 10^7$-fold reduction.

To our knowledge, no previous assay depicting PAS with virulent phages has shown an effect as pronounced as these on colony counts, with a single study reporting eradication (a 9-log reduction), but only with 8× MIC antibiotics (Akturk et al., 2019), while others found 2-log–3.3-log reductions (Kamal & Dennis, 2015; Knezevic et al., 2013; Oechslin et al., 2017) at their endpoints with subinhibitory antibiotics. However, the timing for each of

these is different, making direct comparisons challenging, especially considering our own 1- and 1.5-h exposures resulted in comparatively modest 2–3 $\log_{10}$ reductions, none of which reached statistical significance from the phage-alone control (Figure S1).

Our results revealed a dose-dependent synergy, with the number of survivors increasing with decreasing antibiotic concentrations. The highest concentration that yielded survivors, ½ MIC, still gave rise to a >7-log reduction in the number of survivors at endpoint. The clearest evidence for a strong synergistic effect upon using phage HK97 with ciprofloxacin was obtained by comparing the observed data to the expected multiplication of the independent effects of phage and antibiotics (Figure 3B). Our approach yielded in a massive synergy peaking at ½ MIC, resulting in a 9.7×10^7-fold reduction in CFU/mL, which is 4.5×10^5-fold higher than the expected effect. We observed the same promising effect across lower concentrations than those of MIC.

This combination treatment could be potentiating the killing effect of the antibiotic or that of the phage. If it is the latter, then it could simply be traditional PAS. If known mechanisms of PAS are responsible, then this should be associated with characteristic features of PAS, such as increases in phage titer (Comeau et al., 2007; Kamal & Dennis, 2015; Kim et al., 2018).

2.7.3 Temperate phage-antibiotic synergy is different from traditional PAS

In establishing the mechanistic basis of our tPAS, we first sought to eliminate the known mechanisms behind the well-studied phenomenon of traditional PAS. In these cases, the presence of antibiotic is associated with increased phage production, often including

increases in both plaque size and phage burst size (Comeau et al., 2007; Kamal & Dennis, 2015; Kim et al., 2018).

To determine whether temperate PAS was driven by increase in phage production, the plaque size of HK97 was visualized in the presence of sublethal concentrations of ciprofloxacin. While the phage alone produces small plaques, the plaque size increases with the addition of the antibiotic in a dose-dependent manner (Figure 4A), consistent with previous studies on traditional PAS (Comeau et al., 2007; Kamal & Dennis, 2015; Kim et al., 2018).

In addition to examining plaque size, we also quantified phages in filtrates obtained from the assay in Figure 3A to determine whether this synergistic interaction was associated with increased phage production. Our results demonstrated a positive correlation between synergy and phage titer, but the effect was minimal, with the only statistically significant change being a 3-fold increase at ½ MIC (Figure 4B). By comparison, much higher fold increases in phage titer were observed in traditional PAS studies. Ryan et al. (2012) showed a positive correlation between antibiotic challenge concentration and phage titer, with a maximum of >5-log fold increase. Complementary to these results, Comeau et al. (2007) demonstrated 9- and 11-fold increases in phage T4 titer with 200 ng/mL mitomycin C and 30 ng/mL cefotaxime, respectively. These findings suggest that our synergy is distinct from the traditional phage antibiotic synergy and that the demonstrated reduction in the number of survivors is not driven by a greater number of phages.

Multiple studies exploring the mechanism of traditional PAS have suggested that antibiotics promote accelerated phage-mediated lysis, supported by the observation that

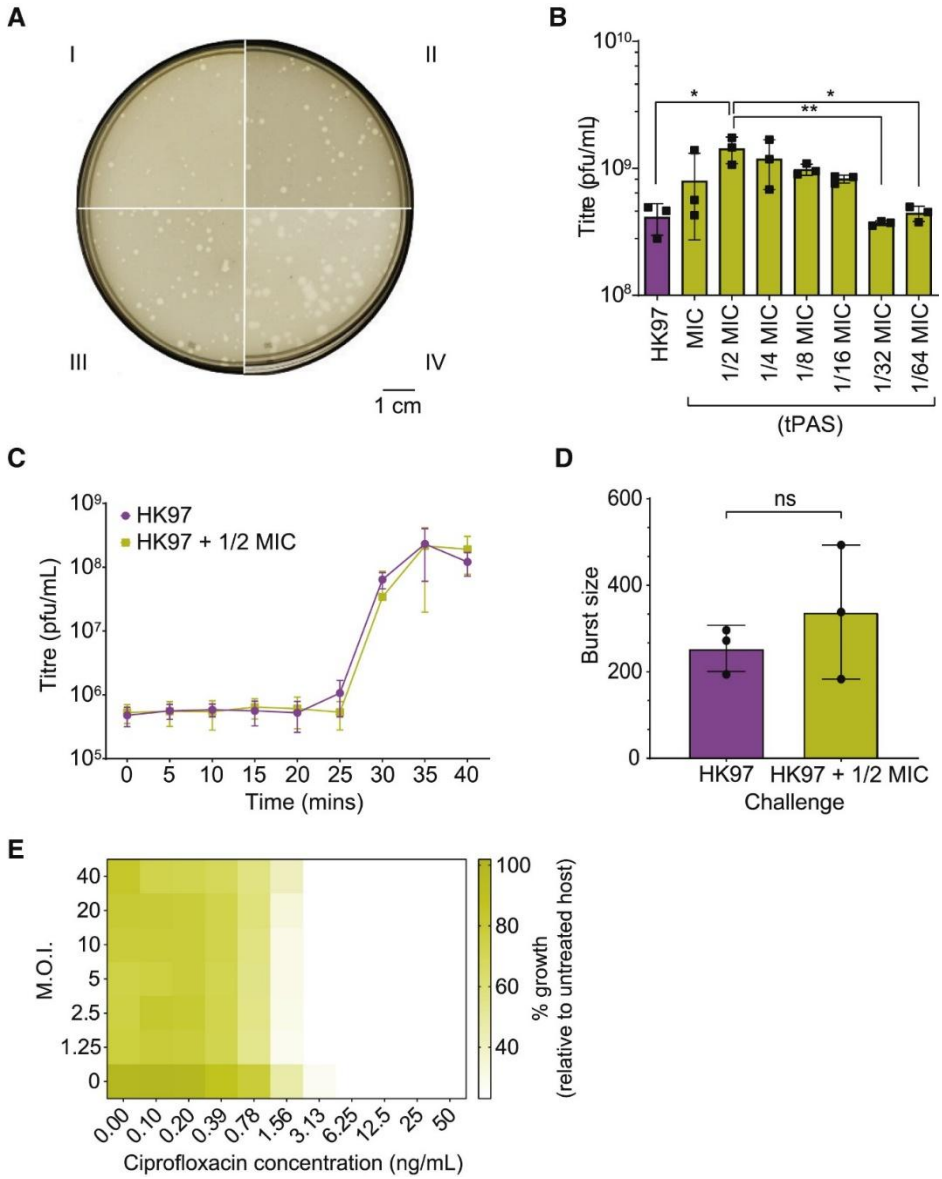


Figure 4. Temperate phage-antibiotic synergy is not driven by traditional PAS. **(A)** Illustrative image of phage HK97 plaque size on *E. coli* K-12 in the absence and presence of ciprofloxacin. I) phage alone, II) phage + 1/16 MIC, III) phage + 1/8 MIC, IV) phage + ¼ MIC. The scale bar depicts 1 cm. **(B)** Phage quantification of tPAS challenges. Points show an average number of plaque-forming units ± SD (n = 3). Phage titer at each antibiotic challenge was compared by 1-way ANOVA and Tukey post hoc test, with $^{*}p \leq 0.05$. and $^{**}p \leq 0.01$. **(C)** HK97 replication curve in *E. coli* K-12 (n = 3). Each point indicates average ± SD. **(D)** Average burst size ± SD in the absence and presence of sublethal concentration of ciprofloxacin at ½ MIC. Average burst size compared using unpaired t test, with ns, not significant ($p = 0.2117$). **(E)** Checkerboard assay of HK97 and ciprofloxacin in *recA* mutant. Endpoint growth relative to untreated bacterial control, averaged among 3 biological replicates, plotted as a heatmap.

sublethal concentrations of β-lactams and fluoroquinolones have been shown to result in cellular filamentation (Comeau et al., 2007; Kamal & Dennis, 2015). Accelerated lysis could be a result of cell wall damage that increases sensitivity to phage lysis proteins and/or increase in the number or expression of phage receptors on a filamented cell surface (Comeau et al., 2007; Kamal & Dennis, 2015). In contrast, Kim et al. (2018) demonstrated that the increased phage production observed in traditional PAS is a result of additional time available for particle assembly due to a longer phage latency period (Kim et al., 2018).

To determine whether the antibiotic has any effects on the replicative parameters of the temperate phage, we carried out a one-step growth curve of phage HK97 in the absence/presence of a sub-inhibitory concentration of ciprofloxacin (Ellis & Delbrück, 1939). In the absence and presence of ciprofloxacin, the latency period of phage HK97 remains unaffected by the antibiotic (Figure 4C). Unlike traditional PAS, tPAS is not driven by any changes in the time of lysis. Burst size was calculated as the number of new phage particles released per infected bacterium. There was a high degree of variability in the calculated burst size in the absence and presence of the antibiotic (Figure 4D). The average fold difference was 1.45 ± 0.96-fold (n = 3, mean ± SD), and not significantly different—falling short of reported increases in burst size for PAS (e.g., >10.5-fold increase observed with ciprofloxacin and 7-fold with cefotaxime) (Comeau et al., 2007; Kim et al., 2018). Even our largest burst-size difference within biological replicates was only 4.1-fold.

Finally, to further distinguish our synergy from PAS, where the bacterial SOS response is not required for synergy (Comeau et al., 2007; Kim et al., 2018), we challenged an *E. coli recA* mutant with phage and antibiotics (Figure 4E). The mutant was more

sensitive to the antibiotic alone, consistent with the role of RecA (Recacha et al., 2017) (Figure 4E versus Figure 2C). However, the synergy previously observed (Figure 2C) was lost entirely in this genetic background.

Overall, we could not attribute the strong synergistic effect to either accelerated or delayed lysis. We also did not see evidence of a significant increase in phage production or burst size. The reduction in the number of survivors observed at higher antibiotic concentrations is likely not the result of increased phage titer. Furthermore, the requirement for RecA further suggests that tPAS is not like traditional PAS described for virulent phages and works through a distinct mechanism.

2.7.4 Temperate phage-antibiotic synergy depletes the lysogen population

To provide further evidence that tPAS is mechanistically distinct, we investigated whether the observed effect is driven by the prevention of lysogeny. To determine the efficiency of our synergy in reducing the frequency of lysogeny, the integration of HK97 into the host chromosome was confirmed by colony PCR using primers designed to detect the phage-host junction. Results of this assay showed that challenging the host overnight reduced the percentage of lysogeny from 98% (n = 55) without antibiotic and 100% (n = 52) with ⅛ MIC down to 32% (n = 52) at ½ MIC (Figure 5A) – the highest concentration at which we obtained any survivors to test (Figure 3A).

We confirmed phage resistance by re-challenging all 52 survivors from ½ MIC (Figures S2A and S2B), all of which were unaffected by re-exposure to the phage. For four purified non-lysogens tested, we determined that resistance was due to a failure of phages to adsorb (Figure S2C), which is characteristic of surface receptor mutants that most

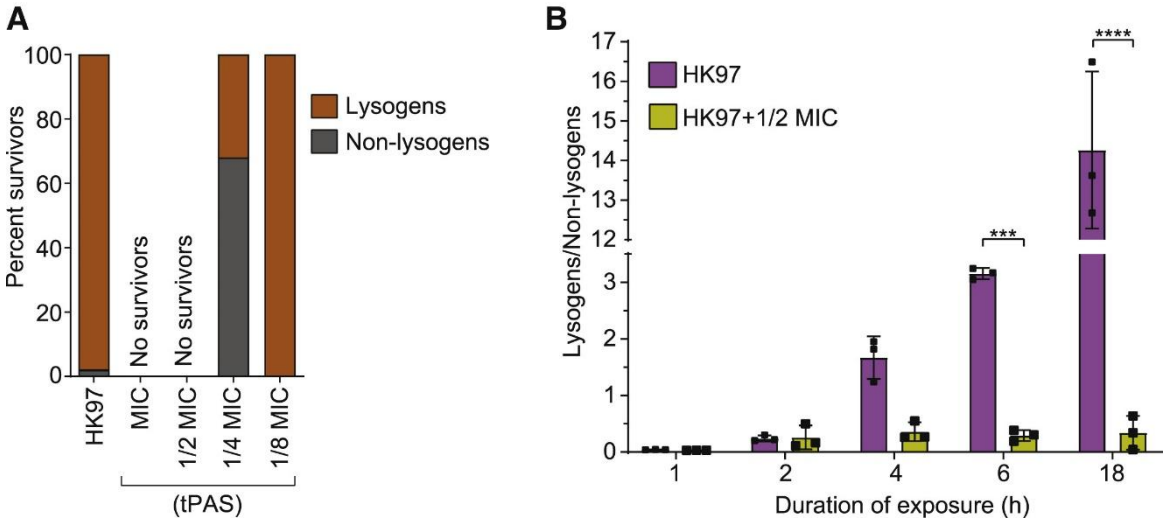


Figure 5. Temperate phage-antibiotic synergy is a result of a depletion of lysogens. (A) Percentage of lysogen and non-lysogen survivors after overnight HK97 and ciprofloxacin challenges. Further characterization of survivors is found in Figure S2. **(B)** Ratio of lysogens to non-lysogens tracked over time for the HK97 or the HK97 and ciprofloxacin challenge using qPCR at 5 time points (n = 3 performed in technical triplicates) for ½ MIC antibiotics. The same assay with MIC antibiotics is found in Figure S3. Error bars represent SD. Lysogen/non-lysogen ratio compared using 2-way ANOVA and Bonferroni multiple comparison tests. $^{***}p \leq 0.001$ and $^{****}p \leq 0.0001$.

commonly arise from a virulent phage challenge in *E. coli* under laboratory conditions (Luria & Delbrück, 1943; Werts et al., 1994). The mechanism of resistance of the other lysogens was not determined, but HK97 is known to provide both superinfection immunity and superinfection exclusion against related phages (Bondy-Denomy et al., 2016; Cumby et al., 2012; Fogg et al., 2010). As the lysogens remained sensitive to λvir (Figure S2), which bypasses these two protections, we have no reason to assume that any other resistance mechanisms are at play.

The ciprofloxacin concentration-dependent bias toward non-lysogen survivors could be a result of the antibiotic biasing the initial lysis-lysogeny decision made by the temperate phage at the time of infection, or a result of lysogens forming but then

subsequently being induced by the antibiotic, as lysogens are known to be more susceptible to antibiotics due to induction (Ronayne et al., 2016).

To disentangle these two not mutually exclusive hypotheses, we used qPCR to detect the frequency of lysogens and non-lysogens over time using primers designed to detect the presence or absence of the phage-host junction in the bacterial genome. Furthermore, as we were detecting phage-host junctions in genomes and not surviving bacteria, we could perform our assays even at the concentrations at which our tPAS proved successful in eradicating bacteria.

Five time points were sampled based on results from the tPAS growth curves in Figure 2B. At the initial 1- and 2-h marks, the ratio of lysogens to non-lysogens remains unchanged (Figure 5B), which is consistent with the lack of detectable synergy in killing assays after 1 and 1.5 h of exposure (Figure S1). This suggests that the presence of the antibiotic does not bias the initial lysis-lysogeny decision made by the temperate phage.

Over 18 h, there is an increasing fraction of lysogens when challenged with phage alone (Figure 5B), as would be expected given selection acting on the phage-mediated protection from subsequent infections. In contrast, when challenged with phage in the presence of the antibiotic, the ratio of lysogens to non-lysogens remains steady after 2 h. There is a 1.3 ± 0.2-fold, a 1.2 ± 0.6-fold, a 5.1 ± 1.7-fold, an 11.7 ± 4.3-fold, and a 130 ± 166.6-fold (mean ± SD, n = 3) difference in the ratio of lysogens to non-lysogens in the presence of the antibiotic at 1, 2, 4, 6, and 18 h, respectively. The same trends were also observed at MIC with a more pronounced effect (Figure S3). These findings suggest that

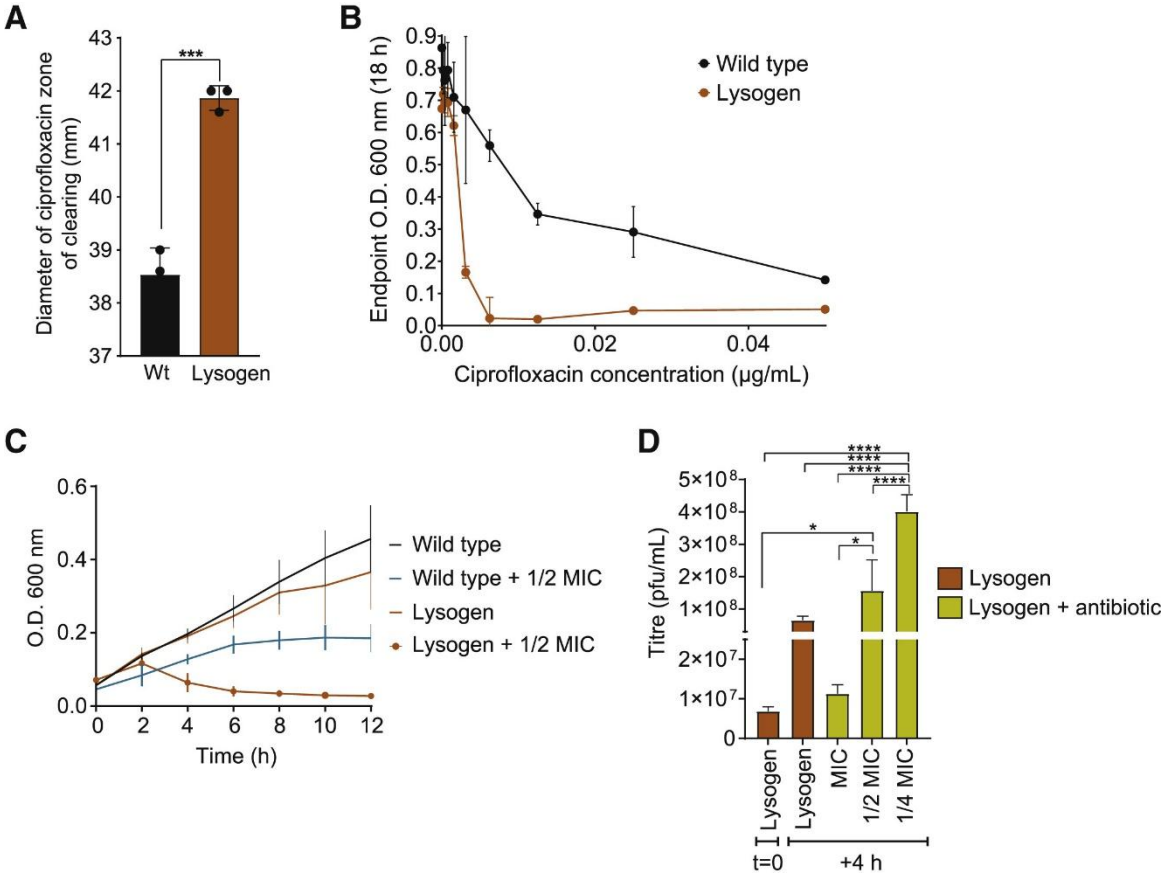


Figure 6. Ciprofloxacin sensitivity and prophage induction of HK97 lysogen. (A) Lysis zone obtained from Kirby-Bauer ciprofloxacin disc diffusion assay was averaged among 3 biological and technical replicates ± SD, plotted, and compared using unpaired t test, with $^{***}p \leq 0.001$. **(B)** MIC in liquid culture for wild-type *E. coli* K-12 and lysogen control was tracked after challenging with serial dilutions of ciprofloxacin (means ± SD, n = 3). MIC is ~0.05 µg/mL for wild type and 0.00625 µg/mL for lysogen. **(C)** Growth curves in liquid culture of wild-type *E. coli* K-12 and lysogen control tracked in the absence and presence of ciprofloxacin at ½ MIC, averaged among 3 biological replicates ± SD. **(D)** Phage quantification at time 0 for lysogen control and after 4 h when challenged with serial dilutions of ciprofloxacin, averaged among 3 technical replicates ± SD. Titer was compared using 2-way ANOVA and Tukey multiple comparison tests. $^{*}p \leq 0.05$ and $^{****}p \leq 0.0001$.

the antibiotic prevents the formation of lysogen colonies over time, by selecting against their expansion.

To confirm that HK97 lysogens behave canonically (Goerke et al., 2006; Soberón et al., 2007) in such a way that ciprofloxacin would select against their expansion, we compared the antibiotic sensitivity of the lysogen to the parent bacterium. Kirby-Bauer

assays confirmed an increased sensitivity to ciprofloxacin (Figure 6A), which, by MIC determination curves, was found to be 4-fold relative to the parent bacterium (Figure 6B), consistent with the clear synergy we observe down to ½ MIC (Figure 3). To ensure this corresponded to phage induction, we observed the characteristic rise-and-fall induction growth curve of the lysogen exposed to ½ MIC antibiotic (Figure 6C), accompanied by an increase in HK97 phage titer at 4 h post-induction at both ½ and ¼ MIC (Figure 6D). The antibiotic concentrations we use in our tPAS would induce lysogens.

2.8 Conclusion

Here, we have evaluated, with phage HK97 and ciprofloxacin, an *in vitro* proof-of-principle of the potential of temperate phages in combination with an antibiotic for therapeutic use. While this synergistic interaction is both antibiotic and phage dose dependent, it is far more sensitive to the antibiotic. Complete bacterial eradication can be achieved with the correct sub-inhibitory antibiotic concentrations. This synergy is unlike that previously described with virulent phages, as it works through a distinct mechanism in which the antibiotic does not merely increase phage production, and acts through the RecA protein, a key element of the bacterial SOS response.

Moreover, our data show that the antibiotic does not bias the initial phage decision toward lysis. The synergy is instead far more pronounced at time points well after the initial infection would be completed (Figure 3 versus Figure S1), and the fraction of lysogens versus non-lysogens, as detected by qPCR, only displays significant changes in the presence of ciprofloxacin after 6 h - a full ~12 generations into the experiment (Figure 5B). Instead, the underlying mechanism is likely a result of lysogeny occurring followed by the

presence of the antibiotic causing prophage induction and selecting against lysogens (Figure 6). The end result is that the synergy eradicates *E. coli* and prevents the survival of lysogens, the emergence of which is the biggest hurdle in the current use of temperate phages in therapy.

While we were able to demonstrate a powerful synergy between temperate phage model HK97 and ciprofloxacin in host *E. coli* K-12, we believe that this is a general phenomenon that exploits the sensitivity of the phage repressor, responsible for superinfection immunity, to specific bacterial stressors. Confirming this would entail testing other phage-host and phage-antibiotic pairings. Synergy also needs to be tested with other antibiotics such as β-lactams, which have been previously tested in traditional PAS and are known to cause phage induction (Meader et al., 2013; Nale et al., 2018; Oechslin, 2018). While we focused particularly on antibiotics due to their effects on health, multiple studies have identified several non-antibiotic agents that also result in phage induction (Boling et al., 2020; Oh et al., 2019; Sutcliffe et al., 2021), which could be tested for synergy with temperate phages.

This is the first demonstration of synergy between temperate phages and antibiotics. If tPAS is broadly applicable, as is suggested by its mechanism of action and what is known of phage induction in temperate phages, then this may allow for the use of these phages in therapy. We are proposing the use of these phages as adjuvants that reach peak efficiency when current antibiotics fail to maintain dosing higher than MIC, which is always unavoidable, due either to poor dosing, failure to adhere to dose timing, or the emergenc of resistant isolates now experiencing sub-MIC antibiotics (He et al., 2016). With our results

demonstrating bacterial eradication at sub-inhibitory concentrations through tPAS, this synergy may provide an effective approach toward overcoming antimicrobial resistance.

2.9 Material and Methods

2.9.1 Experimental model and subject details

The lambdoid phage HK97 was used as model for this study. *Escherichia coli* K-12 (*Ymel mel*-1 supF58) was used as host for phage HK97. The host and phages were obtained from Félix d'Hérelle Reference Center for Bacterial Viruses under the accession HER 1382 and HER 382, respectively, with λ-vir (HER37) propagated on the same host. The *E. coli* BW25113 *recA* mutant was obtained from the Dharmacon KEIO collection through Horizon Discovery (Cambridge, UK). Bacterial culture was grown in 10 mL of lysogeny broth (LB) at 37°C with shaking at 130 rpm (Ecotron, Infors HT, Quebec, Canada). Solid media was prepared by adding 1% (wt/vol) agar to LB broth and soft agar was prepared by adding 0.75% (wt/vol) agar. For same day use, overnight cultures were diluted 1:100 in LB broth and grown to OD_{600nm} 0.2, measured using the Thermo Fischer Scientific Spectronic 20D+ (Waltham, MA, USA).

2.9.2 Phage propagation and titration

Phage lysates were obtained by inoculating frozen bacterial and phage stocks in 10 mL LB broth growing for a maximum of 18 h or inoculating 50 μL of previously prepared phage lysate into 10 mL of grown culture followed by incubation at 37°C for up to 6 h. Cultures were then passed through a 0.45 μm filter to obtain a phage lysate. Phage titration was carried out using the double agar overlay technique (Kropinski et al., 2009). Bacterial culture and ten-fold serial dilutions of the lysate prepared in 1X phage buffer were

mixed in a 3:1 ratio into molten soft agar and distributed onto solid LB agar (Wiegand et al., 2008). Plaques were counted as zones of clearing in bacterial lawn after overnight incubation. Multiplicity of infection (MOI) was determined using the formula $\frac{\text{phage titre } \left(\frac{\text{pfu}}{\text{mL}}\right) \times \text{phage volume (mL)}}{\text{colony forming unit } \left(\frac{\text{cfu}}{\text{mL}}\right) \times \text{bacterial volume (mL)}}$.

2.9.3 MIC determination

MIC of ciprofloxacin hydrochloride (Cayman chemicals, MI, USA) was determined using a slightly modified broth dilution method (Wiegand et al., 2008). Briefly, 100 µL of freshly grown culture, a volume of ciprofloxacin stock solution, and nuclease free water were combined in a microtiter plate to obtain a final volume of 250 µL, and antibiotic working concentration ranging 0.78-100 ng/mL. The microtiter plate was incubated for 18 h at 37°C overnight with double orbital shaking using Epoch 2 microplate spectrophotometer (BioTek Instruments, Inc., VT, USA). MIC determination was performed in triplicate and end point OD_{600nm} was measured after 18 h. The MIC would be the lowest concentration of ciprofloxacin in which the final OD was equal to the initial read at time zero. Given variability between stocks and preparations of the antibiotic, a new MIC determination was performed for each new batch of the antibiotic.

In solid media, the MIC determination was performed by a modified agar dilution plates method (J. M. Andrews, 2001). An inoculant of 100 µL of freshly grown culture, a volume of ciprofloxacin stock solution, and nuclease free water were combined and added to 5 mL of 0.75% molten agar. An antibiotic working concentration ranging 0.78-100 ng/mL was tested and plates were incubated at 37°C overnight. MIC determination was performed in technical and biological triplicates and detected after 18 h. The MIC

would be the lowest concentration of ciprofloxacin in which the plate resulted in no visible growth of bacteria. This typically resulted in an MIC half that of the MIC determined in broth.

2.9.4 Kirby Bauer disc diffusion assay

To investigate the antimicrobial activity of ciprofloxacin in wild type compared to HK97 lysogen, disc diffusion assay was used. A sterile cotton swab was dipped in a bacterial suspension of a standard turbidity compared to 0.5 McFarland standard. Subsequently, the swab was streaked three times at 45° angles over the surface of Mueller-Hinton agar to obtain a complete lawn of bacterial growth. A 5 μg ciprofloxacin disc was placed on top of the agar using a sterile forceps. Plates were incubated overnight and growth inhibition diameter around the disc was measured to the nearest mm using ImageJ (Rasband, W.S., ImageJ, U. S. National Institutes of Health, Bethesda, Maryland, USA).

2.9.5 PAS in solid media

We used double agar overlay assays to enumerate phage populations. One hundred microliters of grown culture were infected with 100 μL of phage lysate at a MOI of at least 10, ciprofloxacin at ½ MIC concentration or with both phage and antibiotic. Each of the three challenge infections and 100 μL of unchallenged bacteria were plated separately using the double agar overlay method. The plates were then incubated overnight at 37°C and growth morphologies of different challenges were compared to unchallenged host.

2.9.6 Broth growth curve PAS

Growth curves in liquid culture for challenged and non-challenged wild-type *E. coli* K-12 were recorded as follows. Freshly grown cultures were infected at time

zero with a MOI of at least 10 for phage challenges and different concentrations of antibiotic ranging 0.78-100 ng/mL. Cultures were then incubated overnight with double orbital shaking and readings were taken every 15 min with the Epoch 2 microplate spectrophotometer.

2.9.7 Checkerboard assay

Fifty to hundred microliters of a grown culture were transferred into wells in a 96-well plate containing 100-125 µL of phage lysate to achieve target MOIs on the vertical axis with a final volume of 200 µL. Two-fold serial dilutions of ciprofloxacin were then added horizontally. Infections were performed in triplicate and OD_{600} was followed using an Epoch 2 microplate spectrophotometer with a measurement every 15 min. Percent growth relative to the untreated host was calculated as follows: $\frac{\mathrm{OD}_{600}\text{ of each challenge}}{\mathrm{OD}_{600}\text{ of untreated host}} \times 100\%$ and results were graphically represented in a heat map.

2.9.8 Overnight quantification assay

One hundred microliters of freshly grown cultures was transferred into wells in a 96-well plate containing 100 µL of phage lysate for a final MOI at least 10 and two-fold serial dilutions of antibiotics. Cultures were then incubated overnight with shaking. Subsequently, a 10-fold serial dilution of each trial was prepared, inoculated in 5 mL of LB soft agar and then incubated overnight. Survivors from each challenge were counted and the actual number of survivors in 1 mL broth was calculated. Subsequently, fold reduction compared to the untreated host was estimated as follows: $\frac{\text{Actual } count\ of\ untreated\ host}{\text{Actual count of each challenge}}$.

2.9.9 Adsorption assay

A volume of 1 mL of either freshly grown culture or LB broth control were incubated with shaking for 30 min with 100 μL of diluted λ-vir phage lysate of titer 10^4 pfu/mL. Samples were then centrifuged to sediment bacteria, and the supernatant was filtered using 0.45 μm filters. One hundred microliters of each filtrate was mixed with 300 μL of host overnight culture, inoculated in 5 mL of molten LB soft (0.75%) agar and then overlay plates were prepared. Plates were examined for plaques after overnight incubation.

2.9.10 Plaque size

Same day culture was infected with a fixed quantity of phage diluted in 1× phage buffer using the double agar overlay method in the presence/absence of antibiotic. Antibiotic was added to both the solid and soft agar layer in 2-fold dilutions starting at MIC. The plates were incubated overnight at 37°C and plaque morphology was compared to host challenged with phage alone.

2.9.11 Phage titer after overnight challenge

To determine phage concentration arising from each phage-antibiotic synergy challenge and compare it to untreated phage challenge, the number of phage particles was determined using a phage plaque assay. Overnight cultures from the three challenges (phage alone, antibiotic alone, and phage + antibiotic) were filtered using 0.45 μm filters to obtain phage lysates. Ten-fold serial dilution was carried out in 1× phage buffer in a final volume of 2 mL. Lysates were titerd using the standard double agar overlay technique.

After overnight incubation, plaques were counted to calculate the number of phage particles in pfu/mL.

2.9.12 One-step growth curve

Freshly grown culture was challenged with phage at a MOI of 0.1 in the absence and presence of antibiotic at ½ MIC in a final volume of 1 mL. Challenges were incubated at 37°C for 5 min to allows phage adsorption to occur. Cells were pelleted by centrifugation, resuspended in 5 mL of LB broth, and ten-fold serial dilution was carried out in LB broth. Antibiotic at ½ MIC was added back to the phage and antibiotic condition only. Dilutions were incubated and sampled at regular time intervals. Dilutions were titered on a same day culture grown to O.D. 0.3-0.4 using the double agar overlay technique.

2.9.13 Lysogeny detection

The integration of HK97 into the host chromosome was confirmed via Polymerase Chain Reaction (PCR). Individual surviving colonies arising from PAS challenge with ¼ MIC, where we started seeing our first survivors, were purified by streaking. This was followed by colony PCR in which primers were designed to amplify the phage-host junction. Each 25 µL PCR reaction contained 1 µl of each primer, 2.5 µL 10x DNA polymerase buffer, 0.5 µL dNTPs, 0.25 µL Taq DNA polymerase, a single survivor colony and the remaining volume was completed with nuclease free water. All PCR reagents were obtained from FroggaBio (NY, USA). Primer sequences are available in Table S1. PCRs were performed in duplicate for confirmation.

2.9.14 Quantitative PCR

Freshly grown culture was challenged with phage at a MOI of at least 10 in the absence and presence of antibiotic at ½ MIC in a final volume of 1 mL in quintuplicate. Challenges were incubated with shaking. One replicate of each of the two challenges was removed after 1, 2, 4, 6, and 18 h of exposure. To remove free floating DNA from lysed cells, challenges were treated with DNase for 10 min at 37°C, followed by addition of 0.5 M EDTA and heat inactivation at 75°C for 10 min. Genomic extraction was carried out using the Monarch Genomic DNA Purification Kit (New England Biolab, MA, USA). *E. coli* housekeeping gene *cysG* was used as a control for quality of the DNA extraction in that sample. Each sample was amplified using primers designed to detect *cysG*, lysogens, and non-lysogens. Nuclease free water, instead of template DNA, was used as a negative control. PowerUp SYBR Green Master Mix (Applied Biosystems, MA, USA), BioRad CFX96 Touch Real Time Detection System, and CFX Manager 3.1.1517.0823. (California, USA) were used to carry out qPCR. Primer sequences are available in Table S1. QPCR cycling mode as follows; 2 min at 95°C followed by 40 cycles of denaturation at 95°C for 15 s and annealing/extension at 60°C for 1 min. Melt curve generated by heating from 65°C to 95°C in 0.5°C increments per second.

2.9.15 Quantification and statistical analysis

All of the statistical details of experiments can be found in the figure legends, figures and results. N is used to identify replicates and represents independent biological replicates from an independently grown bacterial culture. Quantitative values were expressed by mean ± SD. They were compared by t test, one-way ANOVA, two-way

ANOVA, Tukey post hoc and Bonferroni test when appropriate with P value ≤ 0.05 is considered significant. All statistical analysis was done using GraphPad Prism 8.3.0 (GraphPad Software, Inc., CA, US). Software details are available in Table S1.

Chapter 3 - Temperate phage-antibiotic synergy is widespread – extending to *Pseudomonas* - but varies by phage, host strain, and antibiotic pairing

3.1 Preface

With demonstrated synergy between model *E. coli* phage and a DNA damaging antibiotic to be operating uniquely through biasing away from lysogeny, we next sought to investigate if this phenomenon is broadly generalizable across phages, antibiotics, and host in the clinically relevant pathogen *P. aeruginosa.*

The work presented in this chapter is currently in press to mBio and is available as a pre-print (https://www.biorxiv.org/content/10.1101/2024.08.20.608816v1).

Fatima, R.*, Hynes, A. P. (2024). Temperate phage-antibiotic synergy is widespread – extending to *Pseudomonas* – but varies by phage, host strain, and antibiotic pairing. mBio, *in press*.

3.2 Author contributions

R.F. performed all the assays. A.P.H conceived the study. All authors contributed to the writing of the manuscript.

3.3 Abstract

Bacteriophages (phages) are bacterial-specific viruses that can be used alone or with antibiotics to reduce bacterial load. Most phages are unsuitable for therapy because they are “temperate” and can integrate into the host genome, forming a lysogen that is protected from subsequent phage infections. However, integrated phages can be awakened by stressors such as antibiotics. Supported by this interaction, here we explore the potential use of combined temperate phage and antibiotic against the multi-drug-resistant pathogen, *Pseudomonas aeruginosa*. In all, thirty-nine temperate phages were isolated from clinical strains, and a subset was as screened for synergy with six antibiotics (ciprofloxacin, levofloxacin, meropenem, piperacillin, tobramycin, and polymyxin B), using checkerboard

assays. Interestingly, our screen identified phages that can synergize with each antibiotic, despite their widely differing targets; however, these are highly phage-antibiotic and phage-host pairing specific. Screening across multiple clinical strains reveals that temperate phages can reduce the antibiotic minimum inhibitory concentration up to 32-fold, even in a resistant isolate, functionally re-sensitizing the bacterium to the antibiotic. Meropenem and tobramycin did not reduce the frequency of lysogens, suggesting a mechanism of action independent of the temperate nature of the phages. By contrast, ciprofloxacin and piperacillin were able to reduce the frequency of lysogeny, the former by inducing phages – as previously reported in *E. coli*. Curiously, synergy with piperacillin reduced lysogen survivors, but not by inducing the phages, suggesting an alternative mechanism for biasing the phage lysis-lysogeny equilibrium. Overall, our findings indicate that temperate phages can act as adjuvants in clinically relevant pathogens, even in the presence of antibiotic resistance, thereby drastically expanding their therapeutic potential.

3.4 Importance

The recent discovery that otherwise therapeutically unusable temperate phages can potentiate the activity of antibiotics, resulting in a potent synergy, has only been tested in *E. coli*, and with a single model phage. Here, working with clinical isolates of *Pseudomonas* and phages from these isolates, we highlight the broad applicability of this synergy- across a variety of mechanisms, but also highlight the limitations of predicting the phage, host, and antibiotic combinations that will synergize.

3.5 Introduction

The widespread use of antibiotics has selected for resistance, resulting in a decline in their effectiveness and a rise in untreatable infections (Ventola, 2015). As a result, there has been a renewed interest in treatments with alternative modes of action such as bacteriophage (phage) therapy. Phages are bacterial-specific viruses (Brüssow et al., 2004) that hijack the host cell machinery and redirect it to synthesize phage components, resulting in host cell lysis and release of new infectious phage progeny (Drulis-Kawa et al., 2012). These can also synergize with multiple classes of antibiotics, known as "Phage-Antibiotic Synergy" (PAS), which is associated with changes in phage replication and an increase in phage production (Comeau et al., 2007; Davis et al., 2021; Kamal & Dennis, 2015; Kim et al., 2018).

Most phage therapy work to date has been with "virulent" (strictly lytic) phages. In contrast with these, temperate phages can undergo an additional replication cycle known as lysogeny, a dormant state involving the integration of the phage genome into the bacterial host and replication along with it (Howard-Varona et al., 2017). The integrated phage is referred to as a prophage and a bacterium carrying a prophage is a lysogen. In addition to choosing between lysis and lysogeny at the time of infection, prophages are capable of exiting lysogeny and switching to lytic replication in response to external stressors in a process known as induction (Howard-Varona et al., 2017). This decision between lysis and integration, at the initial time of infection or later during dormancy, is facilitated by phage encoded proteins in many well-studied temperate phage models (e.g. cI repressor in *Escherichia coli* phage Lambda) (Ptashne, 1987; Villanueva et al., 2015).

Even though this switch is genetically encoded, much of this decision is responsive to environmental factors (Erez et al., 2017; Silpe & Bassler, 2019; Stokar-Avihail et al., 2019; Trinh et al., 2017; L. Zeng et al., 2010). Of these, one of the most well known are antibiotics that trigger the bacterial SOS response (e.g. mitomycin C, fluoroquinolones, some beta-lactams) and result in phage induction through subsequent cleavage of the phage repressor protein (Bearson & Brunelle, 2015; Ding et al., 2018; Goerke et al., 2006; López et al., 2014; Maiques et al., 2006; Motlagh et al., 2015; Zhang et al., 2000).

Temperate phages have been overlooked for use in phage therapy because they present concerns of overgrowth of phage-resistant lysogens because of immunity; superinfection immunity (Fogg et al., 2010; Mavrich & Hatfull, 2019), mediated by the phage repressor protein, or superinfection exclusion, mediated by phage proteins that block DNA-entry (Cumby et al., 2012). However, due to the narrow host range of many phages, in instances when lytic phages are difficult to find (Hargreaves & Clokie, 2014; T. Zuo et al., 2018) studies have had to employ virulent variants of temperate phages obtained through genetic engineering for the treatment of bacterial infections (Dedrick et al., 2019; Kilcher et al., 2018). Interestingly, up to 75% of bacteria already contain a prophage in their genome (López-Leal et al., 2022; Touchon et al., 2016), greatly facilitating their discovery. The isolation and use of virulent mutants of temperate phages that can infect a lysogenic host have also been proposed for *P. aeruginosa* infections (Bourkal'tseva et al., 2011). These can also be of particular interest for pathogens such as *Clostridioides difficle* where strictly lytic phages have not been identified to date (Hargreaves & Clokie, 2014).

Studies examining the potential of temperate phages in therapy have been few and far between. Temperate phages of *Burkholderia cepacia* complex show synergistic interaction with other phages where their therapeutic potential inversely correlates with the frequency of lysogeny (Lauman & Dennis, 2023). *Burkholderia* temperate phage KS14 also synergizes with several antibiotic classes, as measured by an increase in plaque size and phage titer(Kamal & Dennis, 2015). In addition, temperate phages isolated from clinical strains of *Pseudomonas aeruginosa* were shown to decrease twitching motility, important for virulence, in lysogens (Chung et al., 2012). Administration of a temperate phage was able to reduce bacterial load and prevent toxin production in an *in vitro* human colon model of *C. difficle* infection (Meader et al., 2013). However, the study also reported increased spore formation with potential for increased risk of re-emerging infection. Several studies have also proposed the use of temperate phage cocktails for *C. difficle* (Nale et al., 2016, 2018). In a hamster model, Nale et al. (2016) reported that the phage cocktail not only reduced the colonization load of *C. difficle* but could also delay symptoms by 33 h (Nale et al., 2016).

While temperate phages are currently not ideal for monotherapy, the use of adjuvants that can bias their decision away from lysogeny and toward lytic replication can be promising for compassionate last resort cases. A four-temperate phage cocktail combined with Ca2+ or Zn2+ reduced methicillin-resistant *S. aureus* load by 2.64-fold compared to the phage cocktail alone in a mouse model; however, the frequency of lysogeny was not reported (Li et al., 2022). Knezevic et al. (2013) demonstrated that temperate phage σ-1 combined with ¼ minimum inhibitory concentration (MIC)

ceftriaxone reduced *P. aeruginosa* counts by ≥ 2 logs (Knezevic et al., 2013). The same effect was not observed with ciprofloxacin; a fluoroquinolone, gentamicin; a protein synthesis inhibitor, and polymyxin B; an outer membrane-targeting antibiotic. The study briefly noted a potential involvement of antibiotic-mediated phage induction.

Supported by the idea that SOS-response inducing antibiotics can act as phage inducers, we previously demonstrated that the combination of temperate phage HK97 and sublethal ciprofloxacin can synergistically reduce *E. coli* survivor count up to 10^8-fold after an 18 h treatment, largely by inducing any lysogens that formed. This was dubbed temperate phage antibiotic synergy (tPAS) (Al-Anany et al., 2021) and was generalizable to other antibiotics, including quinolones, anti-folates, and mitomycin C - all known to induce the bacterial SOS response (Al-Anany et al., 2024). Interestingly, protein-synthesis inhibitors of several classes also show comparable synergy in this model, although these were determined to act by biasing the phage away from lysogeny during the initial infection, rather than by inducing lysogens (Al-Anany et al., 2024).

With the reported efficacy of tPAS in *E. coli* demonstrating substantial reduction in lysogeny, the major concern in the therapeutic use of temperate phages, here we systematically investigate its effectiveness across phages, hosts, and antibiotics in the clinically relevant pathogen *P. aeruginosa.*

3.6 Results and Discussion

3.6.1 Temperate phages that synergize with ciprofloxacin are readily isolated from clinical strains

To establish tPAS in *P. aeruginosa*, we isolated 39 temperate phages that could infect the strain *P. aeruginosa* PA14 from filtrates of overnight cultures of 191

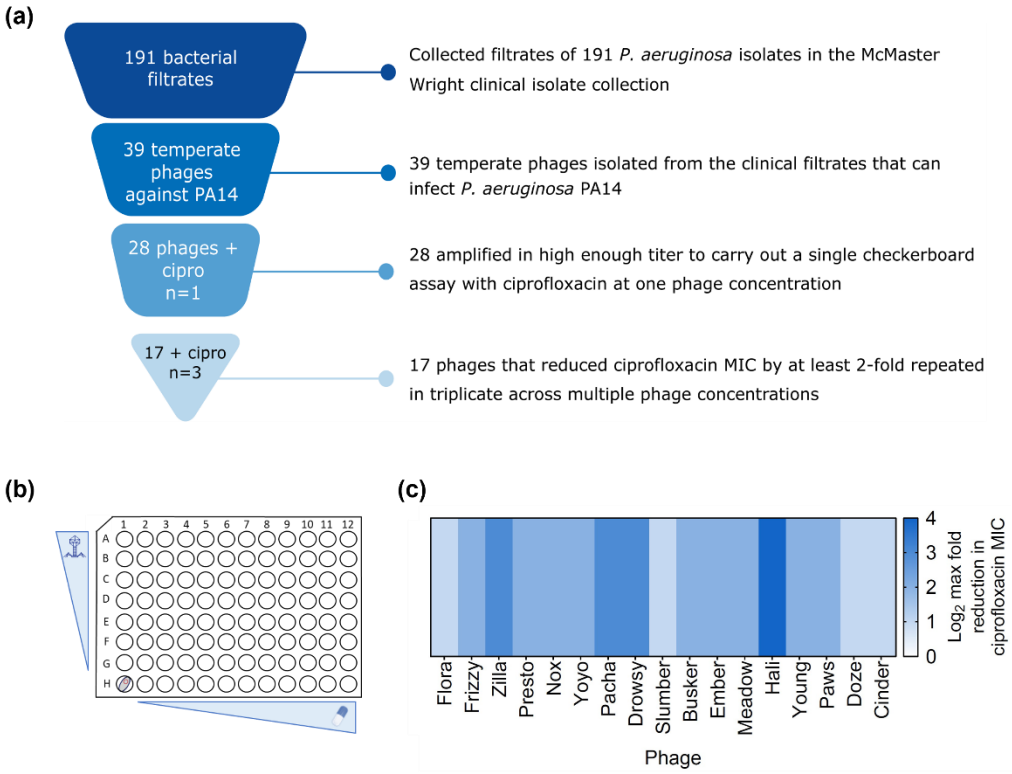


Figure 7. Temperate phages that synergize with ciprofloxacin can readily be isolated from clinical strains. (a) Workflow of isolation of temperate phages infecting *P. aeruginosa* PA14 from clinical strain collection and screening for synergy with ciprofloxacin in PA14. **(b)** Illustrative representation of checkerboard assay screening for synergy between a temperate phage and antibiotic. **(c)** Log_2 maximum reduction in ciprofloxacin MIC achieved with the addition of a panel of temperate phages relative to no phage added control against *P. aeruginosa* PA14, plotted as a heat map (n=3 biological replicate for all except n=5 for phage Flora). The multiplicity of infection (MOI) range of 1.25 – 40 was tested in two-fold increments. The data represented are the maximum MIC reduction regardless of phage dose.

P. aeruginosa clinical isolates, highlighting the abundance and ease of isolation of these phages (Figure 7a). This is consistent with many reports of isolation of temperate phages from clinical strains of *P. aeruginosa* and *C. difficile*, often aided by mitomycin C (Ambroa et al., 2020; Budzik et al., 2004; Goh et al., 2005).

Of the 39 phages, 28 were amplified on *P. aeruginosa* PA14 to a high enough titer to screen for synergy with ciprofloxacin using a checkerboard assay in a single replicate

(Figure 7a-b). In all, 17 phages that reduced the antibiotic MIC by at least two-fold (limit of detection) were then repeated in biological triplicates. A summary of the screening with ciprofloxacin is shown in Figure 7c as maximum fold reduction in the ciprofloxacin MIC achieved with the addition of the phage, regardless of the phage dose. All 17 phages reduced the ciprofloxacin MIC by at least two-fold, with the highest (16-fold) reduction achieved with phage Hali. These findings highlight that synergy between temperate phage and antibiotics can be achieved in *P. aeruginosa* with multiple phages, at least with ciprofloxacin, a known bacterial DNA-damaging phage inducer.

The 17 phages that exhibited a synergistic interaction with ciprofloxacin were further characterized using whole-genome sequencing and bacterial host range analysis. Recognizable phage genomes were obtained for all phages except phage Drowsy. Genome annotation predicted a transposase in 15 out of the 16 phages (not present in phage Flora) and these phages cluster closely with other *Pseudomonas* transposable temperate phages (Figure 8a). Host range analysis was carried out using 96 *P. aeruginosa* clinical isolates reveal that these phages exhibit broad host range, with phage Busker able to infect 31 out of the 96 strains tested (Figure 8b). While phage Drowsy and phage Slumber exhibited a similar host range, the efficiency of plaquing for 4 out 29 strains differed (Figure 8c). Despite variations in host ranges - some of which can presumably be attributed to host-controlled variation, based on sequence similarity and phylogenetic analysis of phages Yoyo, Pacha, Slumber, Busker and Ember, phage Ember was kept as a representative of that cluster.

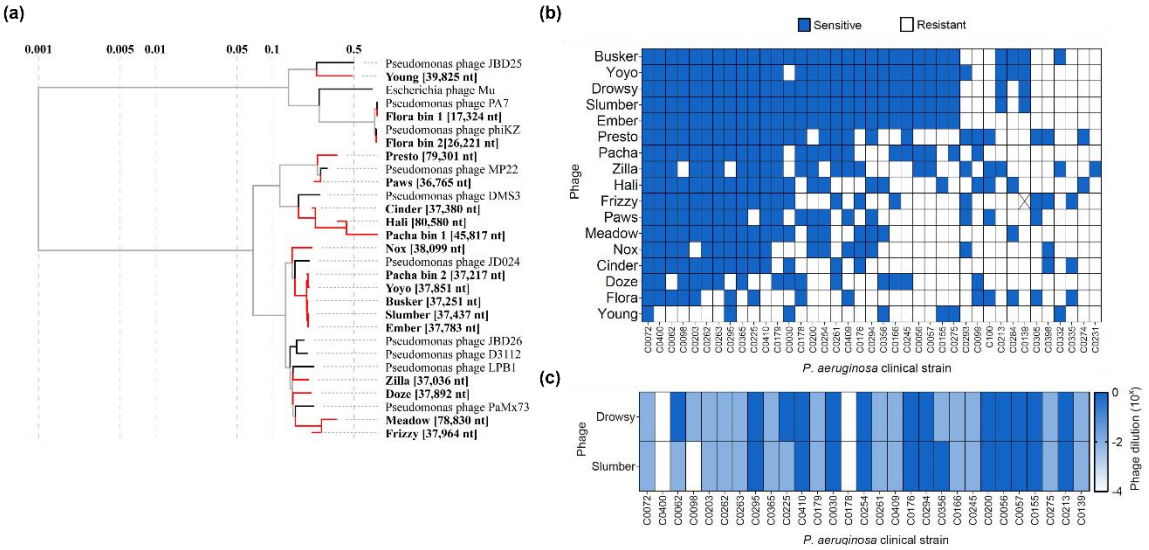


Figure 8. Newly isolated PA14 temperate phages are unique. (a) Phylogenetic tree of the isolated PA14 temperate phages. The tree was generated using ViPTree with a subset of related genomes. Branch lengths are indicated on a log scale. **(b)** Host range of the newly isolated PA14 temperate phages tested against 96 *P. aeruginosa* clinical strains. Each row represents a phage, ordered in largest to smallest host range, and columns represent a single *P. aeruginosa* isolate, ordered based on phage susceptibility. Blue denotes phage susceptible. **(c)** Host range comparison of phage Drowsy and phage Slumber. The heat map depicts the 10-fold phage dilution on which plaquing was observed on a specific host, represented by the columns.

3.6.2 tPAS with ciprofloxacin results in bacterial eradication through induction

While checkerboard assays do establish a clear synergistic effect, revealed as a reduction of the antibiotic MIC, they do not provide enough resolution to quantify this effect and determine the mechanism of bacterial growth inhibition. To better quantify this synergy over a range of antibiotic concentrations, we challenged *P. aeruginosa* PA14 with phage Hali and ciprofloxacin in liquid media. Survivors present after an 18 h challenge were plated with no selection for another 18 h. Fold reduction in survivor count relative to the untreated host was calculated for each challenge. There was a less than five-fold reduction in survivor count when challenged with phage Hali alone and a more pronounced dose-dependent ~10^4-fold reduction with antibiotic at the highest concentration (Figure 9a).

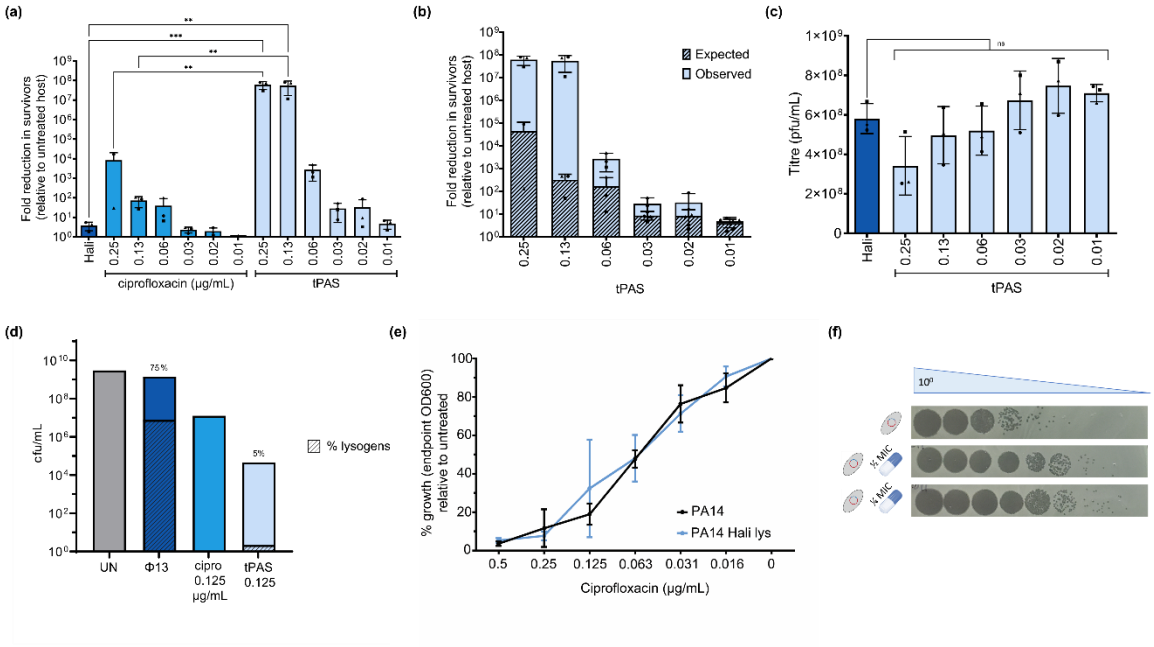


Figure 9. Phage Hali and ciprofloxacin synergize to eradicate *Pseudomonas* through induction. (a) Fold reduction in PA14 survivors after phage Hali [multiplicity of infection (MOI) of 15] and ciprofloxacin treatment relative to untreated host (mean ± SD). In the case that no survivors were detected, the data were set to the limit of detection (1 colony). Each point indicates a biological replicate, denoted by the different shapes, counted in technical triplicates. Values were compared by a one-way ANOVA and Tukey post hoc test, with ****P*-value 0.0001 - 0.001 and ***P*-value 0.001 - 0.01 **(b)** Observed fold reduction in survivors (solid) versus the expected (diagonal line) effect. Expected effect was calculated by multiplying the phage alone reduction with antibiotic reduction for the corresponding antibiotic concentration. **(c)** Phage quantification from the overnight challenges (PFU/mL, mean ± SD). Each point indicates a biological replicate, denoted by the different shapes, counted in technical triplicates. All challenges were compared to phage alone using one-way ANOVA and Bonferroni's test, with "ns" denoting not significant. **(d)** Survivor quantification (cfu/mL) of untreated PA14, challenged with phage Hali [multiplicity of infection (MOI) of 15] ± 0.125 ug/mL ciprofloxacin, where the strongest synergy was observed in a repeat experiment. Striped bars indicate the percentage of lysogens as determined using a lysogen stamp test, values also indicated above the bar. Twenty survivors of phage ± ciprofloxacin were streaked purified for lysogen testing. The height of the % lysogen bar is proportional to the height of the total cfu/mL bar. **(e)** Ciprofloxacin MIC curve of PA14 and PA14 phage Hali lysogen represented as percent growth (endpoint OD600) relative to untreated host, plotted as mean ± SD (n=3 biological replicates, each in technical duplicates, except no antibiotic which was technical triplicates). **(f)** Representative phage quantification of PA14 phage Hali lysogen challenged with ½ and ¼ MIC ciprofloxacin.

These results highlight the poor efficacy of the phage and antibiotic alone in eradicating PA14.

By contrast, PA14 challenged with phage Hali in the presence of ciprofloxacin at the two highest concentrations resulted in approximately a 10^8-fold reduction in survivors, corresponding to complete eradication. The number of survivors then decreased in a dose-dependent manner as the antibiotic concentration decreased. However, the clearest evidence for a synergistic effect is obtained by comparing the observed data to the expected multiplication of the independent effects of phage and antibiotic (Figure 9b). Phage Hali and ciprofloxacin resulted in the strongest synergistic effect at sublethal 0.13 µg/mL with an observed reduction that is roughly 10^5-fold higher than the expected effect. This effect is much higher than that observed with temperate phage σ-1 and ceftriaxone in *P. aeruginosa* ATTC 9027 (Knezevic et al., 2013). However, it agrees with our previous work performed in *E. coli* with model temperate phage HK97 and ciprofloxacin (Al-Anany et al., 2021), although across a smaller range of antibiotic concentrations.

To elucidate the mechanism through which phage Hali-ciprofloxacin pairing results in bacterial killing, we quantified phages in the filtrates of the phage-alone and tPAS challenges from the overnight survivor quantification assay (Figure 9c). We observed no significant difference in phage titer in the presence of the antibiotic compared to the phage alone challenge suggesting that phage Hali-ciprofloxacin synergy is not a result of a substantial increase in phage replication.

To determine whether tPAS was instead biasing the phage lysis-lysogeny decision, we repeated the assay across a shorter range of antibiotic concentrations, purifying

survivors from phage alone and a single antibiotic concentration where we observed the strongest synergy with regard to reduction in survivor count (Figure 9d). Phage alone resulted in 75% lysogens, which reduced to 5% in the presence of ciprofloxacin. The interaction works at the level of biasing the phage lysis-lysogeny equilibrium, potentially at the level of induction since antibiotics are well known to result in induction.

To confirm whether PA14 lysogen of phage Hali can be induced with sublethal ciprofloxacin, we challenged wild type and lysogen with ciprofloxacin and quantified phages in the filtrates. Unlike in prior *E. coli* phage HK97 work (Al-Anany et al., 2021), the PA14 phage Hali lysogen is not more sensitive to ciprofloxacin than the wild type (Figure 9e), despite the roughly 100-fold increase in phage titer, relative to baseline spontaneous induction, in filtrates of lysogen grown with sublethal ciprofloxacin (Figure 9f). The disagreement between this and lack of increase in phage titer observed in overnight challenges could be explained by previous reports that the inherent frequency of lysogeny is typically very low. For example, for Lambda in nutrient broth at 37^{o}C it is typically <1% (Knoll, 1979), but it is the regrowth of these lysogens that result in large resistant populations. This means that even by forcing all the phage to adopt a lytic cycle, the phage titer would only be expected to increase by almost 1%, which is well below our limit of detection.

Overall, our results indicate phage Hali and ciprofloxacin synergy results in bacterial killing by preventing the expansion of lysogen colonies likely through induction, where lysogens form but are subsequently induced in the presence of sublethal antibiotic, like that reported in *E. coli* with phage HK97 and ciprofloxacin (Al-Anany et al., 2021).

3.6.3 tPAS can be achieved even with delayed antibiotic administration

With a clear synergistic interaction observed with the co-administration of phage Hali and ciprofloxacin, we sought to investigate whether both agents need to be simultaneously present to achieve synergy. To first test whether the antibiotic needs to be present when the phage infects, we monitored bacterial growth after simultaneous phage and antibiotic administration (Figure 10a) or delayed antibiotic treatment by 4 h where the antibiotic likely only has lysogens to interact with (Figure 10b). In these assays, we report the functional change in the MIC of the antibiotic in the presence of the phage but do not claim that the phage is affecting the antibiotic – the relative increase (or decrease) in the potency of the antibiotic is almost certainly a result of the interaction of the antibiotic with the phage, and not the other way around.

Delaying the antibiotic by 4 h results in an increase in antibiotic MIC compared to administering it alone at time zero (Figure 10b). However, a comparable level of synergistic reduction in ciprofloxacin MIC was still observed in the presence of phage Hali when the antibiotic was delayed. Ciprofloxacin does not necessarily need to be present when the phage infects to achieve the same levels of tPAS as co-administration. Taken together with the frequency of lysogeny (Figure 9d), the results highlight that phage Hali and ciprofloxacin synergy works through biasing lysis-lysogeny decision at the level of induction.

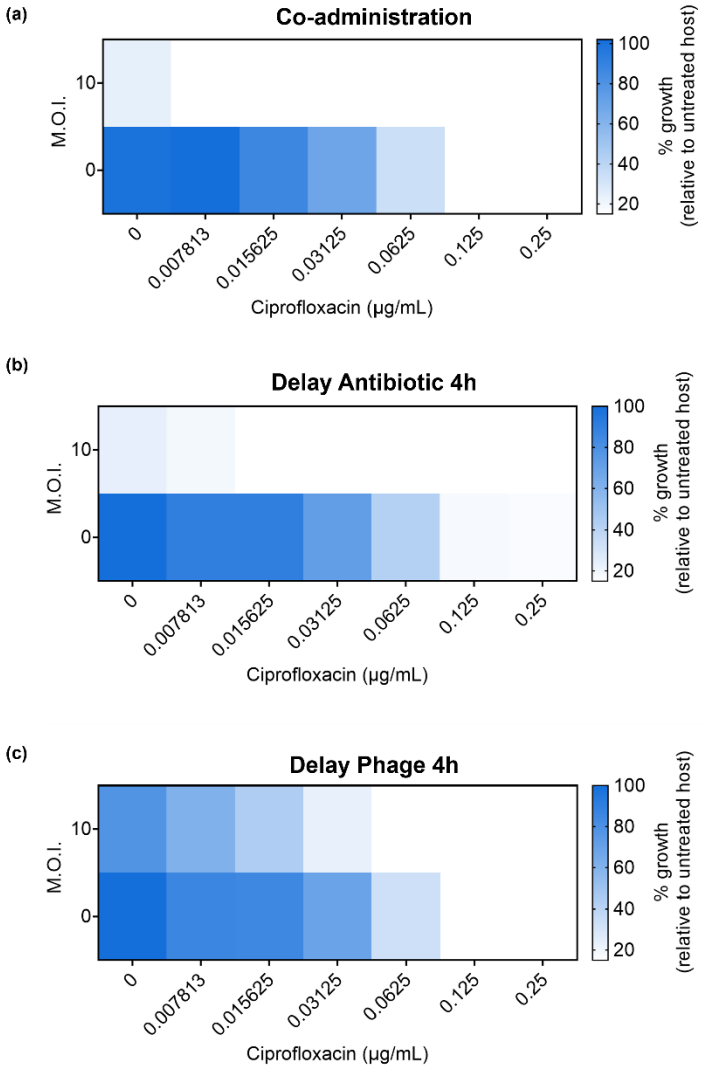


Figure 10. Temperate PAS can be achieved by delaying the antibiotic. Checkerboard assay of phage Hali [multiplicity of infection (MOI) of 10] and ciprofloxacin in PA14 with **(a)** simultaneous, **(b)** delayed antibiotic by 4h, or **(c)** delayed phage by 4 h. Endpoint growth (OD600) relative to untreated host, plotted as a heatmap (n=3 biological replicates, each in technical triplicates).

We also tested the effects of delaying the phage to investigate whether pre-stressing the bacteria with antibiotic prior to phage treatment would result in synergy. Delaying the phage by 4 h results in only a two-fold reduction in ciprofloxacin MIC (Figure 10c). While the antibiotic does not necessarily need to be present at the time of phage infection, the

opposite is not true. By delaying the phage, we drastically reduce the range of antibiotic concentration at which tPAS is observed.

It is important to note that the efficacy of delayed administration is likely dependent on the phage-antibiotic pairing. *P. aeruginosa* virulent phage JG024 demonstrates synergy with ciprofloxacin even with a 1 h delay of either agent (Nikolic et al., 2022). The effect is lost if the treatment occurs after 6 h. By contrast, with ceftriaxone, only delaying phage JG024 by 1 h can inhibit bacterial growth and delayed antibiotic has no effect. Delaying ciprofloxacin (8 x MIC) and gentamicin (1 x MIC and 8 x MIC) 6 h post-lytic phage treatment also reduced viable cell count $>10^2$ cfu/mL (limit of detection), better than sequential treatment, in 48 h mono species *P. aeruginosa* biofilm (Akturk et al., 2019). With regards to tPAS, the extent to which one of the agents can be delayed would presumably depend on the rate at which lysogens form in a specific host.

3.6.4 tPAS is generalizable across phage-antibiotic pairings

Having established this interaction with ciprofloxacin, we sought to test the generalizability across antibiotics, covering three more antibiotic classes in addition to fluroquinolones (Figure 11). These antibiotic classes were selected for their reported ability to either induce phages or their clinical relevance as anti-pseudomonal drugs. Beta-lactams are cell wall synthesis inhibitors that bind to penicillin-binding protein (PBP) to prevent crosslinking (Bush & Bradford, 2016). While these have been shown to result in phage induction in multiple bacterial hosts (Maiques et al., 2006; X. Zeng & Lin, 2013), the exact mechanism through which they induce phages, directly or indirectly through the bacterial SOS response (Christine et al., 2004; Clarke et al., 2021; Tatiana et al., 2005), remains

unclear. The beta-lactams, meropenem and piperacillin, are commonly used in the clinic to treat *Pseudomonas* infection, where the latter is combined with a beta-lactamase inhibitor, tazobactam (Sader et al., 2018).

In addition, aminoglycosides inhibit protein synthesis by binding to the A site of 16S ribosomal RNA, inhibiting translocation resulting in protein mistranslation (Krause et al., 2016). Since phages need host protein machinery for replication, protein synthesis inhibitors are not expected to synergize with antibiotics. Kanamycin antagonizes replication of *E. coli* phage T3, demonstrated as a decrease in efficiency of plaquing, bacterial growth, and biofilm biomass, by decreasing phage burst size independent of changes in phage adsorption (P. Zuo et al., 2021). Similarly, both kanamycin and apramycin reduced efficiency of plaquing of temperate phage Lambda by up to 1000-fold (Kever et al., 2022). Selected protein synthesis inhibitors were also reported to antagonize *P. aeruginosa, S. aureus, and Enterococcus faecium* phages (Coyne et al., 2024). Contrary to these studies, gentamicin and kanamycin both synergize with phage HK97 in *E. coli* by biasing the initial phage decision toward lysis at the time of infection (Al-Anany et al., 2024). To investigate whether a similar synergy between aminoglycoside and temperate phages can be observed in *P. aeruginosa*, we performed our assays with tobramycin, frequently used as an inhaled treatment for cystic fibrosis patients (Sloan et al., 2024). Our screen also includes polymyxin B, an older outer membrane-disrupting antibiotic, which works through binding to lipopolysaccharide (Zavascki et al., 2007), that has regained interest as one of the last resort drugs for severe infections.

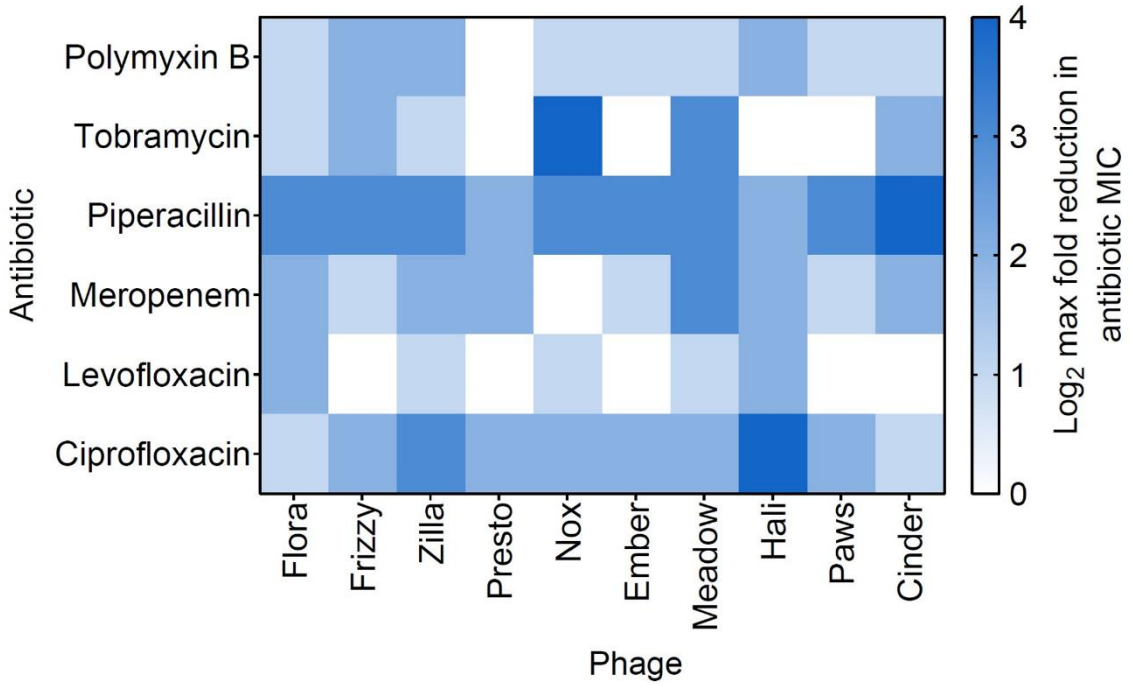


Figure 11. PAS is generalizable to other temperate phage-antibiotic pairings. Ten PA14 temperate phages were screened with two fluoroquinolones (ciprofloxacin and levofloxacin), two beta-lactams (meropenem and piperacillin), an aminoglycoside (tobramycin), and polymyxin B. Ciprofloxacin data are reproduced from Figure 7c as a reference. Log_2 maximum reduction in antibiotic MIC achieved in the presence of PA14 temperate phages relative to no phage control, plotted as a heat map (n=3 biological replicates, with a few n=4 exceptions). Data are represented is the maximum MIC reduction regardless of phage dose [multiplicity of infection (MOI) range of 1.25 – 40 was tested in two-fold increments].

We observed synergy with all antibiotics, despite their vastly different bacterial targets, with most of the phages working particularly well in combination with piperacillin (Figure 11). While some of these antibiotics have not been previously reported to interact specifically with temperate phages, a strong synergy between virulent phages and several cell wall synthesis inhibitors (16/25 antibiotics tested), including piperacillin and meropenem, and protein synthesis inhibitors (5/25 antibiotics tested), including tobramycin, has been previously reported in *P. aeruginosa* (Holger et al., 2022; Uchiyama et al., 2018). Polymyxin B has also been reported to work in combination with virulent

phages for treating *S. aureus* (Jo et al., 2016). However, we did not observe a consistent synergistic pattern with antibiotics of the same drug class, indicating that PAS here is phage-antibiotic pairing specific, previously also reported with a lytic phage and several antibiotic classes in extraintestinal pathogenic *E. coli* (Liu et al., 2020). Nonetheless, this drastically expands the library of compounds that can be used in combination with temperate phages for therapeutic use, even with antibiotics not previously known to induce phages.

3.6.5 Synergy with piperacillin reduces the frequency of lysogeny, but not through induction

With a clear synergy observed with several antibiotics, we looked to confirm whether the interaction with these other classes of antibiotics also operates at the level of biasing the phage lysis-lysogeny equilibrium. To test this, we purified survivors from our overnight survivor quantification assay performed for three other phage-antibiotic pairings. Phage Nox combined with tobramycin was able to reduce survivor counts by 3-4 logs; however, there was only a 15% reduction in lysogeny observed at the highest concentration tested (Figure 12a). Meropenem combined with phage Meadow synergistically reduced survivor count but did not reduce the frequency of lysogeny (Figure 12b). In comparison, while there was not an impressive reduction observed in survivors for phage Cinder-piperacillin, there was a clear reduction in lysogeny from 60% for phage alone down to 25% in the presence of sublethal piperacillin (Figure 12c). The contrasting results of meropenem and piperacillin, belonging to the same drug class, further support that tPAS is phage-antibiotic pairing specific.

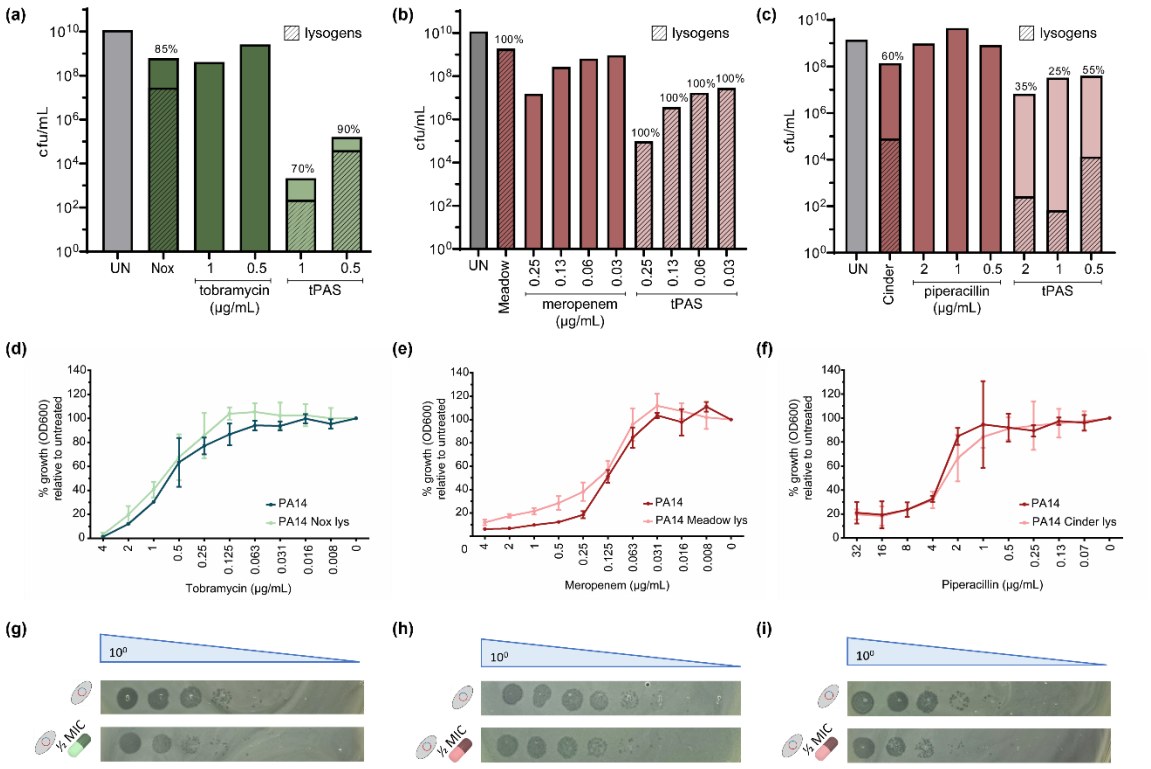


Figure 12. Piperacillin reduces the frequency of lysogeny but not through induction. **(a-c)** Survivor quantification (cfu/mL) of PA14 challenged with individual and combination (a) phage Nox [multiplicity of infection (MOI) of 2.5] + tobramycin, (b) phage Meadow [multiplicity of infection (MOI) of 15] + meropenem, and (c) phage Cinder [multiplicity of infection (MOI) of 15] + piperacillin. Striped bars represent the percentage of lysogens as determined using a lysogen stamp test, values also indicated above the bar. Twenty survivors of phage ± antibiotic were streaked purified for lysogen testing. The height of the % lysogen bar is proportional to the height of the total cfu/mL bar (n=1 biological replicate). **(d-f)** Antibiotic sensitivity of wild type PA14 and (d) phage Nox lysogen with tobramycin, (e) phage Meadow lysogen with meropenem, and (f) phage Cinder lysogen with piperacillin. MIC is represented as percent growth (endpoint OD600) relative to untreated host, plotted as mean ± SD (n=3 biological replicates for tobramycin and piperacillin, n=4 for meropenem, each in technical triplicates). **(g-i)** Representative phage quantification ± ½ MIC antibiotic of PA14 (g) phage Nox lysogen with tobramycin, (h) phage Meadow lysogen with meropenem, and (i) phage Cinder lysogen with piperacillin (n=3 biological replicates, each with single technical replicate).

To confirm whether the observed synergy and reduction in lysogeny could be a result of the ability of the antibiotic to result in induction, we tested the sensitivity of the wild type and the lysogen for their respective antibiotic (Figure 12d-i). None of the lysogens were more sensitive to the antibiotic or released more phages at sublethal doses relative to spontaneous induction after 18 h. This explains the lack of reduction in lysogeny observed with meropenem or tobramycin, where we hypothesize the synergy observed is likely driven by mechanism of traditional PAS instead. By contrast, synergy with piperacillin does operate by biasing the lysis-lysogeny decision; however, it is not at the level of induction. Piperacillin potentially works at the level of biasing the decision at the initial time of infection, like that reported with *E. coli* phage HK97 and gentamicin (Al-Anany et al., 2024). The observed effect with piperacillin could also be attributed to its selective affinity for PBP3, unlike meropenem which preferentially binds PBP4 (Montaner et al., 2023), and/or its ability to result in cell filamentation (Fonseca et al., 2004; Fredborg et al., 2015; Glen & Lamont, 2024), where increased cell volume shows a decrease in the probability of lysogeny in *E. coli* phage Lambda (St-Pierre & Endy, 2008). This is the first report that we know of piperacillin's ability to influence the phage lysis-lysogeny balance. This provides further proof that tPAS can be achieved with non-phage-inducing antibiotics.

3.6.6 tPAS is generalizable across clinical strains, even antibiotic-resistant ones

To establish whether the synergy is generalizable across hosts, we identified the six strongest phage-antibiotic pairings, one for each antibiotic in Figure 11, and tested for synergy across multiple clinical strains, including both antibiotic sensitive and resistant ones. Figure 13a shows the maximum reduction in antibiotic MIC achieved with the

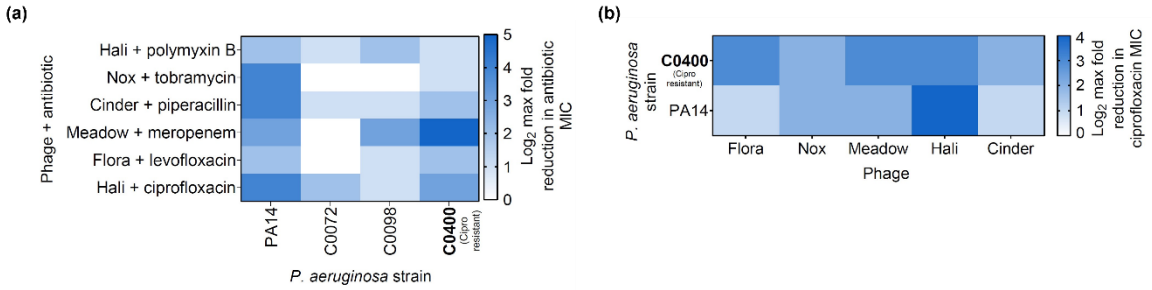


Figure 13. PAS with temperate phages works in clinical isolates. (a) Six of the strongest pairings from PA14 screen (phage Hali + ciprofloxacin, phage Flora + levofloxacin, phage Meadow + meropenem, phage Cinder + piperacillin, phage Nox + tobramycin, phage Hali + polymyxin B) were tested for synergy in *P. aeruginosa* clinical strains based on host range in Fig 2b. Result reported as $\log_2$ reduction in antibiotic MIC calculated from checkerboard assay (n=3 biological replicates). **(b)** Log_2 reduction in ciprofloxacin-resistant strain C0400 MIC with the addition of five temperate phages, regardless of phage dose [multiplicity of infection (MOI) range of 1.25 – 40 was tested in two-fold increments]. Average maximum reduction (n=3 biological replicates) in MIC shown as heat map as determined from checkerboard assays. Isolate in bold is classified as antibiotic-resistant strains in the clinical database using standard laboratory reporting. (a-b) PA14 screen data from Figure 7c and (b) C0400 phage Hali data from panel a are reproduced as reference.

addition of the phage across three isolates that were sensitive to all phages used. We also carried this out with five other clinical strains that were sensitive only to some overlapping phages (Figure S4).

Our findings show that the phage-antibiotic combinations that were initially identified to synergize in PA14 expand across multiple clinical isolates of *P. aeruginosa*, even in the multi-drug-resistant strain C0400. The reported ciprofloxacin resistance of this strain is consistent with our data, as the strain showed four-fold higher MIC than PA14 (Figure S5a). We observed a reduction in ciprofloxacin MIC of this isolate even when combined with four other phages at levels much higher than those observed in the initial PA14 screen (Figure 13b). The co-administration of temperate phage results in as high as a 16-fold reduction in MIC, bringing it down to levels comparable to PA14, re-sensitizing the isolate to the antibiotic. This is in line with previous observations that PAS can be

achieved independent of the antibiotic-resistant nature of the bacteria (Engeman et al., 2021; Holger et al., 2022; Kamal & Dennis, 2015; Kebriaei et al., 2020; Liu et al., 2020).

Strain C0400 is predicted by the Comprehensive Antibiotic Resistance Database Resistance Gene Identifier software to be ciprofloxacin resistant due to the presence of a resistance-nodulation-cell division antibiotic efflux pump (Alcock et al., 2023). Since many antibiotic targets also play a crucial role in phage replication and induction, the role of the exact mechanism of antibiotic resistance should be considered when investigating phage-antibiotic synergy. For example, efflux pumps can serve as phage receptors; hence, the development of phage resistance at the level of the surface receptor can result in re-sensitization to the antibiotic (Chan et al., 2016). This evolutionary tradeoff was used to successfully treat a 76-year-old male patient with *P. aeruginosa*-infected aortic graft using a combination of lytic phage OMKO1, which binds efflux pump, and ceftazidime.

However, the phages isolated in this study are temperate in nature where regrowth of lysogens presents a major concern as opposed to surface receptor mutants. Despite originally being resistant to ciprofloxacin, host C0400 Hali lysogen displays a higher antibiotic sensitivity, compared to wild type C0400 and PA14 (Figure S5a) with significant increases in phage titer at sublethal ciprofloxacin (Figure S5b). The increase in sensitivity of C0400 lysogen but lack of difference in PA14 lysogen ciprofloxacin sensitivity (Figure 9e) could potentially be explained by host-specific factors. Since transposable phages are notorious for different integration sites, we also tested ciprofloxacin induction in independent lysogens, where our earlier findings suggest that it is the main phenotype driving tPAS with ciprofloxacin. We continued to detect ciprofloxacin-triggered induction

in other isolated C0400 Hali lysogens (not shown). These findings further support the hypothesis that Hali-ciprofloxacin synergy prevents lysogen overgrowth through antibiotic-mediated induction, regardless of host. Our results highlight that tPAS can be achieved in clinical strains of *P. aeruginosa*, irrespective of their antibiotic-sensitivity profile.

3.6.7 The frequency of lysogeny and antibiotic mediated induction does not predict the ability of a temperate phage to synergize with an antibiotic

With proof that tPAS is working through biasing of the phage lysis-lysogeny equilibrium and a panel of phage-antibiotic pairings that showed efficacy in various hosts, we investigated whether there are factors that can predict tPAS. We hypothesized that the frequency with which a phage lysogenizes its host or its susceptibility to induction with the antibiotic would serve as predictors of tPAS.

To determine whether the ability of a phage to form stable lysogens in a specific host correlates with the level of synergy, measured as a reduction in MIC in the checkerboard assays, we infected host PA14 with seven phages and host C0400 with five phages in solid media and quantified frequency of lysogeny in the survivors of the phage challenge (Figure S6a). The frequency of lysogeny varied in PA14 ranging from as low as 26% for phage Meadow to 100% for three other phages (phage Zilla, Drowsy, and Cinder). In comparison, all five phages exhibited 100% lysogeny in host C0400. However, there was no correlation between the frequency of lysogeny and the level of synergy observed with ciprofloxacin. It is important to note here that the frequency of lysogeny among survivors does not reflect only the actual frequency of such an event, but only the relative frequency within survivors. If the host cell has a low-cost alternative for resistance, we

would find a low frequency of lysogeny among survivors, even if the actual rate at which lysogeny events occurred were quite high. The frequency of lysogeny here was also tested on solid media and therefore may differ if tested in broth.

In addition, we also investigated the ability of sublethal ciprofloxacin to induce these prophages (Figure S6b). All tested lysogens of PA14 and C0400 were ciprofloxacin inducible, resulting on average in 10-1000-fold increase in phages released, except for the C0400 phage Flora lysogen. Similarly, when correlated with level of synergy as measured by decrease in MIC, level of ciprofloxacin mediated phage induction did not correlate with the magnitude of the synergy observed. This was only tested at one sublethal antibiotic concentration which may not be the optimal dose for induction with a given lysogen-antibiotic pairing. Induction was also measured at endpoint after 18 h, where the affect may have been entirely lost in some cases as a result phage re-adsorption.

Our results show that neither the frequency of lysogens among survivors nor the extent to which the antibiotic results in induction correlate strongly with levels of synergy observed and therefore cannot predict if and how strongly a temperate phage will synergize with a particular antibiotic.

3.7 Conclusion

Here we evaluate the generalizability of tPAS against *P. aeruginosa*. Temperate phages can be easily isolated from clinical strain collections and synergize with multiple clinically relevant antibiotics, irrespective of antibiotic target. This synergy can functionally lower antibiotic MICs up to 32-fold, and can do so even in resistant strains, functionally sensitizing them to the antibiotic. Mechanistically, some of the observed

synergies are not temperate-phage specific, while others – notably ciprofloxacin, work at the level of induction in agreement with previous reports in a single phage in *E. coli* (Al-Anany et al., 2021) . Excitingly, piperacillin can also bias phage lysis-lysogeny equilibrium but appears to be doing so by biasing the initial lysis-lysogeny decision – in a manner akin to that reported for protein synthesis inhibitors for a single phage in *E. coli* (Al-Anany et al., 2024). While temperate phages have been discarded in therapy due to their ability to lysogenize, the use of sublethal antibiotics can serve to bias away from lysogeny. This is both phage-host and phage-antibiotic pairing specific, and difficult to predict using factors inherent to the interaction between the two players. More work deciphering the mechanisms underlying these interactions would be necessary for any practical applications, in order to guide antibiotic and phage selection.

3.8 Material and Methods

3.8.1 Bacterial strains and growth conditions

P. aeruginosa strain PA14 was kindly gifted to us by Dr. Lori Burrows at McMaster University. Clinical strains of *P. aeruginosa* were obtained from the McMaster IIDR Wright clinical isolate collection. Bacterial strains were grown in 10 mL of lysogeny broth (LB) at 37^{o}C with 130 rpm shaking (Ecotron, Infors HT, Quebec, Canada). For growth on solid media, 1% (w/v) of LB agar and 0.75% (w/v) of LB soft agar was used. All plates were incubated at 37^{o}C from overnight growth.

For each experiment, same day culture was grown to an optical density (OD_{600}) of 0.2 from a 1:100 dilution of an overnight culture. OD_{600} was measured using Thermo Fischer Scientific Spectronic 20D+ (Waltham, MA, USA).

3.8.2 Phage isolation, propagation, and titration

One hundred and ninety-one *P. aeruginosa* clinical strains were grown overnight at 37°C in LB broth in a 96 well plate from frozen stocks. The plates were filtered using the Millipore MultiScreen$_{HTS}$ vacuum manifold (Catalog MSVMHTS00, Darmstadt, Germany) with a Millipore Sigma MultiScreen$_{HTS}$ High Volume 96-well 0.45 µm filter plate (Catalog MVHVN4525, Darmstadt, Germany). Approximately 2 µL of the undiluted filtrates were spotted on a 1% LB agar Nunc™ Omnitray™ single well plate with a 15 mL 0.75% agar overlay containing 1 mL overnight culture of PA14. This assay would capture all antimicrobial components.

To identify phages, any filtrates that resulted in clearing were then confirmed by spotting serial dilutions where a phage would dilute to a single plaque as opposed to other bactericidal entities. Briefly, an agar overlay of 300 µL of PA14 overnight culture into 3 mL of 0.75% molten agar was spread onto 1% LB agar petri plate. Ten-fold dilutions (10^0 – 10^{-7}) of the filtrates was prepared in 1x phage buffer and 3 µL was spotted on overlay. The phages were amplified in LB broth to increase the titer. Primary amplification was carried out using frozen stock of the bacteria and phage inoculated in 10 mL of LB broth and incubated for 18 h. Secondary amplification was carried out by challenging same day grown culture at OD_{600} 0.2 with 50 µL of the primary amplification. Phages were titered on the respective host using a spot test and standard plaque assay.

To determine multiplicity of infection (M.O.I.), an OD_{600} vs colony forming unit (cfu/mL) standard curve was carried out for each bacterial strain. Optical density of a same day culture was measured at 30 min intervals and 50 µL of culture was sampled at every

hour, diluted 10-fold in LB, and 100 µL was plated on 1% LB plate using glass beads. M.O.I. was calculated as $\frac{\text{phage titer } \left(\frac{\text{pfu}}{\text{mL}}\right) \times \text{phage volume (mL)}}{\text{bacterial titer } \left(\frac{\text{cfu}}{\text{mL}}\right) \times \text{volume(mL)}}$.

For the clinical isolate screen, phages were amplified on the host being tested if the efficiency of plaquing was 2 $\log_{10}$ or lower relative to PA14. If not, lysate prepared on PA14 was used for the checkerboard screens.

3.8.3 Minimum inhibitory concentration

The minimum inhibitory concentration of each antibiotic was determined using a modified microtiter assay. Ciprofloxacin (hydrochloride) was obtained from Cayman Chemicals (Catalog 14286-5, Ann Arbor, Michigan, USA), levofloxacin from Cedarlane (Catalog 20382-1, Burlington, ON, Canada), meropenem from Sigma-Aldrich (Catalog PHR1772, Oakville, ON, Canada), piperacillin from Sigma-Aldrich (Catalog PHR1805, Oakville, ON, Canada), tobramycin from Sigma-Aldrich (Catalog PHR1079, Oakville, ON, Canada), and polymyxin B sulfate from EMD Millipore (Catalog D46530, Oakville, ON, Canada). In a narrow 96 well plate (Corning, Product Number 3370, ME, USA), 100 µL of same day culture was combined with antibiotic stock and LB broth in a final volume of 250 µL. The plate was incubated for 18 h at 37°C with no agitation. The endpoint OD_{600} was measured using the Epoch 2 microplate spectrophotometer (BioTek Instruments, Inc., VT, USA) with a 10 sec double orbital shake before read. MIC was the lowest antibiotic concentration that resulted in no growth and was re-evaluated for every new batch of antibiotic stock prepared.

3.8.4 Checkerboard assay

Same day culture grown to OD 0.2 was challenged with two-fold dilutions of the phage on the vertical axis and two-fold dilution of the antibiotic on the horizontal axis in a narrow 96-well plate. M.O.I. range tested for each phage was 40 – 1.25. The antibiotic concentrations tested changed depending on the MIC. For each checkerboard performed, the culture, phage, and antibiotic volume were fixed to 25 µL, 62.5 µL, and 12.5 µL, respectively, to achieve the desired M.O.I. and antibiotic concentration. Untreated host and LB-only were used as growth controls. Plates were incubated for 18-20 h at 37°C. OD_{600} was used to measure growth in each plate using a BioTek Epoch 2 microplate spectrophotometer. The lowest antibiotic concentration that resulted in no growth (approximately 15% or less OD_{600} compared to the untreated control) was used as MIC. Checkerboard assay data was represented as the maximum reduction in antibiotic MIC that was achieved with the addition of the phage, regardless of M.O.I.

3.8.5 Phage genome sequencing and analysis

Phage DNA extraction, DNA library preparation, sequencing and analyzes were carried out as described in (Nair et al., 2024). Briefly, 500 µL of phage lysate ($\geq 10^8$ pfu/mL) was treated with DNase I, RNase, and DNase I reaction buffer and incubated at 37°C for 30 min followed by DNase and RNase inactivation at 65°C for 10 min. Next, proteinase K and 2% final volume SDS was added and incubated for 1 h at 37°C to denature protein and break open phage capsid. Following incubation, mixture was split into two aliquots and equal volume of phenol-chloroform was added. The mixture was centrifuged for 10 min at >13 000 rpm, supernatant was collected and treated with $1/10^{th}$ vol of

ammonium acetate and one volume of -20^{o}C isopropanol, followed by another round of 10 min centrifugation. The pellet was resuspended in -20^{o}C 70% ethanol and centrifuged for 10 min. After decanting the supernatant, the new pellet was air dried for 15 min and rehydrated in elution buffer overnight at 4^{o}C. The DNA concentration was measured using Invitrogen Qubit 4 Fluorometer (ThermoFisher Scientific, Waltham, MA, USA).

DNA libraries were prepared for sequencing using the NEBNext® Ultra™ II DNA Library Prep Kit (New England Biolabs, catalog no. E7645S, Massachusetts, USA) with a modified version of the Derakhshani *et. al.*, (2020) protocol as previously outlined (Nair et al., 2024). Sequencing was carried out using MiSeq with paired-end 2 x 300 reads at the McMaster Metagenomics Facility (Ontario, Canada). Genome assembly and analysis was carried out using the pipeline previously outlined (Nair et al., 2024). Briefly, quality assessment on raw reads was carried out using FastQC v0.11.8 (S. Andrews, 2010) before and after trimming with Trimmomatic v0.38 (Bolger *et al.*, 2014). The trimmed reads next go through a series of steps in which *de novo* assembly is performed using metaSPades v3.13.0 (Meleshko, 2017) and the sequence is predicted as phage or not. The last step in the pipeline involves analysis which includes within sample comparison to determine how similar samples are to each other and genome annotation using RASTtk v1.3.0 (Brettin et al., 2015). The tree was generated using VipTree version 4.0 (https://www.genome.jp/viptree/) using the default setting after which a smaller subset of phages was selected to regenerate a smaller tree.

3.8.6 Host range analysis

Phage host range was carried out as described previously (Nair et al., 2024)**.** Briefly, a micro plaque assay was carried out using the Singer Rotor HDA. Ninety-eight clinical isolates of *P. aeruginosa* were inoculated from frozen in 1 mL LB in a deep 96 well plate and grown overnight. Forty-five microliters of each isolate were transferred into a 384-well plate such that each of the four quadrants of the 384 well plate are replicates. One 384-well plate was prepared for each phage where each quadrant contains 45 µL of either undiluted, 10^{-2}, 10^{-4}, and 10^{-6} phage lysate dilution prepared in LB broth. Stamping plates were prepared with 25 mL of 1.5% LB agar per plate. Using the Singer Rotor HDA, culture was stamped first, followed by phage directly on top at a 1536 density (four replicates per phage dilution-host pairing). One plate with only culture stamped was used as a control. The plates were wrapped in plastic bags and incubated for 18 h at 37ºC. Phage sensitivity and efficiency of plaquing, the lowest dilution at which plaques were observed, was noted for each phage-host pairing.

3.8.7 Survivor quantification assay

Overnight challenge survivor quantification assay was performed as previously described (Al-Anany et al., 2021). Briefly, same day culture of PA14 was challenged with phage (M.O.I. of 15 for phage Hali, Meadow, and Cinder, and M.O.I. of 2.5 for phage Nox) and/or two-fold dilutions of antibiotic in a final volume of 1 mL. Untreated host was used as a growth control. Challenges were incubated at 37ºC with 130 rpm shaking for 18 h, following which 100 µL of 10-fold dilutions carried out with LB broth was added to 5 mL of 0.75% LB and spread onto an empty petri plate. Survivors were counted after an

overnight incubation to determine the fold reduction in survivor count relative to the untreated host. The expected synergistic effect was calculated by multiplying phage alone reduction with the appropriate antibiotic alone challenge. For frequency of lysogenization characterization, twenty survivors for phage and all phage + antibiotic challenged were streaked purified thrice on 1% LB and lysogen detection was performed as described below.

3.8.8 Overnight challenge phage quantification

For each survivor quantification assay performed, half the volume after the 18 h challenge was filtered using a Millipore MultiScreen$_{HTS}$ vacuum manifold (Catalog MSVMHTS00, Darmstadt, Germany) vacuum manifold and Millipore Sigma MultiScreen$_{HTS}$ High Volume 96-well 0.45-micron filter plate (Catalog MVHVN4525, Darmstadt, Germany). Phages in the filtrates were quantified using a standard plaque assay using PA14 as the host.

3.8.9 Lysogen isolation & detection

Spot tests of phage Flora, Zilla, Nox, Drowsy, Meadow, Hali, and Cinder were carried out on PA14 and phage Flora, Nox, Meadow, Hali, and Cinder on *P. aeruginosa* clinical strain C0400. All lysates were previously amplified on host PA14 in broth. Regrowth on the plate from the phage challenge was streaked out on to 1% LB agar plate. Twenty colonies for each phage-host pair were streak purified three times and inoculated in 1 mL LB broth overnight in a deep 96 well plate. Wild type culture, not exposed to the phages, and LB broth were added as control. The plates were incubated for 18-24 h at 37^{o}C with no agitation. The following day, the cultures were stamped on an agar overlay of either

PA14 or C0400, depending on the original host used for the spot test, using a disposable pin replicator. Cultures that resulted in the clearing of the wild type host were classified as lysogen. Frequency of lysogeny was calculated as percent of the total number of survivors that were characterized as lysogens for each phage-host pairing. One lysogen for each phage-host pair was randomly selected for ciprofloxacin induction.

3.8.10 Antibiotic induction

Same-day culture of wild type host and the lysogen were grown in the presence of two-fold dilutions of antibiotic, prepared in nuclease free water, in a narrow 96 well plate in 250 µL final volume. Nuclease free water was added to the no antibiotic growth control. The plates were incubated for 18 h at 37^{o}C with no agitation. The following day, the plate was filtered using a vacuum manifold with a 0.45-micron filter plate. The phages in the filtrate of the untreated host (wild type and lysogen), and the host treated with antibiotic were quantified using a spot test using the wild type as the host.

3.8.11 Delay administration

Same-day PA14 culture was treated with phage Hali amplified on PA14 (M.O.I. of 10), ciprofloxacin (2-fold dilution MIC – 1/16 MIC), and combination phage Hali + ciprofloxacin in a final volume of 250 µL in a narrow 96 well plate. To test the effects of delayed administration, either antibiotic or phage was withheld for 4 h. Co-administration was tested on the same plate for each delayed administration tested. Each condition was set up in triplicates. LB broth was used as a negative control. Growth curves were carried out using OD_{600} for 4 h at 30 min intervals using the Epoch 2 microplate spectrophotometer. For delayed antibiotic, no treatment control and phage challenge were treated with nuclease

free water. For delayed phage, LB broth was added to the no treatment and antibiotic alone challenge. After the addition of the delayed agent, growth in the plate was measured for another 18 h at 30 min intervals. Endpoint growth (OD600) was calculated relative to untreated culture and plotted as a heatmap.

3.8.12 Statistical tests

Details of the statistical tests can be found in the figure legend. N denotes a biological replicate performed from an independently grown culture. Quantitative values are represented by mean $\pm$ SD. All statistical analysis was done using GraphPad Prism 8.3.0 or 10.3.0 (GraphPad Software, Inc., CA, US), with P value ≤ 0.05 is considered significant.

Chapter 4 - Temperate phages increase antibiotic effectiveness in a *Caenorhabditis elegans* infection model

4.1 Preface

Thus far, we have established the tPAS is broadly generalizable but is both phage-host and phage-antibiotic pairing specific. With an extensive panel of pairings that synergize, we sought to investigate their potential *in vivo* as i*n vitro* effectiveness is not always a sufficient indicator of *in vivo* efficacy (Hulsart-Billström et al., 2016; Kotani & Ito, 2023). To quickly iterate and screen large number of pairings, we aimed to develop a small animal model using *C. elegans* to test *in vivo* efficacy of tPAS.

The work presented in this chapter is available as a pre-print (https://www.biorxiv.org/content/10.1101/2024.10.28.620739v1).

Fatima, R.*, MacNeil, L.T., Hynes, A. P. (2024). Temperate phages increase antibiotic effectiveness in a *Caenorhabditis elegans* infection model. Biorxiv, 2024.10.28.620739.

4.2 Author contributions

R.F. performed all the assays, and, jointly with A.P.H., conceived the study. L.T.M. provided the worm strains, *E. coli* OP50, and all the *C. elegans* experiments were performed in L.T.M.'s laboratory. R.F. and A.P.H contributed to the writing of the manuscript.

4.3 Abstract

The bactericidal nature of obligately lytic bacterial viruses (phages) is of increasing interest for the treatment of drug-resistant bacterial infections, either administered alone or in combination with antibiotics. In contrast, temperate phages are largely ignored in a therapeutic context due to their ability to lie dormant within the bacterial host. However, these phages often undergo a lytic cycle. Furthermore, even in their dormant state, their

carriage can be a considerable burden to the bacterium – most famously by their ability to switch to lytic replication in response to environmental triggers, such as antibiotics, that stress the bacterial host. Recent reports of antibiotics synergizing uniquely with temperate phages *in vitro*, termed "temperate phage antibiotic synergy" (tPAS), presents a potentially scalable opportunity to make use of these abundant entities for the treatment of bacterial infections. Here we employ *Caenorhabditis elegans* as a robust *in vivo* animal model for testing the efficacy of temperate phages as adjuvants to antibiotics. *In vivo*, the antibiotic ciprofloxacin can abolish dormancy of temperate phage Hali - infecting a ciprofloxacin resistant *Pseudomonas aeruginosa* clinical strain - while in the absence of the antibiotic, dormancy events explain 60% of the bacterial survivors. The phage Hali-ciprofloxacin pairing increased the lifespan of *P. aeruginosa* infected worms to that of the uninfected control, at doses where neither the phage nor the antibiotic had any effect alone. Complete rescue was also observed in worms infected with a phage-carrying strain treated with the otherwise ineffective antibiotic, supporting that the phage - even in its dormant form - can greatly enhance antibiotic effectiveness. This illustrates potential "accidental" phage therapy when antibiotics are normally prescribed. Our work establishes *C. elegans* as a suitable model for studying the *in vivo* efficacy of tPAS and is the first *in vivo* demonstration of this synergy, greatly expanding the therapeutic potential of temperate phages.

4.4 Introduction

In light of the growing antimicrobial resistance crisis, bacteriophages (phages) are increasingly being explored as adjuvants or alternatives to antibiotics. Most of this work

has focused on a particular type of phage called virulent or strictly lytic, given their immediate bactericidal nature (Akturk et al., 2019; Aslam et al., 2020; Chan et al., 2018; Chaudhry et al., 2017; Comeau et al., 2007; Gainey et al., 2020; Henriksen et al., 2019; Kamal & Dennis, 2015; Kebriaei et al., 2020; Kim et al., 2018; Liu et al., 2020; Rodriguez-Gonzalez et al., 2020). However, it is estimated that 50% - 75% of all bacteria contain at least one dormant phage, termed temperate or lysogenic (López-Leal et al., 2022; Touchon et al., 2016). Much of the resistance to the use of temperate phages in therapy comes from concerns of further spread of fitness-benefitting genes within the bacterial population, especially the spread of toxins or antibiotic resistance cassettes. While on average 3.06 $\pm$ 1.84 dormant phages were predicted per clinical isolate of *Pseudomonas aeruginosa*, they were not associated with antibiotic resistance of the bacteria (Chang et al., 2024) and the prevalence of phage-encoded antibiotic resistance genes appears to be overestimated due to inherent biases in bioinformatic based virome studies (Enault et al., 2016). In addition, temperate phages are not inherently better transducers than virulent phages, many of which are capable of generalized transduction (Canosi et al., 1982; Petty et al., 2007; Wilson et al., 1979).

A key concern specific to temperate phages comes at the level of the integrated phage (prophage) conferring immunity to subsequent phage infections through a variety of mechanisms (Bondy-Denomy et al., 2016; Mavrich & Hatfull, 2019). This leads to a rapid regrowth of phage-generated resistant ‘mutants’ and could readily contribute to treatment failure. However, carrying a large genome such as a prophage, especially if it does not confer direct fitness benefit, can pose a heavy metabolic burden on the bacteria. Lysogens

of several *Pseudomonas aeruginosa* phages demonstrate defects in bacterial motility, swarming and twitching (Chung et al., 2012). As a result, the prophage can have a lasting influence on their host physiology, virulence, and pathogenesis, which could be exploited for the treatment of bacterial infections.

One of the most common physiological changes in the prophage-carrying lysogen is the one from which it derives its name; the prophage can be highly responsive to environmental cues that stress their bacterial host in a process referred to as induction (Howard-Varona et al., 2017). In the well-characterized phage model Lambda infecting *Escherichia coli,* the phage can sense host DNA damage to switch from lysogenic to lytic replication, resulting in cell lysis (Sauer et al., 1982). In the gastrointestinal tract of monoxenic mice, 1 to 2% of Lambda lysogenic bacteria were lysed *per* generation due to spontaneous prophage induction (De Paepe et al., 2016). Hence, prophage excision itself likely presents a higher burden to phage-carrying bacteria, especially in environments of high stress. Lysogens can exhibit increased sensitivity to antibiotics, monitored as decreases in minimum inhibitory concentration (MIC), compared to the parent strain (Al-Anany et al., 2021; Fatima & Hynes, 2024; Goerke et al., 2006). Temperate phages of *Burkholderia cepacia* (Kamal & Dennis, 2015), *E. coli* (Al-Anany et al., 2021, 2024), and *P. aeruginosa* (Fatima & Hynes, 2024) can also function synergistically with antibiotics, resulting in increases in phage production (Kamal & Dennis, 2015) or a decrease in the functionally effective dose of the antibiotic (Al-Anany et al., 2021). When the mechanism of action specifically interacts with the phage lysis-lysogeny decision, this is referred to as temperate phage-antibiotic synergy (tPAS). Given that temperate phages are already

abundant within the human body (Shkoporov et al., 2019), there is a strong case in favour of their therapeutic use - they are likely already an influential player in health and treatment outcomes.

There are currently several popular animal models for studying efficacy of phage therapy. Specifically for temperate phages, *Burkholderia* phage AP3 was able to significantly increase survival of wax moth larvae (15% survival at 72 h compared to 0% for untreated at 48 h) (Roszniowski et al., 2017). In a hamster model, a four-phage cocktail targeting *Clostridioides difficile* reduced the colonization load and delayed the onset of symptoms (Nale et al., 2016). More recently, temperate phage of *Acinetobacter baumannii*, vB_AbaM_ABMM1, reduced mortality in a zebrafish model (Mardiana et al., 2023). While useful, these models present several scalability concerns, each animal requiring individual attention or manipulation.

In this study, we develop the nematode *Caenorhabditis elegans* as an infection model for studying the *in vivo* efficacy of a temperate phage-antibiotic combination against the multidrug-resistant bacteria *P. aeruginosa*. Due to its short and measurable lifespan and the ability to generate large, germ-free synchronous populations in the lab, *C. elegans* is a well-established model for the study of bacterial pathogenesis (Corsi, 2006). Many human pathogens, including *P. aeruginosa* (Darby, 2005) are also pathogenic to *C. elegans*. Accordingly, *C. elegans* are also commonly employed as an *in vivo* model for studying the efficacy of new antibacterial drugs (Palacios-Gorba et al., 2020; Son et al., 2016; Yao et al., 2022). Pathogenesis of *P. aeruginosa* in *C. elegans* has been studied in three contexts. Agar based methods are differentiated by fast and slow killing and are dependent on media

composition (Tan et al., 1999). Fast killing occurs within hours, does not require live bacteria, and is mediated by bacterial secreted toxic phenazines (Cezairliyan et al., 2013; Tan et al., 1999). In contrast, slow killing occurs over the course of several days with active intestinal colonization being a key determinant (Tan et al., 1999). A third *P. aeruginosa*-mediated *C. elegans* killing method is observed in liquid, with the same formulation as that of slow killing medium (SKM) (Kirienko et al., 2013). While not as well understood, this is independent of phenazine production or intestinal colonization, however, is reported to be mediated by *P. aeruginosa* secreted siderophore pyoverdine which induces a hypoxic response and subsequent worm death.

Only a few studies have used *C. elegans* to investigate phage therapy, focusing on virulent phages of *B. pseudomallei* (Wang et al., 2022), *Salmonella* Enteritidis (Augustine et al., 2014), *Staphylococcus aureus* (Glowacka-Rutkowska et al., 2019), *E. coli* (Manohar et al., 2022), *Klebsiella pneumoniae* (Manohar et al., 2022), *Enterobacter cloacae* (Manohar et al., 2022), with only one specifically for *P. aeruginosa* (Manohar et al., 2022). The primary outcome measured in these studies was the ability of phages to reduce bacterial load and/or increase survival post infection. However, none of these studies tested antibiotics alone or in combination with their phage of interest in this model. In addition, *in vitro* results are not always sufficient indicators of *in vivo* efficacy (Hulsart-Billström et al., 2016; Kotani & Ito, 2023), especially in cases of antibiotic potency which can be highly sensitive to media composition, pathogen, and host factors (Heithoff et al., 2023; Radlinski & Conlon, 2018). As a result, here we employ a simple animal model to be able to quickly

iterate and screen a large set of temperate phage-antibiotic pairings that synergized *in vitro* against *P. aeruginosa* (Fatima & Hynes, 2024).

4.5 Results and Discussion

4.5.1 Validation of *C. elegans* as an infection model

To investigate if co-administration of temperate phage and antibiotics can result in bacterial killing in an *in vivo C. elegans* model, we first validated *P. aeruginosa* infection with a clinical strain of interest, C0400. C0400 is a ciprofloxacin resistant strain where co-administration of temperate phage Hali and ciprofloxacin could re-sensitize the strain to the antibiotic *in vitro* (Fatima & Hynes, 2024). The strain PA14 is commonly used in this model for studying *P. aeruginosa* pathogenesis (Cezairliyan et al., 2013; Tan et al., 1999) and served as our control. For our experiments, we employed the *glp*-4 (*bn2*) worm strain that carries a temperature sensitive defect in germ line proliferation such that animals maintained at a restrictive temperature of 25^{o}C are sterile (Tekippe & Aballay, 2010). In slow killing assays, dependent on host colonization, both *P. aeruginosa* strains tested colonized the intestine at levels higher than *E. coli* OP50 control, with no differences between PA14 and C0400 (Figure S7a). Similarly, while OP50 did not result in worm killing, *P. aeruginosa*-fed worms maintained on SKM began dying after three days, with complete population death observed by day 10 (Figure S7b). The lifespan data closely matches previously reported survival data of the *glp-4* strain exposed to OP50 and PA14 (Tekippe & Aballay, 2010), validating the clinical *P. aeruginosa* strain C0400 as being suitable for investigating tPAS in this *in vivo* model.

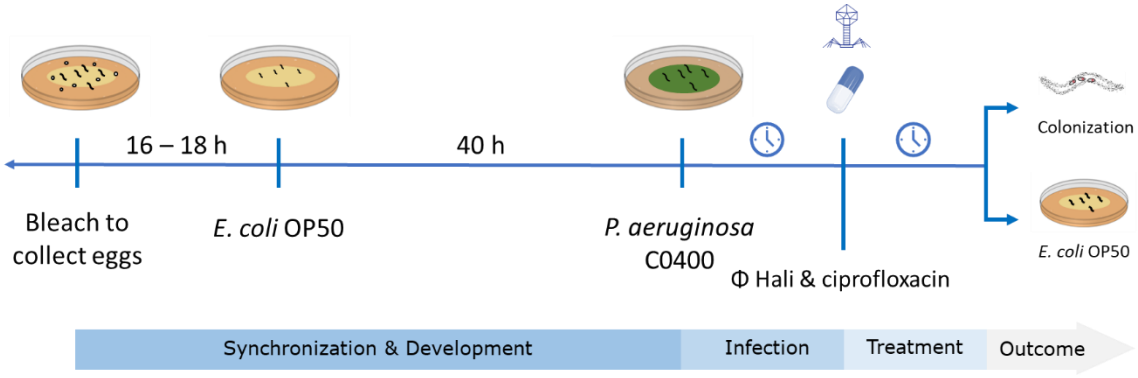


Figure 14. Representation of the assay used for testing efficacy of temperate PAS in a *C. elegans* model of *P. aeruginosa* infection. Worms are bleached to collects eggs which are hatched and synchronized to L1 stage. Animals are developed on *E. coli* OP50 and infected with *P. aeruginosa*, followed by phage and/or antibiotic treatment. Outcome of treatment is monitored by bacterial colonization and lifespan on regular bacterial diet. Each assay was done in independent biological replicates, with technical triplicates plated for assessing bacterial load.

4.5.2 Ciprofloxacin selects against lysogens *in vivo*

To test whether tPAS can reduce *P. aeruginosa* loads *in vivo*, we used two treatment durations, 4 h and 18 h after infection on SKM (Figure 14, Figure 15). We treated the worms with phage Hali and ciprofloxacin across a range of antibiotic concentrations (0.25 µg/mL – 100 µg/mL), alone and in combination, only showing the data for highest concentration (100 µg/mL) in Figure 15a as there was no effect observed at lower doses. There was no reduction in colonization when treated with phage alone and only 1-2 $\log_{10}$ reduction observed with the highest ciprofloxacin concentration tested. Similarly, there was no synergistic reduction in bacterial load for tPAS for either treatment duration.

Despite no reduction in bacterial loads observed, it was still possible that tPAS biased the survivor profile, selecting against lysogens. The phage alone results in an increase in lysogens from 20% to 60% as treatment duration increased (Figure 15b). This regrowth of lysogens was expected as they are immune to further phage infection due to

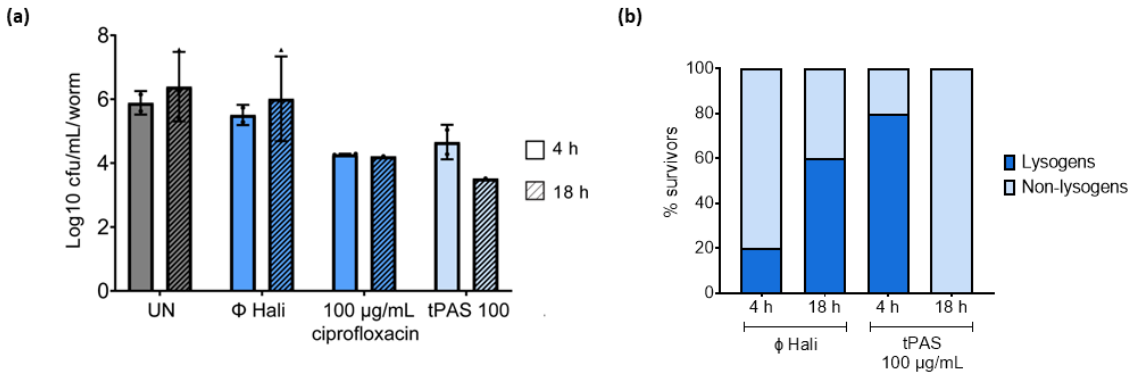


Figure 15. Phage Hali and ciprofloxacin selects against lysogen survivors *in vivo*. (a) *C. elegans* intestinal colonization measured as log10 cfu/mL/worm after 48 h C0400 infection followed by 4 h or 18 h treatment with phage Hali (1×10^9 pfu/mL) ± ciprofloxacin. Data shown as mean ± SD, each data point is a biological replicate plated in technical triplicates, except ones denoted by triangle which are single technical replicate, with of ~50-100 worms each condition. The two treatment durations were tested in independent trials. Values were compared using two-way ANOVA with a multiple comparison Tukey test with conditions only compared within their own treatment duration. All comparisons are not significant. **(b)** Frequency of lysogeny within survivors of phage Hali alone and phage Hali + ciprofloxacin (tPAS). Twenty survivors were tested for each condition.

superinfection immunity, and this finding aligns very closely with our previous report that phage Hali results in 75% lysogens after an overnight challenge *in vitro* (Fatima & Hynes, 2024). In comparison, tPAS resulted in primarily lysogen population within 4 h treatment (80% compared to 20% for phage alone) and a reduction in frequency of lysogeny to zero with longer treatment duration. Compared to administering phage by itself, tPAS selectively biases the phage away from lysogeny *in vivo,* completely preventing the regrowth of lysogens that would otherwise form 60% of the population. This formation of lysogens (4 h) that are then depleted is consistent with the previously reported mechanism of this interaction to be operating through induction (Fatima & Hynes, 2024).

In short, while all treatments had – at best – modest effects on the bacterial load, combining phage and antibiotic drastically altered the nature of those bacteria, selecting

against lysogens. We sought to determine whether this bias was enough to rescue the worms after treatment.

4.5.3 Combined temperate phage and ciprofloxacin rescues *P. aeruginosa* infected worms

To test if selecting for a different survivor population would be enough to rescue the worms, we carried out the same assay but moved the animals to their standard *E. coli* OP50 diet after 18 h treatment to measure lifespan. We performed the lifespan assay with a shorter 4 h infection as we did not observe any rescue from even the antibiotic at high doses with a longer infection duration, suggesting that worms were moribund (data not shown). With a shorter infection, when left untreated, the worms died within maximum thirteen days compared to eighteen plus days for the uninfected OP50 control as they reach the end of their lifespan (Figure 16). Treatment with phage Hali alone was not only ineffective, but it also showed a worse survival outcome compared to untreated in two of the three trials (Figure 16). Ciprofloxacin alone at low antibiotic dose of 2 µg/mL did not reliably rescue alone or in combination with phage (Figure S8a). At a much higher antibiotic dose, 92 µg/mL, the antibiotic alone was sufficient to rescue (Figure S8b). However, we could not reliably discern the effect of tPAS as both antibiotics alone and tPAS survival curve matched the uninfected OP50 control in two trials.

We were primarily interested in antibiotic concentrations that were lower than the effective dose and was therefore insufficient to increase survival. Ciprofloxacin alone at 32 µg/mL could not rescue survival (Figure 16). In comparison, tPAS increased lifespan to levels comparable to the uninfected OP50 control in two of the three trials, and a marked improvement in survival over either treatment alone in the third. In short, the *in vitro* work

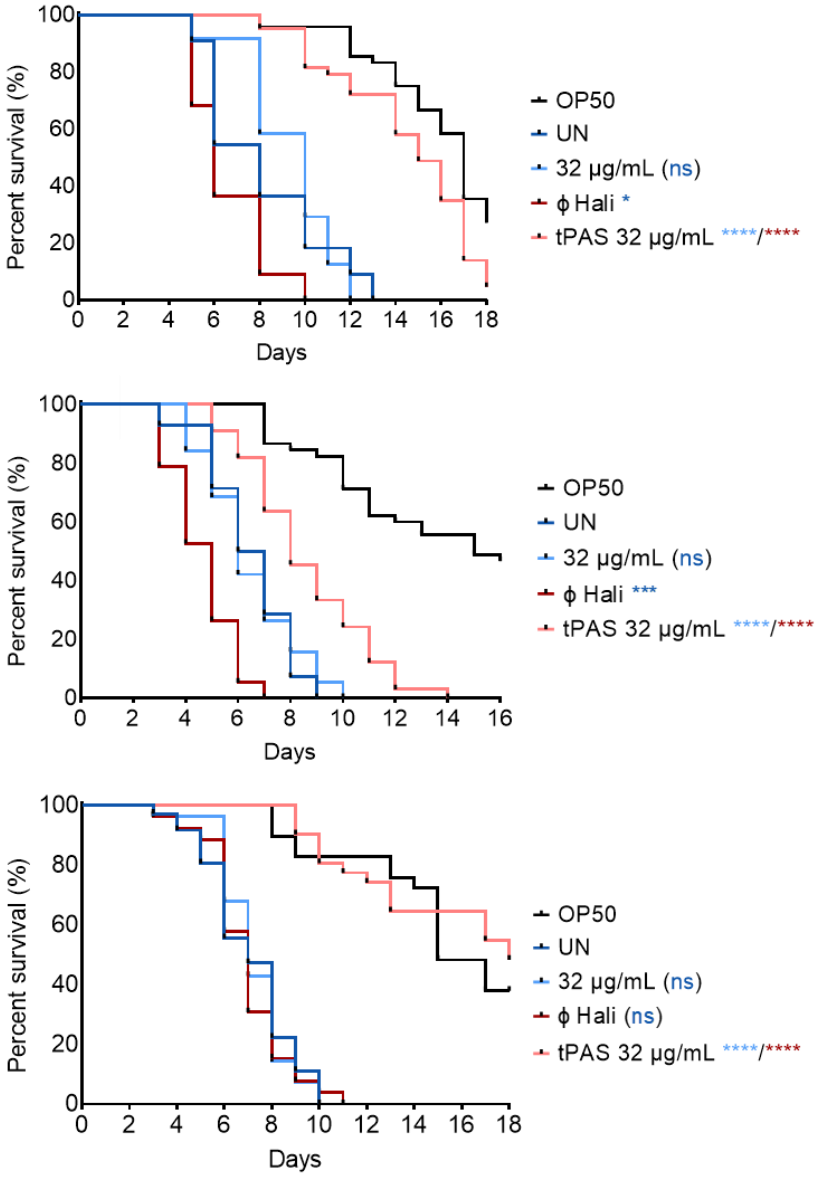


Figure 16. Phage Hali and low dose ciprofloxacin increase survival of *P. aeruginosa* infected worms on standard *E. coli* OP50 diet after treatment. Representative life span of worms on OP50 after 4 h *P. aeruginosa* infection followed by 18 h phage Hali ± ciprofloxacin treatment**.** Approximately 50 worms were added to each condition at the time of treatment, tested in three biological replicates. Survival curves were compared using the Log-rank (Mantel Cox) test with one pair compared at a time, ns = not significant, * $P \leq 0.05$, *** $P \leq 0.001$, and **** $P \leq 0.0001$. Phage alone and antibiotic alone were compared to the untreated, and tPAS to the phage alone and antibiotic alone, as denoted by colour of the significance value.

that had shown synergy between phage Hali and ciprofloxacin translated to a robust, reproducible increase in worm survival at concentrations where neither agent alone had any detectable effect.

We wished to correlate survival with bacterial colonization assays as carried out earlier (Figure 15) but found that treatment outcomes for these shorter 4 h infections did not correlate to bacterial loads (Figure S9). For instance, 32 µg/mL ciprofloxacin with or without phage were both successful in eradicating *Pseudomonas*, even though the antibiotic-alone treatment had no effect on lifespan (Figure S9). The most likely explanation is that the phage-antibiotic combination is benefiting the worms through either faster eradication, qualitatively different endpoints for the bacterium (phage-mediated lysis rather than antibiotic-mediated death), or that the presence of transient lysogeny is in some way mitigating the virulence of the strain.

4.5.4 The lysogen is more sensitive to ciprofloxacin *in vivo*

To determine whether the formed lysogens were differentially virulent, we infected worms with wild type C0400 and a C0400 phage Hali lysogen, followed by ciprofloxacin treatment and subsequently monitoring survival on the standard *E. coli* OP50 diet. Without treatment, the pathogenicity of the lysogen and wild type strains did not differ (Figure 17a).

Intriguingly, lysogen-infected worms could be completely rescued with 32 µg/mL ciprofloxacin, which was insufficient to rescue worms infected with the wild type C0400. This strongly supports the notion that the tPAS observed by co-administration of the phage and antibiotic is primarily driven by the interaction between the prophage and the antibiotic,

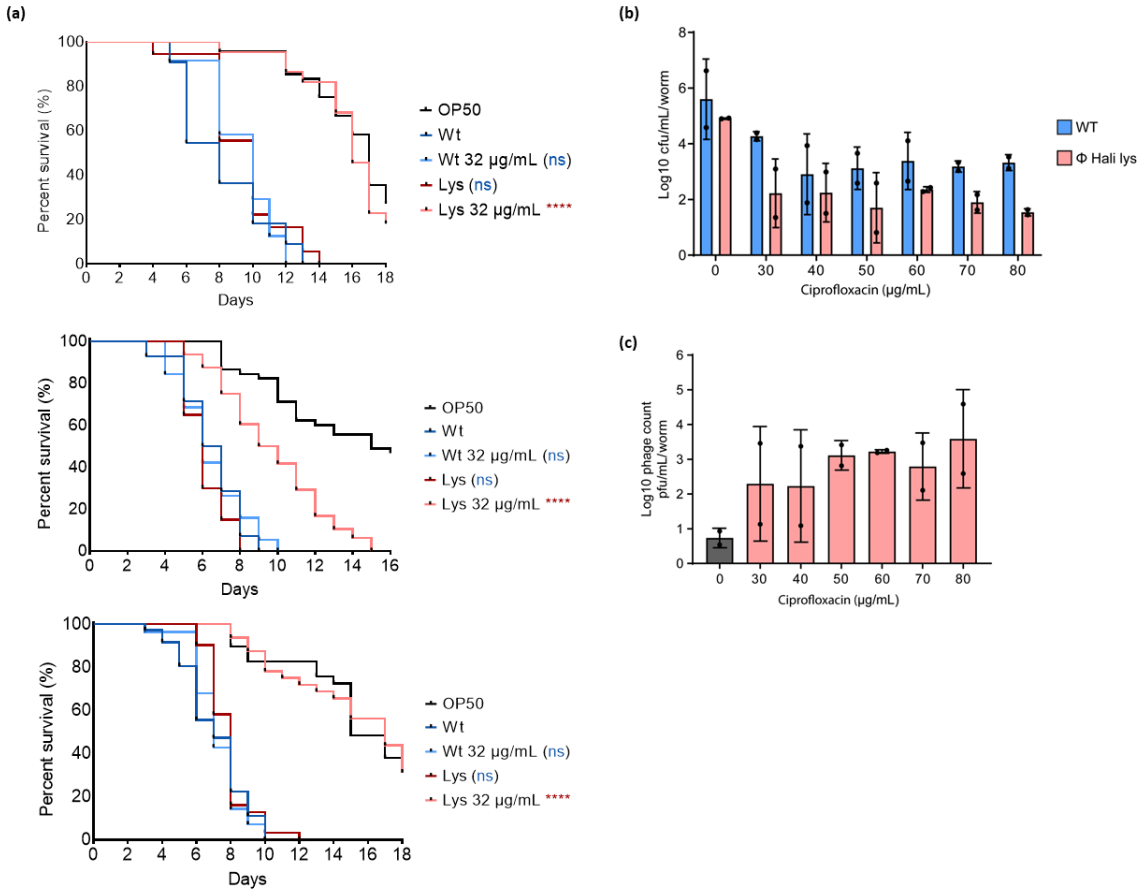


Figure 17. Phage Hali lysogen is more sensitive to ciprofloxacin *in vivo*. (a) Representative life span of worms on OP50 after 4 h *P. aeruginosa* C0400 wild type and phage Hali lysogen infection followed by 18 h ciprofloxacin treatment**.** Approximately 50 worms were added to each condition at the time of treatment, tested in three biological replicates. Survival curves were compared using the Log-rank (Mantel Cox) test with one pair compared at a time, ns = not significant, * $P \leq 0.05$, *** $P \leq 0.001$, and **** $P \leq 0.0001$. Lysogen untreated was compared to wild type untreated, and antibiotic treatment was compared to its respective no antibiotic control, as denoted by colour of the significance value. **(b)** *C. elegans* intestinal C0400 wild type and phage Hali lysogen colonization measured as log10 cfu/mL/worm after 48 h infection followed by 18 h treatment with ciprofloxacin. **(c)** Corresponding phage quantification shown as log10 pfu/mL/worm for the lysogen infected, and ciprofloxacin treated worms. All bar graph data are shown as mean ± SD where each data point is a biological replicate. Colonization assays were carried out with roughly 100 worms per condition.

as any phages induced (spontaneously or by the antibiotic) would have little effect on the lysogens, which are immune to superinfection.

This finding is particularly important mechanistically, because one possible explanation for tPAS would be that the phage administration results in a modest reduction of the bacterial load prior to the regrowth of lysogens, and that at this lower bacterial density the bacteria are more sensitive to the antibiotic – a concept known as density-dependent antibiotic sensitivity (Gutierrez et al., 2017; Udekwu et al., 2009). However, here the phage and antibiotic are truly synergistically interacting. This similar rescue observed to tPAS (Figure 16) also means that irrespective of whether the phage is co-administered or if it exists as a prophage, it can increase the effectiveness of the antibiotic if it is antibiotic inducible.

Considering that lysogens are often more sensitive to environmental stressors because they are carrying prophages that are responsive to these triggers (Al-Anany et al., 2021; Fatima & Hynes, 2024; Goerke et al., 2006), we assessed if carrying a prophage substantially burdens the bacteria *in vivo*, especially in the presence of antibiotic, by measuring intestinal bacterial colonization of worms infected with wild type or lysogen and treated with ciprofloxacin. Ciprofloxacin results in a higher reduction in bacterial load in lysogen infected animals compared to wild type (Figure 17b). This was associated with a higher phage count inside the worms after antibiotic treatment, consistent with induction (Figure 17c). These data are consistent with *in vitro* report that phage Hali lysogens shows 4-fold lower ciprofloxacin MIC compared to wild type, and that this is due to prophage induction (Fatima & Hynes, 2024).

The increased survival of phage Hali lysogen-infected worms treated with ciprofloxacin, taken together with a greater reduction in bacterial load observed for the lysogen, suggests that carrying an inducible phage likely imposes a major fitness cost under mild antibiotic stress. Given the abundance of prophages within bacterial hosts (López-Leal et al., 2022; Shkoporov et al., 2019) and that pathogens are well-known reservoirs of prophages (Ambroa et al., 2020; González de Aledo et al., 2023), this effect is likely already taking place during antibiotic treatments, in a form of "accidental phage therapy". It is important to note that this is likely phage dependent, not just with regards to its integration site and receptor, but especially its ability to respond to antibiotic triggers. The *P. aeruginosa* LES strain common in cystic fibrosis patients is known to contain several prophages that can be induced with ciprofloxacin *in vitro* (Fothergill et al., 2011). In addition, from patient data, the induction of these phages can play a crucial role in regulation of the pathogen in CF lungs (James et al., 2014). However, it is challenging to correlate the effects to long term antibiotic prescription since the data are incomplete. However, James et al. (2014) do propose that treatments that result in phage induction could be promising approaches to tackling *P. aeruginosa* load in these patients (James et al., 2014).

4.6 Conclusion

In phage therapy, temperate phages are discarded due to their ability to lysogenize. However, these have an enormous untapped potential due to their ability to respond to host stressing environmental cues, such as antibiotics, to switch into lytic replication, killing its host in the process. Here we show that *C. elegans* is a suitable model for studying *in vivo*

efficacy of tPAS, but this can easily be extended to traditional PAS with virulent phages. While we did not observe synergistic reduction in bacterial load, ciprofloxacin strongly selects against phage Hali lysogens. In addition, even if lysogens do arise, they can be more sensitive to the antibiotic *in vivo*, compared to the parental strain. While phage Hali is completely insufficient to rescue the worms, phage and ciprofloxacin combined increased the lifespan of *P. aeruginosa* infected worms to levels comparable to the uninfected control, at a similarly ineffective antibiotic dose. Complete rescue can be seen for the lysogen-infected worms treated with the antibiotic. The phage, even in its prophage form, greatly enhanced antibiotic effectiveness. This suggests to us that this kind of 'accidental' phage therapy already often occurs when antibiotics are prescribed.

4.7 Material and Methods

4.7.1 Bacterial strains and growth conditions

P. aeruginosa strain PA14 was obtained from Dr. Lori Burrows, McMaster University and *P. aeruginosa* C0400 was from the McMaster IIDR Wright clinical isolate collection. Bacterial strains were grown in 10 mL of lysogeny broth (LB) at 37ºC with 250 rpm shaking (Ecotron, Infors HT, Quebec, Canada). For growth on a solid medium, 1% (w/v) of LB (lysogeny broth) agar and 0.75% (w/v) of LB soft agar was used. All plates were incubated at 37ºC from overnight growth.

To prepare *C. elegans* plates, bacterial strains were streaked on 1% LB plates and incubated overnight. A single colony was inoculated into 10 mL LB broth, followed by overnight incubation with shaking. To seed plates, 200 µL of overnight culture was used

for 100 mm plates and 100 µL was used for 60 mm plates. The plates were incubated overnight at 37^{o}C prior to transferring worms on to them.

4.7.2 Phage propagation and titration

Phage Hali infecting *P. aeruginosa* PA14 and C0400 was used in this study. Phage lysates were prepared either using a primary or secondary amplification. Briefly, primary amplification was performed by inoculating 10 mL LB broth with bacteria and phage from frozen glycerol stocks for amplification overnight. Secondary amplification was performed as needed to increase phage titer by inoculating 10 mL of same day culture prepared in LB with 50 µL of primary amplification. Phages in filtrate were quantified by a standard spot test or full plate plaque assay.

Multiplicity of infection (M.O.I.) was calculated based on titer and volume of bacteria and phage being combined using the following formula: $\frac{\text{phage titer } \left(\frac{\text{pfu}}{\text{mL}}\right) \times \text{phage volume (mL)}}{\text{bacterial titer } \left(\frac{\text{cfu}}{\text{mL}}\right) \times \text{volume(mL)}}$. The bacterial titer was determined as previously described (Fatima & Hynes, 2024).

4.7.3 *C. elegans* strains and growth conditions

All experiments were performed using *C. elegans* SS104, a temperature sensitive sterile strain, *glp-4* (*bn2*) available from the *Caenorhabditis* Genetic Center (CGC, https://cgc.umn.edu/). Worms were maintained at 15^{o}C on 100 mm nematode growth medium (NGM) plates (Porta-de-la-Riva et al., 2012) seeded with overnight cultures of *E. coli* OP50, prepared as indicated above. Eggs were collected by hypochlorite treatment (Stiernagle, 2006). Eggs were rocked approximately 18 h at 20^{o}C to synchronize to L1 larval stage. L1 worms were plated on 100 mm NGM plates seeded with *E. coli* OP50 and

developed to young adults at 25^{o}C, a restrictive temperature at which they are sterile, for approximately 40 h.

4.7.4 Antibiotic stock preparation

Ciprofloxacin (hydrochloride) was obtained from Cayman Chemicals (Catalog 14286-5, Ann Arbor, Michigan, USA). Working stocks were prepared in nuclease free water. The minimum inhibitory concentration of each newly prepared batch was re-evaluated prior to use as reported previously (Fatima & Hynes, 2024).

4.7.5 *C. elegans P. aeruginosa* infection

For *P. aeruginosa* infection, young adults were washed off NGM OP50 plates with 10 mL M9 and washed three times to remove excess bacteria. Worms were transferred to 100 mm Slow Killing Media plates (SKM; 0.35% peptone, 50 mM NaCl, 2% agar, 1 mM $CaCl_2$, 5 µg/ml cholesterol, 1 mM $MgSO_4$, 20 mM KH_2PO_4, 5 mM K_2HPO_4) seeded with different *P. aeruginosa* strains for appropriate infection duration as indicated in figure legends. Worms were also plated on *E. coli* OP50 for the life span assay as a control.

4.7.6 Preparing *P. aeruginosa* infected worms for treatment

After infection with *P. aeruginosa*, worms were washed off SKM plates with 10 mL M9, followed by another three M9 washes. Each wash step consisted of pelleting the worm 2 min at 1200 rpm and removing the supernatant, except for the third wash in which they were allowed to settle by gravity. To further dilute the bacteria, 1 mL of M9 with 10 µg/mL of tobramycin was added and worms were pelleted using centrifugation, followed by transfer to an unseeded 100 mm 10 µg/mL SKM tobramycin plate for 45 min in the biosafety cabinet to allow remove surface attached bacteria. After crawling, they were

washed off with SKM tobramycin plates with 10 mL M9 tobramycin and subsequently washed another three times with 10 mL M9 tobramycin and final three times with just M9 to dilute out the tobramycin.

4.7.7 Colonization assay

To measure the efficacy of tPAS on bacterial colonization, approximately 100 infected worms were manually added to 2 mL tubes, along with ciprofloxacin (final concentration tested 0.5 µg/mL – 32 µg/mL at 2-fold increments, then 32 µg/mL - 100 µg/mL in increments of 10 µg/mL) and/or phage Hali at 1×10^9 pfu. The final volume was topped up to 1 mL using SKM broth. SKM alone was used a negative control. Tubes were incubated for 18 h at 150 rpm horizontal shaking, after which colonization assays were carried out.

Following treatment, worms were settled by centrifugation for 2 min at 1200 rpm or by gravity when working with more than 10 samples. After removing the supernatant, worms were washed three times with 1 mL M9, followed by once with 1 mL 10 µg/mL M9 tobramycin. To remove surface attached bacteria, they were transferred to 60 mm 10 µg/mL SKM tobramycin plates for 45 min. At this point, the total number of animals for each condition was counted.

Worms were washed off plates with 2 mL 120 µM levamisole in M9 (LM buffer). The number of worms remaining on the plate was counted to determine the number of animals per condition going into the lysis step. Animals were washed with 1 mL 10 µg/mL tobramycin LM buffer; once for the 4 h treatment and three times for the 18 h treatment.

To dilute out the tobramycin, worms were additionally washed three times with 1 mL M9. In the last wash, 500 µL of supernatant was transferred into a new tube.

Approximately 10 sterile silica carbide beads were added to each tube and worms were lysed for 1 min at speed 5 using the Bead Mill 4 (Fisherbrand, catalog 15340164). Lysed fractions and the last wash supernatants were serial diluted 10-fold in LB up to 10^{-8} and 3 µL was spotted onto a 1% LB plate in technical triplicates, along with one replicate for the last wash. LB only and M9 with beads were spotted as control. Plates were incubated at 32^{o}C to prevent overgrowth and colony forming units were counted after an overnight incubation. Bacterial load was determined by subtracting the counts from the last wash and the cfu/mL was normalized to the number of animals per condition.

4.7.8 Frequency of lysogeny

Frequency of lysogeny in the survivors was determined as previously described (Fatima & Hynes, 2024). Twenty colonies for each condition were streak purified three times on 1% LB plate and inoculated in 200 – 250 µL LB broth overnight in a narrow 96 well plate. Wild type culture, C0400 phage Hali lysogen, and LB broth were added as controls. The plates were incubated for 18-24 h at 37^{o}C with no agitation. The inoculated plates were stamped on an agar overlay of wild type C0400 using a disposable pin replicator. Plates were also stamped onto 1% LB with no agar overlay to serve as growth controls. Frequency of lysogeny was calculated as percent of the total number of survivors that resulted in clearing of the wild type host.

4.7.9 Survival on OP50 life span assay

C. elegans were infected with *P. aeruginosa* strains, or OP50 as a control, for 4 h and washed off as indicated above. The volume of infected worms per strain was adjusted to 1 worm/µL and approximately 100 worms were sorted into a 96 well plate using the COPAS FP (Union Biometrica, Holliston, MA). These were then transferred to a 2 mL tube, along with 50 µL of 20× ciprofloxacin stock (final concentration tested 0.25 µg/mL – 32 µg/mL at 2-fold increments, then 32 µg/mL - 100 µg/mL in increments of 10 µg/mL), phage Hali at 1×10^9 pfu. Nuclease free water was used as substitute for antibiotic and LB in place for phage. The final volume was topped up with SKM broth to 1 mL. The worms were incubated in treatment for 18 h shaking horizontally at 150 rpm.

Following treatment, animals were washed with 1 mL M9 three times, allowed to settle by gravity in the third wash, and once with 1 mL 10 ug/mL M9 tobramycin. They were transferred to 60 mm SKM tobramycin plates for 45 min to crawl, after which they were washed off with 2 mL M9 tobramycin. They were washed once more with 1 mL M9 tobramycin, ending with final three washes with M9 alone.

After the last wash, animals were moved to 60 mm SKM plates seeded with OP50. Plates were incubated at 25^{o}C and starting at day 3, were scored daily for survival, with dead worms being removed each day. Survival was plotted using the Kaplan–Meier method.

4.7.10 Statistical tests

Details of the statistical tests can be found in the figure legend. N denotes a biological replicate performed from an independently grown culture with appropriate

technical replicates indicated in the figure caption. Quantitative values are represented by mean ± SD. All statistical analysis was done using GraphPad Prism 10.3.0 (GraphPad Software, Inc., CA, US), with P value ≤ 0.05 is considered significant.

Chapter 5 - Conclusion

5.1 Summary

While temperate phages are currently discarded in therapy due to their ability to lysogenize, in this thesis, I present an approach to leverage their potential using a combination temperate phage-antibiotic for bacterial infections. In chapter 2, we showed that *E. coli* K-12 killing by temperate phage HK97, a Lambda-like phage, is drastically enhanced in the presence of ciprofloxacin (Al-Anany et al., 2021). Co-administration of HK97 and ciprofloxacin reduced the antibiotic MIC by up to 32-fold in a checkerboard assay and resulted in complete bacterial eradication after overnight exposure. This functioned by preventing lysogen overgrowth through antibiotic-mediated RecA-dependent induction, mechanistically distinct from traditional PAS. In parallel published work on which I was the second author, we also reported that gentamicin, a protein synthesis inhibitor, shows comparable reduction in survivors and frequency of lysogeny with HK97 (Al-Anany et al., 2024). However, synergy with gentamicin operated at the initial time of infection, biasing the phage away from lysogeny and towards lysis, the first reported way to block entry into lysogeny.

With demonstrated synergy between a model temperate phage and ciprofloxacin, in chapter 3 I explored the generalizability of tPAS in a multi-drug resistant, clinically relevant bacterium *P. aeruginosa*. Here I outlined that temperate phages can be easily isolated from clinical collections and broadly show synergy with six antibiotics (ciprofloxacin, levofloxacin, meropenem, piperacillin, tobramycin, polymyxin B), irrespective of their target. Meropenem and tobramycin were effective, but did not reduce the frequency of lysogens. In contrast, ciprofloxacin and piperacillin were able to reduce

the frequency of lysogen survivors by 93% and 58%, respectively, relative to phage alone. While ciprofloxacin can induce these prophages, piperacillin does not. This is the first reported instances of piperacillin's ability to influence the lysis-lysogeny equilibrium and that this is independent of phage induction, potentially at the time of initial infection. Interestingly, the same tPAS effect can be accomplished when ciprofloxacin administration is delayed, however delaying the phage results in loss of the synergy, further supporting the mechanism to be operating at the level of induction. Time course assays should be conducted in the future to establish exact point of interaction. Ciprofloxacin also worked in combination with multiple phages, even in antibiotic resistant clinical host, functionally re-sensitizing the bacterium to the antibiotic.

With demonstrated synergy in an antibiotic-resistant background, I looked to investigate this phenomenon in an *in vivo* animal model. In chapter 3, I developed *C. elegans* as a suitable infection model for investigating tPAS. Using phage Hali and ciprofloxacin, I demonstrated that while tPAS did not result in substantial reduction in bacterial load, it did select against lysogen survivors *in vivo*. Complementary to our *in vitro* findings from chapter 3, this appeared to be operating at the level of phage induction *in vivo* as well. Interestingly, lysogen infected worms were also completely rescued with antibiotic treatment alone, at a concentration that was insufficient to rescue non-lysogen infected animals, demonstrating that temperate phage even in their prophage form contribute to antibiotic sensitivity and providing further insight into the mechanism of tPAS. A common hypothesis for tPAS might be that the phage substantially reduces bacterial density initially which increases antibiotic effectiveness in a density dependent

manner. However, the finding that the same effect can be observed with the lysogen does not support this hypothesis, suggesting that the phage and antibiotic truly do synergistically interact. In addition, these findings highlight that even if lysogens escape treatment, they can still be antibiotic sensitive because of carrying a prophage that is responsive to external stressors. Granted that most bacteria already contain prophages, these phages likely also play a role in antibiotic success in the clinic. The dual mechanism - antibiotic action combined with phage-mediated lysis - could amplify the bactericidal effect. The newly released phage particles that infect and kill nearby susceptible bacteria could further enhance the antibiotic's impact.

Interestingly, some antibiotics potentially evolved to be a form of bacterial warfare strategy that induce prophages in competitor. Pyocyanin, a redox-active blue/green pigment with antibacterial properties produced by *P. aeruginosa*, can selectively induce prophages of competing *S. aureus*, which often co-colonizes the same infection niches (Jancheva and Böttcher, 2021). Sublethal concentrations of environmentally produced antibiotics, such as tetracycline and colistin, could induce *Pseudomonas* prophage Pf4 which superinfects neighboring bacteria, resulting in fitness defects (Bucher et al. 2024). These studies highlight the evolutionary advantage of producing antibiotics to gain a competitive advantage through prophage induction. The bacteria likely only need to produce these in enough amounts to sufficiently trigger a stress response in its neighbouring cell that its resident prophage can detect.

5.2 Limitations and Future Work

In chapter 2, we showed that antibiotics directly influence the lysis-lysogeny equilibrium, and hence tPAS is unique and mechanistically distinct from traditional PAS. This was concluded on the basis that reported mechanisms of traditional PAS such as increased phage production and changes in phage replication cycle (Comeau et al., 2007; Kamal & Dennis, 2015; Kim et al., 2018) do not appear to be at play with tPAS. However, it is entirely possible that tPAS could be a result of both mechanisms at play, biasing lysis-lysogeny equilibrium and changing phage replication. In this case, a key area to explore would be to determine how much of the tPAS effect is specific to temperate phage and how much is due to traditional PAS. A more direct approach to this would be a series of comparison between virulent and temperate variants of the same phage. This can be made possible by isolating naturally occurring lytic variants of temperate phages that can surpass superinfection immunity and infect the lysogen. We can also genetically remove the phage lysogeny genes such as the repressor and integrase, an approach used previously in several studies (Kilcher et al., 2018; Selle et al., 2020).

In addition, while we show in chapter 2 that the synergy between HK97 and ciprofloxacin is SOS-response dependent, consistent with our understanding of prophage induction in Lambda, we have yet to show this in *Pseudomonas* in chapter 3. Much of the understanding of biasing phage lysis and lysogeny equilibrium comes from understanding of genetic switch of Lambda. However, the *Pseudomonas* phages isolated and tested in chapter 4 are transposable in nature, unlike Lambda, where triggers of the lysis-lysogeny switch remain unclear. We would test tPAS in this model on a strain deficient in the

bacterial SOS response, specifically Δ*recA*. If synergy is still observed, it would mean tPAS here is independent of the host SOS-response and that the phage and antibiotic interact at the level of an alternative bacterial response pathway. In cases where tPAS was operating at the level of induction, such as between phage Hali and ciprofloxacin, this may also allow for the identification of a non-canonical bacterial stress response involved in prophage induction.

While we believe that we have clearly demonstrated that tPAS is unique, and mechanistically distinct from traditional PAS - and have even shown mechanisms through which it can occur, we failed (chapter 3) to predict when tPAS would occur. It is clearly phage, host, and antibiotic specific - and we have not explored the genetic factors that underpin this, but our hypothesis that the frequency of lysogenization or the ability of the antibiotic to result in induction could predict it was proven incorrect. We could not reliably predict if and how strongly a temperate phage would synergize with a particular antibiotic based on these uniquely inherent properties of the host-phage-antibiotic interaction.

In the *C. elegans* model in chapter 4, we were unable to correlate increases in worm survival to reduction in bacterial load especially at 32 µg/mL ciprofloxacin, where both antibiotic and tPAS resulted in no survivors, but only tPAS rescued life span. The lysogen and wild type were not differentially virulent in this model, so this would not explain the rescue phenotype. We are left to hypothesize that kinetics matter here. To investigate this further, we can do time course colonization experiments after treatment to compare how quickly the antibiotic alone versus tPAS kill the bacteria. It is possible that the antibiotic

alone takes longer to eradicate, thereby leaving the *Pseudomonas* more time to cause damage to the animals in the process, leading to the lack of rescue.

Our finding that lysogen infected worms can be rescued with antibiotic alone at a concentration that was insufficient to rescue wild type infected worms (chapter 4) opens another exciting avenue of exploration; how much do prophages contribute to the success of antibiotics in the clinic? Bacterial pathogens are well known to harbour prophages (Ambroa et al., 2020; González de Aledo et al., 2023; Gutiérrez et al., 2016; Shan et al., 2012; Touchon et al., 2016; Weiser et al., 2020) and antibiotics are well-known to induce prophages in many clinical isolates (Fothergill et al., 2011; López et al., 2014; Meessen-Pinard et al., 2012). For example, *P. aeruginosa* LES, the most common cystic fibrosis epidemic strain in United Kingdom, contains six prophages, four of which can produce active phage particles (Winstanley et al., 2009). *In vitro*, ciprofloxacin and norfloxacin, both DNA damaging antibiotics, result in an increase in total phage production from several LES isolates (Fothergill et al., 2011). Interestingly, in lungs of CF patients, James et al., (2014) reported a negative correlation between LES phage-to-bacterium ratio and bacterial density, suggesting that the release of these prophages potentially plays a crucial role in regulation of the pathogen in CF lungs. However, this was not associated with disease exacerbation. Since the complete antibiotic prescription history of each patient is missing, especially during periods of exacerbation, it is challenging to correlate long term antibiotic prescription, phage induction, and pathogen load. Considering the limited antibiotic prescription data provided, most of these patients were prescribed either β-lactams, aminoglycosides, or polymyxin, where phage induction has not been reported. Therefore,

the findings might differ with well known phage inducing antibiotics such as ciprofloxacin. To properly investigate the contribution of prophages on antibiotic effectiveness, we would require detail records of patient antibiotic prescription, along with clinical specimen for direct bacterial and phage tracking before and after treatment.

While *C. elegans* presents a simple enough *in vivo* model to quickly screen a panel of temperate phage-antibiotic pairings in chapter 4, it is not entirely sufficient. The next step would require testing the pairings that were most efficacious in *C. elegans* in mice, which share approximately 80% of their genes with human in conserved synteny (Waterston et al., 2002) and are considered the gold standard *in vivo* model. *C. elegans* are also maintained at room temperature during the experiments which can influence bacterial sensitivity to both phage and antibiotic, and they also lack adaptive immunity (Tran & Luallen, 2024) that is common to both mice and humans.

Mice trials also present an opportunity to investigate safety and dosing strategy, an important consideration in the use of tPAS clinically. In chapter 3, we show that specifically with *Pseudomonas* phage Hali, ciprofloxacin can be delayed by several hours and yet still achieve the same tPAS effect *in vitro*. The same does not hold true for the phage. This would be of interest to test with other antibiotics, especially *in vivo*. We would expect any combination working through induction to display similar effects even if antibiotic is delayed. In contrast, pairings suspected of synergizing through biasing the initial decision at the time of infection, such as the one reported between HK97 and gentamicin (Al-Anany et al., 2024), likely will not show efficacy with delayed antibiotic where the antibiotic will not have many interactions with the already formed lysogens.

Although it is intuitive to find ways to bias the phage away from lysogeny, exploring new triggers that keep the phage in the lysogenic state could be just as interesting, either further promoting entry into the lysogenic replication or supressing exit/prophage induction. This would be of clinical relevance in cases where antibiotics result in induction of virulence factors. These compounds could be prescribed along with antibiotics where there is a risk of phage induction promoting further infection such as in the case of Shiga toxin producing *E. coli* (Zhang et al., 2000) or induction of *S. aureus* pathogenicity islands (Úbeda et al., 2005).

Currently, in cases of severe drug-resistant bacterial infections, strictly lytic phages, combined with antibiotics, are prioritized. If those are difficult to isolate, genetic engineering of temperate phages to improve their killing activity would be next. The use of temperate phages alone in the clinic would likely require additional regulatory hurdles to bypass. However, the difficulty in isolation of strictly lytic phages prompts the need for investigation of approaches to make use of naturally abundant temperate phages. Given their ability to uniquely synergize with antibiotics across phages, antibiotics, and host, they do present as promising adjuvants to antibiotics, especially if we work towards gaining an understanding of how much they already contribute to the success of antibiotic treatments in the clinic.

Appendix A Supplementary Material

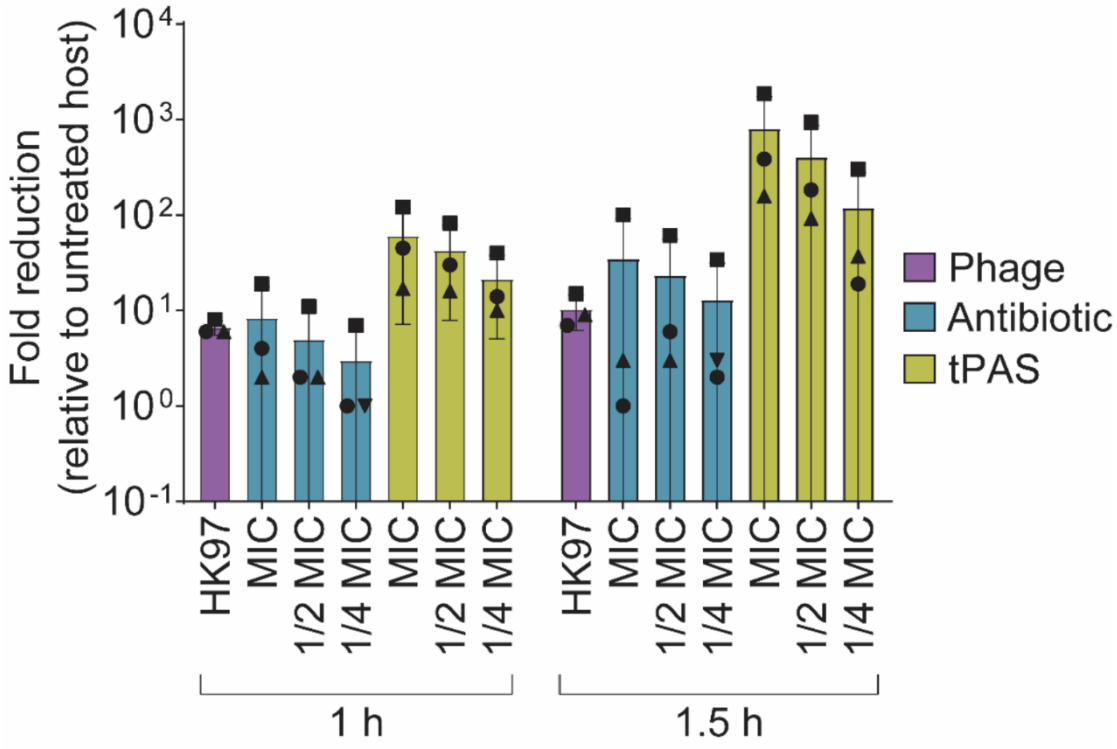


Figure S1. Short-duration HK97 temperate phage ciprofloxacin synergy quantification, related to Figure 3. Fold reduction in bacterial survivors of phage, antibiotic, and tPAS as in Figure 3, but after only 1 h (left) or 1.5 h of exposure to the treatment. Bars show average number of survivors relative to untreated cultures in three biological replicates, each of three technical replicates. Each biological replicate is represented by its own shape; circle, square or triangle. Error bars depict the standard deviation. Data were compared using one-way ANOVA and Tukey post hoc test. No pairwise comparison was found to be significantly ($p \leq 0.05$) different.

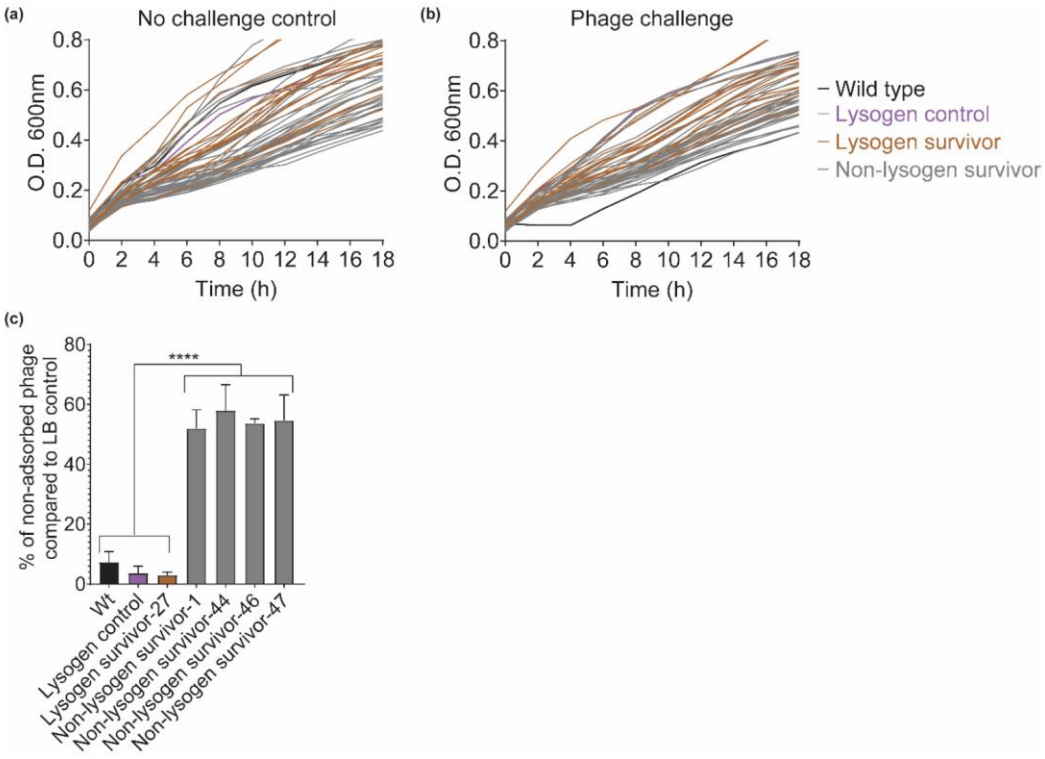


Figure S2. tPAS survivor characterization, related to Figure 5A. (Top) Growth curves in liquid culture for wild type *E. coli* K-12, lysogen control and 52 survivors out of ¼ MIC tPAS challenge were tracked when **(a)** not challenged, **(b)** when challenged with phage HK97 at MOI 10. **(c)** Adsorption assay. Percent of non-adsorbed lambdavir phage, after challenging wild type *E. coli* K-12, lysogen control and 5 survivors out of ¼ MIC tPAS, relative to phage titer incubated in LB control. Results were averaged among three biological and technical replicates ± SD. Results were compared using two-way ANOVA and Tukey multiple comparison tests with **** = $P \leq 0.0001$.

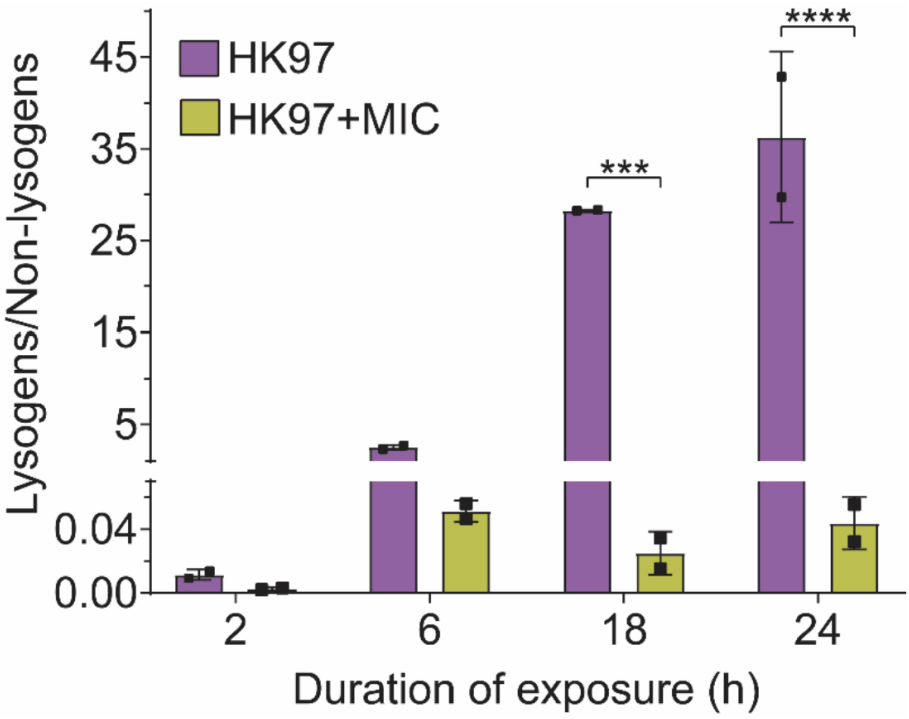


Figure S3. Temperate phage-antibiotic synergy at MIC is also a result of a depletion of lysogens, related to Figure 5B. Ratio of lysogen to non-lysogen detected using qPCR after challenging *E. coli* K-12 with HK97 alone or HK97 and ciprofloxacin at MIC. This assay is identical to that in Figure 5B, but carried out with MIC antibiotic rather than ½ MIC. Each point represents a biological replicate performed in technical triplicates. Error bar represents SD. Lysogen/non-lysogen ratio compared using two-way ANOVA and Bonferroni multiple comparison tests. *** = $P \leq 0.001$ and **** = $P \leq 0.0001$.

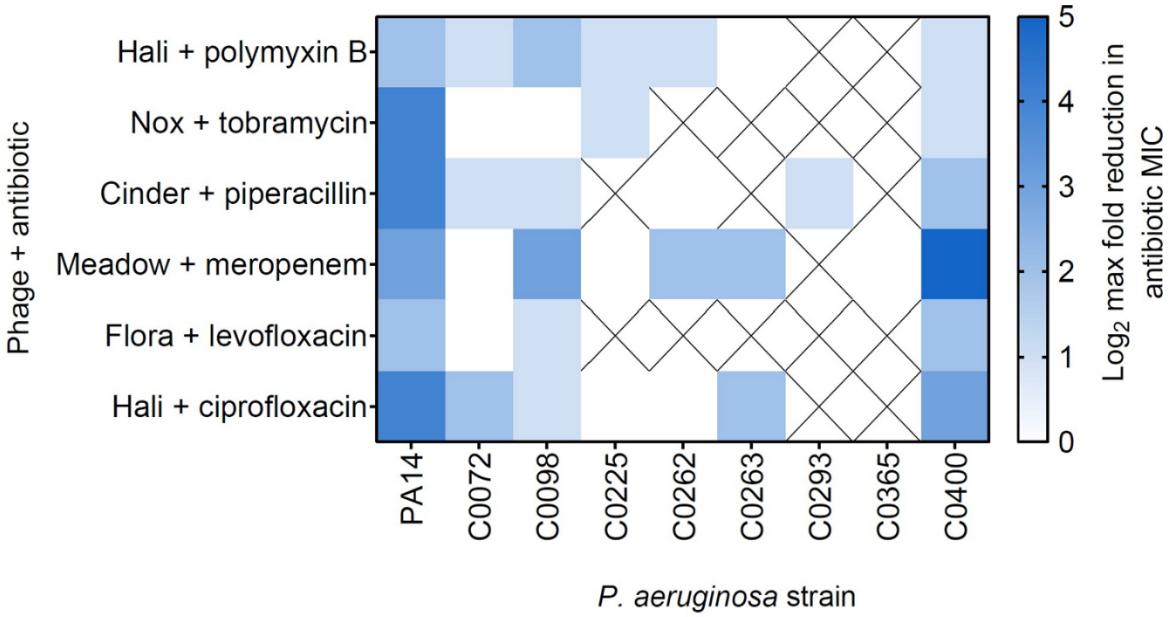


Figure S4. PAS with temperate phages across clinical isolates. Reduction in antibiotic MIC of multiple clinical strains with the co-administration of PA14 temperate phage. Average log2 maximum reduction (n=3 biological replicates, except phage Cinder + piperacillin in C0262 which is n=4) in MIC shown as heat map as determined from checkerboard assays. "X" denotes pairings that were not tested in the specified strain either due to lack of phage sensitivity or inability to obtain a high enough titer for checkerboard assay. Data represented is maximum MIC reduction regardless of phage dose (M.O.I. range of 1.25 – 40 tested in 2-fold increments).

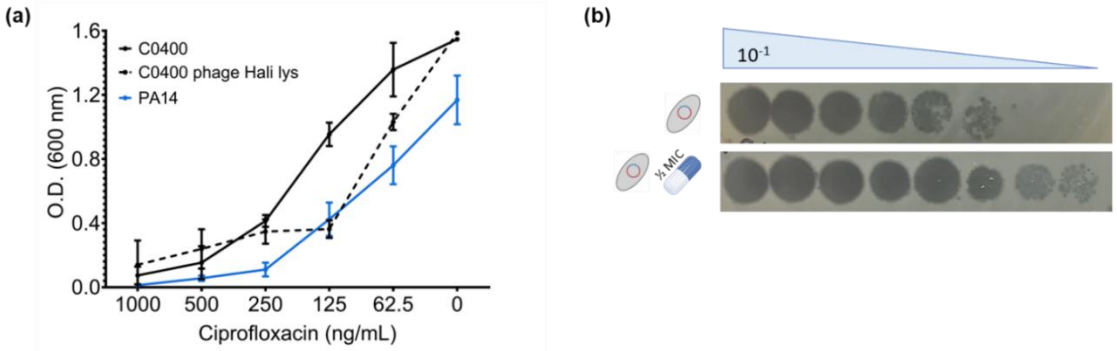


Figure S5. C0400 phage Hali lysogen is ciprofloxacin inducible. (a) End point growth (OD600) of C0400 (black line), C0400 phage Hali lysogen (dotted black line), and PA14 (blue line) across a range of ciprofloxacin concentrations (µg/mL) after 18 h. Data shown as mean ± SD (n=3 biological replicates, each in technical triplicates). **(b)** Representative end point phage titer of filtrates of C0400 phage Hali lysogen ± ½ MIC ciprofloxacin treatment.

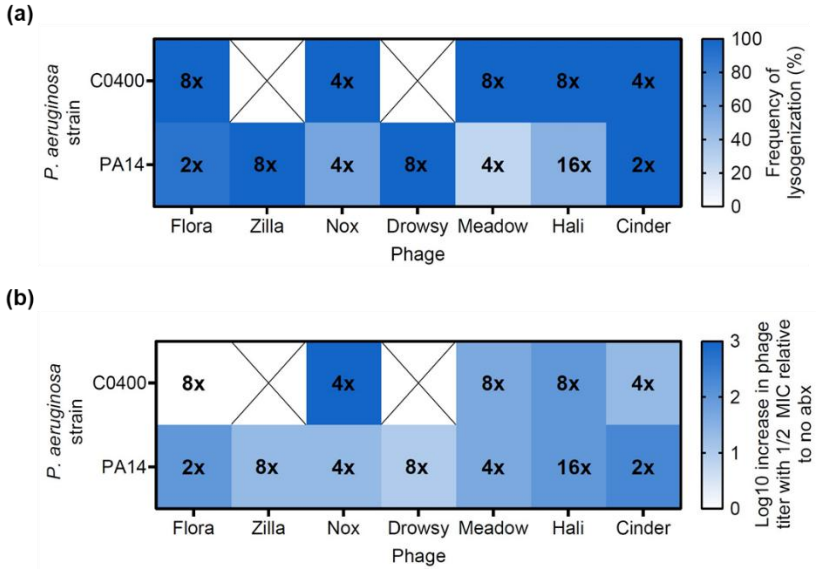


Figure S6. Level of synergy does not correlate with frequency of lysogenization or level of induction. (a) Frequency of lysogenization of temperate phages in host PA14 and C0400 as a heat map. Twenty survivors from phage challenge on solid media were purified and screened for the presence of the phage using a stamp test on wild type host. Number within the colored box indicate the reduction in ciprofloxacin MIC observed in checkerboard assay. **(b)** Log10 average increase (n=3 biological replicate, each in single technical replicate) in phage titer when PA14 or C0400 lysogen were challenged with ½ MIC ciprofloxacin relative to no antibiotic control, represented as a heat map. X denotes combination not tested.

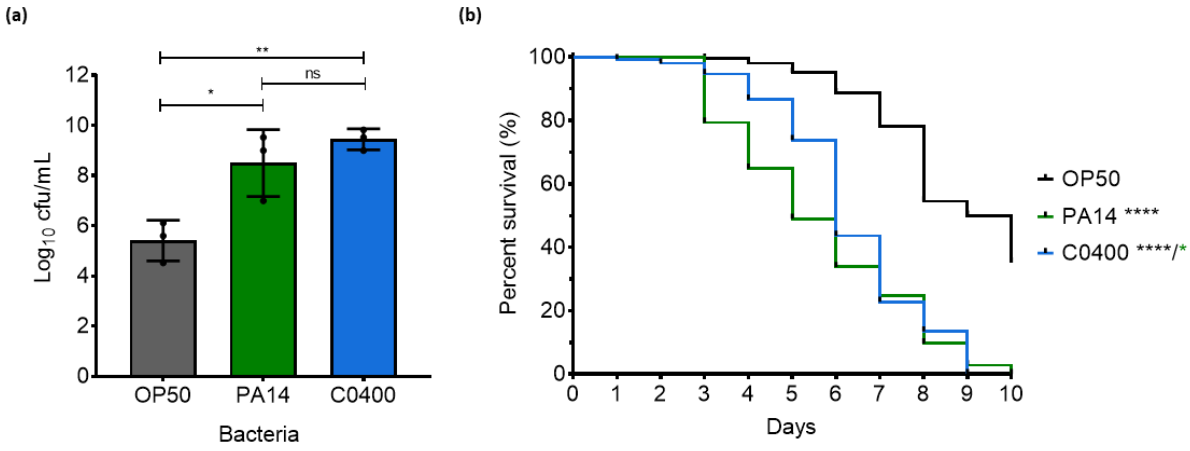


Figure S7. *P. aeruginosa C0400* can colonize and kill *C. elegans*. (a) *C. elegans* intestinal colonization measured as cfu/mL after 24 h growth on *E. coli* OP50 or *P. aeruginosa* strains (PA14 or C0400). Data shown as mean ± SD of three biological replicates, each in single technical replicate. Values were compared using a one-way ANOVA with a Tukey multiple comparison test with *P ≤ 0.05, **P ≤ 0.01, and ns representing not significant. **(b)** Representative survival curve of worms exposed to OP50, PA14, or C0400 in slow killing assay. Curves were compared using the Log-rank (Mantel Cox) test with one pair compared at a time, *P ≤ 0.05 and **** P ≤ 0.0001. Each condition is 150-200 animals. The colour of the significance value represents the comparison performed, with black representing comparison to control and green to PA14.

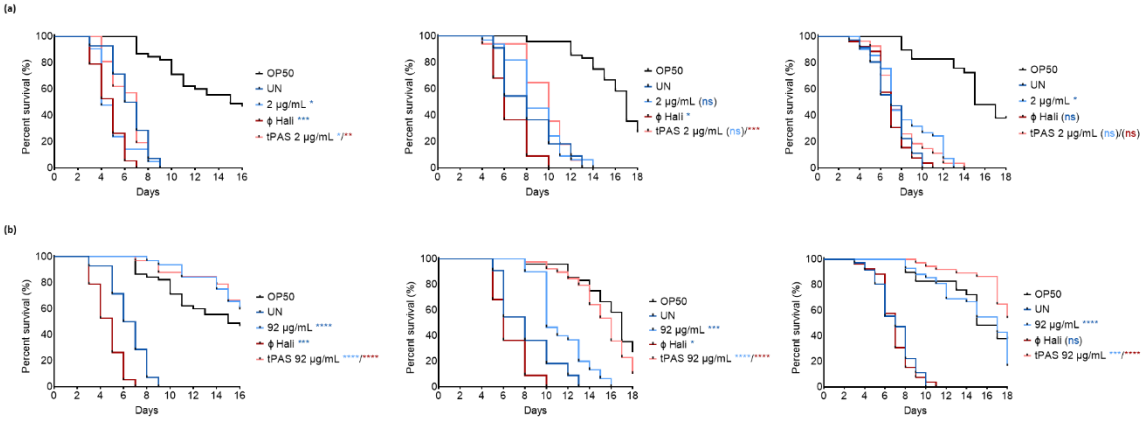


Figure S8. Ciprofloxacin alone at 92 ug/mL could rescue life span of *P. aeruginosa* infected worms on standard *E. coli* OP50 diet after treatment. Survival curve of worms on OP50 after 4 h *P. aeruginosa* infection followed by 18 h phage Hali ± ciprofloxacin treatment, **(a)** low dose 2 µg/mL and (b) high dose 92 µg/mL**.** Approximately 50 worms were added to each condition at the time of treatment. Independent biological replicates are shown for each concentration. Survival curves were compared using the Log-rank (Mantel Cox) test with one pair compared at a time, ns = not significant, * $P \leq 0.05$, *** $P \leq 0.001$, and **** $P \leq 0.0001$. Phage alone and antibiotic alone were compared to the untreated, and tPAS to the phage alone and antibiotic alone, as denoted by colour of the significance value.

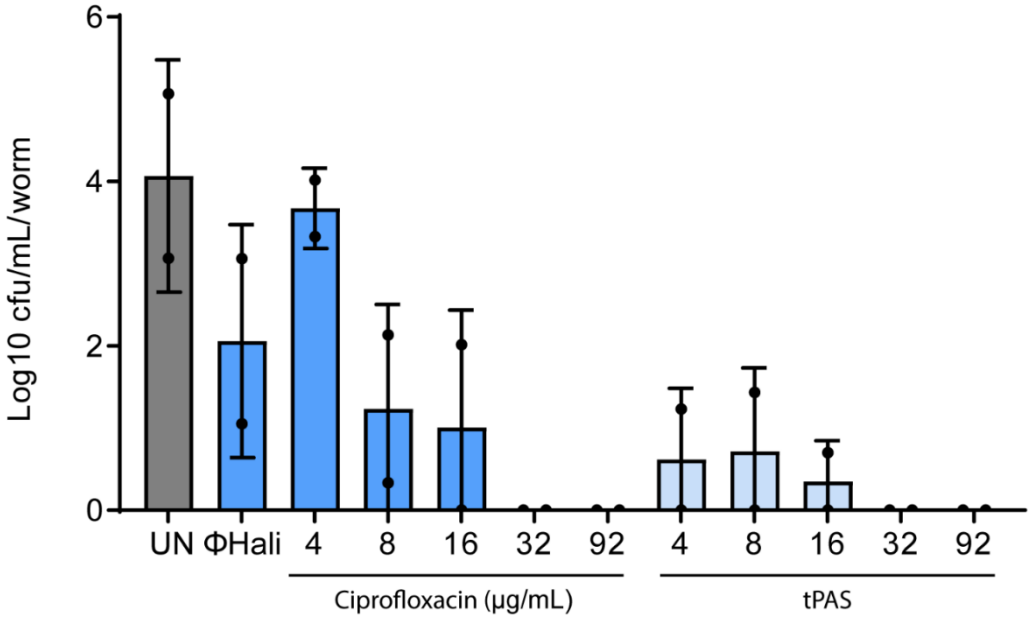


Figure S9. Shorter infection prior to treatment does not provide enough resolution to measuring bacterial colonization. *C. elegans* intestinal colonization measured as log10 cfu/mL/worm after 4h C0400 infection followed by 18 h treatment with phage Hali (1 x 10^9 pfu/mL) ± ciprofloxacin. UN denotes untreated. Data shown as mean ± SD where each data point is a biological replicate (~100 worms each) plated in technical triplicates.

Table S1. Key resources from chapter 2.

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Bacterial and virus strains		
Escherichia coli K-12	Félix d'Hérelle Reference Center for Bacterial Viruses	*Ymel mel*-1 supF58
E. coli BW25113 Δ*recA*	Dharmacon™	KEIO collection
Lambdoid phage HK97	Félix d'Hérelle Reference Center for Bacterial Viruses	λ-like phage
Chemicals		
Ciprofloxacin hydrochloride	Cayman chemicals	Cat #14286-5
Critical commercial assays		
PowerUp SYBR Green Master Mix	Applied Biosystems	Cat # A25742
Deposited Data		
Raw data	This paper; Mendeley Data	http://dx.doi.org/ 10.17632/xtjxj6hz4d.1
Oligonucleotides		
attBF: TGAATCCGTTGAAGCCTGCT	This paper	Primers for lysogen detection
HK97_lys_R: GCGTGTAATTGCGGAGACTT	This paper	Used with attBF for lysogen detection
attBF-veR : GCCTCGATTACTGCGATGTTTAG	This paper	Used with attBF to detect non-lysogens in qPCR
HK97_lys_R2: CGTGATGACAGAGGCAGGG.	This paper	Used with attBF to detect lysogens in qPCR
CysG_F2: AGGGGTTTTTACGTGGATCATTTG	This paper	Primers for *E. coli* cysG detection in qPCR
CysG_R2: GGTGAACTGTGGAATAAACGCT	This paper	Primers for *E. coli* cysG detection in qPCR
Software and algorithms		
ImageJ	(Schneider et al., 2012)	https://imagej.nih.gov/ij/
GraphPad Prism 8.3.0	GraphPad	https://www.graphpad.com/
Adobe Illustrator CC 2019	Adobe	https://www.adobe.com/ca/products/illustrator.html

Appendix B Reprint Approval

Reprint approval of chapter 2 paper

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ELSEVIER

Temperate phage-antibiotic synergy eradicates bacteria through depletion of lysogens

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