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Investigating the potential of phage therapy to tackle *Pseudomonas aeruginosa* infections in dogs

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Declaration

I declare that this thesis has been composed solely by myself and that it has not been submitted, in whole or in part, in any previous application for a degree. Except where stated otherwise by reference or acknowledgement, the work presented is entirely my own.

Shannon Street, 19th September 2023

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List of abbreviations

AMR: antimicrobial resistance
AUC: area under curve
BLAST: Basic Local Alignment Search Tool
CARD: Comprehensive Antibiotic Resistance Database
CF: cystic fibrosis
CFU: colony-forming units
COG: Clusters of Orthologous Genes
CRISPR: clustered regularly interspaced short palindromic repeats
CV: crystal violet
DLA: double layer agar
dsDNA: double-stranded DNA
eDNA: extracellular DNA
EOP: efficiency of plating
EPS: exopolysaccharides
LB: lysogeny broth
LPS: lipopolysaccharide
MDR: multidrug-resistant
MHB: Mueller-Hinton broth
MIC: minimum inhibitory concentration
MOI: multiplicity of infection
NCBI: National Center for Biotechnology Information
OD: optical density
OE: otitis externa
OprM: outer membrane porin M
PBS: phosphate-buffered saline
PFU: plaque-forming units
PHASTER: Phage Search Tool Enhanced Release
PHROG: Prokaryotic Virus Remote Homologous Groups
PNA FISH: peptide nucleic acid fluorescence in-situ hybridization
qPCR: quantitative polymerase chain reaction
RBP: receptor-binding protein
RGI: Resistance Gene Identifier

SM: saline magnesium

SNP: single nucleotide polymorphism

TEM: transmission electron microscopy

TSA: tryptone soya agar

TSB: tryptone soya broth

Abstract

Antimicrobial resistance is an issue of increasing importance worldwide with research attention being dedicated to the development of alternative therapeutics, including phage therapy. This involves the use of bacteriophages to fight bacterial infections. The potential of phage therapy in veterinary medicine has not yet been fully exploited. *Pseudomonas aeruginosa* otitis is a common condition seen in canine veterinary practice and is one of the most challenging ear infections to treat, largely due to resistance of *P. aeruginosa* to multiple antibiotics. This study aimed to isolate novel lytic bacteriophages with activity against canine otitis isolates and investigate their use as a potential treatment. Double layer agar (DLA) assays were performed using *P. aeruginosa* canine otitis isolates to identify and isolate phages from wastewater samples by assessment for plaques. This resulted in the isolation of five phages. Activity of the phages against 24 *P. aeruginosa* canine otitis isolates was evaluated using DLA assays and planktonic growth curves. The host ranges of the five phages were relatively limited, with the broadest host range of any phage being five out of the 24 clinical isolates. Further characterisation of the phages was performed using genome sequencing and transmission electron microscopy. All five phages were assessed to be of Siphovirus morphology. Genome analysis demonstrated that four of these phages were likely to be of a lytic nature. Overall, this study describes five novel phages with activity against *P. aeruginosa* canine clinical isolates and contributes to the ongoing work to develop alternative therapeutics to antimicrobial resistant infections in companion animal veterinary practice.

Lay summary

Many bacterial pathogens that cause clinically important infections in both humans and animals can develop resistance to antibiotics, rendering the infections very difficult to treat with conventional drugs. The prevalence of antibiotic-resistant infections is increasing worldwide at a concerning rate. For this reason, research attention is now being directed at the development of alternative treatments. One of these possible treatments is termed 'phage therapy'. This is where bacteriophages, which are viruses that target bacteria, are used to kill the bacteria causing infections. Bacteriophages, also known as 'phages', are ubiquitous in the environment. They tend to be quite specific for the type of bacteria that they will target and kill. Research and the use of phage therapy is not yet widespread in companion animal veterinary medicine. *Pseudomonas aeruginosa* is a common bacterial cause of ear infections in dogs. These infections can be very difficult to treat due to resistance to multiple antibiotics. This study aimed to find bacteriophages that would be able to kill *P. aeruginosa* bacteria from infected dog ears, with the potential of using these as a new treatment. To find these phages, the bacteria were grown on agar and wastewater samples were added. The plates were then analysed for small holes in the bacterial growth which indicate killing by bacteriophages present in the wastewater. The phages were then isolated from these plates and five phages with killing activity were found. How well these phages killed 24 different *P. aeruginosa* strains from dog ear infections was tested. The sequences of the genetic material in each phage were also assessed to learn more information about the phages. Images were taken of the phage particles using an electron microscope, which is used to examine very small structures, like viruses. Overall, this study characterises five previously unknown phages that can kill some *P. aeruginosa* strains from dog ear infections and contributes to the ongoing research to find alternative treatments for antibiotic-resistant infections in companion animal veterinary practice.

CHAPTER ONE – INTRODUCTION

1.0. Introduction

1.1. *Pseudomonas aeruginosa* infections and clinical significance

Pseudomonas aeruginosa is a ubiquitous Gram-stain-negative bacterium found throughout the environment, including sewage and water,¹ and is a common opportunistic pathogen in humans.¹⁻³ In particular, it is an important pathogen of immunocompromised individuals and people with cystic fibrosis (CF), often causing chronic infections.^{1,2} In canine veterinary practice, *P. aeruginosa* is also an opportunistic pathogen of significance, predominantly in otitis,^{4,5} but can be associated with other conditions including pyoderma,⁶ ulcerative keratitis^{7,8} and urinary tract infections.⁹ *P. aeruginosa* is the most frequently isolated Gram-stain-negative bacterium in canine otitis cases^{4,10,11} and is especially prevalent in chronic cases.^{10,12} The bacterium is generally not present in clinically healthy dog ears^{4,10,13,14} and can be isolated from 28% to 37% of otitis cases.^{11,15,16} A large study of vet practice data in England reported otitis externa (OE) as the most prevalent disorder in dogs with a prevalence of 10%.¹⁷ As canine otitis is such a common condition and *P. aeruginosa* is involved in a notable proportion of cases, infections of this nature pose a significant impact on animal welfare. In addition to this, companion animal *P. aeruginosa* infections present potential zoonotic transmission risk and, although the published evidence of this is limited,¹⁸ there have been some suspected cases.¹⁸⁻²⁰ Therefore, *P. aeruginosa* infections in dogs could also have an important link with human health.

Infections involving this bacterium can be extremely difficult to treat as they are often resistant to conventional antimicrobials. *P. aeruginosa* was one of the six dominant bacterial pathogens associated with antimicrobial-resistance-related human deaths in 2019²¹ and is listed by the World Health Organisation as a ‘priority pathogen’ for the development of new antimicrobial agents.²² Resistance of these infections to antibiotics is also widely documented in companion animals with a summary of some reported antimicrobial resistance (AMR) rates in canine otitis isolates in Table 1.^{12,23-}
²⁸ *Pseudomonas* otitis is often viewed as one of the most challenging ear infections

to treat in dogs^{4,5} and, in severe cases, veterinarians resort to surgery to remove the affected ear canal.⁴

Authors	Region of data origin	Years data collected	% of isolates resistant to antibiotic					
			ERO	CIP	MAR	GM	TIC	CAZ
Bourély <i>et al.</i> ²³	France	2012-2016	67.7	-	-	17.9	-	-
Mekić, Matanović and Šeol ²⁴	Croatia	2007-2009	51.9	8.7	-	43.3	10.6 (+ CA)	0
Martín Barrasa <i>et al.</i> ¹²	Gran Canaria, Spain	Unknown – published 2000	26.3	10.5	0	15.7	15.7	10.5
KuKanich, Bagladi-Swanson and Kukanich ²⁵	Midwestern USA	2018-2020	30.6	-	25.0	7.4	-	0.92
Hariharan <i>et al.</i> ²⁶	Canada	1999-2003	38	-	-	15	-	-
Bugden ²⁷	Australia	2008-2009	36*	-	-	5	-	-

Table 1. Reported *in vitro* antimicrobial resistance rates in *P. aeruginosa* otitis isolates from dog ears.

Abbreviations: ERO = erythromycin; GM = gentamicin; CIP = ciprofloxacin; CAZ = ceftazidime; TIC = ticarcillin; MAR = marbofloxacin; CA = clavulanic acid.

*Surrogate testing used – actually tested against ciprofloxacin or moxifloxacin

1.2. Mechanisms of antimicrobial resistance of *P. aeruginosa*

As mentioned above, *P. aeruginosa* is particularly capable at forming chronic infections and this is due to its ability to adapt to different biological niches and to resist antibiotic treatments via numerous mechanisms.¹⁻³ The main mechanisms with which *P. aeruginosa* is able to resist antibiotics can be split into three categories: intrinsic, acquired and adaptive.² Intrinsic mechanisms are inherently present in a bacterium and *P. aeruginosa* has been shown to contain a high number of these.² Some examples include the production of an AmpC β -lactamase which enables the inactivation of β -lactam antibiotics^{2,29}; restricted outer membrane permeability²; and the expression of efflux pumps, like MexAB-OprM which expels β -lactam and fluoroquinolone antibiotics out of the bacterial cell.^{2,29-31}

Acquired antibiotic resistance refers to mechanisms which are obtained by the bacterium through mutations to the bacterial genetic material or horizontal gene transfer.² Many *P. aeruginosa* strains isolated from the chronically infected lungs of CF patients have been shown to have a 'hypermutable' or 'mutator' phenotype, thought to be due to alterations in the bacterial DNA mismatch repair genes resulting in a high frequency of mutations.³²⁻³⁵ This can lead to mutations that further enhance the ability of the bacteria to resist antibiotics; for example, increased production of β -lactamase and higher expression of the MexCD-OprJ efflux pump.³² A relationship has been found between the presence of 'hypermutable' phenotypes and higher antibiotic resistance levels in CF patients.^{33,35} The 'hypermutable' phenotype appears to be much more prevalent in chronic infections, as opposed to acute infections, which could indicate the importance of high mutation rates for *P. aeruginosa* to adapt and persist in a host long-term.^{33,36} Horizontal gene transfer enables the acquisition of genes conferring antibiotic resistance mechanisms between bacteria via plasmids, transposons, integrons and prophages.^{2,37} This is known to occur frequently in *P. aeruginosa*; for example, the acquisition of metallo- β -lactamases which have been found to be present on some mobile genetic elements, including plasmids and integrons.^{2,38,39} In addition, the study of *P. aeruginosa* prophage sequences, contained within the bacterial genomes, has shown them to contain numerous antibiotic resistance genes.⁴⁰

Adaptive resistance is induced by the presence of an environmental trigger or an antibiotic, which lead to a change in gene and/or protein expression conferring some antibiotic resistance to the bacterium.^{2,37} The main mechanism of this with *P. aeruginosa* is biofilm formation.² *P. aeruginosa* is notoriously known for its production of biofilm which poses important clinical significance,³ especially in infections of CF patients⁴¹ and colonisation of medical implants.⁴² *P. aeruginosa* biofilms are known to reduce the efficacy of antimicrobials.^{3,43} A biofilm is composed of a sessile collection of bacteria embedded within an autogenic matrix of exopolysaccharides (EPS).^{3,44} The matrix makes up 90% of the overall mass of the biofilm and has been shown to contain extracellular DNA (eDNA), protein and lipid, in addition to EPS.^{3,45} Alginate is an important EPS within biofilms produced by *P. aeruginosa* and has been shown to increase the ability of the bacterium to attach and adhere to surfaces.⁴⁶ *P. aeruginosa* strains which produce alginate are often referred to as 'mucoid strains'⁴⁶ or having the 'mucoid phenotype'⁴¹; and are frequently isolated from the lungs of CF patients with associated chronic infections.^{46,47} Alginate has been shown to confer a myriad of benefits for *P. aeruginosa* survival in the CF lung and in the past it has been assumed that alginate is also the predominant EPS within environmental and non-mucoid *P. aeruginosa* biofilms.⁴⁶⁻⁴⁸ However, two *P. aeruginosa* laboratory reference strains (PAO1 and PA14 – both originally clinical isolates from human wounds) have been shown to form biofilms without significant production of alginate.⁴⁷ Subsequently, other EPS molecules (Psl and Pel) have been found to contribute to the biofilm of non-mucoid *P. aeruginosa* instead.⁴⁹⁻⁵² The production of the different EPS molecules – alginate, Psl and Pel – is dependent on the strain of *P. aeruginosa*.^{52,53} Although the genes related to the production of Psl, Pel and alginate have all been detected in canine *P. aeruginosa* isolates to varying degrees of prevalence^{54,55}, to the best of the author's knowledge, the main EPS produced in canine *P. aeruginosa* biofilms has yet to be elucidated.

Despite its considerable ability to form monospecies biofilms, *P. aeruginosa* is known to interact with other bacterial species and form multispecies biofilms.⁵⁶⁻⁵⁸ This is particularly evident as co-infections with *Staphylococcus aureus* associated with CF lungs and wounds.⁵⁶⁻⁵⁸ The relationship between these two bacteria is complex, with both synergism and antagonism evident. For example, the *S. aureus* staphylococcal protein A can bind to the type IV pili and Psl of *P. aeruginosa* inhibiting its ability to

form biofilm.⁵⁸ On the other hand, *S. aureus* can benefit from Pel and Psl produced by *P. aeruginosa* in a dual-species biofilm via the increased protection against antibiotics.⁵⁶ Co-colonisation of *P. aeruginosa* and *S. aureus in vivo* has been shown to increase the severity of disease and can lead to an increase in expression of virulence factors in both species.⁵⁶ Therefore, multispecies biofilms are clinically important and need to be considered when studying the bacteria in the lab. The investigation of co-infections with *S. aureus* is human-based; however, *S. pseudintermedius* is often regarded as the canine counterpart of *S. aureus* and is a common bacterial isolate in canine otitis cases.^{6,14,26} As it has been shown that canine otitis isolates of *S. pseudintermedius* can form biofilms,⁵⁹ this could suggest that there is potential for these bacteria to interact and form multi-species biofilms which could be clinically relevant. However, to the best of the author's knowledge, research into this is lacking.

Biofilms have also been shown to be an issue in canine otitis. In a 2013 study,⁴³ Pye *et al.* found that 40% of *P. aeruginosa* isolates from canine OE cases produced biofilm. Since then, there have been further published studies that have found over 90% of *P. aeruginosa* canine otitis isolates to be biofilm producers,⁵⁹⁻⁶¹ with one study reporting that the otitis isolates produced the most significant biofilms (compared to canine *P. aeruginosa* isolates from other anatomical locations).⁶¹ However, it should be noted that as all these results were obtained *in vitro*, care should be taken with extrapolation of biofilm production to the clinical OE cases. There are many additional factors that could determine whether a biofilm is produced in the ear canal during an infection; including host immune response and ear microflora.⁴³ The same study on canine otitis isolates also reported that the minimum inhibitory concentration (MIC) of all antibiotics tested were significantly higher against biofilm-associated bacteria (compared to their planktonic counterparts).⁴³ This agrees with similar findings for human isolates, although it is worth bearing in mind that many different methods of measuring MIC/response to antibiotics exist and there is no standardised method.⁶² As the MIC is higher against biofilm-associated bacteria, it indicates that biofilm formation could contribute to making OE cases in dogs difficult to treat with conventional antibiotics.

1.3. Bacteriophages

Bacteriophages, often shortened to 'phages', are viruses of bacteria and are thought to be the most numerous biological entities on Earth.^{63,64} There are an estimated 10^{30} to 10^{32} phage particles worldwide and it is speculated that a quarter of the global bacterial population are killed by phages every day.⁶⁵ Phages are ubiquitous and found throughout the environment.^{64,66} They are considered to exist wherever their specific bacterial hosts do.⁶⁴⁻⁶⁷ A phage virion consists of a nucleic acid genome (usually double-stranded DNA [dsDNA]) contained within a protein capsid (Figure 1).^{65,67} Historically, tailed phages, which constitute over 95% of known phages, were members of the order *Caudovirales* and then sub-divided into three families based on tail morphology: the *Myoviridae*, *Siphoviridae* and *Podoviridae*.^{65,67,68} However, recently, the morphology-based taxa were abolished due to extensive evidence that members within these phage families were polyphyletic.⁶⁹ Therefore, the order *Caudovirales* has been replaced with the class *Caudoviricetes*, which constitutes all phages with a dsDNA genome, icosahedral capsid and tail.⁶⁹ These phage members are then assigned a family according to genomic coherence, rather than morphology, and this re-allocation work is still ongoing.⁶⁹

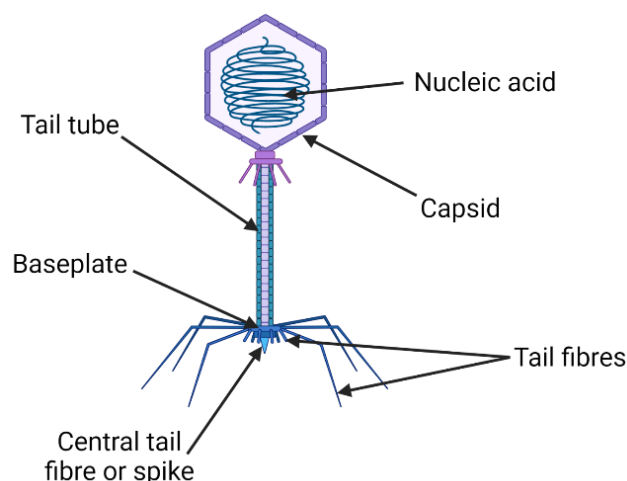


Figure 1. Diagram representing the structure of a bacteriophage virion. The structure is based on the general structure of a phage previously belonging to the *Myoviridae* family, but now known as having the 'myovirus' morphology. Phages with the myovirus structure have long contractile tails. Tails of phages contain tail fibres and tail spikes which can act as a receptor-binding protein (RBP), enabling adsorption onto the host bacterium and ejection of the phage genome into the cell. Produced using Biorender.com based on Nobrega *et al.*, 2018.⁶⁹

The tail usually contains tail fibres used to interact with receptors on the bacterial cell to enable phage entry.^{65,67,68} This adsorption (binding) to the bacteria dictates the specificity of the phage which tends to be for a particular bacterial group or species; with some exhibiting an even higher specificity, only infecting a single strain of bacteria within a species.^{65,67} However, some phages can have a broader host range⁷⁰ and it has also been shown that phages can readily mutate to infect bacteria outwith their original host range.⁷¹⁻⁷³

After adsorption, injection of the phage genome into the bacterial cell occurs.⁶⁵ Following this, generally one of two possible phage life cycles can arise – either a lytic (or ‘virulent’) cycle; or a lysogenic (or ‘temperate’) cycle (Figure 2). In a lytic cycle, the phage genome is transcribed, translated and replicated by the bacterial cell machinery in the cytosol.^{65,67,74} Degradation of the bacterial chromosome typically occurs, usually due to endonucleases carried by the phage.⁷⁵⁻⁷⁷ Once new phage particles are assembled, they are released via lysis of the bacterial cell wall orchestrated by actions of phage-encoded proteins. Most dsDNA tailed phages employ a bi-component system for this – the ‘holin-endolysin dyad’.^{65,78,79} This involves small hydrophobic proteins (the holins) forming pores in the bacterial cytoplasmic membrane, which allow peptidoglycan-degrading enzymes (the endolysins) to access the bacterial cell wall causing subsequent cell lysis.^{65,78,79} The resulting phage progeny can then proceed to infect surrounding bacteria and the cycle can repeat.^{65,67}

In contrast, a lysogenic cycle does not result in immediate bacterial cell death (Figure 2). Instead, the phage genome is integrated into the bacterial genome and remains quiescent. In this state, it is referred to as a ‘prophage’ and can be passed onto bacterial daughter cells via binary fission. If the bacterial cell later undergoes physiological stress, this can induce the prophage to leave the bacterial genome and start a lytic cycle. The resulting temperate phage progeny can undergo either a lytic or lysogenic cycle when infecting new bacteria.^{67,74}

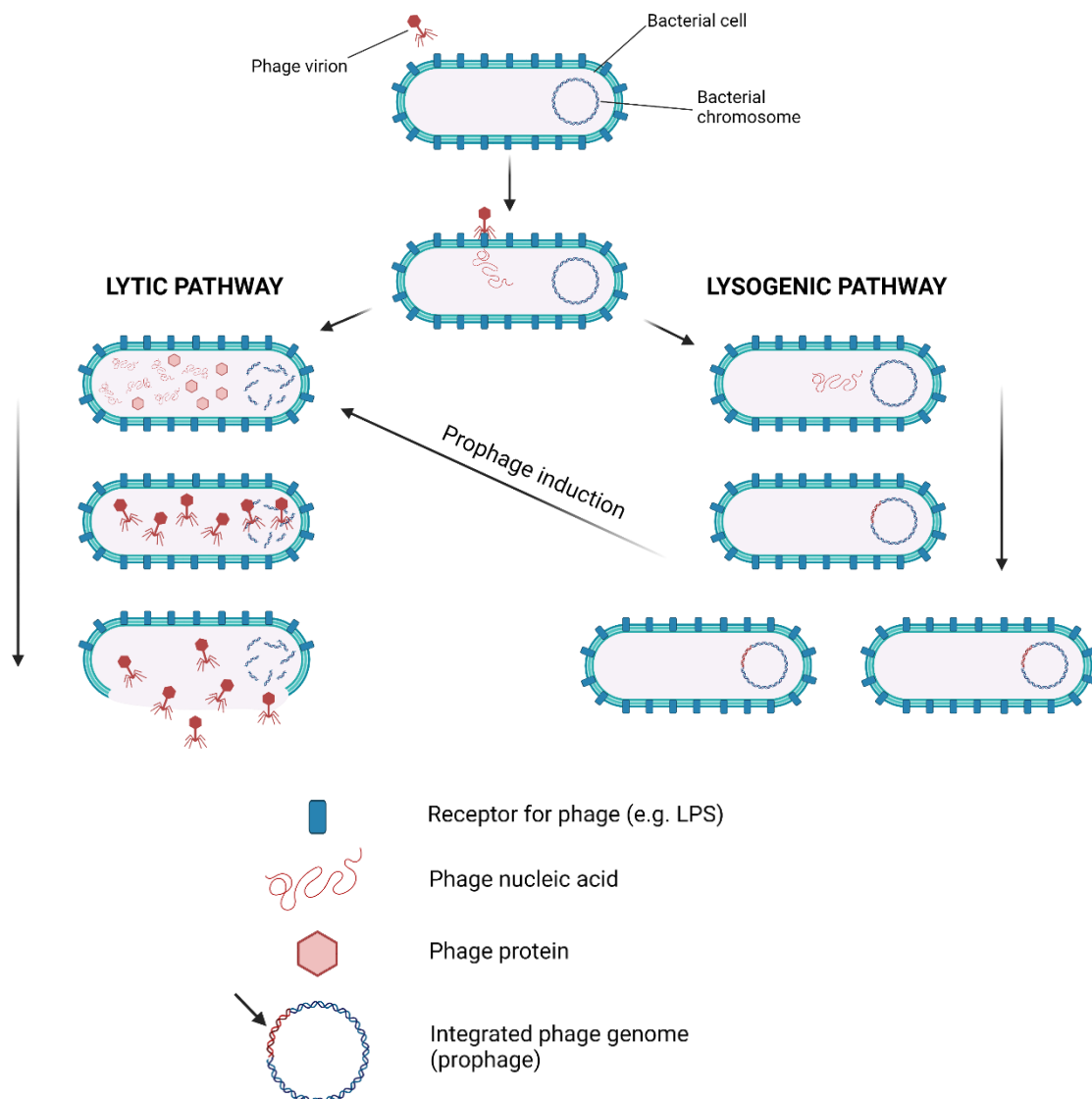


Figure 2. Diagram depicting the two possible cycles a bacteriophage can undertake on infection of a bacterial cell. In the lytic pathway, once the phage nucleic acid is injected into the cell, the bacterial cell machinery is hijacked to replicate the phage genome and produce phage-encoded proteins. Destruction of the bacterial chromosome usually occurs due to phage-encoded endonucleases. Once new phage virion particles have been assembled, the bacterial cell undergoes lysis and the phage particles are released. The cycle can then repeat. In the lysogenic pathway, the phage genome is integrated into the bacterial host genome. It remains there and can be passed onto daughter cells during binary fission. If prophage induction occurs, a lytic pathway begins with replication of the phage genome and production of phage proteins. Produced using Biorender.com. LPS = lipopolysaccharide

1.4. Phage therapy

Phage therapy involves harnessing the bacterial killing capabilities of bacteriophages and utilising these to fight bacterial infections. Phages were first discovered by Twort and d'Hérelle independently in 1915 and 1917 respectively.^{80,81} Initial interest in their potential as therapeutic agents was generally overlooked in the West with the advent of antibiotics.⁸² However, phage therapy trials and research continued in the former Soviet Union resulting in its widespread use in this region and the opening of the bacteriophage institute in Georgia, now known as the 'Eliava Institute of Bacteriophage'.^{82,83} There is now renewed interest in the West due to the emergence of antibiotic resistance and the requirement for therapeutic alternatives, resulting in a surge of phage therapy research.^{82,84} A significant quantity of research and trials have already been performed for phage therapy in humans^{74,83-85} and food production, including established commercial use of phage therapy against food microbial contaminants.⁸⁶⁻⁸⁸ However, the potential of phage therapy in companion animal veterinary medicine has not yet been fully exploited.⁸⁸

Obligate lytic phages (often referred to as 'lytic' or 'virulent' phages) are commonly viewed as the ideal candidates for phage therapy as they should kill bacteria in a rapid fashion, similar to conventional antibiotics; whereas lysogenic phages are more likely to integrate into the bacterial host genome without immediate killing.^{74,85} Another disadvantage to the use of lysogenic phages is that 'superinfection immunity' can occur with them which would be counterproductive in phage therapy.⁸⁵ This is where the integrated prophage confers resistance to the bacterial cell to prevent successful infections by other phage particles of the same immunity type.^{89,90} Furthermore, a prophage can alter the phenotype of the bacterium by the expression of prophage-encoded genes, often resulting in benefit to the host bacterium.^{89,91} This is termed 'lysogenic conversion' and can lead to the acquisition of virulence genes⁹² and/or AMR genes⁹³ which can contribute to a more virulent bacterial population. An important example of this is the Shiga toxin-producing *Escherichia coli* serogroup O157:H7, which is an emerging highly virulent pathogen due to the expression of its prophage-encoded Shiga toxin.⁹⁴ Similarly, it has also been shown that the CTX cytotoxin produced by certain strains of *P. aeruginosa* is prophage-encoded and likely affects the virulence of the bacteria during infections.⁹⁵⁻⁹⁷ Despite lytic phages seeming like the more appropriate option for these reasons, it

should be noted that there is still potential for the spread of bacterial virulence factors with a subset of lytic phages, known as ‘superspreaders’.⁹⁸ These phages have been shown to lack hydrolytic endonucleases, resulting in the release of intact bacterial plasmids into the environment upon cell lysis which can lead to the spread of antibiotic resistance.⁹⁸ Both lytic and lysogenic phages are also capable of generalised transduction (where bacterial DNA is packaged into a phage capsid by accident and can be transferred to another bacterium)⁹⁹⁻¹⁰¹ and so could contribute to the spread of AMR in this way as well.

Phage therapy has many potential advantages over traditional antibiotic therapy and these are summarised in Table 2. However, there are still some limitations of phage therapy. The development of phage resistance in a bacterial population, similar to antibiotic resistance, is typically inevitable.^{74,85,102} Some examples of the methods in which bacteria can resist phage infection include mutation of the phage receptors to prevent phage adsorption; restriction-modification systems used to cleave phage DNA; and the clustered regularly interspaced short palindromic repeats (CRISPR)-Cas system which provides immunity against foreign DNA.¹⁰² However, an important and unique advantage of phages is their ability to mutate alongside their bacterial host to overcome this resistance which can result in an evolutionary ‘arms-race’.^{74,85,102} For example, the genome of the *E. coli*-infecting phage T4 contains modified bases in the form of hydroxymethylcytosine, as an alternative to cytosine, rendering its genome resistant to the restriction-modification systems in the bacterial cell that recognise specific cytosine-containing sequences.¹⁰²

Advantages	Disadvantages
Specific to bacterial species resulting in less off-target effects ^{74,84,85}	High specificity means causative organism must be identified prior to treatment ^{74,85}
Self-replicating (fewer doses required) ^{74,85}	Narrow spectrum ^{74,85}
Reduced risk of allergic reactions (as natural entities that humans and animals already exposed to) ⁸⁵	Less published research and clinical trials – lack of extensive robust evidence for efficacy and safety ^{84,85,103}
Genetic modification potential ¹⁰⁴	Legal framework not yet existent and used usually on ‘compassionate care’ basis ^{74,85,103}
Activity against biofilms ^{74,85}	Some barriers exist to efficient phage production (e.g. prophages in phage propagation strains) ¹⁰³
Numerous and exist in nature – easier to discover? ^{74,85}	
Cheap to produce ⁸⁵	
‘Phage cocktails’ may help overcome resistance and increase spectrum ⁷⁴	
Ability to mutate to overcome development of phage resistance ^{74,85}	
Phage-resistant bacterial mutants can exhibit reduced fitness, reduced virulence and/or increased antibiotic susceptibility ^{74,85}	

Table 2. Advantages and disadvantages of phage therapy compared to conventional antibiotic therapy.

However, it has also been shown that when bacterial mutants are able to resist phages, they can exhibit reduced fitness and/or virulence as evolutionary ‘trade-offs’.^{74,85} Therefore, if chosen carefully, phages could be used to select for bacterial mutants with desired genetic trade-offs.^{74,85} An example of this is the use of phage OMKO1 with multidrug-resistant (MDR) *P. aeruginosa*.¹⁰⁵ It has been demonstrated that *P. aeruginosa* mutants resistant to this phage had increased susceptibility to multiple antibiotics, due to changes in the drug efflux pump systems MexAB-OprM and MexXY-OprM.¹⁰⁵ This resulted from selection against the expression of outer membrane porin M (OprM) as this is a receptor-binding protein (RBP) for the OMKO1 phage.¹⁰⁵ Using phages that induce similar genetic trade-offs could be a strategy to enable sustained use of current antibiotics.

The use of ‘phage cocktails’, where multiple phages of differing host specificity are administered together, has been a common method used in phage therapy thus far. The rationale for this is to combat phage resistance by reducing the risk of the target bacteria developing resistance to all the phages in a treatment concurrently.^{74,85} Furthermore, this method is also thought to increase the spectrum of the treatment.^{74,85} Nevertheless, a disadvantage of cocktails is the potential for selection of ‘broad-spectrum’ phage resistance mechanisms.⁷⁴ This can be combatted by administering phage cocktails in a sequential manner to ensure that the bacteria are frequently confronted with novel phages.⁸⁵ A published clinical example of this was the successful treatment of a 68-year-old man with an MDR *Acinetobacter baumannii* infection using multiple phage cocktails to combat the developing phage resistance.¹⁰⁶

A limitation to the widespread implementation of phage therapy is the lack of formal clinical trials carried out to the current scientific and regulatory standards, which not only leaves the efficacy of the therapy in question, but also the safety.^{84,103} Although phages are expected to be safe for administration to mammals as the viruses should not target mammalian cells, are specific for their host bacteria so have reduced off-target effects on commensal organisms, and there is reduced risk of allergy compared to antibiotics; there is a paucity of evidence to declare this definitively.^{84,85,103} A recent systematic review of published clinical data on phage therapy across multiple human medical specialities found that administration of

phage therapy was relatively safe with very few complications reported, all of which were mild and reversible after cessation of phage administration.⁸⁴ It should be noted that a great number of the published studies are case studies rather than randomised controlled trials which contributes to the general low quality of the clinical data available on phage therapy.⁸⁴ One of the most significant adverse immune system reactions that can be seen with antibiotics is anaphylaxis. However, there have been no published reports of a confirmed anaphylactic reaction in humans due to phage therapy thus far, indicating a diminished risk.^{85,107,108} However, the reduced quantity of trials and published data on phage therapy should be taken into account when considering this. Though phages do not target mammalian cells, there is evidence that they interact with the mammalian immune system.^{85,103} Anti-phage antibodies have been shown to be produced in patients and animals receiving phage therapy.^{103,108,109} These are thought to affect the efficacy of the therapy, although the full clinical significance of this and the effect on safety is not yet known.^{85,103,108,109} Furthermore, despite previous assumptions, there is evidence in the literature that phages are able to interact directly with mammalian cells and even penetrate them allowing spread throughout the body.^{103,110} Although it has been believed that phage therapy would not be effective against intracellular pathogens due to the phage's inability to enter eukaryotic cells,⁷⁴ with this recent revelation, phages could now be considered to treat intracellular infections.^{111,112} This has already been shown possible with *Mycobacterium abscessus* in humans¹⁰⁴ and some *Salmonella* poultry infections.¹¹³ This should be considered when developing a phage therapy and further research is required into this area.^{111,112}

A common theoretical concern with phage therapy is the rapid release of endotoxins from lysed bacteria within the body triggering a dangerous level of inflammatory cytokine response.^{107,108} A few studies have reported adverse reactions with this suspected origin, however conclusive data is lacking.¹⁰⁸ In addition, this possible reaction also exists with some antibiotics and is seldom considered.¹⁰⁷ Nevertheless, endotoxins and other bacterial components could play a role in adverse reactions by their presence as contaminants in phage preparations and for this reason, regulatory bodies should take this into account when licensing phage products for medical uses to ensure high purification requirements.^{107,108}

1.5. Phage therapy research for *Pseudomonas aeruginosa* infections

The first reports of phages specific for bacteria belonging to the *Pseudomonas* genus were published in the mid-twentieth century.⁶⁶ Since then, numerous *P. aeruginosa* phages have been described and sequenced, including lytic and lysogenic phages.^{40,66,97} Their application to phage therapy has been increasingly researched over the decades resulting in many *in vitro* studies.⁶⁶ This is not only the case with human-origin *P. aeruginosa* isolates, but there are also some examples of *in vitro* studies utilising *P. aeruginosa* isolates originally acquired from dogs.^{8,114-116} For example, Santos *et al.* isolated and characterised two phages active against *P. aeruginosa* isolates from canine ulcerative keratitis cases.⁸ Numerous *in vivo* studies have now been carried out on animal models to assess the safety and efficacy of phage therapy with *P. aeruginosa* infections.⁶⁶ These have predominantly been in mice⁶⁶; however there have been a few in other animals including catfish,¹¹⁷ wax moth larvae,¹¹⁸ farmed mink¹¹⁹ and rabbits.¹²⁰ Overall, the results from the *in vivo* studies have been promising and have supported the hypothesis of phage therapy being beneficial to treating *P. aeruginosa* infections.

As *P. aeruginosa* is a naturally occurring cause of otitis in both dogs and humans, this presents an opportunity to trial phage therapy in the treatment of canine cases prior to the application to human patients, without the need for artificial infections.¹²¹ In a published single case report, a dog with chronic bilateral *P. aeruginosa* OE was treated topically with a phage in one ear and then assessed after 27 hours. The ear had improved clinically (when compared to the contralateral ear) and the phages had successfully multiplied within the ear canal.¹²² Following this, a clinical trial was performed on ten dogs with chronic *P. aeruginosa* otitis.¹²³ An assessment of the affected ear of each dog was performed prior to the topical administration of a phage cocktail.¹²³ A re-assessment was carried out 48 hours later and the clinical condition of the treated ears were found to have improved.¹²³ The phage counts had increased, indicating successful replication, and the *P. aeruginosa* bacterial counts had decreased.¹²³ Overall, the results from this trial were deemed promising and considered the first published veterinary clinical trial of phage therapy.¹²³ Subsequently, a preliminary clinical trial was performed on human chronic *P. aeruginosa* otitis cases using a topically-administered phage cocktail.¹²⁴ This was performed after the canine clinical trial, despite the earlier publishing date.¹²¹ The

treated patients had significant clinical improvements compared to the placebo group and decreased *P. aeruginosa* counts after treatment.¹²⁴ Overall, the results were encouraging and indicated the need for progression to a more extensive clinical trial¹²⁴; although to the author's knowledge this is yet to occur. Throughout all these otitis phage therapy trials, there were no adverse effects observed in either canine or human patients.¹²²⁻¹²⁴

Thus far, there have been few clinical trials of phage therapy in humans with *P. aeruginosa* infections meeting modern scientific trial standards. A landmark clinical trial was the 'PhagoBurn' phase 1/2 randomised controlled trial investigating the topical application of a lytic phage cocktail on burn wounds infected *with P. aeruginosa*.¹²⁵ The findings from this study were mixed, as the trial had to be terminated prematurely due to insufficient efficacy of the phage treatment compared to the standard-of-care group.¹²⁵ However, a reduction in bacterial burden did occur in the phage-treated burn wounds, albeit at a slower rate than the standard-of-care, and there were less adverse effects seen in the phage-treated group compared to the standard-of-care group.¹²⁵ A significant issue that occurred during the study was the inadvertent reduction of phage titre after manufacturing of the treatment, resulting in a lower multiplicity of infection existing at the burn wound site than expected.¹²⁵ As phage therapy research progresses, the number of larger-scale official clinical trials is increasing. There is now a phase 1b/2 multi-centre randomised controlled trial ongoing to assess the response of CF patients colonised with *P. aeruginosa* to an intravenous-administered dose of phage therapy in the USA.¹²⁶ As the phage therapy research massively lags behind other fields, for example antibiotic research; it means there are gaps in our knowledge that do lead to limitations in the studies, as seen in the 'PhagoBurn' trial. However, as more *in vitro* and animal model studies are carried out, the hope is that high-quality clinical trials will become more achievable and widespread.

1.6. Bacteriophage study techniques

The double layer agar (DLA) technique is regarded as the 'gold standard' method of phage detection, enumeration and isolation.¹²⁷⁻¹²⁹ The basic principles of this method are what led to the discovery of bacteriophages in the 1910s.⁸⁰ The DLA assay involves a lawn of the host bacterium being formed using two layers of agar.^{127,128}

Both layers are composed of a medium supportive of bacterial growth (e.g. lysogeny broth) and supplemented with agar, usually containing 1-1.5% and 0.4-0.6% agar in the bottom and top layers respectively.^{127,128} The top 'soft agar' layer is mixed with the host bacterium to create the bacterial lawn.^{127,128} The potential phage-containing sample under investigation is added to the top layer of agar.¹²⁸ The plate is then incubated to enable successful host bacterial growth.¹²⁸ Finally, the plate can be examined for detection of phage activity, which is assessed by the appearance of plaques (circular areas of clearance on the lawn).¹²⁸ The number of plaques should be recorded as each single plaque is presumed to indicate the presence of one viable phage infecting a bacterial cell and its subsequent progeny phages infecting the adjacent bacterial cells.¹²⁸ Serial dilutions of the sample can be performed to determine the concentration of lytic phages in plaque-forming units (PFU).¹²⁸

In addition to the quantity of plaques, the nature of the plaque appearance should also be noted as this is important for phage characterisation.^{129,130} The relative opacity of the plaque can indicate the type of bacteriophage detected – a clear plaque is more commonly seen with a lytic phage, while a more turbid appearance can indicate lysogeny.¹³⁰ Plaque diameter has been shown to be correlated to phage morphology, with possible relationships demonstrated with phage head size¹³⁰ and the presence of side tail fibres.¹³¹ However, it should be noted that many other factors have also been shown to influence plaque size, including the specific DLA protocol used.^{129,132} The presence of a 'halo', a region surrounding the plaque that is less turbid than the bacterial lawn but more turbid than the centre of the plaque, can indicate the diffusion and action of phage-encoded hydrolytic enzymes degrading bacterial capsules.^{130,132,133} As halo morphologies tend to be associated with the presence of EPS depolymerases,¹³⁴ it has been hypothesised that halo formation could aid in the rapid identification of phages that would target bacterial polysaccharides and therefore, have potential activity against biofilms.^{130,133}

The DLA technique is a simple method which is relatively cheap to perform and enables enumeration of lytic phage particles.^{128,135} However, it is very labour-intensive and can be difficult to reproduce results precisely due to experimental variability.^{128,135} Contamination from other bacteria and from other phages (including prophages encoded within the bacterial genome) can severely affect results.¹²⁸

Furthermore, not all bacterial species are able to grow as a confluent lawn on agar rendering this assay impractical in investigations involving certain host bacteria.¹²⁹

Transmission electron microscopy (TEM) is another method used to enumerate phages. The high-resolution images produced using electron beams enable visualisation of whole phage particles.¹²⁸ TEM also allows assessment of phage morphology which can aid phage characterisation.¹³⁰ Accurate enumeration can be performed, but a sample with high phage concentration is required (approximately 10^6 particles/mL) and it is still a very laborious method.¹²⁸ The equipment involved is expensive and a highly-trained operator is required.¹²⁸ More recently, another method of phage visualisation and quantification has been developed – NanoSight laser-based optical microscopy.^{128,135} This is a more rapid and less laborious method than the aforementioned techniques, but is very expensive and can only be used with concentrated clear samples (which can be difficult considering many phage investigative studies involve environmental sources, e.g. sewage).^{128,135}

Several molecular techniques for phage detection and enumeration exist involving detection of viral nucleic acid. Quantitative PCR (qPCR) enables relatively quick and precise enumeration and detection of phage nucleic acid in a sample.¹³⁵ This technique has a good reproducibility between laboratories and ability for high throughput.¹³⁵ However, it should be noted that qPCR will also detect nucleic acid from non-infectious phage particles. This can cause higher numbers to be detected compared to a traditional DLA assay. Despite this drawback, the inability to differentiate between infectious and non-infectious phage particles can be seen as an advantage in some investigations, for example when assessing phage particles that have been inactivated in some way (e.g. neutralising antibodies), as a DLA-based assay would not allow their detection.¹²⁸ A limitation of qPCR is the difficulties presented when attempting to detect unknown or novel phages, as primer design is required for this technique and there is no well-conserved gene present on all phage types.^{128,135} Therefore, phage sequencing needs to have been performed to produce a primer and a poorly designed primer can reduce qPCR efficiency.¹³⁵

To combat this constraint of qPCR, whole genome sequencing technology can be used to detect phage sequences and potentially assemble them into complete

genomes without the requirement of prior phage isolation.¹²⁸ Currently, metagenomics (the sequencing of all genetic matter within a sample) is frequently being used to detect novel phages from many different sample origins, including sewage and marine environments.¹³⁶⁻¹³⁹ Generally, this can be very successful with high yields of novel phages; however, many limitations exist. For example, accurate enumeration is not yet optimised¹²⁸ and the bioinformatic processing of the information obtained can be very challenging.^{136,140} As previously stated, there is no well-conserved gene(s) shared between phages^{128,135}; and given the high phage diversity and lack of phage reference genomes, this contributes to the difficulty with assembling phage genomes from the data.¹³⁶ Currently, numerous bioinformatics tools are being developed to attempt to combat these issues.^{136,140}

1.7. Aims and objectives

The overall aim of this study was to investigate the potential of phage therapy as an alternative therapeutic against *P. aeruginosa* infections in canine otitis cases.

The objectives to achieve this were:

- To isolate lytic phages with activity against canine otitis *P. aeruginosa* isolates from environmental samples
- To characterise the isolated phages by determination of the following:
 - Phage morphology
 - Genome sequences
 - Host tropism

CHAPTER TWO – PHAGE ISOLATION

The first objective of the study was to isolate lytic phages with activity against the canine *P. aeruginosa* otitis isolates. To do this, wastewater samples obtained from across Scotland were screened using DLA assays to attempt to find novel phages.

2.0. Materials and Methods

All 'overnight' incubations were approximately 19 hours. A sterility control of 5 ml uninoculated tryptone soya broth (TSB; Oxoid, Basingstoke, UK) was included with every round of incubation. An Ultrospec 2100 pro UV/visible spectrophotometer (GE Healthcare UK Ltd., Little Chalfont, UK) was used for measuring optical density (OD).

2.1. Bacterial isolates

Twenty-five *P. aeruginosa* isolates from canine otitis cases cultured at the Veterinary Microbiology Laboratory at the Royal (Dick) School of Veterinary Studies, University of Edinburgh between 20th November 2017 and 15th September 2022 were included in this study. The majority of these were taken from patients referred to the Hospital for Small Animals, University of Edinburgh; however, two isolates came from external veterinary practices. All isolates originated from either ear canals or middle ears. Each isolate was assigned an alphabetical reference (Table 3). Isolates were identified and their antimicrobial susceptibility profile assessed using VITEK® 2 (bioMérieux, Basingstoke, UK) prior to the start of the study (Table 4).

Study alphabetical reference	Date sample received	Laboratory reference	Source
A	12/01/2022	p69483(111804)	Right ear swab
B	11/02/2022	p71779(115560)	Right ear swab
C	23/02/2022	p72473(116661)	Ear swab
D	11/05/2022	p77749(125230)	Left ear swab
E	25/07/2022	p82544(133293)	Ear swab
F	02/09/2022	p85444(138136)	Ear swab
G	15/09/2022	p86245(139460)	Ear swab
K	18/10/2021	p87671(141846)	Ear swab
L	01/11/2021	p57612(92587)	Ear flush
M	14/08/2020	p63875(102875)	Ear mucus
N	09/10/2020	p64094(103217)	Right ear swab
P	29/10/2020	p65055(104750)	Left ear swab
R	15/02/2021	p36344(57729)	Ear canal swab
S	01/04/2021	p39898(63523)	Ear swab
U	04/05/2021	p41018(65342)	Ear swab
V	05/05/2021	p41127(65549)	Left middle-ear fluid
X	25/10/2019	p41177(65623)	Ear swab
Z	13/07/2018	p47458(75997)	Ear swab
AA	02/08/2018	p50455(80752)	Ear swab
AB	20/02/2019	p51262(82093)	Ear swab
AC	28/03/2019	p52526(84208)	Left ear mucus
AF	20/11/2017	p52690(84467)	Ear swab
AG	10/04/2018	p14625(23278)	Ear swab
AH	13/04/2018	p22684(36176)	Left ear swab
AI	17/05/2018	p28811(45886)	Ear swab

Table 3. Canine otitis *P. aeruginosa* isolates used in this study (n=25). It should be noted that isolate E was eventually excluded from the study due to genetic similarity with other isolates, but was used in the initial phage screening protocol.

Study alphabetical reference	Imipenem	Amikacin	Gentamicin	Enrofloxacin	Marbofloxacin
A	S	S	S	S	S
B	S	S	I	S	S
C	S	I	I	I	I
D	S	S	S	I	S
E	S	S	S	I	S
F	S	S	S	I	S
G	S	R	R	I	S
K	S	S	S	I	S
L	S	S	S	I	S
M	S	R	I	I	S
N	S	S	S	R	R
P	S	S	S	S	S
R	S	S	I	R	R
S	S	S	S	S	S
U	S	S	S	S	S
V	N/A	S	S	I	S
X	S	S	S	R	R
Z	S	R	R	R	R
AA	S	R	R	S	S
AB	S	S	S	I	S
AC	S	S	S	R	R
AF	S	R	R	R	R
AG	S	S	S	R	R
AH	S	I	R	I	S
AI	R	S	S	R	R

Table 4. Antimicrobial susceptibility profiles of the canine otitis *P. aeruginosa* isolates used in this study (n=25). It should be noted that isolate E was eventually excluded from the study due to genetic similarity with other isolates, but was used in the initial phage screening protocol. The antibiotic susceptibility profiles were analysed using VITEK® 2 with Clinical and Laboratory Standards Institute criteria Vet 2017 V8.02 based on M100.S25. Abbreviations: S = sensitive; I = intermediate; R = resistant; N/A

= the susceptibility of isolate V to imipenem could not be read due to insufficient growth on duplicate attempts.

The 25 isolates were submitted to MicrobesNG (Birmingham, UK) for genome sequencing. The resulting sequences were used to form a phylogenetic tree (Figure 3). Initially, there were 34 *P. aeruginosa* isolates originating from canine otitis cases sequenced; however, closely-related isolates were excluded from the study to give a total of 25 (Figure 3). Subsequently, one of these 25 isolates (isolate T) was excluded due to abnormal growth in liquid culture resulting in a total of 24 isolates. The genome sequencing results were not obtained until the study was underway. Therefore, although isolate E was eventually excluded due to genetic similarity with other isolates, it was included in the initial phage screening portion of the study as its genetic similarity with other isolates was not known at this stage. For this reason, it is included in Tables 3 and 4.

Two *P. aeruginosa* laboratory reference strains of human origin were also used in the study: PAO1 (a wound isolate¹⁴¹ – CECT 4122) and NCTC 10662.¹⁴² These were obtained from the National Collection of Type Cultures.

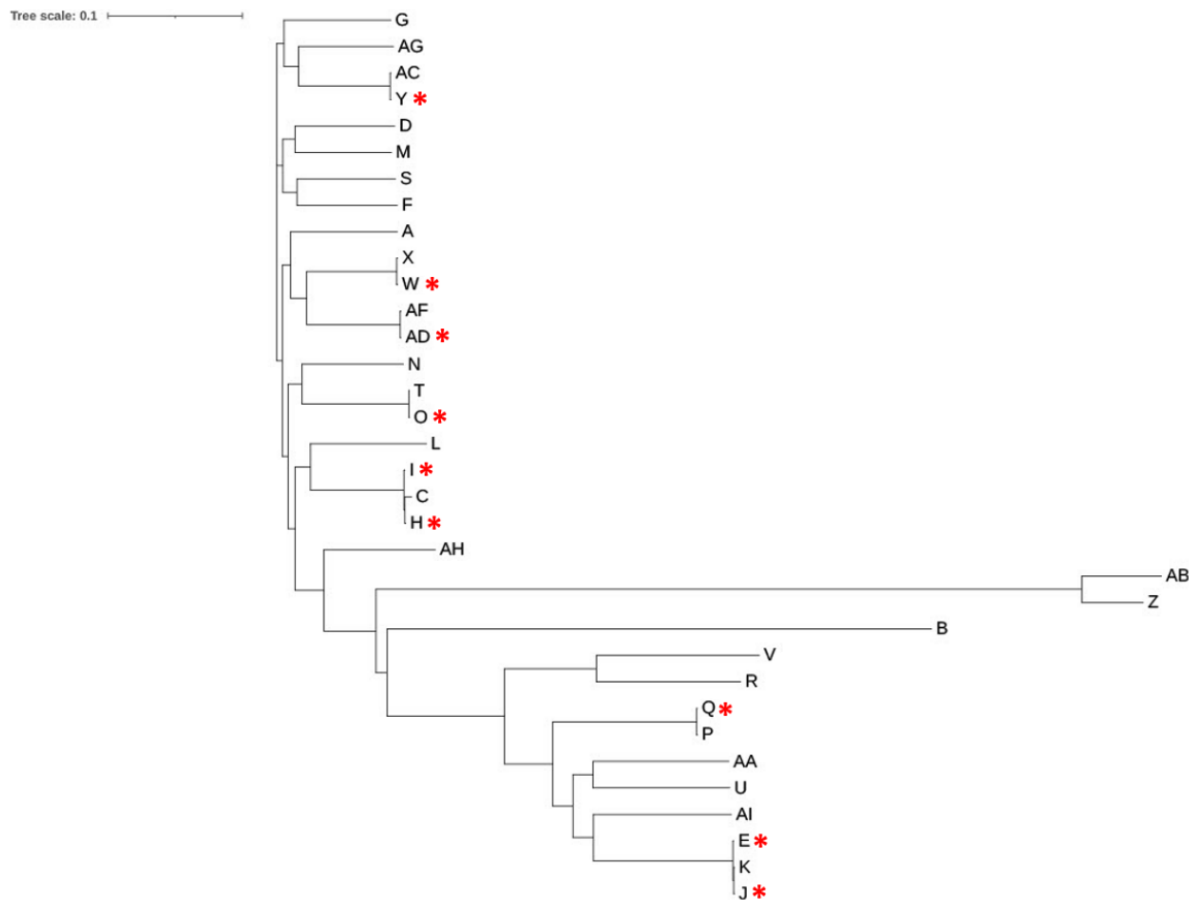


Figure 3. Phylogenetic tree of genome sequenced *P. aeruginosa* isolates from canine otitis cases. Study alphabetical reference used to denote each isolate. Starred isolates (*) were removed from study due to duplication of isolates. An exception is isolate E which was used for the initial phage screening before subsequent exclusion from the study. Created using CSI Phylogeny 1.4 (call SNPs & Infer Phylogeny) <https://cge.food.dtu.dk/services/CSIPhylogeny/>.

2.2. Bacterial isolate maintenance

All bacterial isolates were streaked out onto tryptone soya agar (TSA; Oxoid, Basingstoke, UK) made per manufacturer's instructions. Sometimes pre-poured TSA plates were used instead (E&O Laboratories Ltd., Bonnybridge, UK). The plates were placed in a static incubator maintained at 37 °C overnight. On removal from incubator, the plates were placed in a fridge (4 °C) and kept until needed. This process was repeated weekly to ensure fresh bacterial colonies were available for each experiment, i.e. the bacteria used were sub-cultured no more than seven days prior to experimental use.

2.3. Bacterial lawn production – screening for spontaneous prophage induction

TSA plates supplemented with 5 mM calcium chloride (CaCl₂; Merck, Gillingham, UK) were used in all phage-related experiments. The addition of CaCl₂ was due to the common requirement of divalent ions by phages for successful plaque formation.^{128,129}

Top agar for the overlay method was made using TSB made up according to the manufacturer's instructions with 0.7% (w/v) bacteriological agar (Merck, Gillingham, UK) and 5 mM CaCl₂ added.

A single colony of the bacterial isolate was inoculated into 5 ml TSB in a 30 ml universal container. A sterility control with only 5 ml TSB was also included. The cultures were incubated in a shaking incubator (37 °C, 180 rpm). The OD₆₀₀ of the culture was measured after 3-4 hours using sterile TSB as the blank control. Once the OD₆₀₀ was approximately 1 (between 0.9 and 1.1), 100 µl of the culture was added to 3 ml of top agar (melted and kept at 55 °C) in a 30 ml universal container. This was immediately swirled to ensure good mixing and then poured onto a TSA/CaCl₂ plate. The top agar was allowed to dry. Then, the plate was incubated in a static incubator maintained at 37 °C overnight. The plate was examined the next morning for bacterial lawn growth and bacteriophage plaques.

2.4. Double layer agar assays – screening for lytic phages

Selection of potential host bacterial isolates

Bacterial lawns were produced for the *P. aeruginosa* isolates using the protocol above (section 2.3). If plaques were evident, this indicated spontaneous prophage induction and the bacterial isolate was excluded as a host isolate for phage searching to reduce background lysogenic phage interference. If the bacterial lawn appeared to be free of plaques, the bacterial isolate was deemed to be acceptable for use as a potential phage-screening host isolate.

Bacterial culture preparation for phage screening

A single bacterial colony of the bacterial isolate to be tested was inoculated into 5 ml TSB in a 30 ml universal container. Bacterial cultures were incubated (37 °C, 180 rpm) overnight. After incubation, 50 µl of this culture was inoculated into 5 ml of fresh TSB in a 50 ml tube and incubated for a further 2-3 hours (37 °C, 180 rpm). If more than one bacterial isolate was being tested at the same time, the cultures were pooled by pouring into the same tube after the initial overnight incubation and then 50 µl of this mixture was inoculated into 5 ml fresh TSB and incubated for a further 2-3 hours (37 °C, 180 rpm).

Wastewater sample preparation

Wastewater samples were collected from across Scotland by the Scottish Environmental Protection Agency as part of their monitoring scheme following the COVID-19 pandemic. They were kept in a cold room (4 °C) until required. The details of the ten wastewater samples used in this study are described in Table 5.

Wastewater sample label	Location collected from	Date of collection
U34	Skellyton	26/05/22
U35	Montrose	26/05/22
U39	Newburgh	24/05/22
U40	Peebles	19/05/22
U41	Cumnock	23/05/22
U42	Kinneil	24/05/22
U43	Huntly	27/05/22
U44	Turriff	25/05/22
U46	Lerwick	27/05/22
U47	Fraserburgh	25/05/22

Table 5. Locations and dates of wastewater sample collection for samples used in this study. Samples obtained from Gally laboratory, Roslin Institute.

After agitation of the sample bottle to disturb the sediment, wastewater samples were poured into individual 15 ml tubes. The samples were centrifuged at 5000 rpm for 10 minutes at room temperature (21 °C). The supernatants were then filtered using 0.22 µm syringe filters (Millex-GP filter unit, Merck Millipore Ltd., Cork, Ireland) into new 15 ml tubes.

Phage enrichment stage

The purpose of the enrichment step was to amplify any phages present in the wastewater samples that were able to infect and replicate using the host bacterial isolates.¹³⁴ On removal of the bacterial cultures from the incubator (after 2-3 hours), 500 µl of each filtered wastewater sample was added to each 15 ml tube containing the bacterial cultures. These tubes were then incubated overnight (37 °C, 180 rpm). In addition, a fresh culture of the host bacterial isolate(s) was started and incubated overnight (37 °C, 180 rpm) by inoculating a single colony into 5 ml of TSB in a 30 ml universal container.

After overnight incubation, the enrichment cultures were then centrifuged at 5000 rpm for 10 minutes at room temperature (21 °C). Following this, the supernatants were filtered using 0.22 µm syringe filters into new 15 ml tubes.

DLA plate assays

Petri dishes containing TSA with 5 mM CaCl_2 were used for the DLA assays (section 2.3). First, 100 μl of the host bacterial culture (one colony into 5 ml TSB and incubated overnight at 37 °C, 180 rpm) was added to 3 ml of melted top agar (maintained at 55 °C) in a 30 ml universal container. Then, 100 μl of the filtered enriched wastewater sample was added. The top agar was swirled gently in the container and then poured onto the TSA plate. This process was repeated as required to produce a host bacterial lawn containing each enriched wastewater sample. These plates were allowed to dry before placing in the static incubator for an overnight incubation at 37 °C.

The plates were then examined for bacterial growth and bacteriophage plaques. If any plaques were observed, phages were isolated (section 2.8).

2.5. Plate imaging

All plates from experiments were imaged using a VWR® Gel Imager2 (VWR International, Leicestershire, UK) on the 'white light' setting.

2.6. Bacterial lawn production – revised spontaneous prophage induction screening method

The TSA plates (with 5 mM CaCl_2) and TSB used for this method are described above in section 2.3. A single colony of the bacterial isolate was inoculated into 5 ml TSB in a 30 ml universal container. The cultures were incubated in a shaking incubator overnight (37 °C, 180 rpm).

After the overnight incubation, 100 μl of the culture was added to 3 ml of top agar (melted and kept at 55 °C) in a 30 ml universal container. This was immediately swirled to ensure good mixing and then poured onto a TSA/ CaCl_2 plate. The top agar was allowed to dry. Then, the plate was incubated at 37 °C overnight before examination for bacterial lawn growth and bacteriophage plaques.

2.7. Bacterial lawn production – final method

The method described here was used for all bacterial lawns produced throughout the remainder of the study for phage propagation and assaying. It uses bacteria grown to

a set OD₆₀₀, instead of overnight cultures. This was changed to improve consistency regarding the growth stage/physiological state of the bacterial population used for phage studies. The TSA plates (with 5 mM CaCl₂) and TSB used for this method are described in section 2.3.

A single colony of the bacterial isolate was inoculated into 5 ml TSB in a 30 ml universal container. Bacterial cultures were incubated in a shaking incubator overnight (37 °C, 180 rpm). After incubation, 50 µl of bacterial culture was inoculated into 5 ml of fresh TSB in another 30 ml universal container, to make a 1:100 dilution, and incubated (37 °C, 180 rpm).

The OD₆₀₀ was measured after approximately 3 hours. Once the OD₆₀₀ was approximately 1 (between 0.9 and 1.1), 100 µl of the culture was added to 3 ml of top agar (melted and kept at 55 °C) in a 30 ml universal container. This was immediately swirled to ensure good mixing and then poured onto a TSA/CaCl₂ plate. The top agar was allowed to dry and the plate incubated at 37 °C overnight. The plate was examined the next morning for bacterial growth and bacteriophage plaques.

2.8. Phage isolation and purification

Saline magnesium (SM) buffer was produced by adding 5.8 g of NaCl (Fisher Scientific, Loughborough, UK); 2 g of MgSO₄·7H₂O (Merck, Gillingham, UK); 50 ml of 1 M Tris-Cl (pH 7.5; Merck, Gillingham, UK); and 5 ml of a 2% (w/v) gelatin solution (made up using gelatin powder from Merck, Gillingham, UK) to deionised water resulting in a litre of SM buffer. This was autoclaved before use.

A 1 ml syringe was filled with SM buffer and used to cut out a plaque from the agar. The syringe was checked to ensure that the piece of agar containing the plaque was suspended in the solution. The syringe was left to stand for 20 minutes to ensure the phages became suspended in the buffer. If there were no distinct plaques and lysis was more widespread, 3 ml of SM buffer was poured onto plate and left for 5-10 minutes at room temperature prior to the liquid being removed with a pipette. The contents were then filtered through a 0.22 µm syringe filter into a new 1.5 ml microcentrifuge tube. The tube was labelled with the phage name (host bacterial isolate letter followed by number of wastewater sample; and a lowercase letter added

to the end to denote different phage if there was more than one plaque type evident on one plate) and the purification step number, i.e. 'P1', 'P2' or 'P3'.

Plaques were passaged using the DLA method (using protocol in section 2.7) for three times in total to ensure a pure phage preparation. When not in use, phage preparations were kept refrigerated (4 °C).

To produce DLA plates where plaques were dispersed enough for single well-isolated plaques to be picked, the phage preparations (phages suspended in SM buffer) were serially diluted using SM buffer. The ten-fold dilution series was created by adding 100 µl of the phage preparation to 900 µl of SM buffer in a 1.5 ml microcentrifuge tube. Then, 100 µl of this new solution was added to another microcentrifuge tube with 900 µl of SM buffer and so on, until a dilution series of 10^{-1} to 10^{-8} was produced. Each dilution was then spotted onto bacterial lawns (see section 2.7) of their host isolate by adding 2.5 µl drops onto the top agar surface. After overnight incubation at 37 °C, the spots were assessed to determine the optimum concentration for picking plaques.

If plaques were clear enough on the serial dilution plates and there was no evidence of an impure preparation (i.e. only one plaque morphology), a plaque was picked from this plate for the next purification step. However, if this was not possible, the optimum concentration was selected and a new overlay assay was made using 100 µl of the appropriate phage dilution added to 3 ml top agar, along with 100 µl of the appropriate bacterial host culture. After overnight incubation at 37 °C, plaques could then be picked from this plate as the next purification step. It was ensured that the last 'P3' purification step was carried out in the latter way (with a new overlay assay) to increase the certainty that the phage preparation was pure (i.e. no other types of plaques evident).

It should be noted that to ensure sufficient mixing of each serial dilution of phage when creating the dilution series, but also prior to removing a volume of phage solution for use at any point in the experiments, mixing was executed by flicking the microcentrifuge tube with a finger and inverting them twice. This was carried out instead of using high-setting vortexing or more aggressive mixing methods as these are not recommended when working with phages.¹²⁹

2.9. Assessment of bacterial genome sequences for prophage regions

The assembled genome sequences for the canine isolates from Illumina sequencing at MicrobesNG (Birmingham, UK) and for the two laboratory reference isolates (PAO1 and NCTC 10662; assembled genome accession numbers are NC_002516.2 and CAADJI010000001.1 respectively) were assessed for prophage content using the PHASTER (Phage Search Tool Enhanced Release) webtool.^{143,144} The output of each genome was recorded as the number and predicted completeness of each prophage region detected. Each region is assigned a score by PHASTER based on the number of known phage genes/proteins present. The score is then used to categorise the prophage regions into categories: 'intact' if the score is >90; 'questionable' if the score is 70-90; and 'incomplete' if the score is <70.

3.0. Results and Discussion

3.1. Screening for lytic phages in wastewater samples

Initially, lysogeny broth (LB) and LB agar were trialled as the growth media for the *P. aeruginosa* isolates. However, poor growth was noted when incubating in LB broth. This was in contrast to methods reported in other studies using canine *P. aeruginosa* isolates as some have used LB with no reported issues.^{8,43,114} TSB and TSA were subsequently trialled as alternative growth media and as improved growth was seen, this was the media of choice for the study.

Bacterial lawns were made following the protocol outlined in section 2.3 with a subset of the bacterial isolates to test for spontaneous prophage induction. Of the ten isolates tested, four had plaques present throughout the lawn which were taken to indicate spontaneous prophage induction (Figure 4). These isolates were excluded as candidates for phage isolation due to the issues with background phage interference. Spontaneous prophage induction has been shown to occur frequently with *P. aeruginosa* prophages in previous studies.^{90,145}

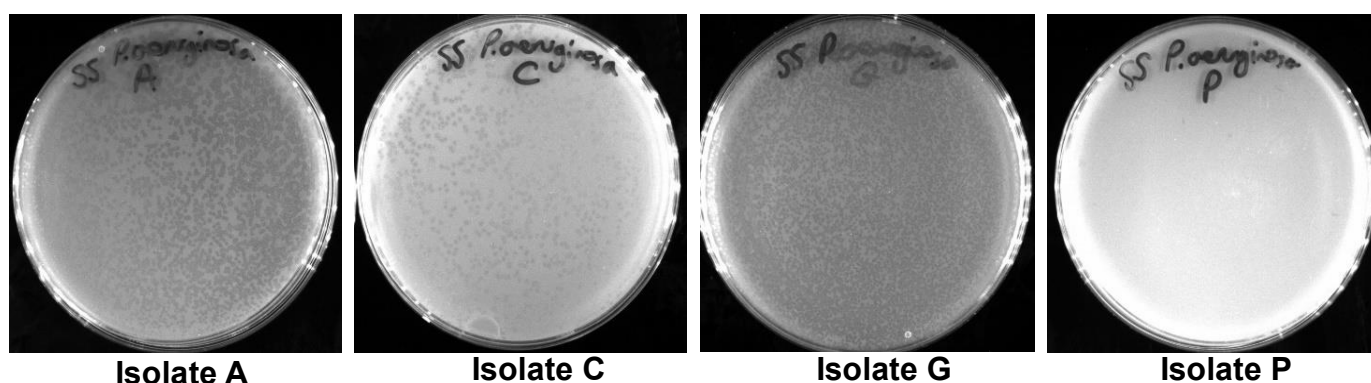


Figure 4. Example images of bacterial lawns produced using *P. aeruginosa* isolates with the DLA method. Bacterial lawns made with isolates A, C and G had plaque formation throughout the plate. In contrast, the bacterial lawn made with isolate P had no evidence of plaque formation or lysis.

The isolates with no plaque formation on bacterial lawns were isolates D, E, K, L, M and P. These isolates were deemed suitable for use as bacterial hosts for phage searching as spontaneous prophage induction was less likely.

Enriched wastewater samples were added to bacterial lawns produced using the six *P. aeruginosa* isolates above (as described in section 2.4). The same ten wastewater samples were tested against each *P. aeruginosa* isolate. Plaques were seen throughout each bacterial lawn made with isolate M with every added wastewater sample (Figure 5). No plaques or lysis were observed on any other bacterial lawn from all the remainder of the bacterial isolate/wastewater combinations tested.

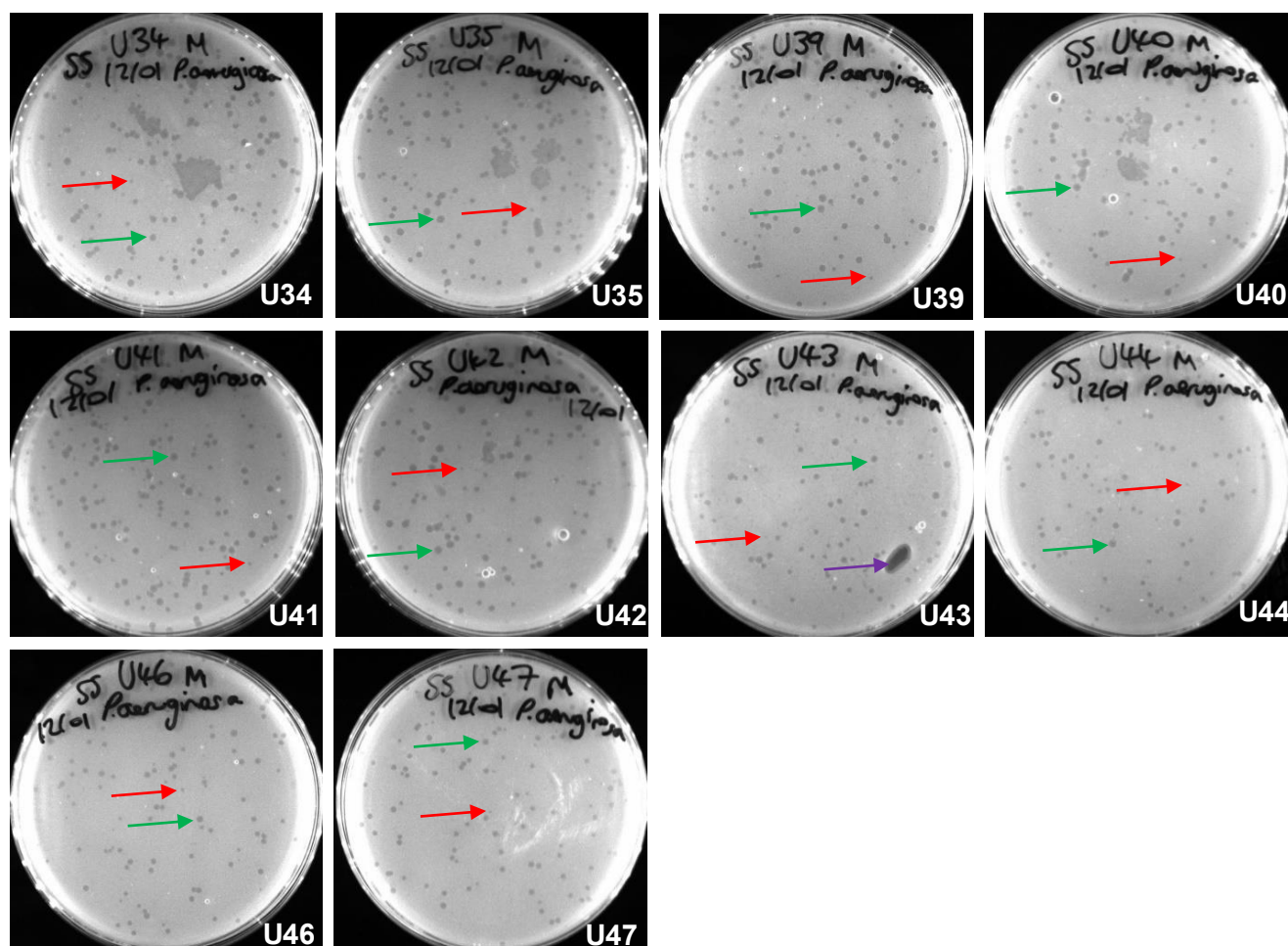


Figure 5. Images of the ten bacterial lawns produced using *P. aeruginosa* isolate M with the DLA method and enriched wastewater added. The labels on the lower right corner of each image indicate which enriched wastewater sample was added to the corresponding plate. All plates produced had plaques throughout the lawns. Plaques of two different morphologies can be seen on each plate – larger diameter plaques (examples indicated by green arrows) and smaller diameter plaques (examples indicated by red arrows). It should be noted that the obvious large clear area on the bottom of the ‘U43’ plate (indicated by a purple arrow) is a defect in the agar overlay due to a technical error when pouring on the molten agar.

There were two different plaque morphologies on each of the M bacterial lawns created with each wastewater sample – larger diameter (approximately 2 mm) plaques

with a turbid appearance in the centre and a clearer region on the outskirts of the plaque; and smaller diameter (approximately 0.5 mm) plaques with a turbid appearance (Figure 5). Both plaque morphologies appeared on all plates produced and all plates looked very similar, despite being created with different wastewater samples. For this reason, it was suspected that the plaques were formed by prophage induction from the host bacterial isolate, rather than by the action of lytic phages contained in the wastewater samples. This hypothesis was further supported by the turbid appearance of the plaques, which is traditionally taken to be more indicative of lysogenic phages, as opposed to the clear plaques of a lytic phage.^{130,146,147} This appearance is due to the survival of lysogenic bacteria within the area of the plaque.^{132,146,147} The morphology of the larger diameter plaques seen – with a more turbid appearance centrally and a clearer peripheral zone – could be due to a higher rate of growth of the lysogenic bacteria within the centre of the plaque, often seen with plaques formed by lysogenic phage.^{146,147} However, it has been shown more recently that factors other than phage properties can affect plaque morphology, including bacteriological media used in the DLA assay.^{132,148} For example, a *S. aureus* phage was found to produce turbid plaques when DLA assays were produced using CaCl₂-supplemented TSA and clear plaques when unsupplemented TSA was used.¹⁴⁸ For this reason, it could not be stated definitively that the plaques on the *P. aeruginosa* lawns were formed by lysogenic phages. The phages were isolated from the plaques and the purification process initiated using the method outlined in section 2.8.

It was noted that the initial bacterial lawns produced for screening for spontaneous prophage induction were made using a slightly different bacterial growth protocol than used in the DLA assays to screen the enriched wastewater samples for phage – a culture incubated overnight (19 hours) was used for the latter and a ~4-hour-incubated OD₆₀₀ ~1.0 culture was used for the former. To rectify this, the initial bacterial lawns screening for spontaneous prophage induction were repeated using the same method of 19-hour-incubated bacterial cultures. Results identical to the first prophage screening plates were obtained for all plates using the new method, with the exception of isolate M. Small (<0.5 mm diameter) turbid plaques were found throughout the lawn in this case. Given this background prophage induction and the other supporting arguments above, the phages isolated from lawns produced using isolate M were

deemed likely to be lysogenic phages and therefore, excluded from further analysis as these would not be ideal candidates for phage therapy.

With no likely lytic phages identified from screening the ten wastewater samples against isolates D, E, K, L, M and P, an additional isolate, AA, was tested. It was found that isolate AA did not show evidence of spontaneous prophage induction on the bacterial lawn produced, so this isolate was deemed acceptable for use in wastewater screening. Five of the ten resulting plates produced had plaques and/or lysis present (Figure 6). All DLA plates made with isolate AA that had evidence of phage activity (i.e. plaques and/or lysis), had phages isolated following the protocol in section 2.8. This resulted in five isolated phages (as there were five different plaque morphologies found on further plating – one morphology per original DLA plate). These were taken forward for phage characterisation.

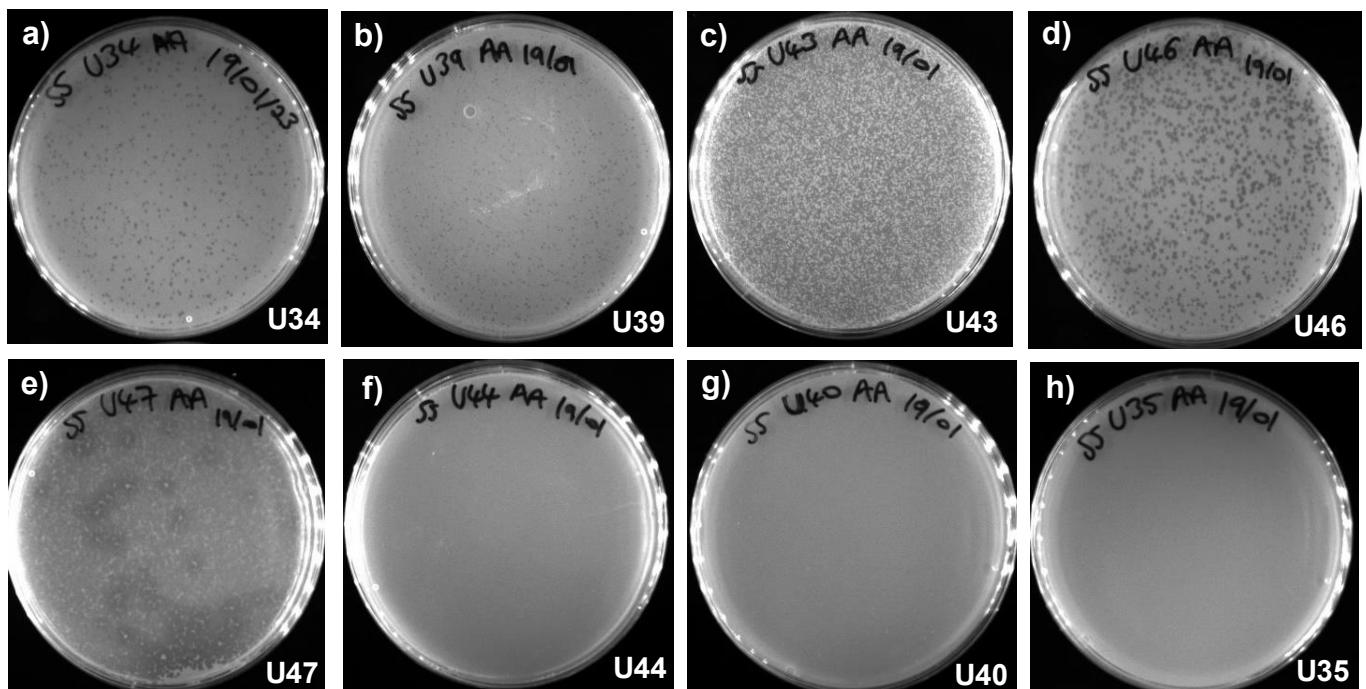


Figure 6. Images of bacterial lawns produced using *P. aeruginosa* isolate AA with the DLA method and enriched wastewater added. The labels on the lower right corner of each image indicate which enriched wastewater sample was added to the corresponding plate. Plates labelled **a-e** have visible plaques and/or widespread lysis. Plates labelled **f-h** have no plaques or lysis visible.

Despite the 70 *P. aeruginosa* isolate/wastewater combinations tested, only five potential lytic phages had been isolated, all with isolate AA as the host. In an attempt to assess the suitability of other isolates for phage-screening, two human-origin *P.*

aeruginosa isolates (PAO1 and NCTC 10662) were used to screen the same ten wastewater samples for phages. PAO1 is commonly used as the host bacterial strain for phage isolation from environmental samples in the literature.¹⁴⁹⁻¹⁵² Bacterial lawns without the addition of wastewater were created to screen for spontaneous prophage induction in these isolates (protocol as described in section 2.6) – both lawns were clear of plaques or lysis so were deemed acceptable for use as potential bacterial host isolates (Figure 7). Then, the ten enriched wastewater samples were added to bacterial lawns using the PAO1 and NCTC 10662 strains as described in section 2.4.



Figure 7. Images of bacterial lawns produced using *P. aeruginosa* strains PAO1 and NCTC 10662 with the DLA method. There was no evidence of obvious plaque formation.

All ten DLA assays made using NCTC 10662 were negative for plaques and lysis. Differing degrees of lysis and/or plaques were seen on PAO1 bacterial lawns with the added wastewater samples U34, U39, U41, U43, U44 and U47 (six out of ten samples tested) (Figure 8). Most of these plates had reduced bacterial lawn growth, taken to indicate widespread lysis activity by potential phage. They also had varying degrees of bacterial colony formation which were suspected to be phage-resistant bacterial colonies. The remaining plates made using the PAO1 strain with the four other wastewater samples had no evidence of plaques/lysis and all had good bacterial lawn growth. These putative phages isolated against PAO1 were not studied further due to time limitations.

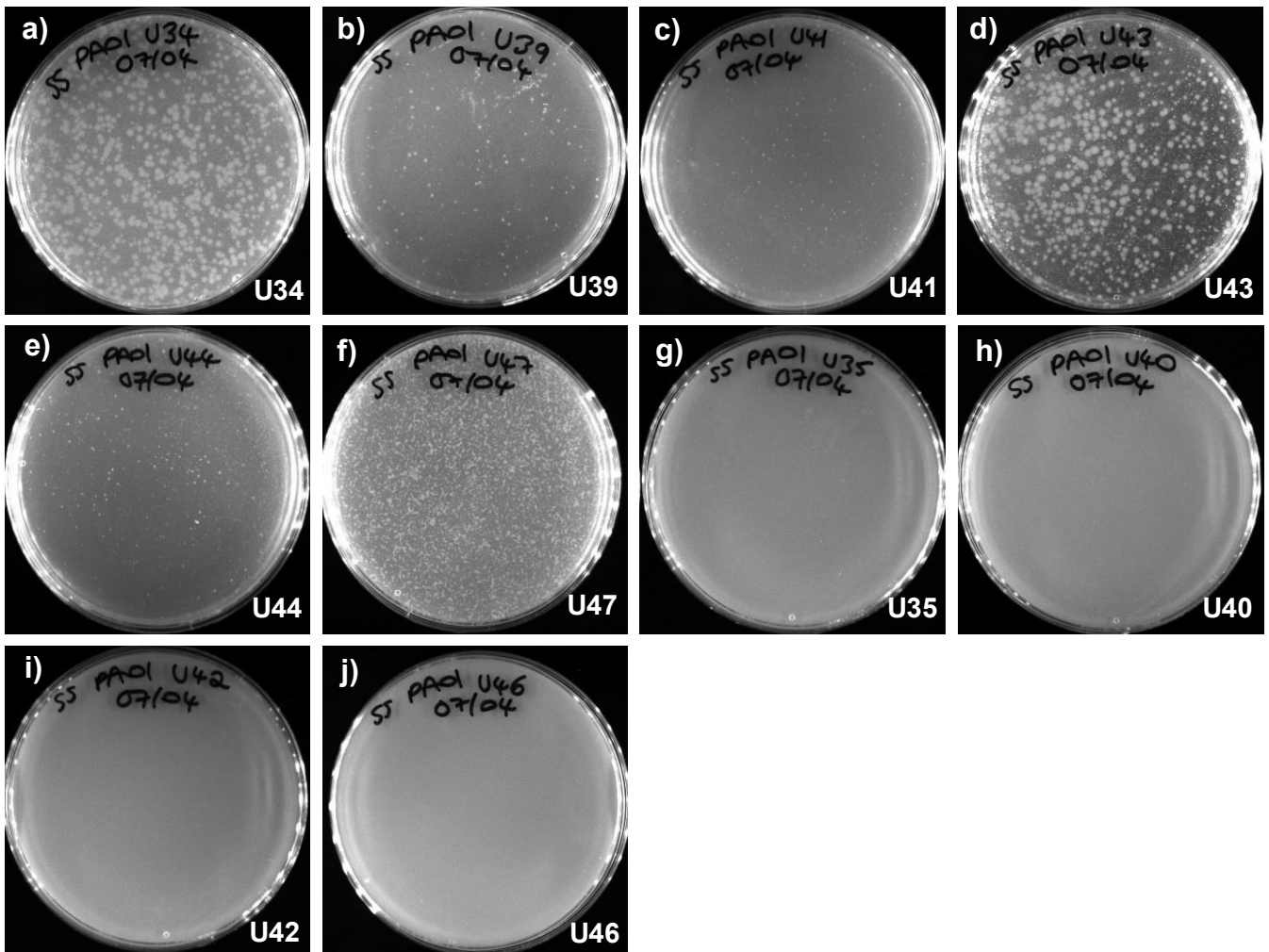


Figure 8. Images of bacterial lawns produced using *P. aeruginosa* strain PAO1 with the DLA method and enriched wastewater added. The labels on the lower right corner of each image indicate which enriched wastewater sample was added to the corresponding plate. Plates labelled **a** and **d** had a similar appearance – complete lysis of bacterial lawn with numerous bacterial colonies (circular white areas on plates) grown throughout plate. Plates labelled **b**, **c** and **e** had a similar appearance – complete lysis of bacterial lawn growth with some bacterial colonies (small pinpoint white areas on plate) present. Plate labelled **f** had clear plaques converging to form widespread lysis throughout plate. Plates labelled **g-j** had no plaques or lysis present, but good bacterial lawn growth (mainly evident through colour and therefore not that obvious on images).

Due to the paucity of identified phages in the screening of canine isolates, there were concerns as to whether this was a suitable protocol to use to isolate *P. aeruginosa* phages. The PAO1 results were taken to indicate that it was possible to find phages in the wastewater samples capable of infecting *P. aeruginosa* with the protocol used. Furthermore, as phages were found relatively easily with a human-origin bacterial strain (multiple wastewater samples showed evidence of phage), it was speculated whether the canine *P. aeruginosa* isolates were more resistant to lytic phages in comparison with the human-origin isolates; although, it should be considered that only two human-origin isolates were tested and this sample size would need to be increased prior to making definitive statements. To the best of the author's knowledge, the degree of relatedness between human- and canine-origin *P. aeruginosa* isolates has not been reported; therefore, it is unknown how different these strains are. The differences seen between the phage susceptibility of the bacterial isolates could be associated with the variability between the bacterial strains irrespective of origin (see further discussion on factors affecting phage susceptibility of isolates in section 6.3).

However, a caveat to these speculations is that although the initial steps of phage isolation were performed (i.e. suspension in SM buffer) to allow future use of the phages isolated using PAO1, there was no purification or further plating of these phages. Therefore, there is no confirmation that these were in fact phages and the lysis/plaques were not due to another mechanism, e.g. detergents in the wastewater inhibiting growth of PAO1. This seems unlikely, given the lack of effect on the other isolates, but needs to be shown formally. The next steps to investigate this would be to serially dilute the SM buffer suspensions and apply these to DLA plates made with PAO1 to assess for true plaques.

Another possibility for the poor lytic phage yield with the canine bacterial isolates could be that the wastewater samples were not the optimum samples to use for phage screening due to a low prevalence of phages able to infect canine isolates. If the source of the samples used for phage screening was the issue, using sources other than wastewater may give an improved phage yield due to the fact that phages are considered to be present wherever their bacterial hosts are.⁶⁴⁻⁶⁷ For example, canine ear canal swabs (though *P. aeruginosa* is not a normal inhabitant of a healthy dog ear^{4,10,13,14}) may be more likely to contain phages capable of lysing canine *P.*

aeruginosa otitis isolates. Another possible sample source could be water or soil samples from dog parks, given that visiting dog parks has been shown to increase the odds of a clinical *P. aeruginosa* infection in canine cases.²⁰ Previous studies have reported isolating phages using *P. aeruginosa* canine isolates as bacterial hosts from dairy farm manure and bovine uterine secretions⁸ and sewage samples.¹¹⁴

Plaque visibility has been reported to be a limiting factor with DLA assays¹²⁷ and this could be a possible explanation for the difficulties with finding lytic phages in the wastewater – perhaps, plaques were present but not detectable. There are a few techniques that have been shown to enhance plaque detection, including replacement of agar with agarose at a lower concentration to enable phage diffusion¹²⁸; including calcium and magnesium divalent ions to aid phage adsorption, of which calcium divalent ions were already present in these DLA assays^{128,129}; and incorporating dyes into the agar overlay.¹²⁹ Adding a combination of glycerol and some antibiotics to the agar substrate has been found to increase the size and contrast of phage plaques.¹²⁷ It should be noted that this effect was seen with other bacterial species and, though *P. aeruginosa* phages were not tested, no difference was seen with the plaques of a *P. fluorescens* phage with the antibiotic/glycerol added.¹²⁷ It has been hypothesised that adding any substance or condition that induces the SOS response (bacterial stress response) in the host bacteria or causes filamentation of the bacteria can increase plaque size by increasing phage production.¹²⁷ As some fluoroquinolone antibiotics have been shown to induce the SOS response in *P. aeruginosa*, for example ciprofloxacin,¹⁵³ it could be hypothesised that adding this to the growth medium of a DLA assay would enhance plaque visibility. However, a significant caveat with this would be that by inducing a stress/SOS response in the bacteria means there would be a high likelihood that prophages would also be induced^{67,74} and complicate the interpretation of the plaques when screening for lytic phages. As it has been shown that ciprofloxacin can increase the rate of prophage induction in a CF *P. aeruginosa* strain,¹⁵⁴ this is likely to be the case if adding this antibiotic to the DLA assay. The same study did show that some antibiotics (for example, azithromycin) can cause a significant reduction of prophage induction below the spontaneous induction level,¹⁵⁴ so perhaps this would be a better antibiotic candidate for improving plaque detection in *P. aeruginosa* DLA assays.

3.2. Prophage content of bacterial genome sequences

The genome sequences of the canine *P. aeruginosa* isolates were run through PHASTER to assess for potential prophage regions, as outlined in section 2.9. At least one 'intact' prophage region was identified in each isolate, except for isolate B which had no 'intact' prophages (Table 6). The bacterial isolate containing the highest number of 'intact' prophage regions was F with five. Isolate M contained one 'intact' prophage sequence. This result is additional support for the hypothesis that the plaques seen on the wastewater screening DLA assays with isolate M were likely a result of prophage induction. However, the PHASTER results also highlighted the potential for the phages isolated using AA as the host bacteria to be lysogenic phage, as AA contained three 'intact' prophage sequences.

Bacterial isolate	Intact	Questionable	Incomplete	Total number of prophage regions
A	2	2	1	5
B	0	1	4	5
C	1	0	3	4
D	3	1	0	4
E	2	2	1	5
F	5	1	2	8
G	2	0	1	3
K	1	1	0	2
L	1	0	1	2
M	1	2	2	5
N	2	0	0	2
P	2	3	1	6
R	4	0	1	5
S	2	5	1	8
U	4	1	1	6
V	2	4	1	7
X	3	0	3	6
Z	3	1	1	5
AA	3	0	2	5
AB	2	0	1	3
AC	1	2	2	5
AF	4	2	2	8
AG	2	0	2	4
AH	3	1	1	5
AI	1	2	2	5
PAO1	1	1	0	2
NCTC 10662	3	1	1	5

Table 6. Results of PHASTER^{143,144} analysis for prophage regions in the bacterial genome sequence of each canine isolate (n=25) and the two human-origin isolates. Each potential prophage region is divided into one of three categories by the program – ‘intact’, ‘questionable’ or ‘incomplete’ – depending on the predicted completeness of the region.

Overall, the canine *P. aeruginosa* isolates used in this study had a high level of predicted prophages present with 96% of the isolates (n=25) having at least one 'intact' prophage present on their genome. This finding is not surprising as a high abundance of prophages in *P. aeruginosa* isolates is reported in the literature.^{40,145,155,156} A recent large bioinformatics study found that approximately 68% of *P. aeruginosa* genomes (n= 5,383; predominantly human-origin isolates with some environmental) contained at least one prophage.⁴⁰ Most genomes containing predicted prophages had one or two prophage sequences present; however, one *P. aeruginosa* genome contained as many as fifteen prophage sequences.⁴⁰ In the study, only prophage sequences with a 'high-confidence' were analysed and those deemed as 'lower-confidence' by the prophage prediction tool used were excluded – the latter were present at a much higher frequency throughout the bacterial genomes.⁴⁰ For this reason, when assessing the PHASTER data from the canine *P. aeruginosa* isolates, the 'intact' prophage regions were considered to be the most relevant to assess as these were more likely to be true temperate phages, in contrast to the 'questionable' or 'incomplete' regions. However, it should be considered that as canine/veterinary isolates are less studied compared to human-origin *P. aeruginosa* isolates and there are less publicly available veterinary isolate genome sequences, lysogenic phages that are specific for veterinary isolates may exist and contain genes that are not present on the current databases. Therefore, there may be more complete prophages in these isolates than predicted by PHASTER. Future investigative steps could include using a prophage induction agent (for example, mitomycin C^{157,158}) with each bacterial isolate to confirm the prophages identified on bioinformatic analysis are functional and, furthermore, potentially identify functional prophages that PHASTER did not.

The sequences for PAO1 and NCTC 10662 *P. aeruginosa* laboratory strains were also assessed for prophage content (Table 6). As PAO1 was shown to contain one likely intact prophage and one questionable region, there is a possibility that the suspected phage activity seen during the wastewater screening assays may originate from prophage induction. Prophages have been reported elsewhere to exist in the PAO1 genome – for example, filamentous phage Pf4.^{155,159,160} It has been shown that though PAO1 is a commonly used reference laboratory strain, it has undergone many genotypic and phenotypic changes through the years.¹⁵⁹ Therefore, there is a chance that the specific isolates used in this study may differ in prophage content, so it may

be worthwhile sequencing the specific isolates used to ensure accurate assessment of this. Along with carrying out further phage purification steps, as explained above in section 3.1, this could help determine the likely nature of the phages identified in the wastewater screening assays with PAO1.

The high prevalence of prophages in the canine *P. aeruginosa* isolates could explain why finding lytic phages able to lyse them in the wastewater samples was challenging. This is because the prophages present could confer superinfection immunity, rendering the bacteria more resistant to successful infections by other phages. It has been shown in the literature that this commonly occurs with *P. aeruginosa* lysogenic phages^{90,145} and there have been several mechanisms identified, for example modification of the O-antigen in the lysogens which some phages require for bacterial cell entry.⁹⁰

3.3. Screening for lytic phages in wastewater samples with isolate B

Following the PHASTER analysis, it was noted that isolate B had no 'intact' prophages identified. There were one 'questionable' and four 'incomplete' regions. Given the limitations of the PHASTER analysis described above (section 3.2), it cannot be stated definitively that isolate B has no complete prophage sequences as the 'questionable' and 'incomplete' sequences may have been labelled as such due to the databases not containing data about some genes/proteins that have not yet been described. Nevertheless, as isolate B was unique in its lack of 'intact' prophages, it was used as a bacterial isolate with the ten wastewater samples in the DLA screening assays (detailed in section 2.4). There were two reasons for this – it was speculated that if the isolate had no prophages present, it may be more susceptible to phage (due to the lack of superinfection immunity) and therefore, more likely to be killed by lytic phages in the wastewater; and there would be reduced risk of spontaneous prophage induction causing issues with interpreting the results.

A bacterial lawn with no wastewater added (protocol in section 2.6) showed no evidence of spontaneous prophage induction, i.e. no plaques or lysis. The ten DLA plates produced using the enriched wastewater samples had no plaques or lysis present, indicating no lytic phage activity (Figure 9).

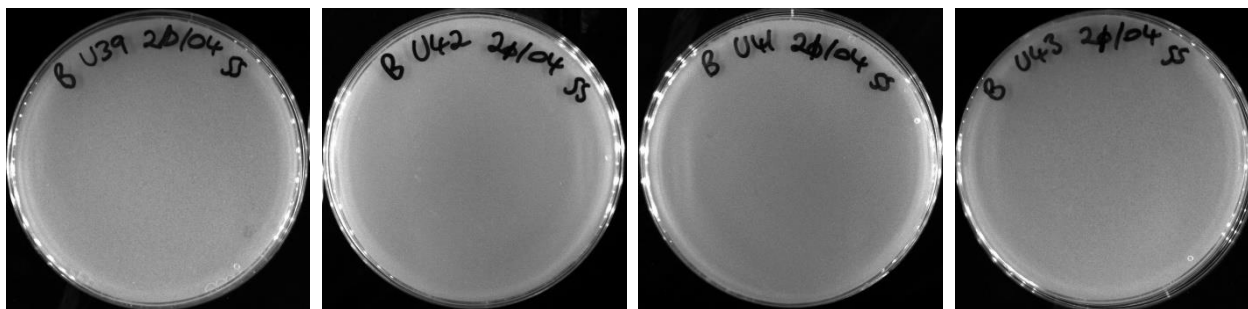


Figure 9. Example images of bacterial lawns produced using *P. aeruginosa* isolate B with the DLA method and enriched wastewater added. All had no evidence of plaque formation or lysis.

Though there was no evidence of spontaneous prophage induction as expected based on the PHASTER results, no phage activity was seen on the plates made with the wastewater samples. This could mean that the isolate has some resistance to phages without the superinfection immunity provided by integrated phage on its genome; or there are some prophages present in this isolate that are conferring superinfection immunity but were not identified using PHASTER. An alternative explanation is a simple lack of phages capable of killing isolate B in the tested wastewater samples.

4.0. Chapter Summary

Overall, the ‘phage hunting’ part of this study was challenging due to the high rates of spontaneous prophage induction seen in the *P. aeruginosa* canine isolates which limited the number of bacterial isolates that could be used to search for phage. Added to this was an apparent scarcity of active phage in the wastewater samples which could also be due to bacterial resistance. In total, five potential lytic phages active against a single *P. aeruginosa* canine otitis isolate were isolated and these were taken forward for further characterisation.

CHAPTER THREE – PHAGE CHARACTERISATION

The second objective of the study was to characterise lytic phages targeting *P. aeruginosa*. Five phages with activity against *P. aeruginosa* canine otitis isolate AA were isolated during the wastewater screening assays and taken forward for characterisation. The initial stage involved propagating the phages and titrating these solutions. Subsequent to this, the phages were imaged to assess morphology and their genomes sequenced to enable many different aspects of their biology to be characterised. Host tropism assays were also performed to evaluate the range of canine otitis *P. aeruginosa* isolates which they could successfully infect. The desired outcome from these characterisation steps was to highlight any of the isolated lytic phages that have potential as novel phage therapy candidates for canine otitis clinical cases.

5.0. Materials and Methods

All 'overnight' incubations were approximately 19 hours. A sterility control of 5 ml uninoculated TSB was included with every round of incubation.

5.1. Phage propagation

A single bacterial colony (from the appropriate host isolate for the phage) was inoculated into 10 ml TSB contained within a 50 ml flat-bottomed flask. The flask opening was covered with aluminium foil and the flask incubated (at 37 °C, 180 rpm) for 90 minutes. Then, 200 µl of the refrigerated phage preparation (P3) was added to the flask. The flask opening was then re-covered with aluminium foil and the flask incubated (at 37 °C, 180 rpm) overnight. After incubation, the contents of the flask were poured into a 15 ml tube and this was centrifuged at 5000 rpm for 10 minutes at room temperature (21 °C). The supernatant was then passed through a 0.22 µm diameter syringe filter into a fresh 15 ml tube. This was then kept refrigerated (4 °C).

5.2. Phage quantification

A ten-fold dilution series of 10^{-1} to 10^{-8} of each propagated phage solution was produced by diluting each solution in SM buffer in 1.5 ml microcentrifuge tubes. The 1.5 ml microcentrifuge tubes were kept in the fridge (4 °C) until needed.

Bacterial lawns were made following the DLA technique (section 2.7) with bacterial isolate 'AA', which is the original host for the isolated phages being characterised. Once dry, 2.5 µl drops of each phage dilution were dropped onto a lawn plate, resulting in one plate per named phage. The plates were then incubated overnight (37 °C) and assessed for plaque formation the following morning. The resulting plaques were used to select which dilutions would be optimal for subsequent plating – the aim was to produce a plate containing approximately 100 well-spaced plaques to enable counting and calculation of the phage concentration of the original solution.

The two dilutions for each phage deemed to be the most appropriate for counting were then plated out in new bacterial lawns with isolate 'AA'. To produce these, 100 µl of 'AA' bacterial culture was added to 3 ml warmed top agar (as per protocol in section 2.7) along with 100 µl of the selected phage dilution. This was used to produce a DLA plate and repeated to produce a technical duplicate plate. The plates were then incubated overnight (37 °C) and assessed the following morning.

For each phage, the most appropriate of the two dilutions was selected for plaque counting depending on the number of plaques (i.e. approximately 100). Once the dilution had been selected, both plates (duplicates) had their plaques counted manually using a Leica Quebec® Darkfield Colony Counter (Milton Keynes, UK). The mean of these two values was taken and used to calculate the phage concentration of the original sample in PFU/ml.

5.3. Transmission electron microscopy

Sample preparation

The purification protocol by Ackermann (2009) from Clokie and Kropinski's Methods and Protocols textbook was used¹⁶¹.

A 1 ml volume of the phage solution (the propagated phage solutions from section 5.1) was added to an Oak Ridge Nalgene centrifuge tube (ThermoFisher Scientific, Loughborough, UK). The tube was then filled with 0.1 M ammonium acetate (pH 7; Merck, Gillingham, UK) to at least 80% of the tube volume to ensure integrity of the tube was maintained (this was approximately 34 ml of ammonium acetate). The tubes were then centrifuged at 25,000 x g for 60 minutes at room temperature (21 °C).

The supernatant was discarded and 1 ml of 0.1 M ammonium acetate was used to re-suspend the visible pellet. Then, the rest of the tube was filled with ammonium acetate (as above). The centrifugation was repeated at 25,000 x g for 60 minutes at room temperature (21 °C).

The supernatant was discarded and 1ml of 0.1 M ammonium acetate was used to re-suspend the visible pellet (as above). This solution was then transferred to a labelled microcentrifuge tube and kept at 4 °C until needed.

This process was carried out for all five phages. The procedure was carried out as close to the day of the TEM imaging as possible to ensure freshness of the samples.

Sample staining and imaging with TEM

Uranyl acetate staining and attachment to grids of the resulting sediment was carried out by Steve Mitchell in the TEM laboratory at King's Buildings, The University of Edinburgh. One droplet of the phage sample was added to a Formvar/Carbon 200 mesh copper grid and left for ten minutes. The edge of the grid was touched briefly with filter paper to remove any excess solution. Then, one drop of 1% aqueous uranyl acetate was added and allowed to sit for one minute. Filter paper was then touched to the edge of the grid to remove the uranyl acetate solution. The grids were allowed to air dry. The samples were then assessed using a JEOL JEM-1400 Plus TEM. Images were obtained using a GATAN OneView camera. Use of the TEM was enabled by the support of the Wellcome Trust Multi User Equipment Grant (WT104915MA).

It should be noted that an initial attempt at imaging led to poor quality images as the phages appeared to be fragmented and damaged. It was decided to be more delicate when handling the phages at subsequent runs of the preparation protocol – as initially more aggressive flicking and disruption of the pellet to re-suspend it had been performed. Furthermore, to improve final phage particle yield in the sample, a sterile Pasteur pipette was used to transfer the final suspension to the microcentrifuge tube to ensure as much of sample was transferred as possible. Prior to this, a Stripette was used which was noted to be leaving some residual suspension at the bottom of the centrifuge tube.

Image processing

Images were opened using the ImageJ software (National Institutes of Health). Measurements of each phage virion were taken using the calibrated scale function in the program. Each phage structure was measured as shown in Figure 10.

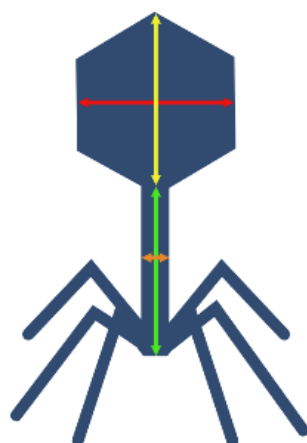


Figure 10. A phage particle with lines denoting measurements taken. Red line = capsid width. Yellow line = capsid height. Green line = tail and baseplate length. Orange line = tail diameter. Created using Biorender.com.

5.4. Phage nucleic acid extraction

The protocol from the 'Norgen Phage DNA Isolation Kit' (Norgen Biotek, Thorold, Canada) was followed using materials included in this kit, unless otherwise stated. This procedure was performed for each propagated phage solution (section 5.1).

Lysate preparation

First, 1 ml of each phage solution was transferred into a 5 ml tube. A DNase treatment step was carried out to prevent bacterial DNA contamination – this is termed an 'optional' step in the published protocol, but as the phages were propagated in TSB with the host bacteria, this step was required. Each tube had 10 μ l of DNase I added and was then incubated at room temperature (21 °C) for 15 minutes. Following this, the tubes were incubated at 75 °C for 5 minutes to inactivate the DNase I enzyme.

After this, 500 μ l of 'lysis buffer B' was added to each tube and the tubes vortexed vigorously for 10 seconds. At this point, 4 μ l of Proteinase K (Merck, Gillingham, UK) was added to each tube and an incubation step of 15 minutes at 55 °C carried out.

This step was also termed 'optional' in the protocol but was performed to increase DNA yield.

A final incubation step of 15 minutes at 65 °C was performed, with intermittent tube inversion to mix the lysate. Lastly, 320 µl of isopropanol was added to each tube and the lysates vortexed briefly.

Sample binding to column

Using the spin columns attached to collection tubes provided in the kit, 650 µl of lysate was added to a column each and centrifuged at 6,000 x g for 1 minute. The flowthrough was then discarded from each collection tube. This process was repeated with the remaining lysate until the full volume of lysate had passed through the column.

Column wash

The wash solution 'A' was added at a volume of 400 µl to each column and centrifuged at 6,000 x g for 1 minute. The flowthrough was then discarded from each collection tube. This process was then repeated 2 more times – resulting in 3 wash stages in total. Finally, the columns were centrifuged at 14,000 x g for 2 minutes to dry the resin. The collection tube was then discarded.

DNA elution

The columns were each placed into a 1.7 ml elution tube and then 75 µl of elution buffer was added to each column. These were then left to sit for 2 minutes at room temperature. Following this, the columns were centrifuged at 6,000 x g for 1 minute.

Extracted DNA quantification

The resulting DNA samples were assessed by Nanodrop ND-1000 Spectrophotometer (ThermoFisher Scientific, Loughborough, UK) and Invitrogen™ Qubit™ 3 fluorometer (ThermoFisher Scientific, Loughborough, UK) following the manufacturer's instructions. This was carried out prior to submission of the samples to MicrobesNG (Birmingham, UK) for genome sequencing by Illumina HiSeq technology.

5.5. Phage sequence analysis

For the following methods, genome sequence assemblies of each phage generated by Illumina HiSeq sequencing obtained from MicrobesNG (Birmingham, UK) were used. All command-line-based software was run by Tom Smith-Zaitlik, a PhD student at the University of Edinburgh.

Genome sequence assembly quality/contamination check

The phage genome sequence assemblies were analysed using CheckV¹⁶² (version 1.0.1, database version 1.5) to assess the quality and completeness of viral genomes. Default settings were used and the full pipeline of analyses were carried out.

The CheckV results enabled the determination of which contig in each genome assembly could be treated as the most likely complete phage genome. All the following methods were carried out on said contigs.

Phage genome % GC content

Each phage genome was entered into a % GC content calculator webtool¹⁶³ (https://jamiemcgowan.ie/bioinf/gc_content.html) to calculate the % GC content.

Determination of phage sequence presence in host bacterial isolate

Each phage genome was analysed using the Basic Local Alignment Search Tool (BLAST®) webtool on the National Center for Biotechnology Information (NCBI) website.¹⁶⁴ The 'standard nucleotide BLAST' (blastn suite) was used, with the phage genome sequence as the 'query sequence' and the bacterial host genome (*P. aeruginosa* isolate AA) as the 'subject sequence'. Default settings were used and the program optimised for 'highly similar sequences/megablast'.

Phylogenetic analysis

The phage genome sequences were entered into ViPTree, a viral proteomic tree server.¹⁶⁵ This server generates a proteomic tree which is based on genome-wide sequence similarities of viral genomes. The genomic similarity scores (S_G) are based on tBLASTx results. Details of the computational method is found in Bhunchoth *et al.*, 2016.¹⁶⁶

ViPTree (version 3.6; version of Virus-Host database RefSeq release 218) was used to generate a proteomic tree which included the phage genome sequences entered and 'related genomes'. The 'related genomes' are reference viral genomes (based on the Virus-Host Database¹⁶⁷) that are sufficiently related to the phage genomes from this study – they fall into one of the following categories:

- Reference genomes that have an $S_G > 0.02$ to at least one of the phage genomes entered
- Reference genomes that have an $S_G > 0.02$ to at least one of the reference genomes that fit into the first category

Genome annotation

The phage genome sequences were analysed using the command-line tool Prokka¹⁶⁸ (version 1.14.6) on default settings, with the Prokaryotic Virus Remote Homologous Groups (PHROG) database¹⁶⁹ added (version 4). Prokka annotates prokaryotic genomes using several databases. The PHROG database was added to this as it consists of proteins originating from genomes of viruses that infect bacteria or archaea.

Once the output was obtained from the analysis, the annotated proteins were manually sorted into nine categories based on those assigned by the PHROG database¹⁶⁹:

- DNA, RNA and nucleotide metabolism
- Head and packaging
- Tail
- Connector
- Lysis
- Integration and excision
- Transcription regulation
- Moron, auxiliary metabolic gene and host takeover
- Other

This was carried out by searching the PHROG number of each annotated protein in the database online and recording the allocated category. For those annotations assigned by other databases including Clusters of Orthologous Genes (COG) by

NCBI, a category was allocated based on the best fit to one of the above nine categories.

Identification of AMR genes

The Resistance Gene Identifier (RGI) main webtool (version 6.0.2) using The Comprehensive Antibiotic Resistance Database¹⁷⁰ (CARD; version 3.2.7) was used to determine if any known AMR genes were present on the phage genome sequences. Default settings were used and the sequence quality was set at 'high quality/coverage'.

5.6. Phage naming

The isolated phages in this study were each given a unique identifier – the host bacterial isolate 'AA', followed by a two-digit number which originated from the label of the wastewater sample from which they were isolated. For formal identification purposes, the phages were named:

- *Pseudomonas* phage AA34
- *Pseudomonas* phage AA39
- *Pseudomonas* phage AA43
- *Pseudomonas* phage AA46
- *Pseudomonas* phage AA47

This was carried out using the guidelines detailed in Adriaenssens and Brister, 2017.¹⁷¹ Published literature was searched for the unique identifiers listed above (AA34, AA39 etc.) using the Phage Name Check webtool¹⁷² (with the 'more stringent' setting) and the NCBI Virus database 'virus name' search tool.¹⁷³ No results were found in the literature for any of the unique identifiers used in this study. Therefore, the proposed unique identifiers were kept as there was no evidence to suggest existing use of these identifiers in the phage scientific field.

5.7. Host tropism determination – double layer agar assays

A fresh ten-fold dilution series was made from each phage solution that was produced via the propagation method (section 5.1). The dilution series technique is described in section 5.2. This was repeated with each phage solution, resulting in all five phages having a dilution series of ten-fold dilutions with the lowest dilution containing an

estimated 10^2 PFU/ml of phages. The estimated concentrations were based on the results from the titration protocol described in section 5.2. The microcentrifuge tubes were kept in the fridge (4 °C) until needed.

It should be noted that one of the initial phage solutions had been estimated to have a concentration of $>2 \times 10^x$ PFU/ml, so a preliminary dilution step was performed to obtain a rough concentration of $\sim 1 \times 10^x$ PFU/ml prior to starting the ten-fold dilution series.

Bacterial lawns were made following the DLA technique (section 2.7) with bacterial isolate 'AA', which is the original host for the isolated phages being characterised. Once dry, 10 µl aliquot drops of each phage dilution were dropped onto a lawn plate, resulting in one plate per named phage. The plates were then incubated overnight (37 °C) and assessed the following morning.

The plates were checked to ensure the dilution series appeared appropriate in regard to lysis/plaque formation. Plaques in the '10³' drop were counted for each plate and the phage concentration calculated. The purpose of this step was to ensure the estimated phage concentrations of the solution and dilutions were correct in preparation for the host tropism assays. This step was repeated for every run of the host tropism DLA assays.

On the same day as the assessment of the serial dilution plates, bacterial lawns were made (section 2.7) using the other *P. aeruginosa* isolates – one isolate per lawn. Once dry, 10 µl aliquot drops of each phage were dropped onto each bacterial lawn at the estimated concentration of 10^6 PFU/ml. Diluted phage stock was selected to try to ensure the titre was not excessively high resulting in false positive results, for example as seen in Carmine de Melo *et al.*, 2019 (see further discussion in section 6.3).¹¹⁶

A positive control plate was made using a bacterial lawn of the bacterial isolate AA with 10 µl aliquot drops of the 10^6 PFU/ml concentration for each phage. A negative control plate was also made for each bacterial isolate which consisted of a bacterial lawn with no phage added and a single 10 µl drop of SM buffer added. This was to assess for any prophage induction in the lawns alone or any induction due to the SM buffer. The

plates were then incubated overnight (37 °C) and assessed the following morning for any evidence of plaques or lysis.

5.8. Host tropism determination – planktonic growth assays

First, to assess the approximate concentration (in colony-forming units [CFU]/ml) of bacteria present in a planktonic culture of each bacterial isolate of interest at an OD₆₀₀ of approximately 1, the following procedure was carried out. A single colony from the relevant bacterial isolate was inoculated into 5 ml TSB in a 30 ml universal container. These were incubated overnight in a shaking incubator (at 37 °C, 180 rpm). After incubation, 50 µl of each bacterial culture was added to 5 ml fresh TSB in a new 30 ml universal container each (to generate a 1:100 dilution). A negative control with TSB only was also included. These were all incubated for approximately three hours (at 37 °C, 180 rpm). Once an OD₆₀₀ of approximately 1 was obtained (range deemed acceptable was 0.9 – 1.1), a dilution series was made by adding 100 µl of the bacterial culture to 900 µl of phosphate-buffered saline (PBS) in a 1.5 ml microcentrifuge tube. Then, 100 µl of this was added to 900 µl of PBS in another 1.5 ml microcentrifuge tube and so on. This was repeated until a ten-fold dilution series was obtained from 10⁻¹ down to 10⁻⁶ of the original culture for each bacterial isolate. Then, 100 µl of each dilution was added to the centre of a TSA plate and spread evenly across using an L-shaped spreader to create a bacterial lawn. All plates were incubated overnight (37 °C). In the morning, the number of colonies were counted on the 10⁻⁶ dilution plates, as these were deemed to be at the best concentration for counting (as near to 100 colonies as possible). The estimated concentrations of the original cultures were then calculated and then used for multiplicity of infection (MOI) calculations.

The day prior to the planktonic growth assay, the concentrations of the phage solutions to be used were assessed using DLA plates with 10 µl aliquot drops of a phage dilution series and the counting of plaques (section 5.7). This was to ensure the concentrations of the phages to be used were as expected. The same ten-fold dilution series (phage suspended in SM buffer) from section 5.7 were used.

On the morning of the planktonic growth assay, a planktonic culture of each bacterial isolate of interest that had been incubated overnight (as detailed above) was diluted 1:100 by adding 50 µl to 5 ml TSB in a fresh 30 ml universal tube. A TSB-only negative

control was included and these were all incubated for approximately 3 hours until the cultures had reached an OD₆₀₀ of approximately 1. At this point, each bacterial culture was diluted in TSB to an appropriate concentration to enable the desired CFU quantity to be added to the plate. These dilutions were based on the estimated concentrations calculated from the previous experiment (as detailed above).

A flat-bottomed 96-well plate was inoculated (Figure 11). Every condition was repeated in technical triplicate on the same plate. There was a triplicate set of 'media only' control wells for each bacterial isolate tested which contained 190 µl sterile TSB and 10 µl SM buffer. The 'bacteria only' control wells contained 180 µl of TSB; 10 µl of the relevant pre-diluted bacterial isolate; and 10 µl of SM buffer (in lieu of phage). All other wells contained 180 µl of TSB; 10 µl of the pre-diluted bacterial isolate; and 10 µl of the appropriate phage solution. The phage solutions used were chosen so that approximately 10⁴ PFU were applied to each well, alongside 10⁶ CFU of bacteria – giving an MOI of 0.01.

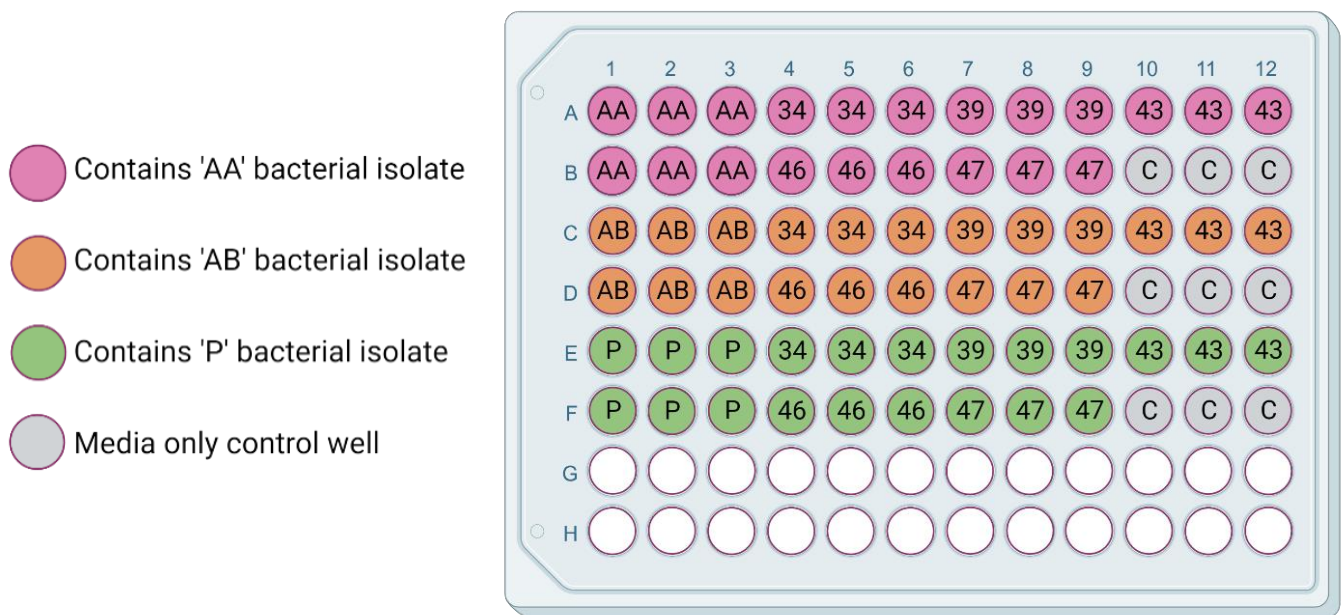


Figure 11. Diagram of a 96-well plate set up for the planktonic growth assay with labels denoting contents of each well. Wells marked with 'AA', 'AB' or 'P' indicate 'bacteria-only' wells which contain the bacteria isolate of interest, TSB and SM buffer. Wells marked with 'C' indicate the 'media only' wells or 'control' wells which contain TSB and SM buffer. Wells marked with numbers contain a bacterial isolate (indicated by colour; see key), TSB and phage (the numbers indicate which phage e.g. '34' = AA34, '39' = AA39 etc.). Created using Biorender.com.

As soon as the phages were added, a gas-permeable sterile optically-clear adhesive plate seal (Azenta Life Sciences, supplied by SLS Ltd., Nottingham, UK) was applied to the plate. The plate was then placed into a Thermo Scientific™ Multiskan™ FC microplate photometer (ThermoFisher Scientific, Loughborough, UK) and the program started. The program was 24 hours in duration and involved constant incubation at 37 °C. Optical density readings were taken every 20 minutes at a wavelength of 620 nm. The incubation was static except for 5 seconds of medium-speed (11 Hz, amplitude 3 mm) shaking immediately prior to each reading.

To ensure the concentration of the bacterial culture was as expected and the estimated MOI was accurate on the day of the experiment, the process outlined above to calculate the concentration of the bacterial culture was also performed at the time of starting the experiment (dilution of the culture and bacterial lawn formation). The next day, colonies were counted on the 10^{-6} dilution plates to confirm the desired inoculum was used.

The OD₆₂₀ outputs were processed using GraphPad Prism (version 9.5.1). The mean was calculated for each triplicate set of results for each well condition. Then, the area under curve (AUC) value for each growth curve graph produced was calculated and then the AUC of each bacterial growth curve with added-phage ($area_{phage}$) was compared to the AUC of the bacterial growth curve of the no-phage control ($area_{control}$) using the following equation:

$$\frac{area_{phage}}{area_{control}} \times 100 = score$$

i.e. the score is the AUC of the phage-added bacterial growth curves as a percentage of the AUC of the appropriate bacteria-only control.

5.9. Biofilm inhibition assay

The protocol used for the biofilm inhibition assay was based on Pye *et al.*, 2013 with some adaptations from Robinson *et al.*, 2019.^{43,60}

The day of the biofilm inhibition assay, the concentrations of the phage solutions to be used were assessed using DLA plates with 10 µl aliquot drops of a phage dilution series and the counting of plaques (section 5.7). This was to ensure the concentrations of the phages to be used were as expected. The same ten-fold dilution series (phage suspended in SM buffer) from section 5.7 were used.

First, the *P. aeruginosa* isolate of interest was inoculated from a fresh TSA plate into 3 ml of Mueller-Hinton Broth (MHB; Oxoid, Basingstoke, UK) until a 0.5 McFarland suspension was produced. This was repeated to ensure a sufficient volume of bacterial culture for plate. Then, a 96-well flat-bottomed plate was inoculated. Every condition was performed in technical triplicate wells. There was a triplicate set of 'media only' control wells for each bacterial isolate tested which contained 150 µl of sterile MHB and 100 µl of SM buffer. The 'bacteria only' control wells contained 150 µl of the appropriate bacterial McFarland suspension and 100 µl of SM buffer (in lieu of phage). All other wells contained 150 µl of the relevant bacterial McFarland suspension and 100 µl of the appropriate phage solution. All five phages were tested against each bacterial isolate. The phage solution used was a 1:10 dilution of a phage solution of

approximately 10^6 PFU/ml, i.e. 10 μ l of the phage solution in 90 μ l of SM buffer. This resulted in an estimated 10^4 PFU of a phage applied to each well.

A gas-permeable sterile optically-clear plate seal (as above) was applied to the plate before an incubation of 24 hours was performed at a temperature of 37 °C. After incubation, the plate seal was removed and the contents of the wells discarded by turning over and flicking the side of the plate. Three wash steps were then performed using PBS and pipetting. This was done as gently as possible to reduce the risk of damage to the biofilm integrity. The plate was re-sealed with a fresh seal and heat-fixed at 60 °C for one hour.

On removal from the incubator, the plate seal was removed and 150 μ l of 0.1% (w/v) crystal violet (CV) solution (Scientific Laboratory Supplies Ltd., Nottingham, UK) was added to each well. The plate was left for 15 minutes at room temperature. The stain was then aspirated from each well with a pipette prior to performing multiple washes with sterile distilled water. This rinsing step was performed until the water removed from each well was clear. The plate was dried at approximately 35 °C for 15 minutes.

Finally, 150 μ l of 95% ethyl alcohol was added to each well to re-solubilise any remaining CV. The plate was then placed in a BioTek Cytation 3 (Agilent, Cheadle, UK) plate reader to have the optical density of each well measured at a wavelength of 570 nm as a single end-point reading.

Each triplicate set of OD₅₇₀ readings for the media-only wells were then averaged (mean) and subtracted from the mean of the other triplicate OD₅₇₀ readings to give a net OD₅₇₀ result for each. These were then compared to the biofilm classification system reported in Pye *et al.*, 2013 (Table 7).⁴³

Biofilm status	Net OD ₅₇₀
Biofilm producer	>0.135
Low biofilm producer	>0.135 but ≤0.27
Moderate biofilm producer	>0.27 but ≤0.54
High biofilm producer	>0.54

Table 7. Classification system for biofilm production levels. The levels were assessed by net OD₅₇₀ values obtained from biofilm crystal violet assay. Classification system from Pye *et al.*, 2019.⁴³

6.0. Results and Discussion

6.1. Phage imaging

All five isolated phages were imaged using TEM (section 5.3 and Figure 12). All phage particles had the morphology of Siphoviruses – i.e. long non-contractile tails.⁶⁸ This was determined by the general appearance and published tail length/diameter classifications (tail lengths >40 nm are not of Podovirus morphology and tail diameters <16 nm are Siphovirus morphology).¹³⁰ Previously this classification was used to assign the phages to taxonomic families; however, due to recent changes, the morphologies of phage particles no longer have taxonomic significance.⁶⁹ The images lacked sufficient resolution to accurately describe the three-dimensional head morphology, but they appeared to be consistent with a typical icosahedral head morphology (except for AA47; Figure 12e – the resolution of the capsids was too poor).

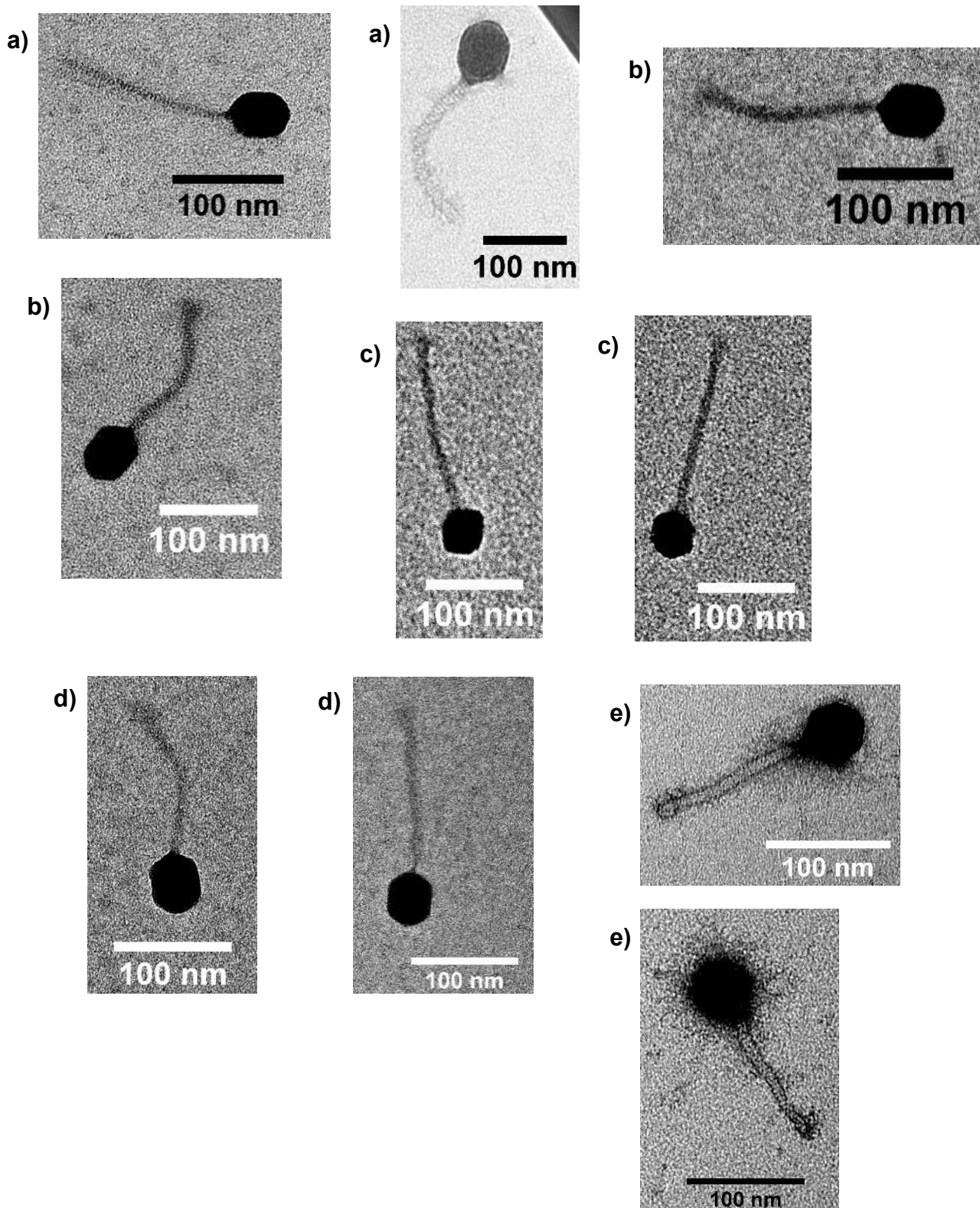


Figure 12. Representative images of phage virions. Phages: a) AA34 b) AA39 c) AA43 d) AA46 e) AA47. Taken using a JEOL JEM-1400 Plus TEM and formatted using ImageJ.

The measurements taken of the phage particles are outlined in Table 8. The mean capsid height of the phages measured was 51.4 nm (37.7 – 67.1 nm) and the mean capsid width was 41.2 nm (29.9 – 59.9 nm). The mean tail and baseplate length was 143.6 nm (108.6 – 169.8 nm). The mean tail diameter was 8.9 nm (6.9 – 10.1 nm).

Phage name	Capsid height (nm)	Capsid width (nm)	Tail and baseplate length (nm)	Tail diameter (nm)	Total length (nm)	Number of particles measured
AA34	57.6	42.0	150.3	6.9	207.9	4
AA39	41.3	29.9	108.6	9.6	149.9	7
AA43	37.7	33.9	136.7	10.1	174.4	2
AA46	53.1	40.5	152.8	7.8	205.8	5
AA47	67.1	59.9	169.8	10.1	236.9	3

Table 8. Summary of the mean measurements of the phage particles imaged using TEM. The number of particles measured (n) is recorded in the final column. All measurements are in nanometres (nm). Raw data can be found in Appendix A.

A limitation to the measurements (Table 8) is the variability in the number of particles measured for each phage. For example, AA43 only had two phage particles measured whereas AA39 had seven measured. The reason for this is due to the differing image qualities produced – some images were useful for morphology identification, but their resolution too low to enable accurate measurement and were thus excluded from measuring. An issue that became apparent to the author with comparison of phage measurements between studies is operator variability. A published study involving phage TEM was read to determine the appropriate measurements to take of each particle in this study (outlined in Figure 10 of section 5.3).¹³⁰ While measuring the phage particles, the difficulty of measuring from identical points on each particle was recognised. Therefore, it can be presumed that each scientist has the potential to carry this out slightly differently and this should be taken into account when comparing phage sizes between studies.

In previous studies, possible correlations have been found between phage morphology and plaque diameter.^{130,131} However, as numerous other factors are known to influence plaque diameter^{129,132} and given the poor reproducibility of DLA

assays between laboratories,^{128,135} relationships between phage measurements and plaque diameters were not assessed here.

Imaging the phages with the TEM not only allowed the assessment of morphology and size, but also acted as a crude method of assessing the phage sample purity. As all phages within a sample appeared similar to each other, this supported the likelihood that the sample contained one type of phage. Some samples had numerous phage particles present to assess, whereas with other samples, the phage particles were scant and took some time to locate with the TEM. It is difficult to state exactly why this was the case; however, it was noted that in samples with sparse particles, there were often capsid heads and tails present separately. This was taken to indicate that particles were damaged during sample preparation. As identical sample preparation protocols were used on each phage, it could be speculated that the phages that had broken particles present are less robust. However, a more definitive way of assessing the relative stability of phage particles would be to perform thermostability and pH-stability experiments.^{8,174} Tail fibres were not visible on any phage particles imaged; however, it should be noted that this may be due to the image resolution and sample quality, rather than indicating a genuine absence.

6.2. Phage sequence analysis

After DNA extraction (as per protocol in section 5.4) and submission for Illumina HiSeq sequencing, genome sequences of sufficient quality were obtained for all phages except AA39. The genome assembly results for phage AA39 had a high level of bacterial contamination (assembly size warning, high % GC content near that of *P. aeruginosa* and a 16S rRNA gene identified consistent with that of *P. aeruginosa*). Due to time constraints, DNA extraction of this phage could not be repeated and, for this reason, AA39 is excluded from further analysis.

6.2.1. Genome sequence assembly quality/contamination check

First, all sequences were analysed using CheckV (section 5.5) to assess for the quality and completeness of the phage genomes. It was noted that each assembly had only one contig >1000 bp and significantly longer than any other contigs present. CheckV identified these contigs for each phage as 'complete' viral genomes with a completeness score of 100% and categorised them as 'high-quality' (as >90%

complete). The completeness method used by the CheckV pipeline for all of these contigs was direct terminal repeats which is the highest-confidence method available on the pipeline. The majority of the remaining contigs for each phage genome assembly had no viral genes detected by CheckV and the few that did, were classified as 'low-quality' and 'genome fragments'. This was interpreted as highly likely to be host bacterial DNA contamination.

A further feature of the CheckV output is the determination of whether the viral genomes are likely to be proviruses or not (by assessing for predicted host boundaries at the 5' and 3' ends of the contig). All contigs were identified as not proviruses. Therefore, this provided support that the isolated phages were indeed lytic phages.

For all further analysis, only the first/longest contig from each phage genome assembly was used as these were deemed highly likely to be the complete phage genomes. Basic features are summarised in Table 9.

Phage name	Total length (bp)	Provirus	CheckV quality summary	CheckV completeness score (%)	Mean coverage	% GC content
AA34	58,551	No	High-quality	100	395.4	57.42
AA43	43,201	No	High-quality	100	654.4	53.57
AA46	58,666	No	High-quality	100	338.3	58.28
AA47	42,516	No	High-quality	100	291.0	62.22

Table 9. Phage genome features. All information based on first/longest contig of each phage assembly. Information obtained from CheckV¹⁶² pipeline output and % GC content calculated using online web calculator (section 5.5). Initial genome assemblies and mean coverage value obtained from MicrobesNG (Birmingham, UK).

Both the genome length and the % GC content for all four sequenced phages sit within the published ranges (2435 bp – 490 kb and 20.3 – 72.7%, respectively) from a large bioinformatics study analysing 4,580 phages, although the proportion of lytic and lysogenic phages in the analysis is unclear.¹⁷⁵ The % GC content of the sequenced phages were lower than that of *P. aeruginosa* (65-67%).^{176,177} This has been shown to be the case with many other lytic phages that have *P. aeruginosa* as their host.^{116,178-184} Furthermore, a bioinformatics study examining a large dataset of phages (n=4,580)

and their bacterial hosts also found that the % GC content of phages tends to be lower than their bacterial host.¹⁷⁵ The reason for this is unknown, but it has been suggested that it could be associated with the increased metabolic costs to synthesise the GTP (guanosine triphosphate) and CTP (cytidine triphosphate) nucleotides.¹⁸⁵ Other possible explanations are lateral gene transfer originating from other bacterial hosts that have lower % GC content, or that the phages only adapted to infect *P. aeruginosa* relatively recently and their initial bacterial hosts had lower % GC content.¹⁸²

6.2.2. Determination of phage sequence presence in host bacterial isolate

The phage genome sequences were run through BLAST (method detailed in section 5.5) against the genome sequence of the host bacterial isolate (AA). No significant similarity was found. This indicated that the phage sequences were not present as prophages on the host bacterial genome, which was further supportive evidence that the isolated phages were not induced prophages.

6.2.3. Phylogenetic analysis

A viral proteomic tree was produced using ViPTree (Figures 13 and 14). This included the genome sequences of the four isolated phages from this study and 'related genomes' (section 5.5).

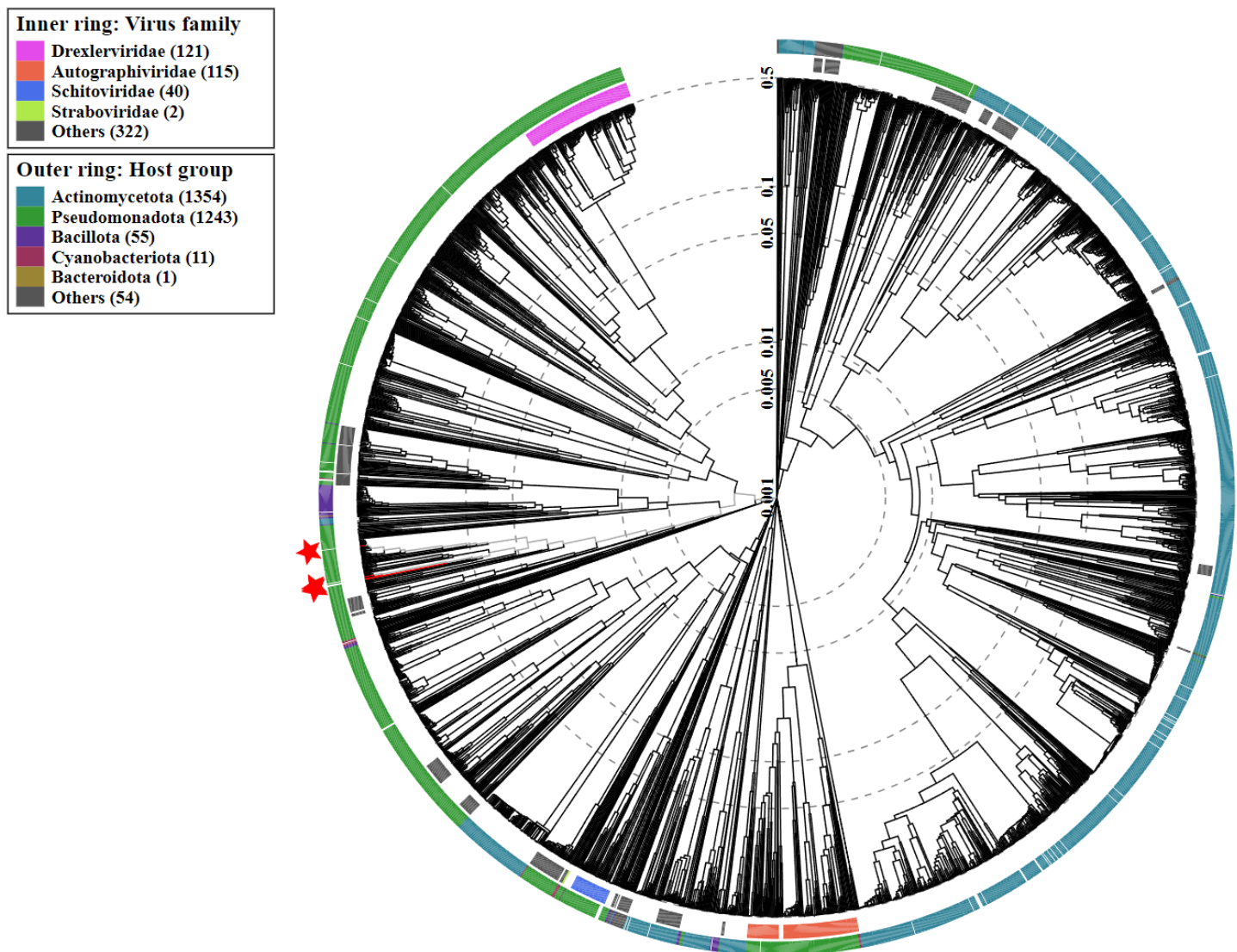


Figure 13. Circular proteomic tree. Produced using ViPTree¹⁶⁵ of the four phage genomes in this study and other related viral genomes. The red stars indicate the phage genomes from this study (AA34, AA43, AA46 and AA47). All four phages sit amongst the viruses who have *Pseudomonadota* bacterial hosts.

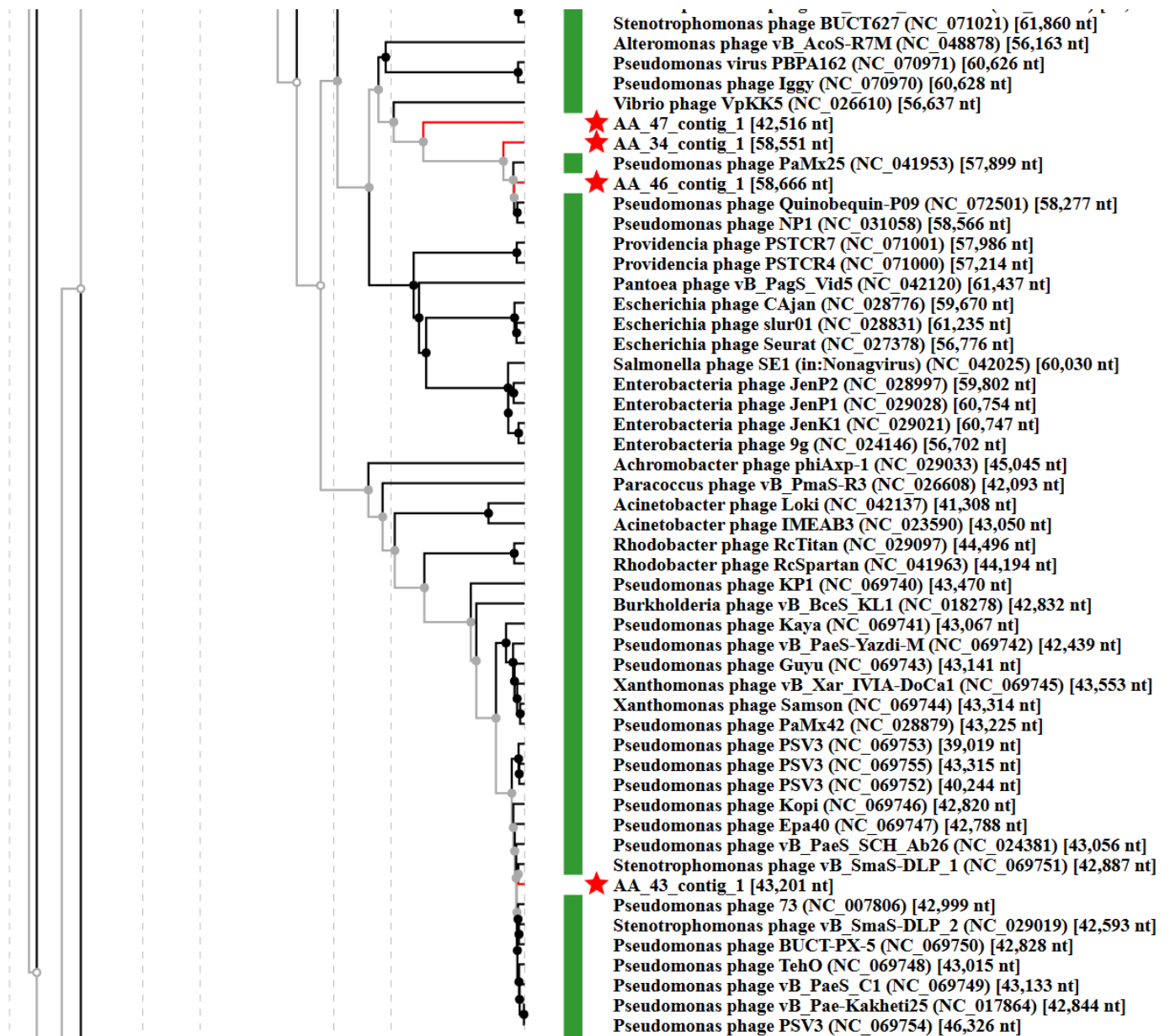


Figure 14. Enlargement of a section of the rectangular proteomic tree. Produced using ViPTree¹⁶⁵ of the four phage genomes in this study and other related viral genomes. The red stars indicate the sequenced phage genomes from this study (AA34, AA43, AA46 and AA47). The green colour indicates that all the related viruses shown in this image have *Pseudomonadota* bacterial hosts.

The viral proteomic trees showed that the viruses closest related to the phages from this study have *Pseudomonadota* bacterial hosts (Figures 13 and 14). This phylum of bacteria was previously known as *Proteobacteria* and includes *P. aeruginosa*.¹⁸⁶ The four phages are split into two clades on this tree – one clade includes AA47, AA34 and AA46; while the other clade consists of AA43 alone. As part of the ViPTree output, genomic similarity scores (S_G) are calculated to allow comparison of phage genomes (section 5.5). The similarity scores between the four phages in this study are shown in Table 10. This showed that all the sequenced lytic phages from this study were distinct from each other.

Phage name	AA34	AA43	AA46	AA47
AA34	1	0.14	0.78	0.31
AA43	0.14	1	0.15	0.15
AA46	0.78	0.15	1	0.29
AA47	0.31	0.15	0.29	1

Table 10. Genomic similarity scores (S_G) assigned to each virus by ViPTree.¹⁶⁵ A score of 1 indicates the viruses in question are identical to each other; whereas a score of 0 would indicate no similarity.

As illustrated in the proteomic tree and reflected in the genomic similarity scores, AA43 was the least closely related among the four sequenced study phages (all S_G scores <0.15), (Figure 14 and Table 10). In terms of related phage genomes, it had S_G scores of approximately 0.9 with 12 phages. All other related phages had S_G scores of <0.8. After further analysis, four of these 12 phages, though submitted to the NCBI virus database, had not yet been associated with any publications and were still awaiting final review by NCBI. For this reason, these were excluded from the following analysis. The known features of the eight closely-related phages in the published literature are outlined in Table 11. The general features of the eight closely related phages were similar to those found for AA43 – the genome sequence length (43,201) is only slightly longer than the range of lengths of these phages (42,593 – 43,055); the % GC content of AA43 (53.6%) sits within the range of % GC contents of these phages (53-54%); and AA43 has a Siphovirus morphology which many of these related phages have also been shown to have (Table 11). All phages were assessed to be likely lytic and had *P. aeruginosa* as their bacterial hosts, except for two phages which had

Stenotrophomonas maltophilia as their host (Table 11).¹⁸⁰ Interestingly, the two *S. maltophilia* lytic phages (vB_SmaS-DLP_1 and vB_SmaS-DLP_2) have been shown to be able to infect and lyse some *P. aeruginosa* strains.¹⁸⁰ Phages with infectivity capabilities across taxonomic orders is not a common finding^{65,67,180} and, as these phages are closely related to phage AA43, it would be worthwhile testing AA43 against *S. maltophilia* isolates to assess whether the host range extends to this bacterial species. *S. maltophilia* is an emerging opportunistic pathogen which is usually extremely resistant to antibiotics and has a high fatality rate in immunocompromised humans.¹⁸⁷ Furthermore, it has been reported to be co-isolated alongside *P. aeruginosa* from human CF patients.¹⁸⁷ Therefore, if phage AA43 had some activity against this bacterial species, it could be potentially useful in a phage cocktail for CF patients.

Phage name	Genome length (bp)	% GC content	Morphology	Reference	NCBI reference
<i>Pseudomonas</i> phage Kopi	42,820	53.0	N/A	Loh <i>et al.</i> , 2021 ¹⁸⁸	NC_069746
<i>Pseudomonas</i> phage Epa40	42,788	53.2	N/A	Campbell <i>et al.</i> , 2021 ¹⁵⁰	NC_069747
<i>Pseudomonas</i> phage vB_PaeS_SCH_Ab26	43,055	53.4	Siphovirus	Essoh <i>et al.</i> , 2015 ¹⁵¹	NC_024381
<i>Stenotrophomonas</i> phage vB_SmaS-DLP_1	42,887	53.7	Siphovirus	Peters <i>et al.</i> , 2015 ¹⁸⁰	NC_069751
<i>Pseudomonas</i> phage 73	42,999	53.6	Siphovirus	Kwan <i>et al.</i> , 2006 ¹⁸² ; Ackermann <i>et al.</i> , 1988 ¹⁸⁹	NC_007806
<i>Stenotrophomonas</i> phage vB_SmaS-DLP_2	42,593	53.7	Siphovirus	Peters <i>et al.</i> , 2015 ¹⁸⁰	NC_029019
<i>Pseudomonas</i> phage TehO	43,015	54.0	N/A	Loh <i>et al.</i> , 2021 ¹⁸⁸	NC_069748
<i>Pseudomonas</i> phage vB_Pae-Kakheti25	42,844	53.7	Siphovirus	Karumidze <i>et al.</i> , 2012 ¹⁸¹	NC_017864

Table 11. Summary of the eight phages closest-related to AA43 with genomes available in the public database and associated with publications. These phages all share a S_G score of ~0.9 with AA43. *P. aeruginosa* is the bacterial host for all phages listed; except *Stenotrophomonas* phage vB_SmaS-DLP_1 and *Stenotrophomonas* phage vB_SmaS-DLP_2, which have *S. maltophilia* as their host. Morphology is recorded as N/A when imaging was not carried out in the description publication.

The two sequenced phages that were the most closely related to each other were AA34 and AA46 with an S_G score of 0.78 (Table 10). Phage AA47 was also relatively similar to AA34 and AA46, but with slightly lower S_G scores than this (0.31 and 0.29 respectively) (Table 10). Phage AA47 had no phage genomes from the public databases with notable genetic similarity to it, with the highest S_G of any phage

genome being 0.3. However, phages AA34 and AA46 both had three closely related phage genomes from the published literature and these were overlapping – AA34 had S_G scores of approximately 0.8 with each of them and AA46 had S_G scores of approximately 0.9 with each (Table 12). All other phages from public databases deemed as related in the VIP tree output had S_G scores of <0.2 , so there were overall much less closely related phages to AA34 and AA46 compared to AA43. Two of the three phages closely related to AA34 and AA46 were deemed to be lytic in nature by the authors (PaMx25 and NP1),^{183,184} but phage Quinobequin-P09 was not specified.¹⁹⁰ The general features of the three closely related phages were similar to those found for AA34 and AA46 – the genome sequence lengths (58,551 and 58,666 respectively) are very similar to the range of lengths of these phages (57,899-58,566); the % GC content of AA46 (58.3%) sits within the small range of % GC contents of these phages (58.3-58.6%), but AA34 has a slightly lower % GC content of 57.4%; and all the related phages have a Siphovirus morphology like the phages in this study (Table 12).

Phage name	Genome length (bp)	% GC content	Morphology	Reference	NCBI reference
<i>Pseudomonas</i> phage PaMx25	57,899	58.6%*	Siphovirus	Sepúlveda-Robles <i>et al.</i> , 2012 ¹⁸³	NC_041953
<i>Pseudomonas</i> phage Quinobequin-P09	58,277	58.3%*	Siphovirus	Hutinet <i>et al.</i> , 2019 ¹⁹⁰	NC_072501
<i>Pseudomonas</i> phage NP1	58,566	58.4%	Siphovirus	Chaudhry <i>et al.</i> , 2017 ¹⁸⁴	NC_031058

Table 12. Summary of the three phages closest-related to AA34 and AA46 with genomes available in the public database and associated with publications. These phages all share a S_G score of ~ 0.8 with AA34 and ~ 0.9 with AA46. *P. aeruginosa* is the bacterial host for all phages listed.

* - % GC content not reported in publication, so obtained using FASTA file from NCBI database and webtool outlined in section 5.5.

A point of interest regarding phage Quinobequin-P09 is that it has been shown to encode enzymes involved in the synthesis of a 7-deazaguanine derivative nucleotide and evidence of the phage containing a nucleoside like this, dG⁺ (2'-deoxyarcheosine), has been reported.¹⁹⁰ This DNA modification system is thought to enable phages to avoid the effects of the restriction systems present in bacteria and is part of the evolutionary arms race between phages and bacteria.^{102,190} Following this discovery, the protein annotations (see section 6.2.4) of the phage genomes from this study were checked for any proteins with obvious involvement with 7-deazaguanine synthesis. It was noted that the gene *queE*, with the protein product function '7-carboxy-7-deazaguanine synthase', was encoded in phages AA34, AA46 and AA47. In addition, phage AA47 also had the *queC* gene present (with the protein product function '7-cyano-7-deazaguanine synthase'). QueE and QueC are both enzymes involved in the synthesis of a key intermediate in this modified base synthesis pathway.¹⁹⁰ This indicates that these phages are likely to also be able to modify their DNA to avoid bacterial restriction enzymes. This could be advantageous

when considering these phages as therapy candidates as it could protect the phages against bacterial resistance mechanisms. This would aid in the reduction of resistant bacterial mutants developing during treatment and/or increase the phage host range by enabling successful infection in bacterial strains that may be resistant to other phages with unmodified bases.

There were no published phages with a S_G score of 1 in relation to any of the phages from this study. Together, with the S_G scores between this study's phages (Table 10), this shows that these are likely four distinct phages that have not been described before.

Phage AA47 had the shortest genome length (42,516 bp), but the highest % GC content (62.22%) of all the phages sequenced in this study (Table 10). This conforms with the published findings that the % GC content of phages tends to increase with shorter genomes; in contrast to bacteria.¹⁷⁵

6.2.4. Genome annotation

The phage genomes were annotated using command-line software as outlined in section 5.5. Once the protein annotations were obtained, they were manually categorised according to the nine categories outlined in section 5.5. The vast majority of the proteins were annotated by PHROG and, therefore, had a PHROG-assigned category. However, there were a few proteins (n=8) across the four phage annotations that had been annotated using different databases e.g. COG, and were, therefore, not easily assigned to PHROG categories. These were assigned categories manually as per the following:

- Adenosylcobalamin-dependent ribonucleoside-triphosphate reductase → DNA, RNA and nucleotide metabolism
- 7-carboxy-7-deazaguanine synthase → DNA, RNA and nucleotide metabolism
- Flavin-dependent thymidylate synthase → DNA, RNA and nucleotide metabolism
- tRNA-Leu(gag) → Moron, auxiliary metabolic gene and host takeover
- 7-cyano-7-deazaguanine synthase → DNA, RNA and nucleotide metabolism

Any 'hypothetical proteins' were not included in the following analysis. It should be noted that the percentage of 'hypothetical proteins' of the total protein annotations of each phage was approximately 38% (31 – 43%) which is a sizeable proportion of encoded proteins, but matches what is reported in the literature for phages.¹⁹¹ This illustrates the limitations of bioinformatics analysis and the potential for many phage proteins to have an unknown function and, therefore, be excluded from analysis. Future work could include using tools, such as InterProScan,^{192,193} to analyse the hypothetical proteins for functional domains to predict the protein functions.

Overall, the most frequent protein function category was 'DNA, RNA and nucleotide metabolism' (Figure 15). The proteins annotated from each phage genome had a similar distribution of functional categories between each phage, with 'DNA, RNA and nucleotide metabolism', 'head and packaging' and 'tail' the most frequent categories for proteins to be assigned to.

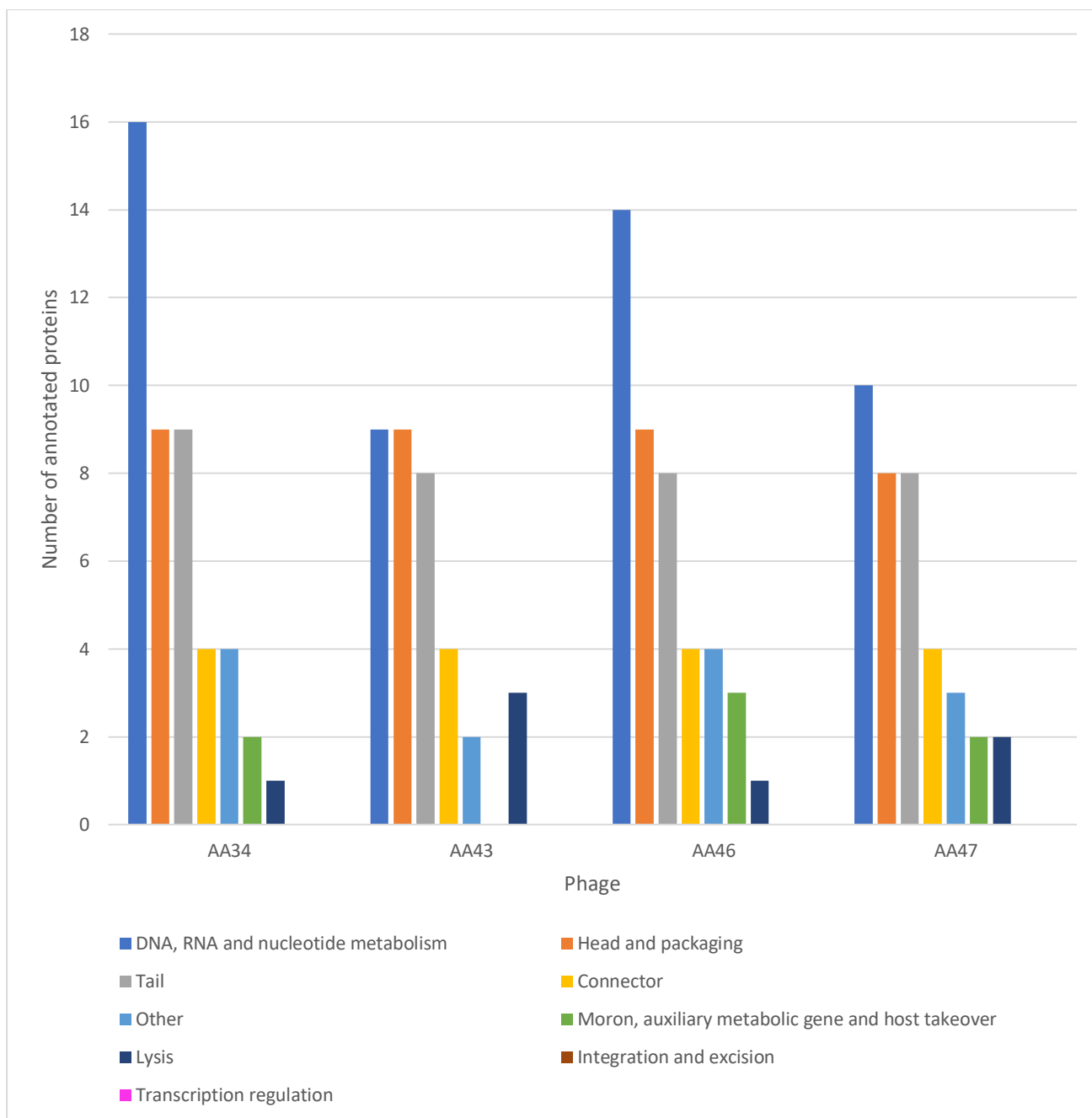


Figure 15. Bar chart showing the number of annotated proteins belonging to each functional category for each phage genome analysed. There were no proteins assigned to the ‘integration and excision’ and ‘transcription regulation’ categories.

It should be noted that every annotated phage genome contained at least one gene associated with ‘lysis’ (Figure 15). Every phage contained a protein labelled as a ‘holin’ which are lysis-associated proteins. Two (AA43 and AA47) of the four analysed phages also contained a protein labelled as an ‘endolysin’ which are also lysis-associated proteins. In addition to this, AA43 contained a ‘Rz-like spanin’ protein. Spanins are phage proteins that are associated with lysis of Gram-stain-negative

bacterial hosts as they are capable of disrupting the outer membrane.¹⁹⁴ The most extensively studied spanins are encoded by Rz and Rz1 genes in the *E. coli* lambda phage – these are two spanin proteins that make up a ‘spanin complex’ (an i-spanin and an o-spanin).¹⁹⁴ Bi-component spanins like this are commonplace in phages with Gram-stain-negative hosts; however, it has also been shown that unimolecular spanins exist, termed u-spanins, and have been found in some *Pseudomonas* phages.¹⁹⁴ As spanins have only been described more recently in the literature, they are less extensively characterised compared to other lysis-associated phage proteins, though seem to be present on the majority of phages with Gram-stain-negative bacterial hosts.¹⁹⁴ For this reason, it is highly possible that the other phage genomes in this study contain genes that encode for proteins with a spanin-function but they have yet to be described in the literature and, therefore, are missed in the current phage annotations.

There were no proteins identified that were assigned to the category of ‘integration and excision’ (Figure 15). In addition to this, the annotated protein outputs for each phage were manually searched for any proteins containing any of the following labels: ‘integrase’, ‘recombinase’ and ‘repressor’ – none were found. As these proteins (integrase, site-specific recombinase and lytic cycle repressors) are commonly associated with lysogeny,^{195,196} the absence of them helped strengthen the proposal that the isolated phages are lytic, rather than lysogenic.

Another protein function searched for manually in the outputs was ‘endonuclease’. Only one phage genome had annotated proteins containing the ‘endonuclease’ label – AA34 contained two endonuclease proteins, ‘endonuclease V N-glycosylase UV repair enzyme’ and ‘HNH endonuclease’. Ideally, phage therapy candidates would have endonucleases present, as it has been previously shown that phages lacking endonucleases have the potential to release intact plasmid DNA into the environment and enable horizontal gene transfer between bacteria, which can include AMR genes.⁹⁸ Therefore, as only AA34 had endonucleases identified on the protein annotation output, care should be taken when considering the use of the other phages as therapy candidates to ensure this ‘superspreader’ phenotype is not present (it should be noted that this term has been disputed in the literature and these phages are considered by some to be ‘superspreading-mediating’, rather than

'superspreaders').¹⁹⁷ To empirically test for this, the phages could each be applied to infect cultures of susceptible *P. aeruginosa* containing plasmids conferring resistance to an antibiotic. Then, the DNA could be extracted from each lysate, transformed into an antibiotic-sensitive *P. aeruginosa* isolate lacking this plasmid and plated on selective media. Bacteria growth can be analysed to assess for the abundance of transformant bacterial cells that possess the antibiotic-resistance-conferring plasmid. This would indicate whether there were high levels of intact plasmids released after lytic infection of the phage.⁹⁸

6.2.5. Identification of antimicrobial resistance genes

Each phage genome sequence was assessed for antimicrobial resistance genes using the webtool RGI on CARD (section 5.5). All phages came back negative for any hits (including perfect, strict or loose). This indicated that there were no known AMR genes present on these phage genomes. Therefore, there was no reason to exclude them as candidates for use in phage therapy based on the risk of transfer of AMR genes to the bacterial pathogen.

6.3. Host tropism determination – double layer agar assays

For the purposes of this assay, the definition of a 'spot' is taken to be a local area of decreased lawn turbidity of a significantly greater diameter than a plaque – usually covering the area of lysate drop application.¹³² The definition of a 'plaque' is taken to be a significantly smaller local area of decreased lawn turbidity likely due to a single phage PFU.¹³²

Before each run of the DLA host tropism assays, the concentration of the phage dilution series was checked using the protocol outlined in section 5.7 (Figure 16). Generally, throughout the experiments, the phage concentrations remained the same, with the exception of phage AA39. The concentration of AA39 reduced by ten-fold over approximately two months. It remained at this concentration for the remainder of the experiments for another month and the appropriate dilution was used accordingly each time to obtain the desired numbers of PFU needed for each experimental run. This reduction in infectivity may indicate that AA39 is a less stable phage than the others, but thermostability and pH-stability experiments would need to be carried out to ascertain this definitively. Despite the frequent phage titrations prior to each

experiment being very labour-intensive, the reduction in concentration of the AA39 phage solution emphasised how important this experimental step is to ensure reproducibility. This has previously been highlighted as an issue affecting phage studies.¹⁹⁸

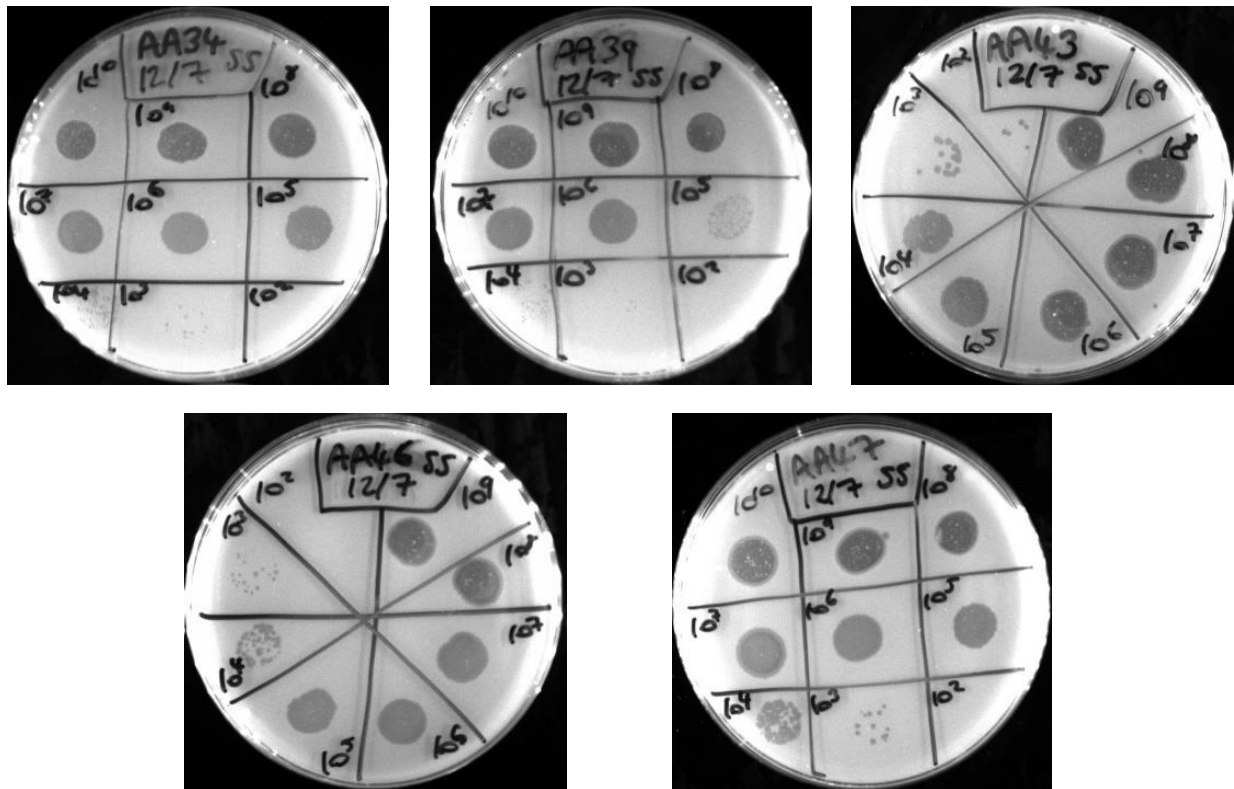


Figure 16. Example images of DLA plates produced using *P. aeruginosa* isolate AA (original host bacterial isolate) with aliquots from each phage dilution series dropped onto the top agar. After incubation, the areas where the drops were applied were assessed to ensure they appeared as expected for each dilution and then the plaques in the 10^3 PFU/ml drop were counted to ensure roughly $\sim 1 \times 10^3$ PFU/ml as expected. It should be noted that some bacterial colonies are present in the centre of the spots – see section 6.6.

The susceptibility of each *P. aeruginosa* canine otitis isolate (n=23; isolate E excluded at this point due to genetic similarity with another isolate) to each phage was tested using the protocol in section 5.7. A positive control plate of a bacterial lawn made with the original host isolate AA and an aliquot of each phage dropped on to the top agar was made for each experimental run (Figure 17). Negative control plates were also made for each bacterial isolate to assess for background spontaneous prophage induction (Figure 18). This allowed comparison with the host tropism DLA assay plates, to aid in the differentiation between spots/plaques caused by the exogenous phage added and spontaneous prophage induction. None of the ‘negative control’

bacterial lawns showed any difference in the region where the SM buffer drop was applied, i.e. regardless of whether there were plaques on the plate or not, the same was seen on the central area of the plate where the SM buffer was present compared to untreated areas of the same plate (Figure 18).

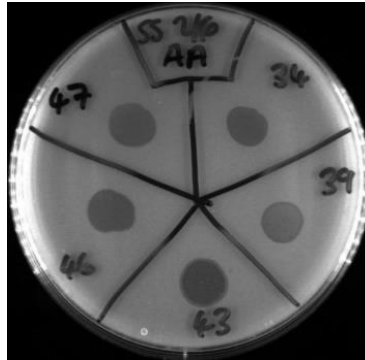


Figure 17. Example plate used as a positive control for the DLA assays to assess host tropism. The bacterial lawn was made using the *P. aeruginosa* canine otitis isolate AA – the original host bacterial isolate for all five phages. 10 μ l aliquots of a 10^6 PFU/ml solution of each phage were applied to the top agar – the numbers on the plate indicate which phage (e.g. '34' = AA34). Clear spots can be seen for all phage aliquots.

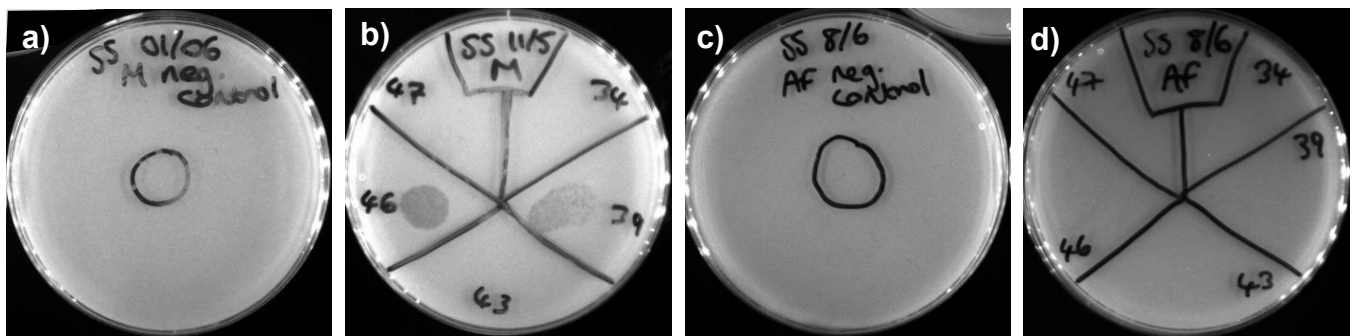


Figure 18. Example images of host tropism DLA plates.

(a) is the negative control bacterial lawn for *P. aeruginosa* isolate M and has a 10 μ l aliquot of SM buffer in the middle of the plate (denoted by the circle). There were small turbid plaques throughout the plate (although this is not that obvious in the image) indicating spontaneous prophage induction. There was no difference seen in the area of the SM buffer aliquot.

(b) is a bacterial lawn made from bacterial isolate M with 10 μ l aliquots of each 10^6 PFU/ml phage solution added (the numbers on the plate indicate which phage e.g. '34' = AA34) – an obvious spot with some plaque formation within it was seen in the area of both AA39 and AA46 aliquots. A background of turbid plaques were present throughout the rest of the plate similar to that of the negative control plate with no added phage shown in (a).

(c) is the negative control bacterial lawn for bacterial isolate AF (produced in the same way to plate (a)). There were no plaques present and no difference seen in the area of the SM buffer spot.

(d) is a bacterial lawn made from bacterial isolate AF with 10 μ l aliquots of each 10^6 PFU/ml phage solution added (similar to plate b). No spots or plaques were seen.

Nine of the 24 bacterial isolates had evidence of spontaneous prophage induction when 'negative control' bacterial lawns were produced. This could have complicated the interpretation of the host tropism DLA assays; however, there was only one bacterial isolate, M, that had background prophage induction and some activity where the exogenous phages had been added (Table 13 and Figure 18). As can be seen in Figure 18, it was still apparent that the two spots were due to the application of phage and the negative control plate allowed comparison to increase the certainty this was the case.

Bacterial isolate	Presence of spots/plaques					Negative control (background prophage induction)
	AA34	AA39	AA43	AA46	AA47	
A	-	-	-	-	-	plaques
B	-	-	-	-	-	clear
C	-	-	-	-	-	plaques
D	-	+	-	+	-	clear
F	-	-	-	-	-	plaques
G	-	-	-	-	-	plaques
K	-	+	-	+	+	clear
L	-	-	-	-	-	clear
M	-	++	-	++	-	plaques
N	-	-	-	-	-	clear
P	-	-	-	-	-	clear
R	-	-	-	-	-	clear
S	-	-	-	-	-	plaques
U	-	-	-	-	-	clear
V	-	-	-	-	-	plaques
X	-	-	-	-	-	plaques
Z	-	-	-	-	-	clear
AA*	+++	+++	+++	+++	+++	clear
AB	+	+++	-	++	++	clear
AC	-	-	-	-	-	clear
AF	-	-	-	-	-	clear
AG	-	-	-	-	-	clear
AH	-	-	-	-	-	clear
AI	-	-	-	-	-	plaques

Table 13. Results of host tropism assessment for each phage using DLA assays made with each *P. aeruginosa* isolate (n=23) and the original host bacterial isolate (AA). ‘-’ = no evidence of spot or plaques. ‘+’ = faint spot and/or plaques in zone. ‘++’

= moderately defined spot and/or plaques in zone. '+++ = very clear spot in zone. The 'negative control' column indicates whether the bacterial lawn plates with no phage added were clear or had plaques throughout – the latter indicating spontaneous prophage induction.

* AA was the host bacterial isolate that the phages were originally isolated against and was used as the positive control in this experiment.

Excluding the bacterial isolate from which the phages were originally isolated (AA), only four of the 23 bacterial isolates had evidence of susceptibility to some of the phages using the DLA assays (Table 13). All of these isolates were free of background plaques on their negative control plates, except for isolate M (as discussed above). With the exception of the original host isolate AA, isolate AB appeared to be susceptible to the highest number of the phages tested compared to the other bacterial isolates, with obvious spots and/or plaques present for four of the five phages (Table 13 and Figure 19).

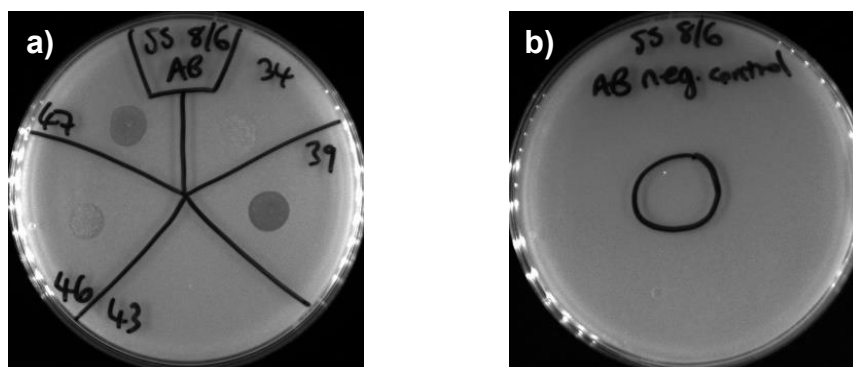


Figure 19. Two host tropism DLA assays using *P. aeruginosa* isolate AB for the bacterial lawn.

(a) has aliquots of the five phages being tested applied to the top agar – the numbers on the plate indicate which phage e.g. '34' = AA34. Spots and/or faint plaques can be seen in all phage aliquot zones except for phage AA43.

(b) is the negative control plate for the bacterial isolate AB – no plaques are present indicating no spontaneous prophage induction and the central area where an SM buffer spot was applied (denoted by the circle) shows no difference.

A point that needs to be thought about when considering why some isolates are susceptible to the phages and not others, is the phage receptors. The receptors that these lytic phages bind to enable bacterial cell entry is unknown. As phages AA39 and AA46 share identical host ranges among these bacterial isolates, this could suggest that they both use the same receptor on the bacterial cells. To determine the receptors, the ability of the phages to lyse mutant strains of *P. aeruginosa* lacking

common phage receptor targets, for example, flagella, pili, LPS or OprM, could be assessed.¹⁹⁹ Once the receptor type is determined, the canine otitis bacterial isolate genome sequences could be analysed to determine which isolates contain these receptors.

Each isolate that showed lysis and/or plaques due to phage application had this evident with at least two of the phages tested. Given that only a few isolates appeared susceptible to the phages and those that did showed susceptibility to multiple phages, it presents the question of whether some isolates are inherently more susceptible or resistant to the lytic phages. This could be a relevant point when considering the results of the wastewater screening assays with the human-origin isolates (section 3.1). One aspect to be considered is the ‘superinfection immunity’ concept of prophages in the bacterial cell conferring resistance against successful infection by other phages.^{89,90} If superinfection immunity was playing an obvious role here, it could be expected that the number of prophages in the bacterial isolates showing susceptibility to the lytic phages would be significantly lower than the isolates showing resistance. However, all the bacterial isolates susceptible to the lytic phages (D, K, M, AB and AA) each contained one to three intact prophages in their genomes (section 3.2, Table 6). These are similar numbers to many of the other bacterial isolates that showed resistance to the lytic phages. In addition, if superinfection immunity was present, it could be anticipated that the only isolate which is potentially intact-prophage-free (isolate B) would be susceptible to some of the lytic phages; however, this is not the case (Table 13). Although there is no apparent evidence that superinfection immunity is present, it cannot be definitively stated that it is not playing a role as there are inherent limitations involved with the bioinformatics analysis of the prophage content of the bacterial isolates (as discussed in section 3.2) which means that some isolates may have lysogenic phages present that are not correctly identified using PHASTER. As superinfection immunity has been shown to be present due to *P. aeruginosa* prophages before,^{90,145} the possibility of this occurring here cannot be excluded. There are many other mechanisms by which bacteria can be resistant to phage, including restriction-modification systems and abortive infection systems (where the bacterial cell is killed but the production of phage progeny is inhibited).¹⁰² To investigate this, the extent of phage defence systems present in the canine otitis bacterial isolates could be

assessed by running their genome sequences through a program like The Prokaryotic Antiviral Defence Locator.^{200,201} The abundance and type of systems present could then be compared between isolates and their susceptibility to different phages could be phenotypically tested. In addition, it has been shown that bacterial plasmids are capable of conferring phage resistance mechanisms and affecting phage infection efficiency.^{102,202} Therefore, further investigations should take the presence of plasmids within the canine otitis isolates into account.

Phages AA39 and AA46 both had the widest host range according to the DLA assays with four bacterial isolates (n=23) showing signs of possible lysis (Table 13). Phage AA47 had two isolates (n=23) showing evidence of lysis and phage AA34 had only one isolate (n=23) with evidence of lysis. Phage AA43 had no susceptible bacterial isolates (other than the original AA host isolate). When selecting a phage for phage therapy, logically the phage with the broadest host range would be chosen as it could be assumed that this would have the potential to kill more strains of *P. aeruginosa* and be effective in more infections. However, it has been shown that phages with a wider host range can incur a fitness cost and therefore, phages with greater host specificity may possess greater bacterial infection efficacy.^{71,203} For this reason, it may be better to consider including multiple phages with efficacy against some *P. aeruginosa* isolates (e.g. all five phages from this study) in a phage cocktail as a treatment – though it should be noted that, due to the restrictive host range of these phages, many *P. aeruginosa* canine otitis isolates would likely not be killed and other phages would need to be added to the cocktail to ensure a sufficiently broad bacterial spectrum. Another possible benefit of such a cocktail, in addition to the increased spectrum, would be the reduced risk of the target bacteria becoming resistant as several distinct phages would be present in the same treatment.^{74,85} Though the limited host range is likely a significant restriction in the use of these phages as therapeutic candidates, a possible method to overcome this is phage training. Phage training, also known as phage pre-adaptation, involves laboratory protocols which select naturally-occurring phage variants (that develop as part of the evolutionary arms race with bacteria) that have an increased efficiency of bacterial lysis capability.^{204,205} These techniques have been used with *P. aeruginosa* phages and have been shown to, not only increase phage efficacy,^{206,207} but also phage host

range.^{204,205,208} This is a current area of research and could be attempted with the phages described here to increase their host range.

An observation was made about the plates of two of the bacterial isolates (D and K) that showed some susceptibility to the phages. In the zones of the phage inoculums of AA39 and AA46 (and also AA47 for isolate K) where lytic activity was seen, it was noted that the morphologies of the plaques were identical in all of these zones on the same plate despite treatment with different phages. As the plaques were small, photos of sufficient quality to visualise this were not possible. Further, these plaques did not match the original plaque morphologies seen when the same phages are plated on bacterial lawns made with the original host isolate AA. In contrast, the effects seen in the phage drops on the DLA assays made with bacterial isolates M and AB appeared more like obvious zones of lysis/spots (rather than individual plaques). This raised doubts as to whether the plaques seen on the D and K plates were related to true infections by the exogenous phage added. Instead, it was speculated that these could have been a result of induced prophage originating from the bacterial isolate itself. Both of these isolates had no spontaneous prophage induction plaques observed on their negative control plates. As the plaques were only present within the areas of the exogenous phage drops, it was thought that the prophages could have been induced by the addition of the phage in the SM buffer. The SM buffer spots on the negative control plates did not induce any plaques, so there was no evidence that the buffer itself acted as a prophage induction agent. Prophage induction can occur due to the triggering of the SOS bacterial response and/or extrinsic factors, for example UV radiation, mitomycin C, antibiotics and changes in pH.^{157,158} Perhaps, the addition of the lytic phages stressed the bacteria and resulted in the activation of prophages; however, to the author's knowledge, there have been no reports of the application of lytic phage inducing prophage in the literature. To determine whether these plaques were due to prophages or the applied lytic phages, the plaques could be picked and the phages isolated (section 2.8). Then, DNA extraction could be performed and the resulting samples sent away for genome sequencing – this would allow comparison with the tested lytic phage sequences and provide a definitive answer to the source of the plaques seen.

Although ideally the isolated lytic phages would have an ability to truly infect and lyse the bacteria of these isolates, there may be an application for these phages regardless. Whether the plaques seen were due to prophage-induced lysis or true infection by the lytic phages, the bacterial cells were still lysed. Therefore, it can be hypothesised that in a *P. aeruginosa* infection, the application of these phages could still reduce the burden of the pathogenic bacteria. Recently, the concept of ‘prophage induction therapy’ has been proposed as an alternative approach to phage therapy – this is where the prophages pre-existing in the pathogenic bacteria would be induced via application of an induction agent, resulting in the elimination of the bacteria.²⁰⁹ Though the proposed induction agent in that model²⁰⁹ is a molecule targeting the CI repressor (a protein involved in the repression of lytic gene expression), in theory, the lytic phages described here could be used in a similar manner to kill the bacteria causing an infection.

An alternative explanation for the distinct plaque morphologies (compared to when the phages are plated on their original host bacterial isolate AA) is that phages have been shown to produce different plaque morphologies depending on the bacterial isolate.²¹⁰ Therefore, it could be that the plaques formed by the lytic phages on the other canine otitis isolates differ to those formed on the original host isolate AA.

The determination of host range using DLA assays in this way ideally should be repeated to produce biological triplicate results in independent experimental runs before anything definitive can be stated and the interpretation of these results should be read with this caveat in mind. Furthermore, there are significant limitations of using the spot formation in these DLA assays to determine host tropism. The generation of spots in the lawns by the application of the lytic-phage lysates cannot be viewed as a definitive indication of bacterial lysis due to phage adsorption.¹³² One of the reasons for this is due to the possibility of other bacterial-growth inhibiting substances existing in the phage lysate, for example bacteriocins and phage endolysins – these can also result in spot formation.¹³² Another possible cause of spot formation is the ‘lysis from without’ phenomenon where bacterial cell lysis can occur when high phage titres are applied causing disruption of the cell walls without phage replication.^{116,132,211} For these reasons, the determination of phage host range with DLA assays in this way should only be seen as a preliminary assessment of

host range and more definitive tests, like Efficiency of Plating (EOP) experiments, should be carried out.^{132,212,213} Although the DLA spot-formation assays show a reduced specificity (i.e. increased rate of false positives), a negative result from these can be viewed as highly indicative that the bacterial isolate in question is outwith of the phage's host range.¹³² As the majority of the canine otitis bacterial isolates tested did not show spots or plaques when the lytic phages were added, these results can be considered more likely accurate. To investigate the host range of the lytic phages in this study further, perhaps EOP experiments could be performed with the isolates where spots or plaques were formed with these DLA assays. Furthermore, it could be interesting to search the bacterial isolate AA and phage genomes for bacteriocins to assess whether the possibility of them being present in the phage lysates is high. A program like BAGEL4 could be used for this.²¹⁴ Any bacteriocins discovered could be of potential value as therapeutic candidates against *P. aeruginosa* infections²¹⁵ and could be investigated in their own right, though this is beyond the scope of this study.

Looking at these preliminary DLA results (Table 13), the phages in this study appear to have a very limited host range. This is in contrast to published studies in the literature that describe phages with activity against a broad spectrum of canine *P. aeruginosa* isolates.^{8,114-116} However, it should be noted that there are significant limitations with the way in which some of these host ranges were assessed. For example, Santos *et al.* (2011) performed DLA assays to assess host range using very high-titre concentrated drops of phage lysate ($>10^{10}$ PFU/ml) and then defined whether a phage was active against the bacterial strain by the presence of a clear 'lytic zone'.⁸ This is likely to have led to false positives and a falsely extensive host range due to the 'lysis from without' phenomenon (as described above). This issue is demonstrated further in another of these studies where when applying undiluted phage to DLA assays, the host range obtained for the phage was higher (11/16 canine isolates) than when diluted phage drops were used (8/16 canine isolates).¹¹⁶ This emphasises how important applying lower-titre phage drops is when using DLA assays to assess host range.

6.4. Host tropism determination – planktonic growth assays

For the planktonic growth assays to assess phage host tropism, the bacterial isolates AA, AB and P were selected (Figures 20, 21 and 22 respectively). This was because AA was the original host bacterial isolate against which all phages are active; AB had shown susceptibility to the highest number of the lytic phages in the DLA host tropism assays; and P had shown resistance to all phages in the DLA host tropism assays and had no evidence of spontaneous prophage induction (Table 13). A few bacterial isolates fit the latter criteria, so P was arbitrarily selected from these.

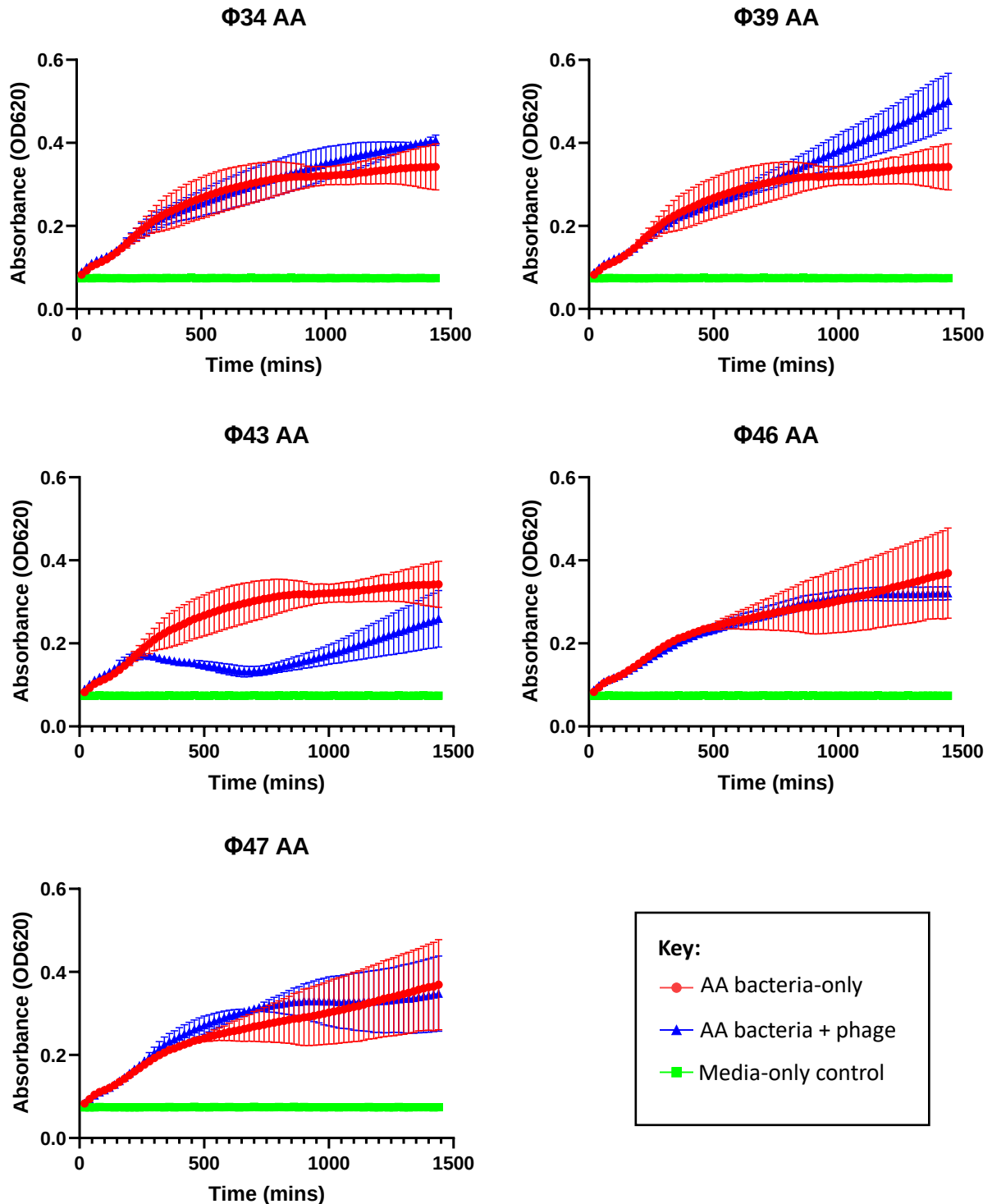


Figure 20. Graphs showing the change in OD₆₂₀ of planktonic cultures of canine otitis *P. aeruginosa* isolate AA over a 24-hour period in the presence and absence of the five lytic phages. ϕ and the number indicates the phage (e.g. ϕ 34 = phage AA34). Red points/line = bacteria-only wells (with SM buffer added in lieu of phage); blue points/line = wells with bacteria and added phage; and green points/line = media-only control wells. Single experiment performed in technical triplicate, data shown (means) with $\pm$ standard deviation plotted.

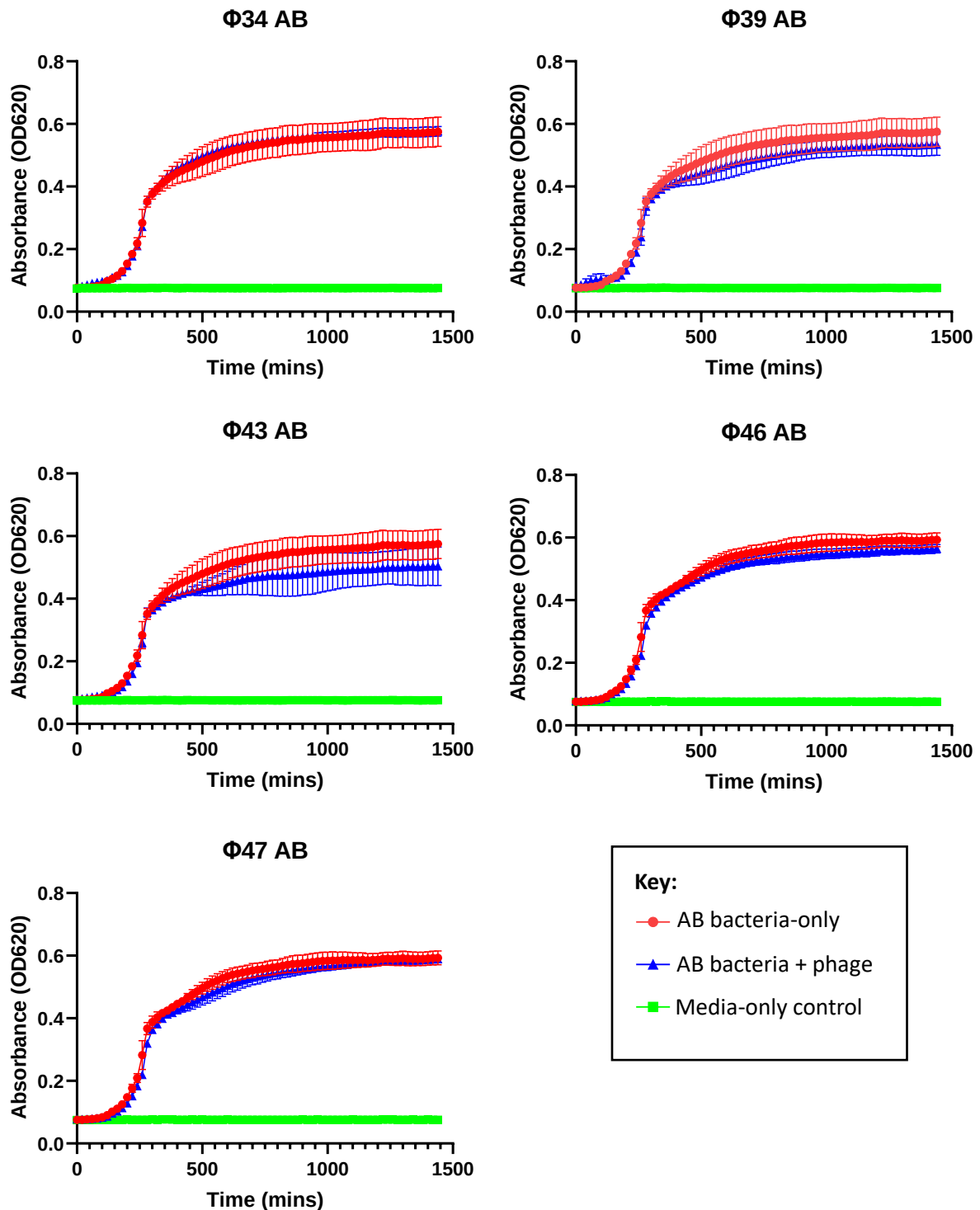


Figure 21. Graphs showing the change in OD₆₂₀ of planktonic cultures of canine otitis *P. aeruginosa* isolate AB over a 24-hour period in the presence and absence of the five lytic phages. ϕ and the number indicates the phage (e.g. $\phi 34$ = phage AA34). Red points/line = bacteria-only wells (with SM buffer added in lieu of phage); blue points/line = wells with bacteria and added phage; and green points/line = media-only control wells. Single experiment performed in technical triplicate, data shown (means) with $\pm$ standard deviation plotted.

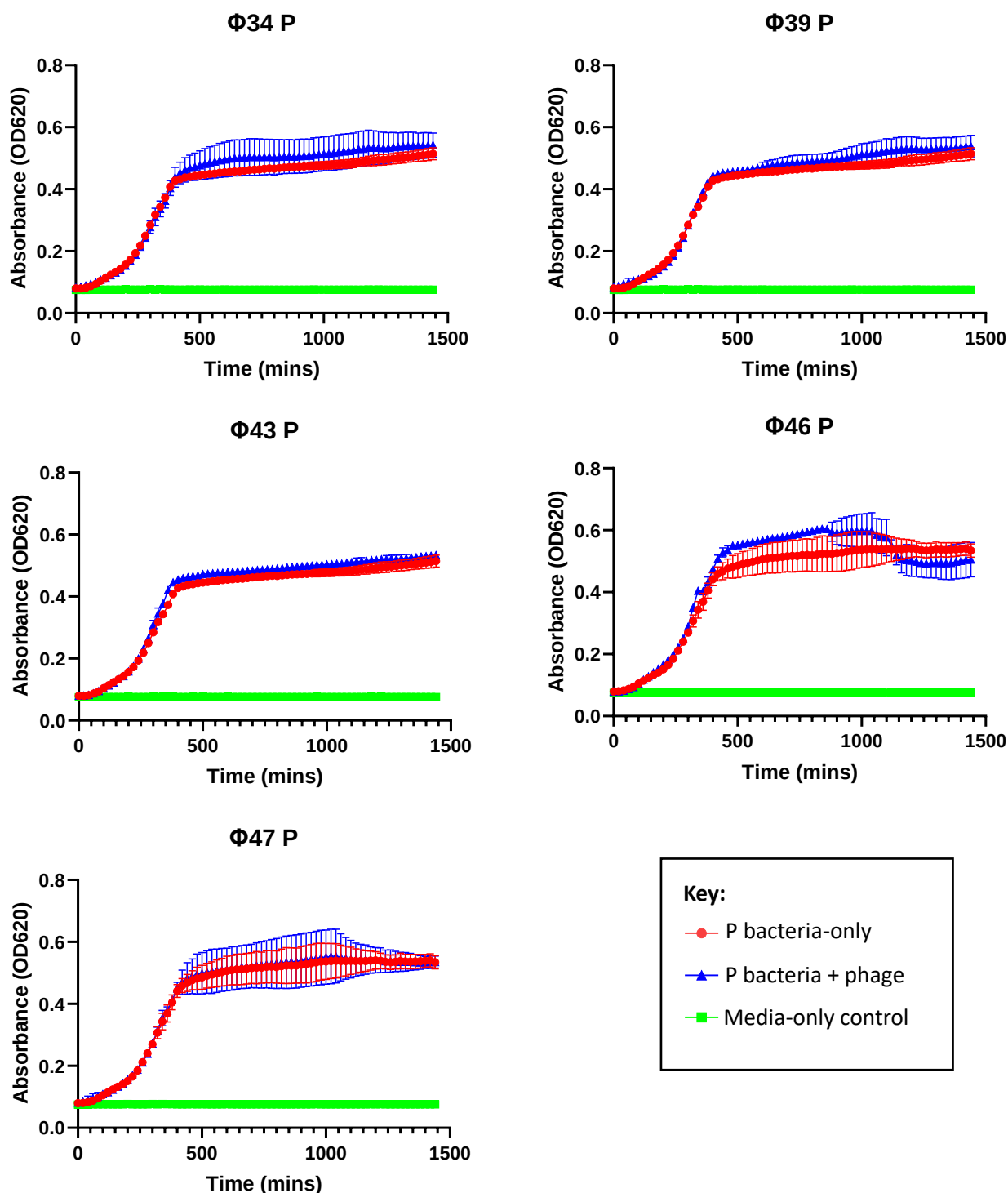


Figure 22. Graphs showing the change in OD₆₂₀ of planktonic cultures of canine otitis *P. aeruginosa* isolate P over a 24-hour period in the presence and absence of the five lytic phages. ϕ and the number indicates the phage (e.g. ϕ 34 = phage AA34). Red points/line = bacteria-only wells (with SM buffer added in lieu of phage); blue points/line = wells with bacteria and added phage; and green points/line = media-only control wells. Single experiment performed in technical triplicate, data shown (means) with $\pm$ standard deviation plotted.

Though performed in technical triplicate wells, ideally the experiment would have been repeated for each bacterial isolate to obtain biological triplicate results in independent experimental runs. However, due to time constraints this was not possible. It is unknown whether any statistical significance exists in the difference between the growth curves. For this reason, the following data and interpretation cannot be considered definitive and only as preliminary results.

The OD₆₂₀ is taken as a proxy for the number of bacterial cells present in the culture and therefore, the change in OD₆₂₀ as representative of the growth/death rates. For bacterial isolate AA, there was no notable inhibition of bacterial growth seen in the presence of any of the phages, except AA43 (Figures 20, 23 and Table 14). In the presence of phage AA43, the AA bacterial growth appeared inhibited with a reduction of growth from around 200 minutes (Figure 20). At approximately 700 minutes, the bacterial growth increased indicating recovery of the bacteria from the initial phage infection. The AUC of the growth curve in the presence of AA43 was reduced by approximately 38% compared to the bacteria-only control curve (Table 14).

Phage/control	Isolate AA	Isolate AB	Isolate P
AA34	105.46	101.06	107.42
AA39	113.43	90.40	104.77
AA43	61.90	85.93	106.35
AA46	98.23	93.02	119.22
AA47	106.65	95.34	101.27

Table 14. Scores for each bacterial isolate-phage combination in the planktonic growth assays. The scores (the AUC of the phage-added growth curves as a percentage of the AUC of the appropriate bacteria-only control) were calculated as outlined in section 5.8. A score of 100 indicates no effect of the phage on bacterial growth. A score of <100 indicates inhibition of bacterial growth with the phage added. A score of >100 indicates increased bacterial growth with the phage added.

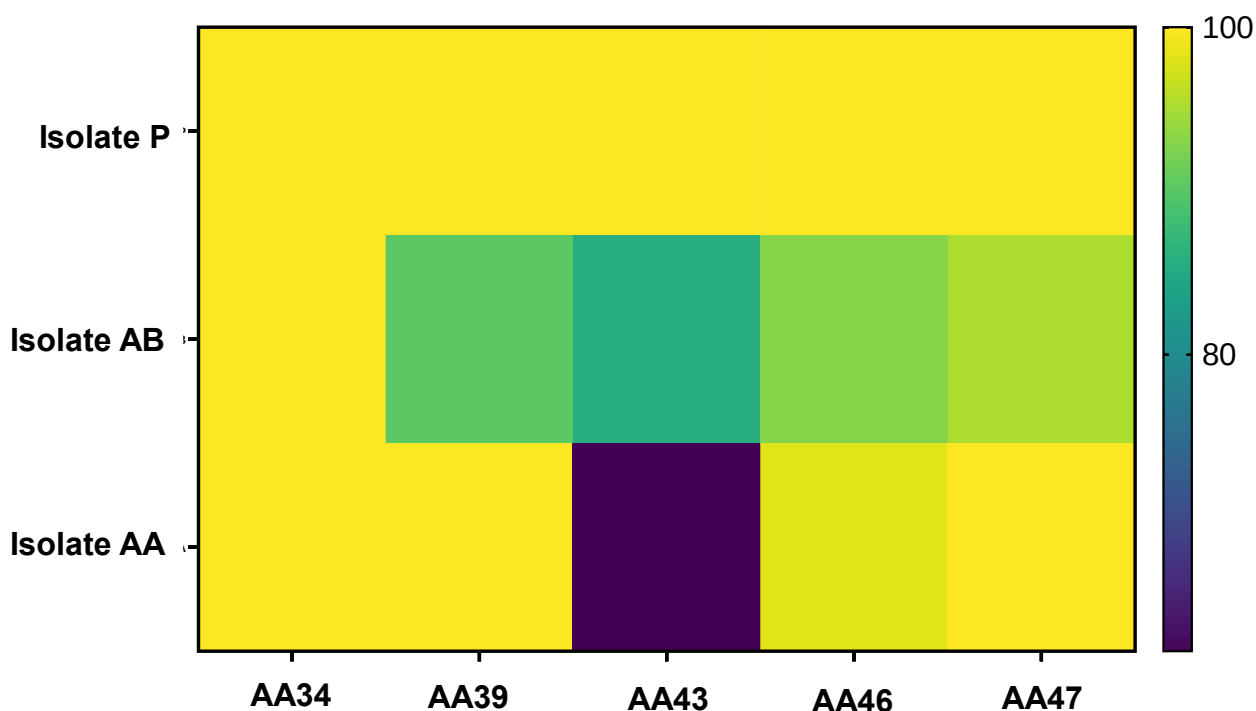


Figure 23. Heat map showing each bacterial isolate-phage combination as a percentage of the AUC of the bacteria-only control in the planktonic growth assays. The results have been adjusted to make 100% the highest possible percentage to enable clearer interpretation of phage effect on bacterial growth. Data used for this heat map is shown in Table 14.

There was only slight inhibition of growth of isolate AB with all phages, except AA34, as seen in the AUC proportion scores (Table 14) and highlighted in the heat map (Figure 23); but, the relative level of inhibition was quite low, varying from 5-14% reductions in the AUC compared to the bacteria-only curves (Table 14 and Figure 21). It is likely that these small differences reflect the normal biological and technical variations inherent in these experiments and, while further replicates are needed, if they produce similar results, they would probably lack statistical significance.

For bacterial isolate P, there was no inhibition of bacterial growth seen in the presence of any phages (Figures 22, 23 and Table 14).

In the presence of phage AA39, the exponential phase of the AA bacterial growth appears to still be occurring at the 24-hour mark whereas the bacteria with no added phage had reached the stationary phase by this point. This was reflected by the AUC values as there was approximately a 13% increase in the AUC of the AA growth with

AA39 added, compared to without (Table 14). Similarly, there is also a notable increase (19%) in the relative growth of P with phage AA46 added (Table 14) though the shape of the growth curve itself is similar to the bacteria-only control in this case (Figure 22). However, the significance of these findings is unknown and with further biological repeats of the experiment, it may be evident that these are not statistically significant increases in growth. If it transpired that these were significant increases in bacterial growth, it is unknown what would cause this as a similar phenomenon does not appear to have been reported in the literature to the best of the author's knowledge. It is hypothesised that the bacterial growth stimulation could either be due to true effects of the phages or due to components from the broth carryover providing nutrition to the bacteria (i.e. the small volume of broth and/or lysed bacteria in the phage dilution used). A possible next investigative step to determine which is true, would be to repeat similar planktonic growth experiments with the phages inactivated (could be carried out via heat treatment or ultraviolet radiation²¹⁶).

Looking at this preliminary set of data, it appears that only phage AA43 had notable success at killing the bacteria in the planktonic culture of isolate AA. However, a rebound growth was seen in this case. This phenomenon suggests the growth of phage-resistant or phage-insensitive bacterial cells, after the initial period of inhibition by the phage, and is commonly seen when phages are tested in planktonic cultures of their gram-stain-negative bacterial host.^{115,116,174,217-219} In the case of AA43 and the bacterial isolate AA, this rebound growth started around 700 minutes/approximately 12 hours, which is a similar time point seen with some other *P. aeruginosa* phages in the literature.^{115,116,174} These findings do suggest the development of phage resistance which is regarded as inevitable when exposing a bacterial population to phages.^{74,85,102} This should be considered when contemplating the use of phage AA43 in phage therapy; however, it is thought that the occurrence of phage resistance is likely very different *in vivo* compared to *in vitro*.^{204,218-220} For example, a study showed that phage-resistant *P. aeruginosa* mutants emerged rapidly *in vitro* in response to a phage cocktail treatment, whereas no resistant mutants developed in the *in vivo* rat model with the same phage cocktail.²²⁰ This was thought to be due to the mutations conferring resistance to the phages affecting bacterial surface structures that were crucial for maintenance of the infection (e.g. pili motility genes).²²⁰ For this reason, AA43 should not be disregarded

as a phage therapy candidate based on the rapid emergence of resistant bacterial mutants *in vitro*. Furthermore, as it is known that phage-resistant bacterial mutants often exhibit significant fitness costs,^{74,85,204,221} this means that even if resistant mutants develop when treating an infection with phage therapy, a rebound growth of bacteria may not be detrimental to the patient if the bacteria have reduced virulence. It also should be remembered that the host immune system will play a role in the clearance of the infection *in vivo*, so a reduction (rather than elimination) of an infection by phage therapy could still be beneficial in allowing the host to overcome the infection, in a similar manner to bacteriostatic antibiotics.^{222,223}

The cause for the disparity of phage host tropism between the DLA assay results and the planktonic growth curve results is unknown. It has been shown that although the DLA-based host tropism assay (using phage drops) can be useful for an initial screen to determine possible host range of a phage, the results cannot be taken as a reliable indication of successful phage infection and replication.^{132,212,213} This type of DLA assay has been shown to overestimate the host ranges of phage when compared to other methods, like EOP and planktonic growth assays²¹³; therefore, it is possible that this has happened here and perhaps the results of the planktonic growth assays should be considered more reliable in reflecting the real phage host range. Having said that, it should be noted that the study²¹³ referred to used much higher phage titres in the drops applied to the bacterial lawns when performing the DLA assays and, as this has previously been shown to result in false positives due to the 'lysis from without' phenomenon,^{116,132,211} the overestimation of DLA drop assays may not be as significant as stated.

Future work could involve performing biological repeats of the planktonic growth assays for the isolate/phage combinations already tested to enable statistically significant conclusions to be made. In addition, it could be interesting to assess the bacterial isolates D, K and M in an identical way as these did show evidence of possible lytic activity when some of the phages were applied during the DLA host tropism assays (Table 13).

6.5. Biofilm inhibition assay

Prior to this study, the set of canine otitis *P. aeruginosa* isolates had not been used in any biofilm assay before; therefore, it was unknown which of these isolates would form biofilms. As previous published studies have reported a high rate of biofilm production among canine otitis *P. aeruginosa* isolates,^{43,59-61} it was suspected that most of the isolates would exhibit some degree of biofilm production. Two bacterial isolates were selected to be used in the biofilm inhibition assay – the original bacterial host for the phages, isolate AA, and a bacterial isolate that had shown resistance to all phages in the two assays thus far (DLA and planktonic growth curve), isolate P. It was hoped that both isolates would produce significant biofilms and the ability of the phages to inhibit their formation could be assessed.

Despite the technical triplicate results, repeats of the experiment should be performed to produce biological triplicate results to enable assessment of statistical significance. Due to time constraints, this was not possible, so the results of this experiment must be interpreted with caution and considered to be preliminary.

Bacterial isolate AA produced a ‘moderate’ biofilm when incubated alone (Table 15). There was still a significant biofilm produced when phages AA34 and AA39 had been added. The biofilm formed when AA34 was added was classified as higher than when the bacteria was incubated alone; however, due to the absence of statistical analysis, it cannot be assumed that this was a significant difference. A notable reduction in biofilm production was seen when incubated with phages AA43, AA46 and AA47, indicating that these phages inhibited the formation of biofilm. Therefore, these phages could have potential to be able to impede biofilm formation if administered in an active canine otitis case. Though previously phages have been shown to have activity against *P. aeruginosa* canine isolates,^{8,114-116,118} the activity of phages against biofilms formed by canine isolates of *P. aeruginosa* has not been reported on to the best of the author’s knowledge. However, there have been extensive studies investigating the ability of phages to impede biofilms formed by other *P. aeruginosa* isolates and these have demonstrated that many lytic phages are able to inhibit biofilms.^{174,224-227} For example, pre-treating medical catheters with a phage cocktail reduced the mean *P. aeruginosa* biofilm density at 48 hours by 99.9%.²²⁵ However, it should be noted that there is no standardised method of

assessing phage-biofilm interactions and this can be problematic as many of the protocols have inconsistencies and limitations, as discussed below¹⁹⁸.

Well contents	Net OD ₅₇₀	Biofilm category
AA only	0.45	Moderate
AA + 34	0.57	High
AA + 39	0.50	Moderate
AA + 43	0.06	No
AA + 46	0.11	No
AA + 47	0.22	Low
P only	0.01	No
P + 34	0.02	No
P + 39	0.06	No
P + 43	0.04	No
P + 46	0.01	No
P + 47	0.06	No

Table 15. Summary of the final biofilm production results in each well condition. The letters 'AA' and 'P' denote the bacterial isolates. The numbers denote the phage added (e.g. '34' = AA34). The net end-point OD₅₇₀ for each well condition was calculated as the mean of the triplicate readings of the media-only wells subtracted from the mean of the triplicate readings for each well condition. Each net OD₅₇₀ was used to classify the biofilm production into the categories outlined in section 5.9.

This experiment indicated that bacterial isolate P is not a biofilm-producer under these specific conditions (Table 15). No biofilm was detected in any incubation condition with this isolate, including in the absence or presence of phage. Isolate P was originally chosen for this experiment due to its demonstrated resistance to the lytic phages in the other assays (DLA and planktonic growth) with the aim of assessing whether the phages could inhibit the bacteria in an alternative way instead i.e. by affecting the formation of biofilms. Nevertheless, as the P isolate failed to produce a biofilm, this question was not answered. It has previously been shown that a 24-hour incubation period is optimal to enable sufficient biofilm production with *P. aeruginosa* canine otitis isolates in a microtitre CV-based plate assay⁶⁰; therefore, adequate time was allowed using this protocol for the P isolate to establish a biofilm. As isolate P was originally selected for the planktonic growth assays as the 'phage resistant' bacterial isolate, it had also been chosen based on its apparent lack of spontaneous prophage induction to reduce the likelihood of interference with potential results. Although beneficial during phage isolation, with the biofilm assay, a lack of spontaneous prophage induction may actually be a disadvantage in experiments. It has been shown that biofilm formation can be promoted by the occurrence of spontaneous prophage induction.^{44,157} This is thought to be due to the release of eDNA on lysis of the bacterial cells which contributes to the biofilm structure stability and, in the case of *P. aeruginosa* PAO1, filamentous phages forming bacterial variants with enhanced attachment capabilities.^{44,157,228} Future work should include different *P. aeruginosa* canine otitis isolates in this assay (e.g. A, C or M) that have previously shown evidence of spontaneous prophage induction, as these may be superior at biofilm formation. Isolate M would be of particular interest as there has been possible susceptibility to the lytic phages seen when the DLA host tropism assays were performed (Table 13). Given the extensive study of biofilm production by human-origin *P. aeruginosa* strain PAO1,^{157,228,229} it may be worth testing the lytic phages against this isolate in the biofilm inhibition assay. If the phages are capable of inhibiting the biofilm production of PAO1, though it may not be clinically useful in the case of phage therapy for canine otitis, it may demonstrate a use for the phages as human *P. aeruginosa* phage therapy and/or provide some insight into possible differences in the biofilms produced by the canine isolates compared to the human isolates, as information about this is lacking in the literature.

As an extension to the assay performed here, future work could include a mature biofilm assay. Clinical application of these phages as therapeutics in an otitis case would most likely involve phages being administered into an ear canal with an established biofilm present. Therefore, a possibly more realistic scenario for the evaluation of phage efficacy in the laboratory is testing against a mature biofilm. For example, the same protocol (section 5.9) could be used but the biofilm allowed to establish over a 24-hour period prior to the addition of phage. Despite this established biofilm being used in an assay, it should be noted that in a canine otitis case much older biofilms will likely be present, especially due to the chronicity of infections.^{10,12} As it is thought that the number of cells with lower metabolic activity levels will rise with increasing biofilm age, it can be predicted that much older biofilms will be significantly less vulnerable to phages.⁴⁴

Even though a mature biofilm assay may be an improvement over the biofilm inhibition assay in terms of clinical relevance, the general limitations of the laboratory-based biofilm assays must not be forgotten. Though frequently used, CV-based assays have been shown to produce results with large variation when performed with *P. aeruginosa* biofilms and the fixation of the biofilms by CV much reduced with this bacterial species, thought to be due to the high water content of the biofilm matrix²³⁰; although it should be noted that another study found little variation with CV assays performed on *P. aeruginosa* biofilms which led them to the conclusion that media composition may have contributed to the variation seen in the other study.²³¹ In the biofilm inhibition assay performed in this study, MHB was the media used. In a study comparing biofilm growth of canine otitis *P. aeruginosa* isolates in different broth media types, no hugely significant differences were found between biofilm production levels in different broths (MHB, TSB and Luria-Bertani enrichment broth) and they determined that any of these three broths were appropriate for *P. aeruginosa* biofilm assays in microtitre plates.⁶⁰

Furthermore, as the CV assays are quantifying biomass, this includes non-viable/dead cells which lack relevance for phage susceptibility studies.²³⁰ To combat this, alternative assays could be performed which assess the metabolic activity and viable cells of a biofilm assay; for example, a tetrazolium salt-based assay.^{224,230,232}

Though significant limitations exist even with these techniques, including a lack of reproducibility.^{44,230} Furthermore, the monospecies biofilms produced in a laboratory setting are likely not representative of naturally occurring biofilms in the canine ear canal, as it has been shown that *P. aeruginosa* frequently forms biofilms with other bacterial species, like *S. aureus* in humans,⁵⁶⁻⁵⁸ and therefore potentially *S. pseudintermedius* in the dog. It has also been demonstrated that the presence of other strains of *P. aeruginosa* can affect phage infection dynamics.²³³ Therefore, studies involving the presence of, not only other bacterial species, but also competing strains should be considered. As more modern techniques like peptide nucleic acid fluorescence in-situ hybridization (PNA FISH) enable the staining of specific bacterial cells within more complex multispecies biofilms,²³⁴ perhaps this would be a worthwhile pursuit to investigate phages interacting with biofilms of the canine otitis *P. aeruginosa* isolates and other bacterial species, like *S. pseudintermedius*. In addition, the spatial organisation of biofilms is also known to be an important factor influencing phage-biofilm interactions^{44,233,235} and techniques like PNA FISH can enable visualisation of this. Another possible protocol to investigate phage-biofilm interactions is outlined in Boas *et al.*, 2019 and involves quantifying the number of viable bacterial cells prior to adding phages at a known MOI to a mature biofilm.²³⁴ Subsequent samples are taken to enumerate the viable bacterial cells (in CFU) and phage virions (in PFU) in the biofilm to assess how well the phage has penetrated the biofilm and infected the associated cells.²³⁴ This also allows a more kinetic analysis rather than a single end-point result (as in many of the biofilm assays¹⁹⁸).

Regardless of the specific protocol used, it should be remembered that all of these biofilm studies are still *in vitro* and there will be several other potential factors affecting biofilm production and structure in the canine ear canal, including host immune response and ear microflora.⁴³ A possible alternative to an *in vitro* experiment, without involving an *in vivo* clinical trial, could be an *ex vivo* biofilm model – canine ear canals could be used to allow *P. aeruginosa* biofilm to establish and then the efficacy of the phage therapy to disrupt this could be assessed. *Ex vivo* biofilm models have been developed using porcine skin with success,^{236,237} though a canine skin or ear model has not been reported in the literature to the author's knowledge. An obvious limitation of this would be the lack of host immune response,

but it would at least otherwise mimic the general conditions in which a biofilm would naturally form.

Previously, it has been shown that an early indicator during phage studies of potential activity against biofilms is the formation of a 'halo' morphology on associated DLA plaques.^{130,133} These are thought to be due to the diffusion of phage-encoded EPS depolymerases^{132,134} and as *P. aeruginosa* biofilms are predominantly composed of EPS,³ phages that produce these enzymes may be effective at disrupting biofilms. Therefore, screening for the halo morphology could be used as a preliminary method of selecting potential phage therapy candidates for *P. aeruginosa* biofilm-associated infections, like canine otitis. Ideally, it was hoped that this study would allow assessment for correlation between halo plaque morphology and phage efficacy against biofilms. However, no halo morphologies were observed in any of the plaques of any phage in the DLA assays. Plates were frequently reassessed for the first few days of storage to ensure no haloes were developing, as it has been shown that haloes can expand out from the edges of the plaque after plaque maturation, even with refrigeration.^{132,134} Though no haloes were ever noted with any of the plaques seen, it does not rule out the possibility of the isolated phages possessing EPS depolymerases, as some phages can contain them but not produce the classical halo morphology.^{127,134,238} Furthermore, some phages lacking depolymerases can still display good efficacy against biofilms, for example the *P. fluorescens* phage ϕ IBB-PF7.^{238,239} This is thought to be possible due to alternative ways in which a phage can access and infect cells within a biofilm. An example of this is the phages travelling through the pores and channels that are naturally present in a biofilm to allow the bacterial cells within each biofilm layer access to nutrients.²³⁹

6.6. Isolation of phage-resistant bacterial mutants

During the phage quantification steps at the start of the host tropism assays (section 5.7) and throughout all the phage work carried out, it was noted that bacterial colonies would be present in the middle of the spots where high phage titres were dropped onto the top agar (Figure 16). This occurred with all five phages when dropped onto DLA assays made with the host bacterial isolate AA. These were suspected to indicate growth of phage-resistant bacterial mutants. Two separate

colonies (each in a different spot) were picked using inoculation loops for each phage and streaked out onto a TSA plate. These were incubated statically overnight at 37 °C, prior to being streaked out again onto fresh TSA plates to single colonies.

These bacterial isolates were tested to assess whether their phage-resistant phenotype was maintained. To do this, bacterial lawns were made (using the protocol outlined in section 2.7) with each 'resistant' bacterial isolate. Once the bacterial lawns were dry, a 10 µl drop of the appropriate 10^6 PFU/ml phage solution (dilution series made in section 5.7 and freshly quantified the day prior to this experiment) was applied to the lawn. The ten plates were then incubated statically overnight at 37 °C. The next day they were assessed for any evidence of plaques/spots. None of the plates had any plaques/spots present, except for the two plates made from the bacterial isolates initially showing resistance to phage AA34 – these both had spots present matching the area of the applied phage drop (Figure 24).

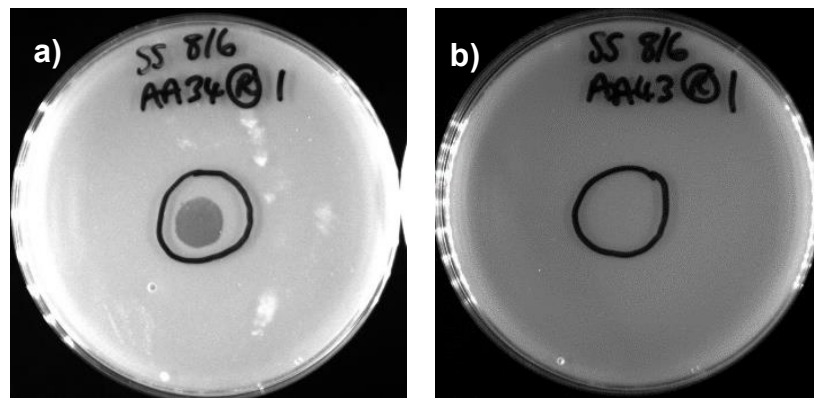


Figure 24. Re-testing of potential phage-resistant mutants. The plates are DLA assays produced using two of the bacterial isolates of AA that had previously shown resistance to **a)** phage AA34 **b)** phage AA43. A 10 µl drop of 10^6 PFU/ml of the appropriate phage solution (AA34 for plate a and AA43 for plate b) was applied to the centre of the plate (within the demarcated black circle). A spot is present in this area on plate a – this indicates successful lysis of bacteria by the phage and, therefore, no obvious phage resistance. No spot is present on plate b – this indicates maintenance of the phage-resistant phenotype.

These results indicated that all the bacterial mutants had maintained their phage-resistant phenotype, with the exception of the bacterial isolate initially resistant to AA34. Occurrence of phage-resistant bacterial mutants is very common in phage

studies of a similar nature, as discussed in section 6.4. The bacterial isolates that had maintained their phage-resistant phenotype were then sent for Illumina HiSeq sequencing at MicrobesNG (Birmingham, UK) – ten bacterial isolates were sent in total; two per phage. On receipt of the sequencing results for the phage-resistant isolates, the intention was to assess these for any single nucleotide polymorphism(s) (SNP) in comparison to the wild-type isolate AA and, on identification of these, assess which genes these were contained within. Taking into account whether the mutations were silent, missense or nonsense, this would allow the potential mechanisms of phage resistance to be elucidated. Analysis of SNP calling of phage-resistant mutants in comparison to the wild-type bacteria is a common method used to investigate the mechanisms of phage-resistance development in *P.*

aeruginosa.^{221,240,241} For example, one study used this method to identify that mutations in genes essential for type IV pili formation in *P. aeruginosa* conferred resistance against some lytic phages.²⁴¹ If the mechanisms of phage-resistance were identified, the information could be used to select any phages that induce bacterial mutants with reduced fitness and/or virulence, as these could be very useful phage therapy candidates.^{74,85} The sequencing and variant calling results were not obtained by the end of this study so need to form part of future work.

7.0. Chapter Summary

The isolated lytic phages were characterised here in terms of morphology, genome sequence, host tropism and activity against biofilms. All five phages had a Siphovirus morphology. The phage genome sequences had strong evidence to suggest they were of a lytic nature and novel – though this cannot be stated for phage AA39, as genome analysis was not performed for this phage due to suboptimal DNA extraction. The host ranges of the phages, as determined by DLA assays, were small, with the phages with the most extensive host ranges (AA39 and AA46) only showing activity against five of the 24 canine otitis isolates they were tested against. The planktonic growth curves indicated that, of the five phages, only AA43 had notable activity against its host bacterial isolate in planktonic culture. Potential action against biofilm formation was seen with phages AA43, AA46 and AA47; though more data would be required before this trend to be definitively declared. After evaluation, there was no single isolated phage that stood out as a superior phage therapy

candidate for canine otitis cases. Though all five phages have potential use in phage therapy, their limited host range presents a major barrier.

CHAPTER FOUR – FINAL DISCUSSION

8.0. Final Discussion

Throughout this study, the high prevalence of prophages in the canine *P. aeruginosa* isolates was apparent, both evident genotypically (Table 6) and phenotypically (Table 13). This complicated the screening DLA assays when looking for lytic phages and the DLA assays when assessing for host tropism. Spontaneous prophage induction not only poses a problem with background prophage interfering with experiments, but also when considering *P. aeruginosa* isolates for use as phage propagation strains. It has been shown that when propagating phages for use in therapeutics, the lysate that is used can contain contaminant lysogenic phages released from the host bacteria due to spontaneous prophage induction.^{103,204} These can lead to horizontal gene transfer and a potential increase in bacterial virulence through the spread of virulence factors.^{89,91,103,204} It can be very challenging to separate these contaminant lysogenic phages from the therapeutic phage, so to eliminate this risk, prophage-free bacterial strains are desired for phage propagation.^{103,204} As many of the canine otitis *P. aeruginosa* isolates in this study are polylysogens, including the host bacterial isolate (AA) of the lytic phages, this presents a limitation to using these as propagation strains. To combat this, a prophage-free *P. aeruginosa* strain could be found that these phages are capable of infecting. For example, a study which isolated lytic *P. aeruginosa* phages from sewage samples used a different *P. aeruginosa* strain (CN573), that had no phenotypic or genotypic evidence of prophages, to study the phages and not the original isolation strain, as a prophage was induced from the latter.²⁴² As it could be challenging to find a suitable prophage-free strain, an alternative option is to eliminate the prophages present in a *P. aeruginosa* strain that is susceptible to the appropriate lytic phage, e.g. isolate AA, via gene modification.^{103,204} To the best of the author's knowledge, the generation of a prophage-free *P. aeruginosa* strain has not yet been reported in the literature, though it has been carried out in a *P. putida* strain.²⁴³ However, a published technique has been developed to genetically remove prophages from *P. aeruginosa* which could be harnessed to produce a prophage-free strain.¹⁵⁵

Though problematic in some regards, the high prevalence of prophages and spontaneous prophage induction in the canine *P. aeruginosa* isolates demonstrates how abundant lysogenic phages are and how readily they can be isolated. Difficulty in isolating strictly lytic phages has been reported in other bacterial species, for example *Clostridioides difficile* (formerly known as *Clostridium difficile*).^{244,245} Given this, the use of lysogenic phages in phage therapy has been a topic of research. One method has been to isolate natural lytic mutants of temperate phages and there have been reports of efficacy using these with a *P. aeruginosa* strain.²⁴⁶ Another method of exploiting temperate phages for therapy is through bioengineering to convert them to a lytic phenotype, by eliminating components that are essential to the lysogenic cycle.^{104,245,247} An example of this is the administration of a phage cocktail containing engineered-temperate phages (for example, one lysogenic phage, ZoeJ, had its repressor gene removed) to a 15-year-old cystic fibrosis patient suffering from a drug-resistant *M. abscessus* infection.^{104,247} However, there has been little research into investigating the use of temperate phages as therapeutics in their natural form. This has been carried out with *C. difficile* due to the lack of strictly lytic phages described and some success has been seen with temperate phage cocktails.^{248,249} Furthermore, there has been evidence of a distinct temperate phage-antibiotic synergy mechanism reported.²⁴⁵ Despite this, research into temperate phage therapy is still limited and ongoing.

The lytic phages isolated in this study are potential candidates for a canine *P. aeruginosa* otitis phage therapy treatment. The genome analysis of four of the phages did not reveal any genes of concern (e.g. AMR genes or genes associated with lysogeny) that would exclude them as therapeutic candidates. Genome sequencing would need to be carried out to determine if this is the case for phage AA39. As previously discussed, their limited host range does pose a hurdle to their efficacy against clinical infections; however, using them in a phage cocktail alongside phages with different host ranges is one possibility, in addition to phage training to attempt to increase their host range and infection efficacy. Using phages in combination with antibiotics when treating infections has frequently been shown to be effective and increase the overall efficacy of the treatment.^{66,74} This has been demonstrated in *P. aeruginosa*⁶⁶; for example, a 10,000-fold greater reduction in bacterial CFU/g was seen with phage-ciprofloxacin combination treatment compared

to the phage or ciprofloxacin monotherapies in an *in vivo* rat model of *P. aeruginosa*-associated endocarditis.²²⁰ In addition, negative cultures were obtained from 64% of the rats after combination treatment; whereas, no rats treated with the phage or antibiotic alone were cleared of the infection.²²⁰ A similar synergistic effect was seen *in vitro*,²²⁰ so it could be interesting to test the ability of the isolated phages from this study to kill the canine otitis bacterial isolates in combination with antibiotics *in vitro* to assess for a potential phage-antibiotic synergy.

Prior to progression of these phages to phage therapy, further characterisation work should be carried out; some examples include pH and thermostability testing, burst size, latent period and phage receptor determination. A point of note is that all the phage experiments here were carried out aerobically, as in most of the literature.²⁵⁰ However, *P. aeruginosa* is known to survive in anaerobic conditions¹ and form anaerobic biofilms in the human CF lung.^{251,252} As *P. aeruginosa* canine otitis isolates tend to form biofilms,^{43,59-61} it could be interesting to carry out the phage characterisation experiments in anaerobic conditions also to observe the effect on phage efficacy. Anaerobic conditions have been shown to affect the infection efficacy of a *P. aeruginosa* phage (PEV2, which has a canine otitis isolate within its host range), resulting in an increase of the latent period and significant reduction of the burst size, though the phage remains successful at infecting the bacteria overall in these conditions.²⁵⁰

During the genome analysis of the four isolated phages, two distinct endolysins were found to be encoded on two of the phages. These lysis-associated proteins are peptidoglycan-degrading enzymes which are currently being researched as potential antibacterial agents in their own right, i.e. direct application of endolysins to the bacteria to induce cell wall lysis without association with the phage particle.^{79,253} Previously, their success in *in vitro* and *in vivo* animal model studies has mainly been against Gram-stain-positive bacterial pathogens, due to the barrier of the outer membrane in Gram-stain-negative bacteria preventing access of the endolysin to the peptidoglycan cell wall.^{79,253} However, this gap in research is closing with different ways of enabling endolysins to lyse Gram-stain-negative bacteria – for example, bioengineering endolysins to be able to work from the outside of Gram-stain-negative bacteria²⁵⁴⁻²⁵⁶ and using endolysins in combination with outer membrane-

permeabilising agents to enable lysis of *P. aeruginosa*.²⁵⁷ Naturally-occurring endolysins that are found in phages targeting Gram-stain-negative bacteria have also been shown to be able to lyse the cell wall when applied exogenously, without any alteration or adjunctive agents.²⁵⁸ There are now numerous published studies exploring the use of endolysins in targeting *P. aeruginosa*, including *in vivo* mouse models, showing promise.^{255,256,259,260} To investigate the therapeutic potential of the endolysins present in the phages isolated here, the next investigative step could be to clone the endolysins in a bacterial expression vector plasmid and over-express the protein in *E. coli*. The preparation from this could then be screened for killing activity against the canine otitis *P. aeruginosa* isolates. Any endolysins of interest highlighted by this screening could then be further purified and fully characterised (e.g. assessment of MIC). In addition to this, endolysins could be searched for in the prophages identified in the canine otitis isolates and investigated in the same way.²⁵⁹ Furthermore, holins and spanins (both of which were found in the phages isolated here) are gaining interest as possible pharmaceutical agents, but research into these proteins is in the early stages.^{194,261}

9.0. Conclusion

Five lytic phages with activity against canine otitis *P. aeruginosa* isolates were successfully isolated from wastewater samples. These were characterised in terms of virion morphology, genome analysis, extent of host range amongst canine otitis isolates of *P. aeruginosa* and activity against planktonic and biofilm-associated bacteria. Though the phages appear promising in terms of their lytic nature and lack of virulence-associated genes, their narrow host range presents a limiting factor when considering their use as phage therapy candidates for the use in clinical canine otitis infections. Future work could include further characterisation of these phages and investigating ways to utilise them to produce a clinical product with a broader host range.

10.0. References

1. Hoiby N, Johansen HK, Moser C, Ciofu O. Clinical relevance of *Pseudomonas aeruginosa*: A master of adaptation and survival strategies. In: Rehm BHA, editor. *Pseudomonas: Model Organism, Pathogen, Cell Factory*. Weinheim, Germany: Wiley-VCH; 2008. p. 25-44.
2. Pang Z, Raudonis R, Glick BR, Lin T-J, Cheng Z. Antibiotic resistance in *Pseudomonas aeruginosa*: mechanisms and alternative therapeutic strategies. *Biotechnology Advances*. 2019;37:177-92.
3. Tran Thi MT, Wibowo D, Rehm BHA. *Pseudomonas aeruginosa* Biofilms. *International Journal of Molecular Sciences*. 2020;21(8671).
4. Barnard N, Foster A. *Pseudomonas* otitis in dogs: a general practitioner's guide to treatment. *In Practice*. 2017:386-98.
5. Pye C. *Pseudomonas* otitis externa in dogs. *The Canadian Veterinary Journal*. 2018;59(11):1231-4.
6. Hillier A, Alcorn JR, Cole LK, Kowalski JJ. Pyoderma caused by *Pseudomonas aeruginosa* infection in dogs: 20 cases. *European Society of Veterinary Dermatology*. 2006;17(6):432-9.
7. Prado MR, Rocha MFG, Brito ÉHS, Girao MD, Monteiro AJ, Teixeira MFS, et al. Survey of bacterial microorganisms in the conjunctival sac of clinically normal dogs and dogs with ulcerative keratitis in Fortaleza, Ceará, Brazil. *Veterinary Ophthalmology*. 2005;8(1):33-7.
8. Santos TMA, Ledbetter EC, Caixeta LS, Bicalho MLS, Bicalho RC. Isolation and characterization of two bacteriophages with strong in vitro antimicrobial activity against *Pseudomonas aeruginosa* isolated from dogs with ocular infections. *American Journal of Veterinary Research*. 2011;72(8):1079-86.
9. Hall JL, Holmes MA, Baines SJ. Prevalence and antimicrobial resistance of canine urinary tract pathogens. *Veterinary Record*. 2013;173(22):1-6.

10. Miller WH, Griffin CE, Campbell KL. Diseases of the eyelids, claws, anal sacs and ears. Muller and Kirk's Small Animal Dermatology. 7th Edition ed. St. Louis: Elsevier/Saunders; 2013. p. 724-73.
11. Colombini S, Merchant SR, Hosgood G. Microbial flora and antimicrobial susceptibility patterns from dogs with otitis media. *Veterinary Dermatology*. 2000;11(4):235-9.
12. Martín Barrasa JL, Lupiola Gómez P, González Lama Z, Tejedor Junco MT. Antibacterial susceptibility patterns of *Pseudomonas* strains isolated from chronic canine otitis externa. *Journal of Veterinary Medicine B*. 2000;47:191-6.
13. Korbelik J, Singh A, Rousseau J, Weese JS. Characterization of the otic bacterial microbiota in dogs with otitis externa compared to healthy individuals. *Veterinary Dermatology*. 2019;30(3):228-e70.
14. Bradley CW, Lee FF, Rankin SC, Kalan LR, Horwinski J, Morris DO, et al. The otic microbiota and mycobiota in a referral population of dogs in eastern USA with otitis externa. *Veterinary Dermatology*. 2020;31(3):225-e49.
15. Cole LK, Kwochka KW, Kowalski JJ, Hillier A. Microbial flora and antimicrobial susceptibility patterns of isolated pathogens from the horizontal ear canal and middle ear in dogs with otitis media. *Journal of the American Veterinary Medical Association*. 1998;212(4):534-8.
16. Petersen AD, Walker RD, Bowman MM, Schott II HC, Rosser Jr. EJ. Frequency of isolation and antimicrobial susceptibility patterns of *Staphylococcus intermedius* and *Pseudomonas aeruginosa* isolates from canine skin and ear samples over a 6-year period (1992-1997). *Journal of the American Animal Hospital Association*. 2002;38(5):407-13.
17. O'Neill DG, Church DB, McGreevy PD, Thomson PC, Brodbelt DC. Prevalence of disorders recorded in dogs attending primary-care veterinary practices in England. *PLOS ONE*. 2014;9(3):e90501.
18. (AHAW) EPoAHaW, Nielsen SS, Bicout DJ, Calistri P, Canali E, Drewe JA, et al. Assessment of listing and categorisation of animal diseases within the framework of

- the Animal Health Law (Regulation (EU) No 2016/429): antimicrobial-resistant *Pseudomonas aeruginosa* in dogs and cats. EFSA Journal. 2022;20(5):1-78.
19. Michl RK, Beck JF, Mainz JG. Cystic fibrosis: patient's best friend? Potential transmission of *Pseudomonas aeruginosa* from a dog. Klinische Padiatrie. 2017;229(4):245-6.
 20. Morris DO, Davis MF, Palmeiro BS, O'Shea K, Rankin SC. Molecular and epidemiological characterization of canine *Pseudomonas* otitis using a prospective case-control study design. Veterinary Dermatology. 2017;28(1):118-25.
 21. Collaborators AR. Global burden of bacterial antimicrobial resistance in 2019: a systematic analysis. The Lancet. 2022;399:629-55.
 22. WHO publishes list of bacteria for which new antibiotics are urgently needed [press release]. World Health Organisation website: World Health Organisation. 2017.
 23. Bourély C, Cazeau G, Jarrige N, Leblond A, Madec JY, Haenni M, et al. Antimicrobial resistance patterns of bacteria isolated from dogs with otitis. Epidemiology and Infection. 2019;147(e121):1-10.
 24. Mekic S, Matanovic K, Seol B. Antimicrobial susceptibility of *Pseudomonas aeruginosa* isolates from dogs with otitis externa. Veterinary Record. 2011;169(125).
 25. KuKanich KS, Bagladi-Swanson M, KuKanich B. *Pseudomonas aeruginosa* susceptibility, antibiogram and clinical interpretation, and antimicrobial prescribing behaviors for dogs with otitis in the Midwestern United States. Journal of Veterinary Pharmacology and Therapeutics. 2022;45(5):440-9.
 26. Hariharan H, Coles M, Poole D, Lund L, Page R. Update on antimicrobial susceptibilities of bacterial isolates from canine and feline otitis externa. The Canadian Veterinary Journal. 2006;47(3):253-5.
 27. Bugden DL. Identification and antibiotic susceptibility of bacterial isolates from dogs with otitis externa in Australia. The Journal of the Australian Veterinary Association Ltd. 2013;91(1-2):43-6.
 28. Nuttall T. Successful management of otitis externa. In Practice. 2016;38(S2):17-21.

29. Kiil K, Binnewies TT, Willenbrock H, Hansen SK, Yang L, Jelsbak L, et al. Comparative Genomics of *Pseudomonas*. In: Rehm BHA, editor. *Pseudomonas: Model Organism, Pathogen, Cell Factory*. Weinheim, Germany: Wiley-VCH; 2008. p. 1-24.
30. Dupont P, Hocquet D, Jeannot K, Chavanet P, Plésiat P. Bacteriostatic and bactericidal activities of eight fluoroquinolones against MexAB-OprM-overproducing clinical strains of *Pseudomonas aeruginosa*. *Journal of Antimicrobial Chemotherapy*. 2005;55(4):518-22.
31. Masuda N, Sakagawa E, Ohya S, Gotoh N, Tsujimoto H, Nishino T. Substrate specificities of MexAB-OprM, MexCD-OprJ, and MexXY-OprM efflux pumps in *Pseudomonas aeruginosa*. *Antimicrobial agents and chemotherapy*. 2000;44(12):3322-7.
32. Mandsberg LF, Ciofu O, Kirkby N, Christiansen LE, Poulsen HE, Hoiby N. Antibiotic resistance in *Pseudomonas aeruginosa* strains with increased mutation frequency due to inactivation of the DNA oxidative repair system. *Antimicrobial Agents and Chemotherapy*. 2009;53(6):2483-91.
33. Maciá MD, Blanquer D, Togores B, Sauleda J, Pérez JL, Oliver A. Hypermutation is a key factor in development of multiple-antimicrobial resistance in *Pseudomonas aeruginosa* strains causing chronic lung infections. *Antimicrobial Agents and Chemotherapy*. 2005;49(8):3382-6.
34. Montanari S, Oliver A, Salerno P, Mena A, Bertoni G, Tummler B, et al. Biological cost of hypermutation in *Pseudomonas aeruginosa* strains from patients with cystic fibrosis. *Microbiology*. 2007;153:1445-54.
35. Oliver A, Cantón R, Campo P, Baquero F, Blázquez J. High frequency of hypermutable *Pseudomonas aeruginosa* in cystic fibrosis lung infection. *Science*. 2000;288(5469):1251-3.
36. Hall KM, Pursell ZF, Morici LA. The role of the *Pseudomonas aeruginosa* hypermutator phenotype on the shift from acute to chronic virulence during respiratory infection. *Frontiers in Cellular and Infection Microbiology*. 2022;12:1-9.

37. Breidenstein EBM, de la Fuente-Núñez C, Hancock REW. *Pseudomonas aeruginosa*: all roads lead to resistance. Trends in Microbiology. 2011;19(8):419-26.
38. Yan J-J, Hsueh P-R, Lu J-J, Chang F-Y, Ko W-C, Wu J-J. Characterization of acquired β -lactamases and their genetic support in multidrug-resistant *Pseudomonas aeruginosa* isolates in Taiwan: the prevalence of unusual integrons. Journal of Antimicrobial Chemotherapy. 2006;58(3):530-6.
39. Khajuria A, Praharaj AK, Kumar M, Grover N. Emergence of NDM-1 in the clinical isolates of *Pseudomonas aeruginosa* in India. Journal of Clinical and Diagnostic Research. 2013;7(7):1328-31.
40. Johnson G, Banerjee S, Putonti C. Diversity of *Pseudomonas aeruginosa* temperate phages. mSphere. 2022;7(1):e01015-21.
41. Malhotra S, Hayes J, Don, Wozniak DJ. Cystic Fibrosis and *Pseudomonas aeruginosa*: the Host-Microbe Interface. Clinical Microbiology Reviews. 2019;32(3):e00138-18.
42. Amankwah S, Adisu M, Gorems K, Abdella K, Kassa T. Assessment of phage-mediated inhibition and removal of multidrug-resistant *Pseudomonas aeruginosa* biofilm on medical implants. Infection and Drug Resistance. 2022;15:2797-811.
43. Pye CC, Yu AA, Weese JS. Evaluation of biofilm production by *Pseudomonas aeruginosa* from canine ears and the impact of biofilm on antimicrobial susceptibility *in vivo*. Veterinary Dermatology. 2013;24:446-e99.
44. Pires DP, Melo LDR, Azeredo J. Understanding the complex phage-host interactions in biofilm communities. Annual Review of Virology. 2021;8:73-94.
45. Yang L, Hu Y, Liu Y, Zhang J, Ulstrup J, Molin S. Distinct roles of extracellular polymeric substances in *Pseudomonas aeruginosa* biofilm development. Environmental Microbiology. 2011;13(7):1705-17.
46. Boyd A, Chakrabarty AM. *Pseudomonas aeruginosa* biofilms: role of the alginate exopolysaccharide. Journal of Industrial Microbiology. 1995;15(3):162-8.
47. Wozniak DJ, Wyckoff TJO, Starkey M, Keyser R, Azadi P, O'Toole GA, et al. Alginate is not a significant component of the extracellular polysaccharide matrix of

PA14 and PA01 *Pseudomonas aeruginosa* biofilms. Proceedings of the National Academy of Sciences. 2003;100(13):7907-12.

48. Ryder C, Byrd M, Wozniak DJ. Role of polysaccharides in *Pseudomonas aeruginosa* biofilm development. Current Opinion in Microbiology. 2007;10(6):644-8.

49. Jackson KD, Starkey M, Kremer S, Parsek MR, Wozniak DJ. Identification of psl, a locus encoding a potential exopolysaccharide that is essential for *Pseudomonas aeruginosa* PAO1 biofilm formation. Journal of Bacteriology. 2004;186(14):4466-75.

50. Byrd MS, Sadovskaya I, Vinogradov E, Lu H, Sprinkle AB, Richardson SH, et al. Genetic and biochemical analyses of the *Pseudomonas aeruginosa* Psl exopolysaccharide reveal overlapping roles for polysaccharide synthesis enzymes in Psl and LPS production. Molecular Microbiology. 2009;73(4):622-38.

51. Franklin MJ, Nivens DE, Weadge JT, Howell PL. Biosynthesis of the *Pseudomonas aeruginosa* extracellular polysaccharides, alginate, Pel, and Psl. Frontiers in Microbiology. 2011;2:1-16.

52. Jennings LK, Storek KM, Ledvina HE, Coulon C, Marmont LS, Sadovskaya I, et al. Pel is a cationic exopolysaccharide that cross-links extracellular DNA in the *Pseudomonas aeruginosa* biofilm matrix. PNAS. 2015;112(36):11353-8.

53. Colvin KM, Irie Y, Tart CS, Urbano R, Whitney JC, Ryder C, et al. The Pel and Psl polysaccharides provide *Pseudomonas aeruginosa* structural redundancy within the biofilm matrix. Environmental Microbiology. 2012;14(8).

54. Plokarz D, Czopowicz M, Bierowiec K, Rypula K. Virulence genes as markers for *Pseudomonas aeruginosa* biofilm formation in dogs and cats. Animals. 2022;12(422).

55. de Sousa T, Garces A, Silva A, Lopes R, Alegria N, Hébraud M, et al. The impact of the virulence of *Pseudomonas aeruginosa* isolated from dogs. Veterinary Sciences. 2023;10(5):1-14.

56. Hotterbeekx A, Kumar-Singh S, Goossens H, Malhotra-Kumar S. *In vivo* and *in vitro* interactions between *Pseudomonas aeruginosa* and *Staphylococcus* spp. Frontiers in Cellular and Infection Microbiology. 2017;7(106).

57. Gambino E, Maione A, Guida M, Albarano L, Carraturo F, Galdiero E, et al. Evaluation of the pathogenic-mixed biofilm formation of *Pseudomonas aeruginosa*/*Staphylococcus aureus* and treatment with limonene on three different materials by a dynamic model. International Journal of Environmental Research and Public Health. 2022;19(3741).
58. Armbruster CR, Wolter DJ, Mishra M, Hayden HS, Radey MC, Merrihew G, et al. *Staphylococcus aureus* protein A mediates interspecies interactions at the cell surface of *Pseudomonas aeruginosa*. mBio. 2016;7(3):e00538-16.
59. Chan WY, Hickey EE, Page SW, Trott DJ, Hill PB. Biofilm production by pathogens associated with canine otitis externa, and the antibiofilm activity of ionophores and antimicrobial adjuvants. Journal of Veterinary Pharmacology and Therapeutics. 2019;42:682-92.
60. Robinson VH, Paterson S, Bennett C, Steen SI. Biofilm production of *Pseudomonas* spp. isolates from canine otitis in three different enrichment broths. Veterinary Dermatology. 2019;30:218-e67.
61. Hattab J, Mosca F, Esmeralda Di Francesco C, Aste G, Marruchella G, Guardiani P, et al. Occurrence, antimicrobial susceptibility, and pathogenic factors of *Pseudomonas aeruginosa* in canine clinical samples. Veterinary World. 2021;14(4):978-85.
62. Macia MD, Rojo-Molinero E, Oliver A. Antimicrobial susceptibility testing in biofilm-growing bacteria. Clinical Microbiology and Infection. 2014;20(10):981-90.
63. Rohwer F. Global phage diversity. Cell. 2003;113(2):141.
64. Clokie MRJ, Millard AD, Letarov AV, Heaphy S. Phages in nature. Bacteriophage. 2011;1(1):31-45.
65. Deresinski S. Bacteriophage therapy: exploiting smaller fleas. Clinical Infectious Diseases. 2009;48(8):1096-101.
66. Pires DP, Vilas Boas D, Sillankorva S, Azeredo J. Phage therapy: a step forward in the treatment of *Pseudomonas aeruginosa* infections. Journal of Virology. 2015;89(15):7449-56.

67. Moineau S. Bacteriophage. In: Maloy S, Hughes K, editors. Brenner's Encyclopedia of Genetics (Second Edition). 2 ed: Academic Press; 2013. p. 280-3.
68. Nobrega FL, Vlot M, de Jonge PA, Dreesens LL, Beaumont HJE, Lavigne R, et al. Targeting mechanisms of tailed bacteriophages. *Nature Reviews Microbiology*. 2018;16(12):760-73.
69. Turner D, Shkoporov AN, Lood C, Millard AD, Dutilh BE, Alfenas-Zerbini P, et al. Abolishment of morphology-based taxa and change to binomial species names: 2022 taxonomy update of the ICTV bacterial viruses subcommittee. *Archives of Virology*. 2023;168(2).
70. Yu P, Mathieu J, Li M, Dai Z, Alvarez PJJ. Isolation of polyvalent bacteriophages by sequential multiple-host approaches. *Applied and Environmental Microbiology*. 2016;82(3):808-15.
71. Duffy S, Turner PE, Burch CL. Pleiotropic costs of niche expansion in the RNA bacteriophage $\Phi 6$. *Genetics*. 2006;172(2):751-7.
72. Duffy S, Burch CL, Turner PE. Evolution of host specificity drives reproductive isolation among RNA viruses. *Evolution*. 2007;61(11):2614-22.
73. Subramanian S, Dover JA, Parent KN, Doore SM. Host range expansion of Shigella phage Sf6 evolves through point mutations in the tailspike. *Journal of Virology*. 2022;96(16):e00929-22.
74. Kortright KE, Chan BK, Koff JL, Turner PE. Phage therapy: a renewed approach to combat antibiotic-resistant bacteria. *Cell Host & Microbe*. 2019;25(2):219-32.
75. Parson KA, Snustad DP. Host DNA degradation after infection of *Escherichia coli* with bacteriophage T4: dependence of the alternate pathway of degradation which occurs in the absence of both T4 endonuclease II and nuclear disruption on T4 endonuclease IV. *Journal of Virology*. 1975;15(1):221-4.
76. Powell IB, Tulloch DL, Hillier AJ, Davidson BE. Phage DNA synthesis and host DNA degradation in the life cycle of *Lactococcus lactis* bacteriophage c6A. *Journal of General Microbiology*. 1992;138(5):945-50.
77. Miller ES, Kutter E, Mosig G, Arisaka F, Kunisawa T, Ruger W. Bacteriophage T4 genome. *Microbiology and Molecular Biology Reviews*. 2003;67(1):86-156.

78. Catalão MJ, Gil F, Moniz-Pereira J, São-José C, Pimentel M. Diversity in bacterial lysis systems: bacteriophages show the way. *Federation of European Microbiological Societies Microbiology Reviews*. 2012;37(4):554-71.
79. Oliveira H, Sao-José C, Azeredo J. Phage-derived peptidoglycan degrading enzymes: challenges and future prospects for *in vivo* therapy. *Viruses*. 2018;10(292):1-18.
80. Twort FW. An investigation on the nature of ultra-microscopic viruses. *The Lancet*. 1915;186(4814):1241-3.
81. d'Hérelle F. Sur un microbe invisible antagoniste des bacilles dysentérique. *Comptes rendus de l'Académie des Sciences*. 1917;165:373-5.
82. Chanishvili N. Chapter 1: Phage therapy - History from Twort and d'Herelle through Soviet experience to current approaches. In: Lobocka M, Szybalski W, editors. *Advances in Virus Research: Bacteriophages, Part B*. 83: Academic Press; 2012. p. 3-40.
83. Kutateladze M, Adamia R. Phage therapy experience at the Eliava Institute. *Médecine et maladies infectieuses*. 2008;38:426-30.
84. Uytendaele S, Chen B, Onsea J, Ruythooren F, Debaveye Y, Devolder D, et al. Safety and efficacy of phage therapy in difficult-to-treat infections: a systematic review. *The Lancet Infectious Diseases*. 2022;22:e208-20.
85. Gordillo Altamirano FL, Barr JJ. Phage therapy in the postantibiotic era. *Clinical Microbiology Reviews*. 2019;32(2):e00066-18.
86. Endersen L, O'Mahony J, Hill C, Ross RP, McAuliffe O, Coffey A. Phage therapy in the food industry. *Annual Review of Food Science and Technology*. 2014;5:327-49.
87. Moye ZD, Woolston J, Sulakvelidze A. Bacteriophage applications for food production and processing. *Viruses*. 2018;10(205).
88. Atterbury RJ, Barrow PA. The use of bacteriophages in veterinary therapy. In: Harper DR, Abedon ST, Burrowes BH, McConville ML, editors. *Bacteriophages: Biology, Technology, Therapy*. Cham: Springer International Publishing; 2021. p. 953-87.

89. Dabrowska K, Gorski A, Abedon ST. Bacteriophage pharmacology and immunology. In: Harper DR, Abedon ST, Burrowes BH, McConville ML, editors. Bacteriophages: Biology, Technology, Therapy. Cham: Springer International Publishing; 2021. p. 295-339.
90. Bondy-Denomy J, Qian J, Westra ER, Buckling A, Guttman DS, Davidson AR, et al. Prophages mediate defense against phage infection through diverse mechanisms. *The ISME Journal*. 2016;10:2854-66.
91. Los J, Zielinska S, Krajewska A, Michalina Z, Malachowska A, Kwasnicka K, et al. Temperate phages, prophages, and lysogeny. In: Harper DR, Abedon ST, Burrowes BH, McConville ML, editors. Bacteriophages: Biology, Technology, Therapy. Cham: Springer International Publishing; 2021. p. 119-50.
92. Fortier L-C, Sekulovic O. Importance of prophages to evolution and virulence of bacterial pathogens. *Virulence*. 2013;4(5):354-65.
93. Haaber J, Leisner JJ, Cohn MT, Catalan-Moreno A, Nielsen JB, Westh H, et al. Bacterial viruses enable their host to acquire antibiotic resistance genes from neighbouring cells. *Nature Communications*. 2016;7(13333):1-8.
94. Allison HE. Stx-phages: drivers and mediators of the evolution of STEC and STEC-like pathogens. *Future Microbiology*. 2007;2(2):165-74.
95. Wood SJ, Goldufsky JW, Seu MY, Dorafshar AH, Shafikhani SH. *Pseudomonas aeruginosa* cytotoxins: mechanisms of cytotoxicity and impact on inflammatory responses. *Cells*. 2023;12(195):1-33.
96. Nakayama K, Kanaya S, Ohnishi M, Terawaki Y, Hayashi T. The complete nucleotide sequence of phiCTX, a cytotoxin-converting phage of *Pseudomonas aeruginosa*: implications for phage evolution and horizontal gene transfer via bacteriophages. *Molecular Microbiology*. 1999;31(2):399-419.
97. Hertveldt K, Lavigne R. Bacteriophages of *Pseudomonas*. In: Rehm BHA, editor. *Pseudomonas: Model Organism, Pathogen, Cell Factory*. Weinheim, Germany: Wiley-VCH; 2008. p. 255-92.

98. Keen EC, Bliskovsky VV, Malagon F, Baker JD, Prince JS, Klaus JS, et al. Novel 'superspreader' bacteriophages promote horizontal gene transfer by transformation. *mBio*. 2017;8(1):e02115-16.
99. Schneider CL. Bacteriophage-mediated horizontal gene transfer: transduction. In: Harper DR, Abedon ST, Burrowes BH, McConville ML, editors. *Bacteriophages: Biology, Technology, Therapy*. Cham: Springer International Publishing; 2021. p. 151-92.
100. Drexler H. Transduction by bacteriophage T1. *Proceedings of the National Academy of Sciences*. 1970;66(4):1083-8.
101. Ely B, Johnson RC. Generalized transduction in *Caulobacter crescentus*. *Genetics*. 1977;87(3):391-9.
102. Labrie SJ, Samson JE, Moineau S. Bacteriophage resistance mechanisms. *Nature Reviews Microbiology*. 2010;8:317-27.
103. Górski A, Miedzybrodzki R, Lobočka M, Glowacka-Rutkowska A, Bednarek A, Borysowski J, et al. Phage therapy: what have we learned? *Viruses*. 2018;10(288):1-28.
104. Dedrick RM, Guerrero-Bustamante CA, Garlena RA, Russell DA, Ford K, Harris K, et al. Engineered bacteriophages for treatment of a patient with a disseminated drug-resistant *Mycobacterium abscessus*. *Nature Medicine*. 2019;25(5):730-3.
105. Chan BK, Siström M, Wertz JE, Kortright KE, Narayan D, Turner PE. Phage selection restores antibiotic sensitivity in MDR *Pseudomonas aeruginosa*. *Scientific Reports*. 2016;6(26717):1-8.
106. Schooley RT, Biswas B, Gill JJ, Hernandez-Morales A, Lancaster J, Lessor L, et al. Development and use of personalized bacteriophage-based therapeutic cocktails to treat a patient with a disseminated resistant *Acinetobacter baumannii* infection. *Antimicrobial Agents and Chemotherapy*. 2017;61(10):e00954-17.
107. Speck P, Smithyman A. Safety and efficacy of phage therapy via the intravenous route. *FEMS Microbiology Letters*. 2016;363(3):1-5.

108. Liu D, Van Belleghem JD, de Vries CR, Burgener E, Chen Q, Manasherob R, et al. The safety and toxicity of phage therapy: a review of animal and clinical studies. *Viruses*. 2021;13(1268):1-20.
109. Dedrick RM, Freeman KG, Nguyen JA, Bahadirli-Talbott A, Smith BE, Wu AE, et al. Potent antibody-mediated neutralization limits bacteriophage treatment of a pulmonary *Mycobacterium abscessus* infection. *Nature Medicine*. 2021;27:1357-61.
110. Nguyen S, Baker K, Padman BS, Patwa R, Dunstan RA, Weston TA, et al. Bacteriophage transcytosis provides a mechanism to cross epithelial cell layers. *mBio*. 2017;8(6).
111. Zaczek M, Gorski A, Skaradzinska A, Lusiak-Szelachowska M, Weber-Dabrowska B. Phage penetration of eukaryotic cells: practical implications. *Future Virology*. 2019;14(11):745-60.
112. Sliwka P, Ochocka M, Skaradzinska A. Applications of bacteriophages against intracellular bacteria. *Critical Reviews in Microbiology*. 2022;48(2):222-39.
113. Wernicki A, Nowaczek A, Urban-Chmiel R. Bacteriophage therapy to combat bacterial infections in poultry. *Virology Journal*. 2017;14(179):1-13.
114. Furusawa T, Iwano H, Higuchi H, Yokota H, Usui M, Iwasaki T, et al. Bacteriophage can lyse antibiotic-resistant *Pseudomonas aeruginosa* isolated from canine diseases. *Journal of Veterinary Medical Science*. 2016;78(6):1035-8.
115. Fujiki J, Furusawa T, Munby M, Kawaguchi C, Matsuda Y, Shiokura Y, et al. Susceptibility of *Pseudomonas aeruginosa* veterinary isolates to Pbnavirus PB1-like phages. *Microbiology and Immunology*. 2020;64(11):778-82.
116. Carmine de Melo AC, da Mata Gomes A, Melo FL, Ardisson-Araújo DMP, Castagna de Vargas AP, Lunkes Ely V, et al. Characterization of a bacteriophage with broad host range against strains of *Pseudomonas aeruginosa* isolated from domestic animals. *BMC Microbiology*. 2019;19(134):1-15.
117. Khairnar K, Raut MP, Chandekar RH, Sanmukh SG, Paunekar WN. Novel bacteriophage therapy for controlling metallo-beta-lactamase producing *Pseudomonas aeruginosa* infection in catfish. *BMC Veterinary Research*. 2013;9(264):1-9.

118. Antoine C, Laforet F, Blasdel B, Glonti T, Kutter E, Pirnay JP, et al. Efficacy assessment of PEV2 phage on *Galleria mellonella* larvae infected with a *Pseudomonas aeruginosa* dog otitis isolate. *Research in Veterinary Science*. 2021;136:598-601.
119. Gu J, Li X, Yang M, Du C, Cui Z, Gong P, et al. Therapeutic effect of *Pseudomonas aeruginosa* phage YH30 on mink haemorrhagic pneumonia. *Veterinary Microbiology*. 2016;190:5-11.
120. Wang J, Meng W, Zhang K, Wang J, Lu B, Wang R, et al. Topically applied bacteriophage to control multi-drug resistant *Pseudomonas aeruginosa*-infected wounds in a New Zealand rabbit model. *Frontiers in Microbiology*. 2022;13(1031101):1-11.
121. Soothill J. Use of bacteriophages in the treatment of *Pseudomonas aeruginosa* infections. *Expert Review of Anti-infective Therapy*. 2013;11(9):909-15.
122. Sivera Marza JA, Soothill JS, Boydell P, Collyns TA. Multiplication of therapeutically administered bacteriophages in *Pseudomonas aeruginosa* infected patients. *Burns*. 2006;32:644-6.
123. Hawkins C, Harper D, Burch D, Anggard E, Soothill J. Topical treatment of *Pseudomonas aeruginosa* otitis of dogs with a bacteriophage mixture: A before/after clinical trial. *Veterinary Microbiology*. 2010;146:309-13.
124. Wright A, Hawkins CH, Anggard EE, Harper DR. A controlled clinical trial of a therapeutic bacteriophage preparation in chronic otitis due to antibiotic-resistant *Pseudomonas aeruginosa*; a preliminary report of efficacy. *Clinical Otolaryngology*. 2009;34:349-57.
125. Jault P, Leclerc T, Jennes S, Pirnay JP, Que Y-A, Resch G, et al. Efficacy and tolerability of a cocktail of bacteriophages to treat burn wounds infected by *Pseudomonas aeruginosa* (PhagoBurn): a randomised, controlled, double-blind phase 1/2 trial. *The Lancet Infectious Diseases*. 2019;19:35-45.
126. Tamma PD, Souli M, Billard M, Campbell J, Conrad D, Ellison DW, et al. Safety and microbiological activity of phage therapy in persons with cystic fibrosis colonized

with *Pseudomonas aeruginosa*: study protocol for a phase 1b/2, multicenter, randomized, double-blind, placebo-controlled trial. *Trials*. 2022;23(1057):1-12.

127. Santos SB, Carvalho CM, Sillankorva S, Nicolau A, Ferreira EC, Azeredo J. The use of antibiotics to improve phage detection and enumeration by the double-layer agar technique. *BMC Microbiology*. 2009;9(148).

128. Ács N, Gambino M, Brondsted L. Bacteriophage enumeration and detection methods. *Frontiers in Microbiology*. 2020;11:1-7.

129. Kropinski AM, Mazzocco A, Waddell TE, Lingohr E, Johnson RP. Chapter 7 - Enumeration of bacteriophages by double agar overlay plaque assay. In: Clokie MMJ, Kropinski AM, editors. *Bacteriophages Methods in Molecular Biology™*. 501: Humana Press; 2009. p. 69-76.

130. Jurczak-Kurek A, Gasior T, Nejman-Falenczyk B, Bloch S, Dydecka A, Topka G, et al. Biodiversity of bacteriophages: morphological and biological properties of a large group of phages isolated from urban sewage. *Scientific Reports*. 2016;6(34338).

131. Gallet R, Kannoly S, Wang I-N. Effects of bacteriophage traits on plaque formation. *BMC Microbiology*. 2011;11(1).

132. Abedon ST. Detection of bacteriophages: Phage plaques. In: Harper D, Abedon S, Burrowes B, McConville M, editors. *Bacteriophages: Biology, Technology, Therapy*. Cham: Springer International Publishing; 2021. p. 507-38.

133. Knecht LE, Velijkovic M, Fieseler L. Diversity and Function of Phage Encoded Depolymerases. *Frontiers in Microbiology*. 2020;10(2949).

134. van Charante F, Holtappels D, Blasdel B, Burrowes BH. Isolation of bacteriophages. In: Harper D, Abedon S, Burrowes B, McConville M, editors. *Bacteriophages: Biology, Technology, Therapy*. Cham: Springer International Publishing; 2021. p. 433-64.

135. Anderson B, Rashid MH, Carter C, Pasternack G, Rajanna C, Revazishvili T, et al. Enumeration of bacteriophage particles. *Bacteriophage*. 2011;1(2):86-93.

136. Shang J, Tang X, Guo R, Sun Y. Accurate identification of bacteriophages from metagenomic data using Transformer. *Briefings in Bioinformatics*. 2022;23(4):1-11.

137. Benler S, Yutin N, Antipov D, Rayko M, Shmakov S, Gussow AB, et al. Thousands of previously unknown phages discovered in whole-community human gut metagenomes. *Microbiome*. 2021;9(78):1-17.
138. Strange JES, Leekitcharoenphon P, Moller FD, Aarestrup FM. Metagenomics analysis of bacteriophages and antimicrobial resistance from global urban sewage. *Scientific Reports*. 2021;11(1600).
139. Coutinho FH, Silveira CB, Gregoracci GB, Thompson CC, Edwards RA, Brussaard CPD, et al. Marine viruses discovered via metagenomics shed light on viral strategies throughout the oceans. *Nature Communications*. 2017;8(15955):1-12.
140. Schackart III KE, Graham JB, Ponsero AJ, Hurwitz BL. Evaluation of computational phage detection tools for metagenomic datasets. *Frontiers in Microbiology*. 2023;14(1078760):1-16.
141. Grace A, Sahu R, Owen DR, Dennis VA. *Pseudomonas aeruginosa* reference strains PAO1 and PA14: a genomic, phenotypic, and therapeutic review. *Frontiers in Microbiology*. 2022;13(1023523):1-15.
142. Cultures NCoT. Bacteria collection: *Pseudomonas aeruginosa*: UK Health Security Agency; [Available from: <https://www.culturecollections.org.uk/products/bacteria/detail.jsp?refId=NCTC+10662&collection=nctc#medDoc>]
143. Arndt D, Grant J, Marcu A, Sajed T, Pon A, Liang Y, et al. PHASTER: a better, faster version of the PHAST phage search tool. *Nucleic Acids Res*. 2016(May 3).
144. Zhou Y, Liang Y, Lynch KH, Dennis JJ, Wishart DS. PHAST: a fast phage search tool. *Nucleic Acids Res*. 2011;39:W347-W52.
145. James CE, Fothergill JL, Kade H, Hall AJ, Cottell J, Brockhurst MA, et al. Differential infection properties of three inducible prophages from an epidemic strain of *Pseudomonas aeruginosa*. *BMC Microbiology*. 2012;12(216):1-10.
146. Christie GE. Propagation of viruses: bacteria. In: Granoff A, Webster RG, editors. *Encyclopedia of Virology (Second Edition)*. Second ed: Elsevier; 1999. p. 1413-8.

147. Shin H, Lee J-H, Yoon H, Kang D-H, Ryu S. Genomic investigation of lysogen formation and host lysis systems of the *Salmonella* temperate bacteriophage SPN9CC. *Applied and Environmental Microbiology*. 2014;80(1):374-84.
148. Ramesh N, Archana L, Royam MM, Manohar P, Eniyan K. Effect of various bacteriological media on the plaque morphology of *Staphylococcus* and *Vibrio* phages. *Access Microbiology*. 2019;1(4):e000036.
149. Wang X, Tang J, Dang W, Xie Z, Zhang F, Hao X, et al. Isolation and characterization of three *Pseudomonas aeruginosa* viruses with therapeutic potential. *Microbiology Spectrum*. 2023;11(3).
150. Campbell RA, Farlow J, Freyberger HR, He Y, Ward AM, Ellison DW, et al. Genome sequences of 17 diverse *Pseudomonas aeruginosa* phages. *Microbiology Resource Announcements*. 2021;10(19):e00031-21.
151. Essoh C, Latino L, Midoux C, Blouin Y, Loukou G, Nguetta S-PA, et al. Investigation of a large collection of *Pseudomonas aeruginosa* bacteriophages collected from a single environmental source in Abidjan, Cote d'Ivoire. *PLoS ONE*. 2015;10(6).
152. Yuanyuan N, Xiaobo Y, Shang W, Yutong Y, Hongrui Z, Chenyu L, et al. Isolation and characterization of two homolog phages infecting *Pseudomonas aeruginosa*. *Frontiers in Microbiology*. 2022;13.
153. Cirz RT, O'Neill BM, Hammond JA, Head SR, Romesberg FE. Defining the *Pseudomonas aeruginosa* SOS response and its role in the global response to the antibiotic ciprofloxacin. *Journal of Bacteriology*. 2006;188(20):7101-10.
154. Fothergill JL, Mowat E, Walshaw MJ, Ledson MJ, James CE, Winstanley C. Effect of antibiotic treatment on bacteriophage production by a cystic fibrosis epidemic strain of *Pseudomonas aeruginosa*. *Antimicrobial Agents and Chemotherapy*. 2011;55(1):426-8.
155. Shmidov E, Zander I, Lebenthal-Loinger I, Karako-Lampert S, Shoshani S, Banin E. An efficient, counter-selection-based method for prophage curing in *Pseudomonas aeruginosa* strains. *Viruses*. 2021;13(336):1-12.

156. Winstanley C, Langille MGI, Fothergill JL, Kukavica-Ibrulj I, Paradis-Bleau C, Sanschagrín F, et al. Newly introduced genomic prophage islands are critical determinants of in vivo competitiveness in the Liverpool Epidemic Strain of *Pseudomonas aeruginosa*. *Genome Research*. 2009;19(1):12-23.
157. Nanda AM, Thormann K, Frunzke J. Impact of spontaneous prophage induction on the fitness of bacterial populations and host-microbe interactions. *Journal of Bacteriology*. 2015;197(3).
158. Miller-Ensminger T, Garretto A, Stark N, Putonti C. Mimicking prophage induction in the body: induction in the lab with pH gradients. *PeerJ*. 2020;8(e9718):1-18.
159. Klockgether J, Munder A, Neugebauer J, Davenport CF, Stanke F, Larbig KD, et al. Genome diversity of *Pseudomonas aeruginosa* PAO1 laboratory strains. *Journal of Bacteriology*. 2010;192(4).
160. Gavric D, Knezevic P. Optimized method for *Pseudomonas aeruginosa* integrative filamentous bacteriophage propagation. *Frontiers in Microbiology*. 2022;12(707815):1-8.
161. Ackermann H-W. Basic phage electron microscopy. In: Clokie MRJ, Kropinski AM, editors. *Bacteriophages: Methods and Protocols, Volume 1: Isolation, Characterization and Interactions*. *Methods in Molecular Biology*. 501. New York: Humana Press; 2009. p. 113-26.
162. Nayfach S, Pedro Camargo A, Schulz F, Eloë-Fadrosh E, Roux S, Kyrpides NC. CheckV assesses the quality and completeness of metagenome-assembled viral genomes. *Nature Biotechnology*. 2021;39:578-85.
163. McGowan J. GC Content Calculator 2023 [Available from: https://jamiemcgowan.ie/bioinf/gc_content.html]
164. Information NCfB. Basic Local Alignment Search Tool (BLAST) National Library of Medicine: NCBI; [Available from: <https://blast.ncbi.nlm.nih.gov/Blast.cgi>]
165. Nishimura Y, Yoshida T, Kuronishi M, Uehara H, Ogata H, Goto S. ViPTree: the viral proteomic tree server. *Bioinformatics*. 2017;33(15):2379-80.

166. Bhunchoth A, Blanc-Mathieu R, Mihara T, Nishimura Y, Askora A, Phironrit N, et al. Two asian jumbo phages, ϕ RSL2 and ϕ RSF1, infect *Ralstonia solanacearum* and show common features of ϕ KZ-related phages. *Virology*. 2016;494:56-66.
167. Mihara T, Nishimura Y, Shimizu Y, Nishiyama H, Yoshikawa G, Uehara H, et al. Linking virus genomes with host taxonomy. *Viruses*. 2016;8(3):1-6.
168. Seemann T. Prokka: rapid prokaryotic genome annotation. *Bioinformatics*. 2014;30(14):2068-9.
169. Terzian P, Ndela EO, Galiez C, Lossouarn J, Pérez Bucio RE, Mom R, et al. PHROG: families of prokaryotic virus proteins clustered using remote homology. *NAR Genomics and Bioinformatics*. 2021;3(3):1-12.
170. Alcock BP, Huynh W, Chalil R, Smith KW, Raphenya AR, Wlodarski MA, et al. CARD 2023: expanded curation, support for machine learning, and resistome prediction at the Comprehensive Antibiotic Resistance Database. *Nucleic Acids Research*. 2023;51(D1):D690-9.
171. Adriaenssens EM, Brister JR. How to name and classify your phage: an informal guide. *Viruses*. 2017;9(4):1-9.
172. Abedon ST. Phage Name Check [Available from: <http://namecheck.phage.org>]
173. Information NCfB. NCBI Virus [Available from: <https://www.ncbi.nlm.nih.gov/labs/virus/vssi/#/find-data/virus>]
174. Guo Y, Chen P, Lin Z, Wang T. Characterization of two *Pseudomonas aeruginosa* viruses vB_PaeM_SCUT-S1 and vB_PaeM_SCUT-S2. *Viruses*. 2019;11(4):1-19.
175. Almpanis A, Swain M, Gatherer D, McEwan N. Correlation between bacterial G+C content, genome size and the G+C content of associated plasmids and bacteriophages. *Microbial Genomics*. 2018;4(4).
176. Klockgether J, Cramer N, Wiehlmann L, Davenport CF, Tummler B. *Pseudomonas aeruginosa* genomic structure and diversity. *Frontiers in Microbiology*. 2011;2.

177. Stover CK, Pham XQ, Erwin AL, Mizoguchi SD, Warrenner P, Hickey MJ, et al. Complete genome sequence of *Pseudomonas aeruginosa* PA01, an opportunistic pathogen. *Nature*. 2000;406(6799):959-64.
178. Cruz-Plancarte I, Cazares A, Guarneros G. Genomic and transcriptional mapping of PaMx41, archetype of a new lineage of bacteriophages infecting *Pseudomonas aeruginosa*. *Applied and Environmental Microbiology*. 2016;82(22):6541-7.
179. Flores V, Sepúlveda-Robles O, Cazares A, Kameyama L, Guarneros G. Comparative genomic analysis of *Pseudomonas aeruginosa* phage PaMx25 reveals a novel siphovirus group related to phages infecting hosts of different taxonomic classes. *Archives of Virology*. 2017;162:2345-55.
180. Peters DL, Lynch KH, Stothard P, Dennis JJ. The isolation and characterisation of two *Stenotrophomonas maltophilia* bacteriophages capable of cross-taxonomic order infectivity. *BMC Genomics*. 2015;16(664):1-15.
181. Karumidze N, Thomas JA, Kvatadze N, Goderdzishvili M, Hakala KW, Weintraub ST, et al. Characterization of lytic *Pseudomonas aeruginosa* bacteriophages via biological properties and genomic sequences. *Applied Microbiology and Biotechnology*. 2012;94:1609-17.
182. Kwan T, Liu J, DuBow M, Gros P, Pelletier J. Comparative genomic analysis of 18 *Pseudomonas aeruginosa* bacteriophages. *Journal of Bacteriology*. 2006;188(3):1184-7.
183. Sepúlveda-Robles O, Kameyama L, Guarneros G. High diversity and novel species of *Pseudomonas aeruginosa* bacteriophages. *Applied and Environmental Microbiology*. 2012;78(12):4510-5.
184. Chaudhry WN, Concepción-Acevedo J, Park T, Andleeb S, Bull JJ, Levin BR. Synergy and order effects of antibiotics and phages in killing *Pseudomonas aeruginosa* biofilms. *PLoS ONE*. 2017;12(1).
185. Rocha EPC, Danchin A. Base composition bias might result from competition for metabolic resources. *Trends in Genetics*. 2002;18(6):291-4.

186. Oren A, Garrity GM. Valid publication of the names of forty-two phyla of prokaryotes. *International Journal of Systematic and Evolutionary Microbiology*. 2021;71(10):1-7.
187. Brooke JS. *Stenotrophomonas maltophilia*: an emerging global opportunistic pathogen. *Clinical Microbiology Reviews*. 2012;25(1):2-41.
188. Loh B, Wang X, Hua X, Luo J, Wen T, Zhang L, et al. Complete genome sequences of bacteriophages Kaya, Guyu, Kopi, and TehO, which target clinical strains of *Pseudomonas aeruginosa*. *Microbiology Resources Announcements*. 2021;10(48).
189. Ackermann H-W, Cartier C, Slopek S, Vieu J-F. Morphology of *Pseudomonas aeruginosa* typing phages of the Lindberg set. *Annales de l'Institut Pasteur*. 1988;139(4):389-404.
190. Hutinet G, Kot W, Cui L, Hillebrand R, Balamkundu S, Gnanakalai S, et al. 7-deazaguanine modifications protect phage DNA from host restriction systems. *Nature Communications*. 2019;10.
191. Boeckeaerts D, Stock M, De Baets B, Briers Y. Identification of phage receptor-binding protein sequences with hidden Markov models and an extreme gradient boosting classifier. *Viruses*. 2022;14(6):1-13.
192. Blum M, Chang H-Y, Chuguransky S, Grego T, Kandasaamy S, Mitchell A, et al. The InterPro protein families and domains database: 20 years on. *Nucleic Acids Research*. 2021;49(D1):D344-D54.
193. Jones P, Binns D, Chang H-Y, Fraser M, Li W, McAnulla C, et al. InterProScan 5: genome-scale protein function classification. *Bioinformatics*. 2014;30(9):1236-40.
194. Kongari R, Rajaure M, Cahill J, Rasche E, Mijalis E, Berry J, et al. Phage spanins: diversity, topological dynamics and gene convergence. *BMC Bioinformatics*. 2018;19:1-26.
195. Howard-Varona C, Hargreaves KR, Abedon ST, Sullivan MB. Lysogeny in nature: mechanisms, impact and ecology of temperate phages. *The ISME Journal*. 2017;11(7):1511-20.

196. Silpe JE, Duddy OP, Johnson GE, Beggs GA, Hussain FA, Forsberg KJ, et al. Small protein modules dictate prophage fates during polylysogeny. *Nature*. 2023.
197. Abedon ST. Phage-provided environmental DNA and superspreading. *Bacteriophages as drivers of evolution*. Cham: Springer; 2022. p. 125-35.
198. Abedon ST, Danis-Wlodarczyk KM, Wozniak DJ, Sullivan MB. Improving phage-biofilm in vitro experimentation. *Viruses*. 2021;13(6):1-19.
199. Garbe J, Bunk B, Rohde M, Schobert M. Sequencing and characterization of *Pseudomonas aeruginosa* phage JG004. *BMC Microbiology*. 2011;11(102):1-12.
200. Payne LJ, Todeschini TC, Wu Y, Perry BJ, Ronson CW, Fineran PC, et al. Identification and classification of antiviral defence systems in bacteria and archaea with PADLOC reveals new system types. *Nucleic Acids Research*. 2021;49(19):10868-78.
201. Payne LJ, Meaden S, Mestre MR, Palmer C, Toro N, Fineran PC, et al. PADLOC: a web server for the identification of antiviral defence systems in microbial genomes. *Nucleic Acids Research*. 2022;50(W1):W541-50.
202. Ngiam L, Weynberg KD, Guo J. The presence of plasmids in bacterial hosts alters phage isolation and infectivity. *ISME Communications*. 2022;2:1-11.
203. Poullain V, Gandon S, Brockhurst MA, Buckling A, Hochberg ME. The evolution of specificity in evolving and coevolving antagonistic interactions between a bacteria and its phage. *Evolution*. 2008;62(1):1-11.
204. Rohde C, Resch G, Pirnay J-P, Blasdel BG, Debarbieux L, Gelman D, et al. Expert opinion on three phage therapy related topics: bacterial phage resistance, phage training and prophages in bacterial production strains. *Viruses*. 2018;10(4):1-15.
205. Abdelsattar AS, Dawooud A, Rezk N, Makky S, Safwat A, Richards PJ, et al. How to train your phage: the recent efforts in phage training. *Biologics*. 2021;1(2):70-88.
206. Morello E, Sausseureau E, Maura D, Huerre M, Touqui L, Debarbieux L. Pulmonary bacteriophage therapy on *Pseudomonas aeruginosa* cystic fibrosis strains: first steps towards treatment and prevention. *PLoS ONE*. 2011;6(2).

207. Friman V-P, Soanes-Brown D, Sierocinski P, Molin S, Johansen HK, Merabishvili M, et al. Pre-adapting parasitic phages to a pathogen leads to increased pathogen clearance and lowered resistance evolution with *Pseudomonas aeruginosa* cystic fibrosis bacterial isolates. *Journal of Evolutionary Biology*. 2016;29(1):188-98.
208. Burrowes BH, Molineux IJ, Fralick JA. Directed in vitro evolution of therapeutic bacteriophages: the Appelmans protocol. *Viruses*. 2019;11(3).
209. Lakshminarasimhan A. Prophage induction therapy: activation of the lytic phase in prophages for the elimination of pathogenic bacteria. *Medical Hypotheses*. 2022;169(110980):1-6.
210. Gelman D, Yerushalmy O, Alkalay-Oren S, Rakov C, Ben-Porat S, Khalifa L, et al. Clinical phage microbiology: a suggested framework and recommendations for the in-vitro matching steps of phage therapy. *The Lancet Microbe*. 2021;2(10):e555-63.
211. Abedon ST. Lysis from without. *Bacteriophage*. 2011;1(1):46-9.
212. Khan Mirzaei M, Nilsson AS. Isolation of phages for phage therapy: a comparison of spot tests and efficiency of plating analyses for determination of host range and efficacy. *PLoS ONE*. 2015;10(3):1-13.
213. Haines MEK, Hodges FE, Nale JY, Mahony J, van Sinderen D, Kaczorowska J, et al. Analysis of selection methods to develop novel phage therapy cocktails against antimicrobial resistant clinical isolates of bacteria. *Frontiers in Microbiology*. 2021;12:1-15.
214. van Heel AJ, de Jong A, Song C, Viel JH, Kok J, Kuipers OP. BAGEL4: a user-friendly web server to thoroughly mine RiPPs and bacteriocins. *Nucleic Acids Research*. 2018;46(W1):W278-81.
215. Blasco L, González de Aledo M, Ortiz-Cartagena C, Blériot I, Pacios O, López M, et al. Study of 32 new phage tail-like bacteriocins (pyocins) from a clinical collection of *Pseudomonas aeruginosa* and of their potential use as typing markers and antimicrobial agents. *Scientific Reports*. 2023;13(117):1-11.

216. Raza S, Wdowiak M, Paczesny J. An overview of diverse strategies to inactivate *Enterobacteriaceae*-targeting bacteriophages. *EcoSal Plus* (published online ahead of print 18 Jan 2023). 2023.
217. O'Flynn G, Coffey A, Fitzgerald GF, Ross RP. The newly isolated lytic bacteriophages st104a and st104b are highly virulent against *Salmonella enterica*. *Journal of Applied Microbiology*. 2005;101(1):251-9.
218. Chang Park S, Shimamura I, Fukunaga M, Mori K-I, Nakai T. Isolation of bacteriophages specific to a fish pathogen, *Pseudomonas plecoglossicida*, as a candidate for disease control. *Applied and Environmental Microbiology*. 2000;66(4):1416-22.
219. Silva YJ, Costa L, Pereira C, Mateus C, Cunha A, Calado R, et al. Phage therapy as an approach to prevent *Vibrio anguillarum* infections in fish larvae production. *PLoS ONE*. 2014;9(12):1-23.
220. Oechslin F, Piccardi P, Mancini S, Gabard J, Moreillon P, Entenza JM, et al. Synergistic interaction between phage therapy and antibiotics clears *Pseudomonas aeruginosa* infection in endocarditis and reduces virulence. *The Journal of Infectious Diseases*. 2017;215(5):703-12.
221. Castledine M, Padfield D, Sierocinski P, Pascual JS, Hughes A, Makinen L, et al. Parallel evolution of *Pseudomonas aeruginosa* phage resistance and virulence loss in response to phage treatment in vivo and in vitro. *eLife*. 2022;11.
222. Nemeth J, Oesch G, Kuster SP. Bacteriostatic versus bactericidal antibiotics for patients with serious bacterial infections: systematic review and meta-analysis. *Journal of Antimicrobial Chemotherapy*. 2015;70(2):382-95.
223. Tacconelli E, Foschi F, Forstner C, Finch RG, Pletz MW. Principles of anti-infective therapy and surgical prophylaxis. In: Cohen J, Powderly WG, Opal SM, editors. *Infectious Diseases*. 2. 4th ed: Elsevier; 2017. p. 1145-61.e2.
224. Pires DP, Sillankorva S, Faustino A, Azeredo J. Use of newly isolated phages for control of *Pseudomonas aeruginosa* PAO1 and ATCC 10145 biofilms. *Research in Microbiology*. 2011;162(8):798-806.

225. Fu W, Forster T, Mayer O, Curtin JJ, Lehman SM, Donlan RM. Bacteriophage cocktail for the prevention of biofilm formation by *Pseudomonas aeruginosa* on catheters in an *in vitro* model system. *Antimicrobial Agents and Chemotherapy*. 2010;54(1):397-404.
226. Yuan Y, Qu K, Tan D, Li X, Wang L, Cong C, et al. Isolation and characterisation of a bacteriophage and its potential to disrupt multi-drug resistant *Pseudomonas aeruginosa* biofilms. *Microbial Pathogenesis*. 2019;128:329-36.
227. Chegini Z, Khoshbayan A, Moghadam MT, Farahani I, Jazireian P, Shariati A. Bacteriophage therapy against *Pseudomonas aeruginosa* biofilms: a review. *Annals of Clinical Microbiology and Antimicrobials*. 2020;19(45):1-17.
228. Rice SA, Hao Tan C, Mikkelsen PJ, Kung V, Woo J, Tay M, et al. The biofilm life cycle and virulence of *Pseudomonas aeruginosa* are dependent on a filamentous prophage. *The ISME Journal*. 2009;3(3):271-82.
229. Periasamy S, Nair HAS, Lee KWK, Ong J, Goh JQJ, Kjelleberg S, et al. *Pseudomonas aeruginosa* PAO1 exopolysaccharides are important for mixed species biofilm community development and stress tolerance. *Frontiers in Microbiology*. 2015;6:1-10.
230. Peeters E, Nelis HJ, Coenye T. Comparison of multiple methods for quantification of microbial biofilms grown in microtiter plates. *Journal of Microbiological Methods*. 2008;72:157-65.
231. Haney EF, Trimble MJ, Cheng JT, Vallé Q, Hancock REW. Critical assessment of methods to quantify biofilm growth and evaluate antibiofilm activity of host defence peptides. *Biomolecules*. 2018;8(2):1-22.
232. Sabaeifard P, Abdi-Ali A, Soudi MR, Dinarvand R. Optimization of tetrazolium salt assay for *Pseudomonas aeruginosa* biofilm using microtiter plate method. *Journal of Microbiological Methods*. 2014;105:134-40.
233. Testa S, Berger S, Piccardi P, Oechslin F, Resch G, Mitri S. Spatial structure affects phage efficacy in infecting dual-strain biofilms of *Pseudomonas aeruginosa*. *Communications Biology*. 2019;2(405).

234. Boas DV, Almeida C, Azevedo N, Sillankorva S, Azeredo J. Techniques to assess phage-biofilm interaction. In: Clokie MRJ, Kropinski A, Lavigne R, editors. Bacteriophages, Methods and Protocols, Volume IV. Methods in Molecular Biology. 1898. New York, NY: Springer Science and Business Media; 2019. p. 137-46.
235. Hansen MF, Svenningsen SL, Roder HL, Middelboe M, Burmolle M. Big impact of the tiny: bacteriophage-bacteria interactions in biofilms. Trends in Microbiology. 2019;27(9):739-52.
236. Lorenz K, Preem L, Sagor K, Putrins M, Tenson T, Kogermann K. Development of *in vitro* and *ex vivo* biofilm models for the assessment of antibacterial fibrous electrospun wound dressings. Molecular Pharmaceutics. 2023;20(2):1230-46.
237. Wilkinson HN, Iveson S, Catherall P, Hardman MJ. A novel silver bioactive glass elicits antimicrobial efficacy against *Pseudomonas aeruginosa* and *Staphylococcus aureus* in an *ex vivo* skin wound biofilm model. Frontiers in Microbiology. 2018;9:1-16.
238. Pires DP, Oliveira H, Melo LDR, Sillankorva S, Azeredo J. Bacteriophage-encoded depolymerases: their diversity and biotechnological applications. Applied Microbiology and Biotechnology. 2016;100(5):2141-51.
239. Sillankorva S, Neubauer P, Azeredo J. *Pseudomonas fluorescens* biofilms subjected to phage phiBB-PF7A. BMC Biotechnology. 2008;8(79):1-12.
240. Koderi Valappil S, Shetty P, Deim Z, Terhes G, Urbán E, Váczi S, et al. Survival comes at a cost: a coevolution of phage and its host leads to phage resistance and antibiotic sensitivity of *Pseudomonas aeruginosa* multidrug resistant strains. Frontiers in Microbiology. 2021;12.
241. Li N, Zeng Y, Wang M, Bao R, Chen Y, Li X, et al. Characterization of phage resistance and their impacts on bacterial fitness in *Pseudomonas aeruginosa*. Microbiology Spectrum. 2022;10(5).
242. Akremi I, Merabishvili M, Jlidi M, Brahim AH, Ali MB, Karoui A, et al. Isolation and characterization of lytic *Pseudomonas aeruginosa* bacteriophages isolated from sewage samples from Tunisia. Viruses. 2022;14(11).

243. Martínez-García E, Jatsenko T, Kivisaar M, de Lorenzo V. Freeing *Pseudomonas putida* KT2440 of its proviral load strengthens endurance to environmental stresses. *Environmental Microbiology*. 2015;17(1):76-90.
244. Hargreaves KR, Clokie MRJ. *Clostridium difficile* phages: still difficult? *Frontiers in Microbiology*. 2014;5.
245. Al-Anany AM, Fatima R, Hynes AP. Temperate phage-antibiotic synergy eradicates bacteria through depletion of lysogens. *Cell Reports*. 2021;35(8):1-9.
246. Bourkal'tseva MV, Krylov SV, Kropinski AM, Pleteneva EA, Shaburova OV, Krylov VN. Bacteriophage phi297, a new species of *Pseudomonas aeruginosa* temperate phages with a mosaic genome: potential use in phage therapy. *Russian Journal of Genetics*. 2011;47(7):794-8.
247. Dedrick R, Guerrero CA, Garlena RA, Pinches RS, Cornely K, Hatfull GF. Mycobacteriophage ZoeJ: a broad host-range close relative of mycobacteriophage TM4. *Tuberculosis*. 2019;115:14-23.
248. Nale JY, Redgwell TA, Millard A, Clokie MRJ. Efficacy of an optimised bacteriophage cocktail to clear *Clostridium difficile* in a batch fermentation model. *Antibiotics*. 2018;7(13):1-15.
249. Nale JY, Spencer J, Hargreaves KR, Buckley AM, Trzepinski P, Douce GR, et al. Bacteriophage combinations significantly reduce *Clostridium difficile* growth *in vitro* and proliferation *in vivo*. *Antimicrobial agents and chemotherapy*. 2016;60(2):968-81.
250. Ceyssens P-J, Brabban A, Rogge L, Lewis MS, Pickard D, Goulding D, et al. Molecular and physiological analysis of three *Pseudomonas aeruginosa* phages belonging to the "N4-like viruses". *Virology*. 2010;405(1):26-30.
251. Yoon SS, Hennigan RF, Hilliard GM, Ochsner UA, Parvatiyar K, Kamani MC, et al. *Pseudomonas aeruginosa* anaerobic respiration in biofilms: relationships to cystic fibrosis pathogenesis. *Developmental Cell*. 2002;3(4):593-603.
252. Hassett DJ, Cuppoletti J, Trapnell B, Lyman SV, Rowe JJ, Yoon SS, et al. Anaerobic metabolism and quorum sensing by *Pseudomonas aeruginosa* biofilms in

- chronically infected cystic fibrosis airways: rethinking antibiotic treatment strategies and drug targets. *Advanced Drug Delivery Reviews*. 2002;54(11):1425-43.
253. Pastagia M, Schuch R, Fischetti VA, Huang DB. Lysins: the arrival of pathogen-directed anti-infectives. *Journal of Medical Microbiology*. 2013;62:1506-16.
254. Lukacik P, Barnard TJ, Keller PW, Chaturvedi KS, Seddiki N, Fairman JW, et al. Structural engineering of a phage lysin that targets gram-negative pathogens. *PNAS*. 2012;109(25):9857-62.
255. Briers Y, Walmagh M, Grymonprez B, Biebl M, Pirnay J-P, Defraigne V, et al. Art-175 is a highly efficient antibacterial against multidrug-resistant strains and persisters of *Pseudomonas aeruginosa*. *Antimicrobial Agents and Chemotherapy*. 2014;58(7):3774-84.
256. Yang H, Wang M, Yu J, Wei H. Antibacterial activity of a novel peptide-modified lysin against *Acinetobacter baumannii* and *Pseudomonas aeruginosa*. *Frontiers in Microbiology*. 2015;6.
257. Briers Y, Walmagh M, Lavigne R. Use of bacteriophage endolysin EL188 and outer membrane permeabilizers against *Pseudomonas aeruginosa*. *Journal of Applied Microbiology*. 2011;110(3):778-85.
258. Lai M-J, Lin N-T, Hu A, Soo P-C, Chen L-K, Chen L-H, et al. Antibacterial activity of *Acinetobacter baumannii* phage Φ AB2 endolysin (LysaAB2) against both Gram-positive and Gram-negative bacteria. *Applied Microbiology and Biotechnology*. 2011;90(2):529-39.
259. Raz A, Serrano A, Hernandez A, Euler CW, Fischetti VA. Isolation of phage lysins that effectively kill *Pseudomonas aeruginosa* in mouse models of lung and skin infection. *Antimicrobial Agents and Chemotherapy*. 2019;63(7).
260. Cui J, Shi X, Wang X, Sun H, Yan Y, Zhao F, et al. Characterization of a lytic *Pseudomonas aeruginosa* phage vB_PaeP_ASP23 and functional analysis of its lysin LysASP and holin HolASP. *Frontiers in Microbiology*. 2023;14.
261. Abeysekera GS, Love MJ, Manners SH, Billington C, Dobson RCJ. Bacteriophage-encoded lethal membrane disruptors: advances in understanding and potential applications. *Frontiers in Microbiology*. 2022;13.

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Appendices

Appendix A – Phage image measurements

AA34

Phage image	Capsid height (nm)	Capsid width (nm)	Tail and baseplate length (nm)	Tail diameter (nm)	Total length (nm)
34_0002	58.47	40.75	162.29	6.8	220.76
34_0003-a	57.25	42.27	152.51	6.85	209.76
34_0003-b	55.2	40.06	145.22	7.04	200.42
34_0007	59.32	44.94	141.15	6.85	200.47
AVERAGE	57.56	42.005	150.2925	6.885	207.8525

AA39

Phage image	Capsid height (nm)	Capsid width (nm)	Tail and baseplate length (nm)	Tail diameter (nm)	Total length (nm)
39_0001	30.37	21.76	82.43	8.5	112.8
39_0003	33.76	24.19	78.05	10.63	111.81
39_0005	45.66	33.16	107.79	9.62	153.45
39_0007	46.36	33.5	112.01	9.19	158.37
39_0008	45.22	30.83	126.06	10.14	171.28
39_0009	43.98	31.47	132.64	9.43	176.62
39_0010	43.51	34.65	121.42	9.32	164.93
AVERAGE	41.26571429	29.93714286	108.6285714	9.547142857	149.8942857

AA43

Phage image	Capsid height (nm)	Capsid width (nm)	Tail and baseplate length (nm)	Tail diameter (nm)	Total length (nm)
43_0002_0407	36.47	35.59	137.4	9.62	173.87
43_0003_0407	38.82	32.26	136.08	10.66	174.9
AVERAGE	37.645	33.925	136.74	10.14	174.385

AA46

Phage image	Capsid height (nm)	Capsid width (nm)	Tail and baseplate length (nm)	Tail diameter (nm)	Total length (nm)
46_0001	53.61	38.71	148.83	8.21	202.44
46_0002	55.44	41.87	135.28	7.08	190.72
46_0004	60.66	50.5	194.41	7.75	255.07
46_0005	42.56	30.77	118.53	8.36	161.09
46_0007	53.02	40.48	166.71	7.66	219.73
AVERAGE	53.058	40.466	152.752	7.812	205.81

AA47

Phage image	Capsid height (nm)	Capsid width (nm)	Tail and baseplate length (nm)	Tail diameter (nm)	Total length (nm)
47_0007	85.97	80.67	199.66	10.14	285.63
47_0008	50.84	41.98	148.15	9.75	198.99
47_0009	64.43	56.92	161.69	10.51	226.12
AVERAGE	67.08	59.85666667	169.8333333	10.13333333	236.913333