Understanding therapeutic phages

In vitro approaches to an improved strategy against bacterial pathogens

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Cover design explanation: Like the watercolors on this image, bacteria and phages are locked in a continuous clash, always fighting each other but always co-existing. The green-blue color comes from a mix of the pigments pyocyanine and pyoverdine produced by *Pseudomonas aeruginosa*, and therefore represents the bacteria. The purple color represents the phages.

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Coco Duizer
Dr. Leonardo Cecotto

A mis abuelos, Goya y Fausto

Table of contents

Chapter 1

General introduction

Bacteriophages

Bacteriophages, also known as phages, are viruses that specifically target and infect bacteria. Phages fill a unique ecological niche in the microbiomes of sea water, soil, plants, animals and even humans[1,2]. They are found in high numbers, with tailed phages existing in five- to ten-fold excess to bacteria in nature and thus making up the absolute majority of biological entities on the globe[3]. In recent years, it has become clear that the human body is not solely made up of its own cells, but instead functions more as a communal living space shared with a variety of micro-organisms, most of which are bacteria and phages. It is estimated that around 2×10^{12} bacteriophage particles exist within the colon of an average human, with our bodies containing up to 3×10^{13} human cells and 4×10^{13} bacterial cells[4,5]. Due to the antibacterial power of some phages, they are currently being brought into the spotlight as one of the new hopes for fighting multidrug resistant (MDR) bacteria.

Phages can be classified according to their genome or their morphology. Three different groups can be identified based on genome type and size. The first corresponds to RNA phages, which can present double-stranded or single-stranded genomes[6]. The second group is made up by phages with small (usually less than 10 kb) single-stranded DNA genomes. The last group is composed of phages with double-stranded DNA genomes that range from 30 kb up to more than 200 kb. Phages with potential for clinical applications often belong to this group, and more specifically to the class *Caudoviricetes*[7]. *Caudoviricetes*, or tailed phages, are non-enveloped phages made up of an icosahedral head and a tail. The head is a structure composed of repeating subunits made up of different proteins. It encloses a single molecule of linear, double stranded DNA. The tail is a protein tube made up of a helix or stacked discs, which usually incorporates spikes, base plates and/or fibers at the distal end (figure 1a). Some phages, in addition, present collar-like structures at the intersection of the head and the tail. Differences in the morphology of the tail were in the past the main criterion to distinguish between the families of viruses that make up this order. More recently, genome sequencing and phylogenetic analysis have allowed to identify other differences between the different species. The taxonomy of phages is nowadays regularly updated; in 2022,

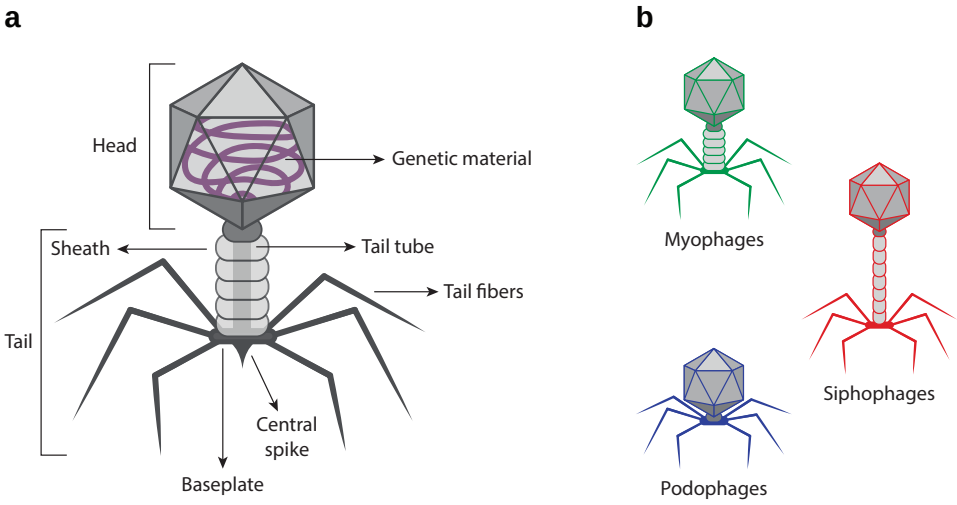

Figure 1: Schematic representation of the morphology of tailed phages. a) Simplified overview of the parts comprising a tailed phage capsid. The head contains the genetic material, which is double-stranded DNA. The tail is made up of a protein tube through which the DNA travels when the phage infects its host. In the case of myophages, the tube is covered by a contractile protein sheath. Attached to the tube can be a baseplate, as well as spikes or fibers in charge of recognizing the receptors on the bacterial surface. b) Different morphotypes of tailed phages. Myophages are characterized by a contractile tail, siphophages present a long, non-contractile tail, and podophages have a short, non-contractile tail.

862 new species and well as several other taxons were created or redefined[8]. Nonetheless, the characteristics of the main different tailed phages morphotypes are still worth highlighting: siphoviruses are characterized by long non-contractile tails, podoviruses present short non-contractile tails, and myoviruses have long contractile tails (figure 1b).

The structures on the terminal end of the phage tail recognize receptors on the surface of bacteria, thereby enabling adsorption of the phage. These receptors are peptide sequences or polysaccharides present on the bacterial surface, and can vary depending on the type of host bacteria. Bacteria can be classified into two main groups, depending on the composition of their cell envelopes: Gram-positive bacteria and Gram-negative bacteria[9]. Gram-positive bacteria have a thick peptidoglycan cell wall which covers an inner membrane. Gram-negative bacteria also have a cell wall and inner membrane, but their peptidoglycan layer is thinner

and they present an additional outer membrane surrounding it. Phages targeting Gram-positive bacteria often recognize receptors found in the cell wall, such as peptidoglycan wall teichoic acids (WTA)[10]. In contrast, many phages targeting Gram-negative bacteria bind to receptors on the outer membrane, like lipopolysaccharide (LPS)[11]. In addition to this, phages can recognize other protruding structures such as capsules, pili or flagella[12]. Phage binding can be either reversible or irreversible, and in some cases, it involves multiple receptors. Generally, phages recognize host receptors with a great degree of specificity, meaning that the host range of a certain phage is often limited. Adsorption of the virion is followed by ejection of the genetic material into the host cytoplasm. Viral peptidoglycan hydrolases partially digest the cell wall and the tail tube, through which the DNA travels, penetrates into the bacterium[13]. Some phages eject their DNA in a stepwise manner, being transcription of a certain segment of viral DNA necessary for the rest of the DNA to be taken up[14]. The capsid of the virion remains outside, although some proteins may be ejected together with the genetic material.

If the infecting tailed phage is virulent, the infection will follow a lytic cycle[15] (figure 2). In this case, the phage hijacks the bacteria's cellular machinery, using it to express its own genes and proteins. For this purpose, some phages rely on the bacterial RNA polymerase, although some others encode their own[16]. In addition, some phages can degrade the host chromosome[17]. The lytic cycle culminates with the expression of late genes, which encode structural proteins of the capsid and genes necessary for bacterial host lysis. This ultimately leads to the production of more viral particles that will eventually be released to infect other neighboring cells. The event of host lysis is highly regulated and requires the presence of two proteins: holins and endolysins[18]. Holins accumulate at the membrane until they reach a high enough concentration, at which point they cause a localized lipid depletion in the inner membrane. The consequence of this is that phage endolysins can reach the peptidoglycan and degrade it, triggering bacterial lysis[19]. In the case of Gram negative bacteria, the release of phage progeny requires a third kind of protein, called spanin, which fuses the outer membrane to the inner membrane[20]. The time that the phage takes from the moment it infects until it causes host lysis is known as the latent period. The latent period and the burst size, or number of

virions produced by infected cell, are dependent on the phage species, as well as resource availability[21].

On the other hand, if the infecting phage is temperate, it may also follow a lysogenic cycle (figure 2). In this case, the viral genome persists within the host cell, either introduced in the bacterial chromosome as a prophage or outside of it forming a plasmid[22]. Whether a temperate phage will follow a lytic or a lysogenic cycle may be dependent on peptide-based communication between the viruses or on host

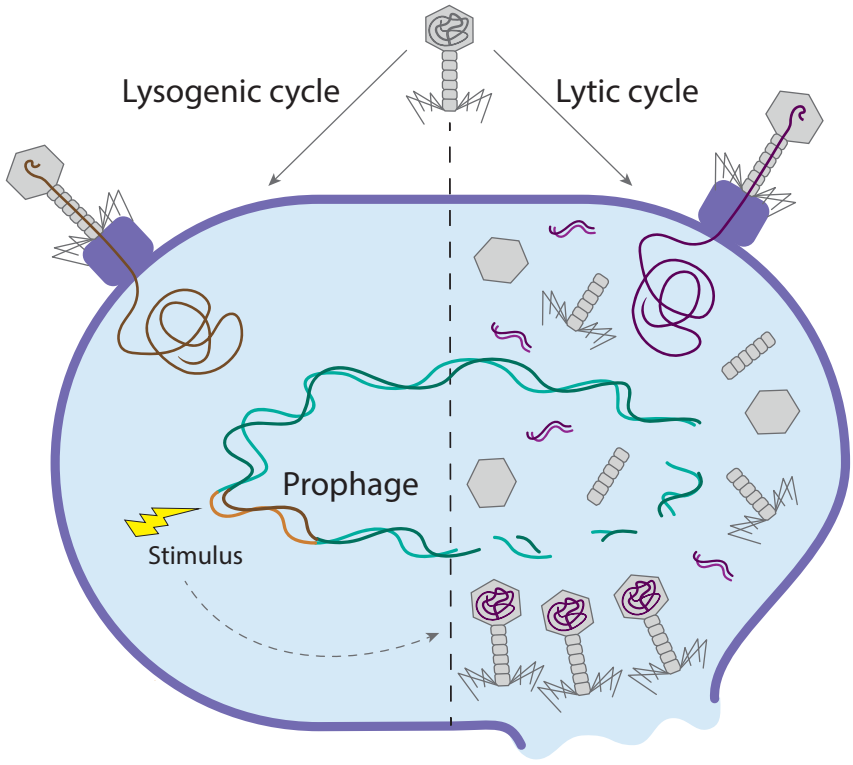

Figure 2: Infection cycles of a phage. Upon infection, temperate phages can follow a lysogenic cycle, where their DNA integrates into the host chromosome as a prophage. Virulent phages follow a lytic cycle. Here, the phage uses the bacterial machinery to replicate its own DNA and translate its proteins, sometimes even degrading the host DNA. After assembly of the new viral progeny is complete, holins and endolysins accumulate at the inner membrane, eventually triggering cell lysis and the release of the new virions. Some phages can follow either cycle depending on the environmental conditions. Prophage excision can also occur in response to different stimuli, after which the phage would transition to a lytic cycle.

repressor genes that form part of a quorum-sensing system[23]. In any case, the density of the bacterial and phage populations at the site of the infection seems to be a determining factor in the lytic or lysogenic fate of the temperate phage. Phages used for therapeutic applications are almost exclusively virulent, as temperate phages can raise concerns regarding horizontal gene transfer amongst their host bacteria[24].

Bacteriophage therapy

The use of phages to treat persistent infections is hardly a recent idea. Despite possibly being the most ubiquitous and abundant organisms on Earth, the discovery of phages did not take place until the beginning of the 20th century. It was F. Twort[25] and F. d'Herelle[26] who independently reported about what we now know as bacteriophages for the first time. The term, coined by d'Herelle himself, means "bacteria eaters". The "invisible microbes" that he described in 1917 had been isolated from stools of patients who had recovered from an infection caused by *Shigella dysenteriae*. This inspired d'Herelle to propose phages as agents for combating bacterial infections in humans. From then on, phages began to be used to treat infections ranging from intestinal diseases like cholera to skin or eye infections[27,28].

The initial interest in phages as potential agents for treatment of bacterial infections faded following the discovery of penicillin two decades later[29]. The complex biology of these organisms, together with limited technical resources to assess their activity, made it difficult to design and implement effective phage therapy approaches. Furthermore, antibiotics offered a more straightforward alternative, as they could be used to target a broader range of bacteria and were easier to produce and administer. Nevertheless, phage therapy still continued to be popular in the former Soviet republics, especially in Georgia. Here, phages continued to be used in clinical practice to treat, for instance, infected wounds and several forms of intestinal infections. In addition, they have been extensively studied up to this date in specific institutions such as the Eliava institute (Tbilisi, Georgia)[30]. As a result,

the use of phage cocktails as medicines is currently common in Georgia and other former Soviet republics like Russia, where they are even available commercially in pharmacies. Still, the decades of experience accumulated by scientists in these countries have not had a big repercussion on the research carried out in Western countries. This is partly due to the inaccessibility of the information, which was predominantly recorded in Russian[31].

In the present time, we are facing an enormous global challenge as the so-called antibiotic crisis continues to become more and more worrisome. In 2015, the World Health Organization issued a "Global action plan on antimicrobial resistance" to try to take steps to tackle the problem. This general state of alarm has once again revitalized the interest in phages. Up to this date, there are already many reports indicating the potential of phages as anti-bacterial treatment. A notorious example of this is the case reported by Schooley *et al.* in 2017, in which a widespread multi-drug resistant *Acinetobacter baumannii* infection was successfully overcome using a personalized phage cocktail previously tested against bacteria isolates from the patient[32]. Phages may also contribute to the beneficial effects of fecal microbiota transplantations, or even of sterile fecal filtrate transfers. A study published by Ott *et al.* in 2017 described how this approach was able to eradicate *Clostridium difficile* infections for a period of at least six months, corresponding with changes not only in the gut microbiome but also in the virome of these patients[33]. Phage therapy can also be successful as an adjuvant to antibiotic treatment, as illustrated by a recent case report in which this combination was used to treat a persistent infection in a toddler, ultimately enabling him to receive a much needed liver transplantation[34]. In a recent pre-print outlining the retrospective analysis of one hundred phage therapy interventions performed in Belgium, Pirnay *et al.* illustrate how using phages as a personalized medicine can increase the chances of therapeutic success in cases where the standard of care is failing[35]. However, to fully evaluate the effectivity of phages, clinical studies should be carried out with proper controls comparing phages to placebo treatments in different patient groups. Although some clinical trials have been carried out which provide evidence of the safety of phages, their efficacy in these settings remains disputed[36]. Even so, phages appear to be a promising aid in the battle against multi-resistant bacteria.

Still phage therapy has to overcome a number of challenges before it can become fully practiced in Western countries. First of all, current health regulations strictly limit the use of phage therapy medicinal products. This is largely due to the lack of clinical evidence for their use and the limited availability of registered phage products. Efforts are being carried out in different countries to implement the use of phages as magistral preparations, put together by a pharmacist in response to the particular needs of each patient[37]. These preparations could also be used to perform research in the form of clinical trials[38]. However, the implementation of this method is not straightforward, as a certain infrastructure is required to produce GMP-like phage products. Nonetheless, the development of phage therapy strategies is encouraged by organizations such as the European Medicines Agency (EMA), which approved in October 2023 a new chapter in the European Pharmacopeia to regulate the use of phages as veterinary medicines[39].

The success of phage therapy can be influenced by many factors. As phages are foreign particles, the human immune system may recognize them and react against them in a way that could potentially limit their activity. Furthermore, phages are locked in an evolutionary arms race with bacteria, in which bacteria have developed multiple mechanisms to oppose the attack of phages. The bacterial immune system, both innate and adaptive via the action of, for example, CRISPR-Cas, can target and neutralize invading phages[40]. In addition, in phage-sensitive bacterial population the appearance of insensitive mutants can take place within hours[41]. Nevertheless, mutations in bacteria in response to a phage challenge may come with a fitness trade-off [42]. If these mutations occur in bacterial surface proteins that play a role in antibiotic resistance, like efflux pumps, porins or toxin secretion systems, they may lead to bacterial mutants that regain antibiotic susceptibility or lose virulence. In any case, our current knowledge about phages and bacteria and the way they interact is significantly richer than it was in the early years of phage therapy. This means that it is not far-fetched to conceive a future where the successful treatment of MDR bacterial infections through phage therapy will be achieved. Still, it is vital to keep delving deeper into the fundamentals of phage biology and to develop new techniques to better assess the activity of phages against pathogens.

Pseudomonas aeruginosa

Pseudomonas aeruginosa is a Gram-negative, non-fermenting, rod-shaped flagellated bacterium of the class Gammaproteobacteria. It is an opportunistic pathogen that causes infections primarily in immunocompromised individuals, or in patients with pre-existing conditions like cystic fibrosis or severe burn wounds. *P. aeruginosa* thrives in moist environments and can also survive in conditions of low oxygen. Because of this, it is often found in hospital settings and is a prevalent cause of nosocomial infections, that can range from superficial skin infections to endocarditis, pneumonia, or sepsis. These infections are often complicated by the fact that *P. aeruginosa* can be resistant to a large number of antibiotics. The bacteria present characteristics such as low membrane permeability and a high expression of efflux pumps that make them intrinsically resistant to certain antibiotics. In addition, in chronic infections hypermutable populations are often present, which can rapidly develop resistance to multiple antimicrobials[43]. Diversity in *P. aeruginosa* genomes is also heavily driven by horizontal gene transfer, which can influence both antibiotic resistance and the expression of virulence factors[44]. Another element that complicates treatment is that *P. aeruginosa* tends to form biofilms on surfaces that it colonizes. Biofilms are communities of bacteria that exist embedded in a matrix of extracellular polymers. In these structures, bacteria are physically shielded from antimicrobials. In addition, persister cells, or antibiotic-tolerant cells with temporarily halted metabolism, may be more likely to appear in a biofilm[45]. All these characteristics make *P. aeruginosa* a very good candidate for treatment with phage therapy, as conventional antibiotics are often insufficient to clear persistent infections[46].

The relationship of *P. aeruginosa* with phages, and especially with temperate phages and prophages, plays an important role in the pathogenic presentations of this bacterium. The diversity and abundance of prophages harbored in *P. aeruginosa* genomes is very high[47]. Prophage drive evolution in bacteria and can be sources of genes encoding for virulence or fitness-associated factors. These genes are sometimes named “morons”, as they add “more on” the genome of the prophage and by extension of its host bacteria[48]. Morons help bacteria adapt to

their environment and overcome different selective pressures, enabling a symbiotic relationship between bacterial lysogens and their prophages. Yet this is not the only way in which *P. aeruginosa* benefits from its interactions with temperate phages. A filamentous phage, Pf4, can promote the survival of *P. aeruginosa* in harsh environments by producing a liquid crystalline structure that encapsulates the bacteria[49]. Nonetheless, the relationship between bacteria and phages is not always peaceful. Like any other bacteria, *P. aeruginosa* is in constant battle against virulent (or lytic) phages, as evidence by the high number of anti-phage defense systems found in clinical isolates[50].

Phages in the human body

The human body is a reservoir for bacteria, and in most cases, wherever bacteria are present phages can be expected. This is clear from the presence of prophages in strains isolated from different locations in human body, such as the vagina or respiratory tract[51]. In addition to prophages, virulent phages also coexist with us, even occupying different niches within our organism. For example, skin has been found to contain a strong core phageome[52], which could contribute to the prevention of disease caused by bacteria such as *Propionibacterium acnes*[53]. Most of the human phageome can be found in the intestinal tract. A study of the phageomes of individuals from around the world revealed the existence of a healthy gut phageome, where around 62% of the healthy participants shared a set of core phages[54]. In contrast, the prevalence of these phages in the genomes of ulcerative colitis and Crohn's disease was reduced to 42% and 54% respectively. Similarly, the phageome of patients suffering from type II diabetes or childhood obesity was seen to be significantly different and more diverse than that of healthy individuals[55,56]. These observations suggest that the composition of the gut phageome is an important indicator of human health. Additionally, research suggests that phages act as an immune barrier in the gut, where phages accumulate at the intestinal mucosal layer and execute a policing function against invading bacteria[57]. From there, phages can also cross the gut epithelial layer by transcytosis[4], reaching the bloodstream, lymph and organs.

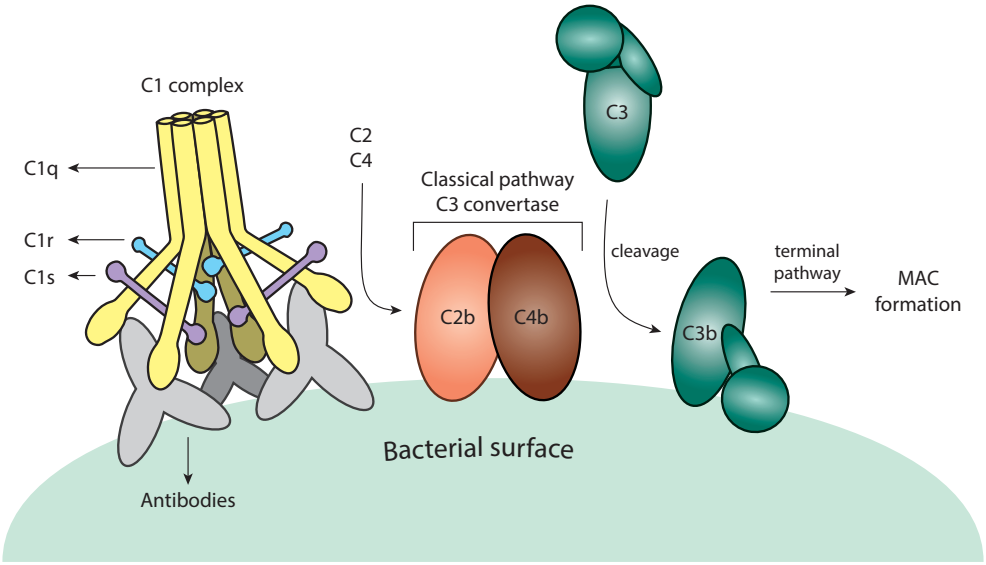

Figure 3: Schematic representation of the early stages of the classical complement pathway. The C1 complex, made up of C1q and two copies each of C1r and C1s, recognizes antibodies on the surface of pathogens. Through its proteolytic activity, C1 cleaves C2 and C4. The fragments C2b and C4b deposit on the surface to form the C3 convertase (C4b2b). This complex cleaves C3, inducing a conformational change in the subunit C3b which allows it to also bind to the surface. After this, downstream events are triggered in the cascade, eventually leading to the formation of the membrane attack complex (MAC).

Once phages are inside the human body, they can interact with the immune system. The immune system protects against pathogens by differentiating between self and non-self, either through recognition by the innate immune system or specific recognition of antigens by the adaptive immune system[58]. One of the first barriers posed by the innate immune system is the complement system. The complement system is composed of a series of more than 30 proteins that circulate in plasma. Upon recognition of pathogens, the complement system is activated, which leads to the proteolytic cleavage of complement proteins. This can happen through three different pathways: the classical pathway is dependent on recognition of the pathogen by antibodies, the alternative pathway relies on spontaneous hydrolysis of complement C3, and the lectin pathway works through the recognition of surface carbohydrates[59]. In the classical pathway, the first step is the binding of the C1 complex, composed of C1q, C1r and C1s (figure 3). All pathways converge in the

formation of a C3 convertase that catalyzes the cleavage of C3, thereby triggering C3b deposition on the surface of the pathogen. Complement components like C4b and C3b can act as opsonins, making the pathogen more readily recognized by immune cells and therefore susceptible to phagocytosis. In the case of some Gram-negative bacteria, complement can lead to direct killing via the formation of the membrane attack complex (MAC). The complement system can also target viruses in different ways. Viral neutralization by complement can be achieved through opsonization, MAC formation on infected cells or directly on the virion[60]. Recognition of C3b labeled viruses can also trigger their uptake by cells, after which intracellular sensing of C3 leads to a pro-inflammatory response and to degradation of the viruses by the proteasome[61]. Although most of the knowledge on interactions between phages and complement is focused on eukaryotic viruses, inactivation of phages by complement has also been described for *E. coli* phage T4[62]. On the other hand, it seems like in some cases the complement system and phages could work together in clearing bacterial infections, as described by Abd El Aziz *et al.* in a study carried out on mice infected with *P. aeruginosa*[63].

Another function of the complement system is to release chemoattractants, which recruit cellular components of the immune system, like neutrophils, macrophages and dendritic cells, to the site of infection. These cells recognize pathogen associated molecular patterns (PAMPs) through the action of Toll-like receptors (TLRs). Recognition by TLRs can result in further signaling inside and outside the cell through the release of cytokines. This could happen in response to phages, as double-stranded RNA (dsRNA) derived from phages can activate receptors on peripheral blood mononuclear cells (PMBCs), inducing the production of different cytokines[64]. In another instance, phages infecting members of the bacterial genera *Lactobacillus*, *Escherichia*, and *Bacteroides* were seen to stimulate IFN-gamma production by interacting with TLR-9[65]. Immune cells, and particularly macrophages, can also remove phages from circulation via phagocytosis[66].

Recognition of pathogens (or phages) by dendritic cells can also cause the immune system to transition to an adaptive response. Dendritic cells process and present antigens via MHC class II, subsequently activating CD4+ (helper) or CD8+ (cytotoxic)

T-cells in the lymph nodes. Helper T-cells will in turn activate B-cells, causing them to start producing antibodies. Evidence of the interactions of phages with T-cells is sparse, but the humoral response (antibody-based) against phages has been more studied in phage therapy settings and is better understood. The induction of IgM and IgG against bacteriophage ϕX-174 after primary, secondary and tertiary administration was described already more than 30 years ago[67]. Antibodies against phages of, for instance, *Staphylococcus aureus*[68] or *P. aeruginosa*[69] can also be found in the sera of healthy individuals. Some of these antibodies could potentially neutralize the phage infectivity. To test this, as well as complement-dependent neutralization, Dąbrowska *et al.* (2014) immunized mice with proteins from the capsid of phage T4. The resulting sera of mice immunized with major head protein (gp23*) and highly antigenic outer capsid protein (Hoc) contained specific IgG and IgM antibodies and inhibited phage activity[70]. Interestingly, active complement further boosted phage inactivation in this study. In clinical settings, inactivation of phages administered as part of treatment has also been described[71,72]. All in all, the interactions of phages with the human immune system are multi-faceted, and may be different for each individual patient.

Aim of this thesis

In this thesis, we approach phage biology from different angles to try to understand the factors that may influence the success of phage therapy. Our aim was to provide tools to facilitate research in the context of phage therapy, as well as to generate knowledge to help develop phage therapy applications against *P. aeruginosa*. We have developed and validated a set of rapid, high-throughput assays for monitoring phage activity *in vitro*. Next, we have applied these assays to study the influence of the complement system on phage infection. Here, we have identified that the complement system can inhibit some, but not all, phages, highlighting the need for thorough phage screenings in physiological conditions. Furthermore, we have investigated the synergistic potential of phages and antibiotics, showing that certain antibiotic classes may be more efficacious than others in boosting phage activity. Finally, we discuss the mechanisms behind anti-phage defense and resistance and

their relevance in a clinical context. The work presented in this thesis constitutes a rational take on phage therapy and may contribute to further improving phage therapy strategies.

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Chapter 2

Monitoring phage-induced lysis of Gram-negatives in real time using a fluorescent DNA dye

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Abstract

Bacteriophages (phages) are viruses that specifically attack bacteria. Their use as therapeutics, which constitutes a promising alternative to antibiotics, heavily relies on selecting effective lytic phages against the pathogen of interest. Current selection techniques are laborious and do not allow for direct visualization of phage infection dynamics. Here, we present a method that circumvents these limitations. It can be scaled for high-throughput and permits monitoring of the phage infection in real time via a fluorescence signal readout. This is achieved through the use of a membrane-impermeant nucleic acid dye that stains the DNA of damaged or lysed bacteria and new phage progeny. We have tested the method on *Pseudomonas aeruginosa* and *Klebsiella pneumoniae* and show that an increase in fluorescence reflects phage-mediated killing. This is confirmed by other techniques including spot tests, colony plating, flow cytometry and metabolic activity measurements. Furthermore, we illustrate how our method may be used to compare the activity of different phages and to screen the susceptibility of clinical isolates to phage. Altogether, we present a fast, reliable way of selecting phages against Gram-negative bacteria, which may be valuable in optimizing the process of selecting phages for therapeutic use.

Introduction

Bacteriophage (phage) therapy has recently regained interest as an alternative to antibiotics, due to the increased prevalence of multidrug resistant bacteria and the limited availability of new antibacterial compounds[1,2]. This strategy is based on using phages, viral predators of bacteria, to treat bacterial infections in patients[3]. Phages are the most abundant biological entities on the planet, and they are immensely varied[4]. This diversity can constitute an advantage when searching for a phage to target a certain pathogen. On the other hand, phages have a narrow host range and often only infect a single species or even a specific strain. This means that therapeutic preparations must be tailored to specifically target the causative pathogen. Therefore, therapeutic phage preparations generally consist of a cocktail of different phages, with the aim of broadening the host range and overcoming any resistance that might arise[5,6]. Overall, selecting the optimal mix of phages for anti-bacterial therapy can be a challenging task. Hence, it is important to have methodologies that allow us to compare the activity of different phages.

Phages can follow different types of infection cycles. Some, referred to as temperate phages, can lysogenize the bacterial host, meaning that they are able to integrate their genome into the bacterial chromosome[7]. In contrast, phages used in therapy are often obligately lytic[8]. These phages first inject their genetic material into the host and then hijack the host's cellular machinery to replicate and assemble into new viral particles. After enough progeny has been produced, the new phages will burst out of the host cell, causing it to lyse.

In phage therapy, an important step is the identification of phages that are able to infect the pathogen of interest. Traditionally, this is performed via a plaque assay[9], in which phage suspensions are spotted onto a bacterial lawn[10]. Where the phages are causing bacterial growth inhibition or lysis, a clear area, named plaque, appears in the bacterial lawn. Another variation of this method, known as double-layer agar (DLA) assay, consists in mixing bacteria with phages in soft agar and overlaying that on a solid agar plate[11]. In both cases, the number of infective phages can be determined as plaque forming units by counting the single plaques that are obtained.

These classical methods, while broadly accepted and inexpensive, cannot look at the dynamics of phage infection, and only provide results after overnight incubation. Furthermore, they are difficult to scale up for high-throughput screening, and they are not always precise or reproducible[12].

In an effort to overcome these limitations, different ways to assess killing by bacteriophages have been described[13]. One of these is to measure the optical density of a bacterial culture and study how it changes over time as the phage infection progresses[14,15]. While this type of assay does allow for real-time monitoring of the infection, it is an indirect determination of bacterial damage since it only measures turbidity of the bacterial culture. Approaches based on bacterial respiration have also been explored[16,17]. Another assay that may be used is a one-step growth curve. In this technique, bacteria and phages are incubated together and aliquots of free phage, taken at different time-points, are plated on double-layer agar containing the host bacteria[18]. This assay is considered a golden standard when it comes to characterizing phages. It can be used to determine their latent period (time to generate new progeny), and the number of new phages produced per bacterium (burst size). However, this assay is very labor intensive, and, like the DLA assay, it is difficult to scale up. Therefore, it seems like complementing these assays with new high-throughput methods could aid the selection of phages for therapeutic use.

With this study, we aimed to develop a robust assay to monitor phage infections in real time in a multi-well plate setting. We use Sytox green, a membrane-impermeant nucleic acid dye that stains DNA of lysed bacteria and new phage progeny, producing a fluorescent signal as phage infection progresses. This tool has been extensively used to evaluate viability in bacteria[19], but, to our knowledge, never to monitor killing by phages. Here, we show this application on *Pseudomonas aeruginosa* and *Klebsiella pneumoniae*, two ESKAPE pathogens against which phage therapy is particularly relevant[20,21]. We demonstrate that the developed assay correlates with standard assays, such as plaque assays and bacterial viability on plate, and propose a way of optimizing the method for phage screening in a clinical setting.

Results

Signal increase in fluorescent DNA dye assay reflects phage-mediated killing

In order to measure phage infection in real time and in a high-throughput manner, we developed a fluorescence-based assay using the dye Sytox green. This dye cannot permeate through intact bacterial membranes, but it can enter both damaged bacteria and the capsid of non-enveloped bacteriophages (figure 1a)[19,22]. Thus, as a phage infection takes place, Sytox green will bind to free DNA, DNA of lysed or damaged bacteria, and phage DNA, emitting a strong fluorescent signal. The assay, hereafter referred to as fluorescent DNA dye assay, can be performed in a microplate reader.

To confirm the potential application of our fluorescent DNA dye assay for monitoring phage-induced damage, we looked at the infection of *P. aeruginosa* strain PAO1 by the strictly virulent myophage PB1. Phage PB1 was first identified by Bradley in 1966[23] and it gives name to a very widely spread genus of phages infecting *P. aeruginosa*, Pbunavirus[24,25]. Some PB1-like viruses, like phage 14-1, have been included in phage therapy cocktails, making Pbunavirus an interesting genus of phages to assess in our assay[5].

The assay was set up by mixing the host bacteria with phage PB1 and the DNA dye Sytox green (figure 1a). The phage preparation used here was pre-treated with DNase and RNase to reduce background staining. The fluorescence intensity of the dye was monitored over time to assess the progression of the infection process. We used bacteria incubated with heat-inactivated PB1 and bacteria alone as a control. When measuring over time a sigmoid curve was obtained for the conditions containing active phages (figure 1b, figure S1). Both control conditions showed no increase in fluorescence, indicating that the signal obtained in the conditions with active phages is specific to phage-induced damage. For active PB1 at MOIs of both 10 and 1 we observe a latent period of approximately 60 minutes, corresponding to the time it takes for new phage progeny to be produced until the new virions cause host lysis. After this time, the fluorescence intensity signal starts steadily increasing until it reaches a plateau after 100 minutes. The height of this plateau

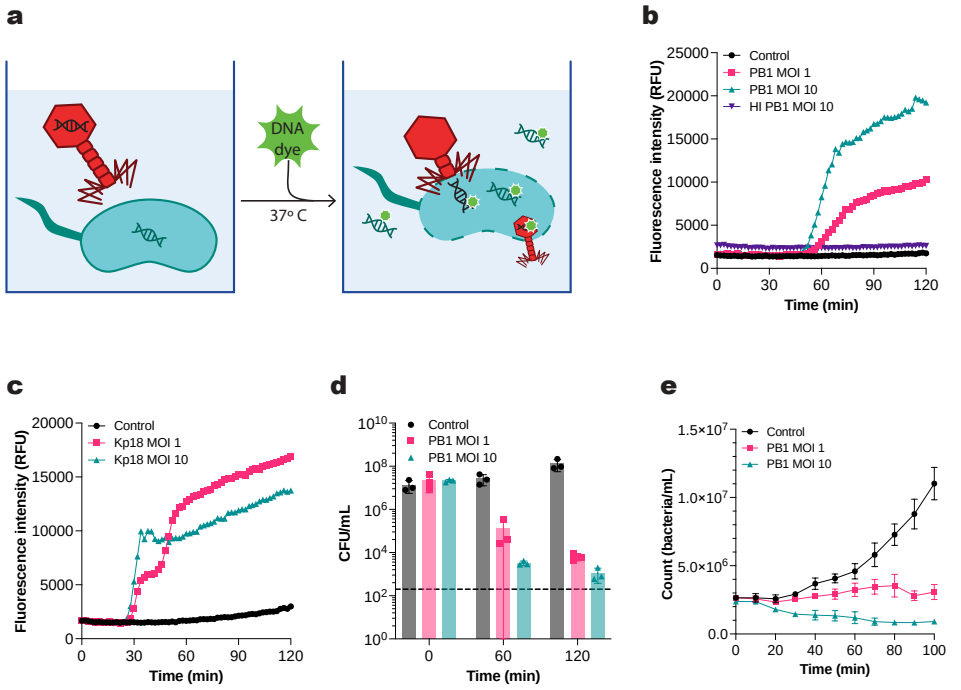

Figure 1. Fluorescent DNA dye assay detects phage-mediated killing. a) Phage infection can be monitored in a 96-well plate setting using a membrane-impermeant DNA dye. The dye is added to a plate containing a phage-infected bacterial culture, which is then incubated at 37°C. As the infection progresses, Sytox green will bind to phage DNA, DNA of damaged bacteria, and free bacterial DNA. The fluorescent signal of the DNA dye is measured over time. b) Fluorescence intensity over time of *P. aeruginosa* infected with phage PB1 at an MOI of 1 and 10. Control are uninfected bacteria. Bacteria infected with heat-inactivated (HI) PB1 at an MOI of 10 are shown as a further control. c) Fluorescence intensity over time of *K. pneumoniae* infected with phage Kp18 at an MOI of 1 and 10. Control are uninfected bacteria. d) *P. aeruginosa* infected with phage PB1 at an MOI of 1 (pink) and 10 (green), or not infected (control, black) were plated at the initial time-point (0 minutes), after 60 minutes and after 120 minutes. Number of recovered colonies per plated volume is expressed as colony forming units per mL (cfu/mL). e) Bacterial concentration (count/mL) of a GFP-positive *P. aeruginosa* population in the first 100 minutes after infection with phage PB1 at an MOI of 1 or 10, as determined by means of flow cytometry. The concentration of an uninfected population is shown as control. b,c) A representative graph of at least three independent experiments is shown. d,e) Data represent mean ± SD of three independent experiments.

was lower when using an MOI of 1. Although the dye Sytox green can bind to phage DNA, it was not seen to have any effect on the infectivity of phage PB1 (figure S2). Similar results were obtained when performing the assay on a clinical strain of *K.*

pneumoniae with podophage vB_KpP_FBKp18 (ϕKp18) of the *Autographiviridae* family (figure 1c, figure S3, figure S4), indicating the applicability of the assay to other Gram-negatives. However, in this case, a second increase in fluorescent signal can be observed with an MOI of 1 after the first plateau is reached, which could correspond to a second cycle of phage infection.

Next, we assessed whether the increase in fluorescent signal reflects killing of bacteria. We incubated bacteria with phages under the same conditions as for the fluorescent DNA dye assay and evaluated their viability by colony forming units at different time points (figure 1d). We could detect that, for both MOIs tested, phages caused a reduction in viability already after 60 minutes, coinciding with the initial increase in fluorescence intensity (figure 1b). However, it should be noted that the assessment of colony formation could show confounding results in this case, as phage infection will be ongoing during the overnight incubation. As a further confirmation, we assessed the concentration of intact bacteria in an infected culture of *P. aeruginosa* (PAO1) in real time by means of flow cytometry (figure 1e). To do so, we inoculated a culture of bacteria expressing green fluorescent protein (GFP) with phage PB1 at different MOIs, after which we measured aliquots at different time-points. Here, we could observe that the concentration of bacteria infected with an MOI of 10 was reduced to half. In addition, up to 20% of the bacteria remaining showed membrane damage, as indicated by Sytox blue influx (figure S5). By comparison, the control population increased steadily over the assessed period of time and remained impermeable to Sytox. These observations indicate that an increase in DNA dye fluorescence indeed reflects bacterial killing.

Staining of bacterial DNA drives signal increase in fluorescent DNA dye assay

As Sytox green can stain intact phages, the total signal might correspond not only to the DNA of damaged or lysed bacteria but also to newly produced phages. Therefore, we estimated how much the DNA of newly produced phages contributes to the fluorescent signal. We first determined the burst size of phage PB1, defined as the amount of new virions produced from each single infected bacterium[26]. This parameter can be obtained from a one-step growth curve, as described by

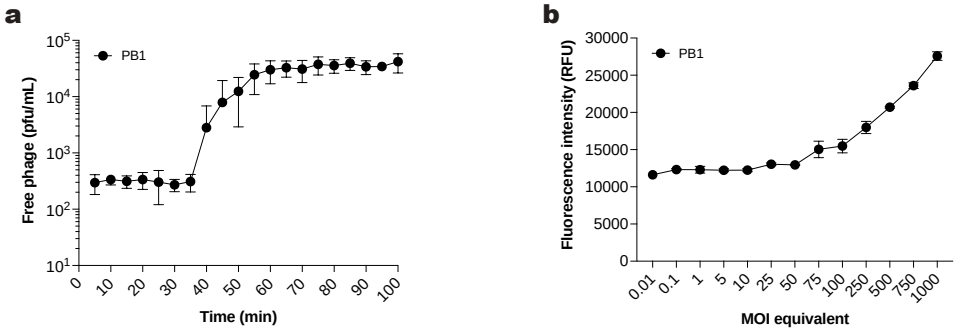

Figure 2. Staining of phage DNA is not the main driver of signal increase in the fluorescent DNA dye assay. a) One-step growth curve of phage PB1. Concentration of phage is expressed as plaque-forming units per mL (pfu/mL). Data represent mean ± SD of three independent experiments. b) Sytox green fluorescence intensity of phage PB1 at different concentrations. The background signal of the buffer is depicted as Bg. Data represent mean ± SD of three technical replicates.

Kropinski (2018)[18]. A one-step growth curve looks at the amount of free infective virions produced during the first cycle of phage replication after bacteria are infected. We obtained the one-step growth curve for PB1 (figure 2a) and based on this we calculated a burst size of 165 virions per bacterium (figure S6). In the fluorescent DNA dye assay, the initial bacterial concentration is of 2 x 10^7 bacteria/mL. Therefore, in an ideal situation where all bacteria lyse after the first infection cycle, the maximum concentration of phages obtained would be 3.3 x 10^9 PFU/mL.

We then checked how much fluorescent signal would derive from staining phages in a similar concentration. Using Sytox green, we stained phage PB1 at several different concentrations between 10^8 PFU/mL and 10^{10} PFU/mL and measured fluorescence intensity (figure 2b). Fluorescence intensity increased with phage concentration, indicating that Sytox green indeed stains encapsidated phage DNA. However, the phage-derived signal was at most three-fold higher than the background signal for all the concentrations assayed. In contrast, the maximum signal obtained in the fluorescent DNA dye assay after 2 hours when bacteria are present is almost ten-fold higher than the background (figure 1b). This shows that the main contributor to the fluorescence signal in our assay is stained bacterial DNA (figure S7), and not DNA of newly produced phages.

Fluorescent DNA dye signal correlates with a loss of bacterial metabolic activity

To check if an increase in fluorescence signal corresponds to loss of bacterial viability in real time, we compared the results obtained in the fluorescent DNA dye assay with metabolic activity measurements. For this, we used *P. aeruginosa* with genomically integrated *lux* reporter genes[27]. These bacteria express the luciferase operon constitutively, and thus produce a luminescent signal if they are viable and metabolically active. The *lux* system, much like the fluorescent dye assay, allows for monitoring the state of the bacteria in real time by means of a plate reader. In addition, bacteria expressing the *lux* system can also be directly used in the fluorescent DNA dye assay. In this way, fluorescence and luminescence measurements can be obtained from the same well, which we used to further validate our fluorescent DNA dye assay.

We incubated bacteria expressing the *lux* system with phage PB1 at different concentrations in the presence of Sytox green. Fluorescence measurements

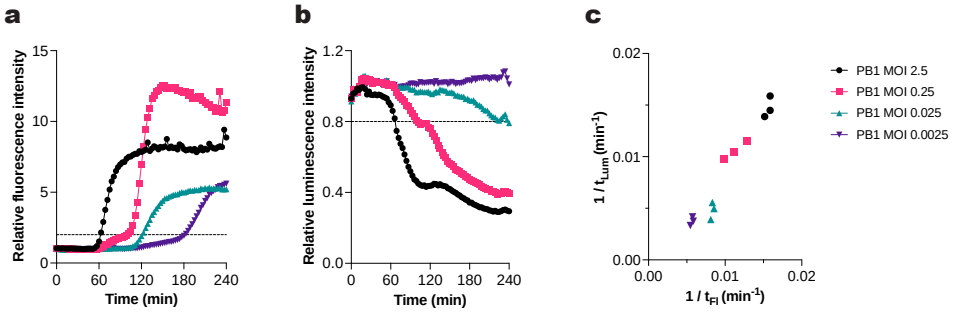

Figure 3. The fluorescent DNA dye signal correlates with a loss of bacterial metabolic activity. PAO1 expressing a luciferase reporter system was incubated with phage PB1 (different MOIs) at 37°C in the presence of Sytox green. a) Sytox green fluorescence intensity was measured using a fluorometer. Values were divided by the control signal (uninfected bacteria) to obtain relative fluorescence intensity. Black dotted line represents the threshold for phage-mediated damage (relative fluorescence = 2). b) Luminescence intensity of samples was measured and divided by the signal of the control. Black dotted line represents threshold for phage-mediated damage (relative luminescence = 0.8). c) Correlation between time to reach phage-mediated damage threshold as determined through fluorescent DNA dye staining and luminescence measurements for phage PB1 at different MOIs (Pearson's r = 0.97, two-tailed P value <0.0001). Data are represented as 1/t, where higher values indicate a faster infection. Data points represent individual results of three independent experiments. a,b) A representative graph of at least three independent experiments is shown.

revealed a concentration-dependent effect, where higher MOIs caused an earlier signal increase (figure 3a, figure S8). Luminescence measurements showed a similar MOI-dependent effect, where the highest MOI of 2.5 achieved a complete reduction of the luminescence signal, while the lower MOIs showed an intermediate effect (figure 3b, figure S9). To more closely compare both signals, we established an arbitrary threshold for an increase in fluorescence and decrease in luminescence. When the signal crosses this threshold, we consider that we are detecting phage-induced damage. Based on this, we analyzed the time at which the phage-induced damage was detected by the fluorescent DNA dye assay (t_{Fl}) and by luminescence measurements (t_{Lum}). For each MOI, we plotted the inverse of t_{Fl} against the inverse of the corresponding t_{Lum}, in such a way that higher values indicate a faster infection (figure 3c). This revealed a clear correlation between both types of measurements. We can thus conclude that a signal increase in the fluorescent DNA dye assay indicates loss of metabolic activity of the host bacteria.

Bacterial metabolic activity correlates with fluorescent DNA dye assay for different *Pseudomonas* phages

After confirming that the fluorescent DNA dye assay correlates with metabolic activity measurements in bacteria infected with PB1, we performed this analysis on a broader set of phages. We selected three additional well-described *Pseudomonas* lytic phages: 14-1[24], LKD16[28] and LUZ19[29]. When comparing all four phages using the fluorescent DNA dye assay, the podophages (LKD16 and LUZ19) caused a more rapid rise in fluorescence than the myophages (PB1 and 14-1) (figure 4a, figure S10). Analysis of bacterial metabolic activity showed similar results (figure 4b, figure S11). Phages 14-1, LKD16 and LUZ19 at an MOI of 2,5 caused metabolic activity to decrease to background levels, but the same was not observed for phage PB1. Lower MOIs of these phages caused slower and less pronounced changes in fluorescence and luminescence (figures S12, S13).

We compared the time at which phage-induced damage was observed across all different conditions (t_{Fl}, t_{Lum}). For the fluorescent DNA dye assay, this revealed that the timing of damage induction depends on the MOI and on the type of phage. At

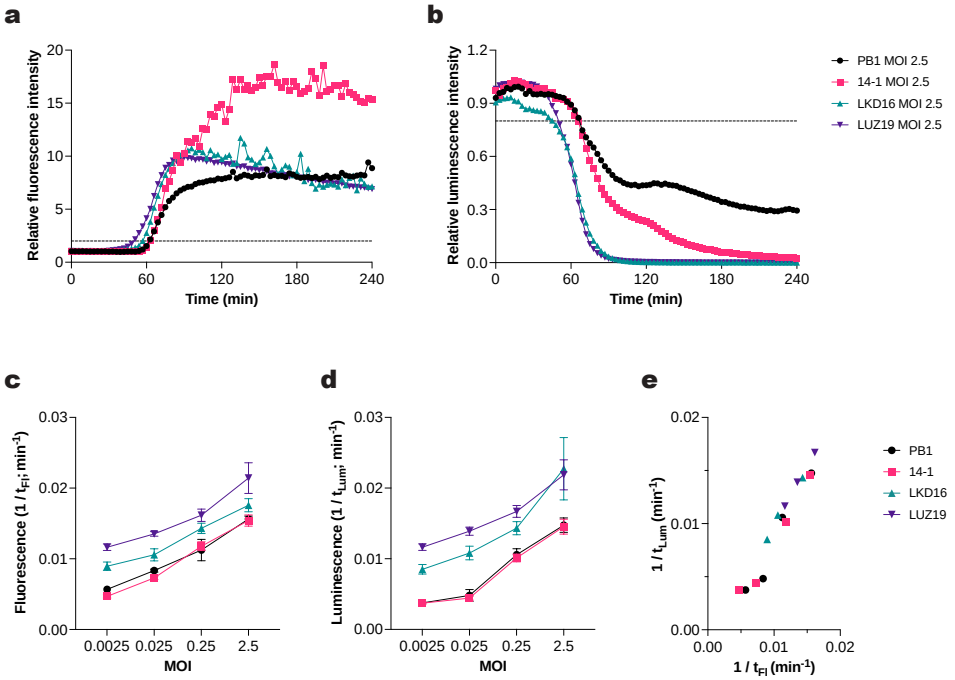

Figure 4. The fluorescent DNA dye assay correlates with bacterial metabolic activity for different *Pseudomonas* phages. PAO1 expressing a luciferase reporter system was incubated with phages PB1, 14-1, LKD16 and LUZ19 in a range of MOIs at 37°C in the presence of Sytox green. a) Sytox green fluorescence intensity was measured using a fluorometer. Values were divided by control signal to obtain relative fluorescence intensity. Black dotted line represents threshold for phage-mediated damage (relative fluorescence = 2). b) Luminescence intensity of samples was measured and divided by signal of the control. Black dotted line represents threshold for phage-mediated damage (relative luminesence = 0.8). c) Time of damage induction of the different phages as detected by the fluorescent DNA dye assay (t_{Fl}). Data are plotted as $1/t_{Fl}$ and represent mean ± SD of three independent experiments. d) Time of damage induction of the different phages as detected by luminescence measurements (t_{Lum}). Data are plotted as $1/t_{Lum}$ and represent mean ± SD of three independent experiments. e) Correlation between t_{Fl} and t_{Lum} for phages PB1, 14-1, LKD16 and LUZ19 at MOIs of 2.5, 0.25, 0.025 and 0.0025 (Pearson's r = 0.97, two-tailed P value <0.0001). Data represent mean of three independent experiments and are plotted as 1/t, where higher values indicate a faster infection. a,b) A representative graph of at least three independent experiments is shown.

higher MOIs, damage was detected faster for all the different phages (figure 4c). The same trend was observed when looking at loss of bacterial viability in terms of luminescence signal production (figure 4d). Therefore, we performed a correlation analysis between the time of phage-induced damage detected by both methods,

including data from the four different phages at five different concentrations (figure 4e). Indeed, we found a significant correlation between these two parameters. This further confirms that an increase in fluorescence is related to a loss in bacterial metabolic activity. In addition, this analysis showcases the differences in kinetics between the four phages tested. Altogether, both methods appear to be suitable for assessing and comparing virulence of different phages.

Fluorescent DNA dye assay facilitates susceptibility profiling of clinical isolates

Given the suitability of the fluorescent DNA dye assay for monitoring phage infections in bacteria, we analyzed if this method is suited to screen clinical isolates for susceptibility to different phages. To standardize the assay and make it more suited to a diagnostics laboratory workflow, we switched from using an overnight bacterial culture to using bacteria re-suspended directly from plate. This modification removes the need for overnight incubation and reduces the time needed to carry out the assay.

We then determined the susceptibility of a panel of 21 clinical *P. aeruginosa* isolates to different phages. These strains were obtained from patients suffering from chronic infections. We tested 5 phages of different taxa and targeting different receptors: 2 myophages targeting LPS (PB1 and 14-1)[24] and 3 podophages targeting type 4 pili (LKD16, LUZ19 and PAXYB1)[28–30]. For each of the strains, we monitored phage-induced damage over the course of 4 hours (figures 5a, S14) and analyzed the time of phage-induced damage (t_{FI}). Based on this parameter, we defined phage sensitivity as $1/(t_{FI})$ x 1000 (figure 5c). We compared these results with a screening based on plaque assays (figures 5b, S15). Here, we spotted a high concentration of each of the phages on clinical strains to determine sensitivity in a coarse way. We scored phage sensitivity in a binary way depending on whether plaques were obtained (figure 5d).

For 95 out of the 110 phage-bacteria combinations tested (86%) both approaches yielded a similar result, where plaque formation corresponded to a positive fluorescent signal. No clear relationship was found between the timing of the fluorescent signal

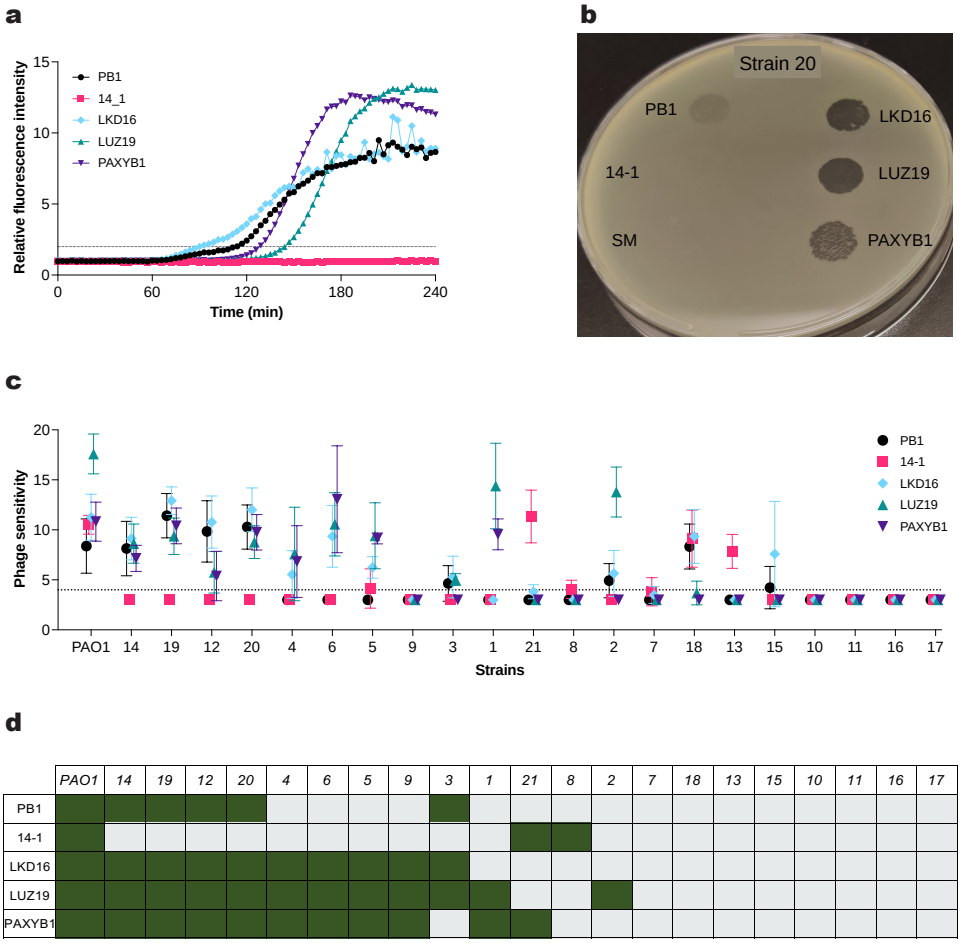

Figure 5: Phage susceptibility profiling of several *P. aeruginosa* clinical strains. a) Strain 20 of the panel was incubated with phages PB1, 14-1, LKD16, LUZ19 or PAXYB1 at an MOI of 1 at 37°C in the presence of Sytox green. Fluorescence intensity was measured using a fluorometer, and values were divided by control signal to obtain relative fluorescence intensity. Black dotted line represents threshold for phage-mediated damage (relative fluorescence = 2). A representative graph of at least three independent experiments is shown. b) Strain 20 of the panel was inoculated into top agar and overlayed on a plate. Phages PB1, 14-1, LKD16, LUZ19 and PAXYB1 (106 pfu/mL, 5 mL) and SM buffer (5 mL) were spotted to detect plaque formation after overnight incubation at 37°C. c) Summary of the susceptibility of *P. aeruginosa* strains to 5 different phages found with the fluorescent DNA dye assay. Phage sensitivity was defined as 1/time of damage * 1000 (min^{-1}). A higher value reflects faster induction of phage-mediated damage. Black dotted line represents time detection limit in this assay (240 minutes). Data represent mean ± SD of three independent experiments. d) Summary of the susceptibility to 5 different phages determined through plaque assays. Green indicates sensitivity to the phage (plaques were found), gray indicates no sensitivity (no plaques or barely detectable).

and the morphology or number of plaques. For instance, strain 20 seemed more quickly damaged by phage PB1 than by phage LUZ19 in the fluorescent DNA dye assay. However, this strain presented more turbid plaques for phage PB1 than for phage LUZ19. In addition, some discrepancies were found between both assays. Strains 2, 8, 13 and 15 showed an evident increase in fluorescent signal for several phages which did not seem infective in the plaque assays. Strikingly, the opposite effect was found for strain 9, which showed plaques for all 3 podophages and no increase in fluorescent signal. Another interesting observation is that phages with the same receptor were not necessarily able to infect the same strains, as seen for example for strain 1 (sensitive to LUZ19 and PAXYB1, but not LKD16) and strain 21 (sensitive to 14-1, but not to PB1). In conclusion, both assays could detect differences in the phage susceptibility profile of the clinical strains, producing matching but not completely overlapping results.

Discussion

Finding suitable phages for specific clinical isolates can be a bottleneck in the process of administering phage therapy. Traditional methods, such as the DLA technique, are inefficient and offer very limited information regarding how the phage infection progresses. To circumvent this $OD_{600\ nm}$ measurements are often used. Although these can be collected in high-throughput and in real time, they do not directly measure damage and can therefore be difficult to interpret[13]. Other fast methods, such as the OmniLog™ system and surface plasmon resonance have been adapted to monitor phage infections, but these require specific equipment, making it more challenging to implement their use[31,32]. In sum, there is still room for improvement in methods for determining phage sensitivity, as there is currently no clear standard.

The present study tackles this problem by showing a way in which the DNA dye Sytox green can be used to monitor a lytic phage infection in real time. The application of DNA dyes as a marker for cell death is widely accepted, both for eukaryotic and prokaryotic cells[33]. One example in which this dye has been used in bacteria is to

evaluate damage to Gram-negative outer membranes caused by the human innate immune system, especially the complement system[34]. This situation, however, is different to phage-mediated killing in that with the latter membrane damage is expected to occur from within, even leading to violent lysis[35]. Nonetheless, our results indicate that Sytox green can also have a valuable application for detecting phage-induced damage. A comparable finding was reported by Harhala *et al.*, where Sytox green was used to effectively evaluate bacteriolytic activity of phage endolysins[36], but this had not yet been shown for whole phage particles. Similarly, other nucleic acid dyes like SYBR gold and Syto 13 have been used to stain and detect phages and phage-infected bacteria, although these studies did not focus on assessing infection dynamics[37,38].

We have shown that the fluorescent DNA dye assay presented here correlates with colony plating assays and measurements with bacteria expressing a *lux* reporter. Furthermore, we consider that our assay complements classical phage assessment methods in several aspects. First of all, it can follow the infection process from the moment bacteria and phages come in contact, although at low MOIs the resolution of the assay might not be sufficient to detect the first infection cycles. By monitoring phage-induced damage in time, infection dynamics of different phages can be compared. This information could potentially be useful for selecting phages for therapeutic cocktails, although further characterization of the phages would be necessary. Combining phages with different infection strategies may increase effectiveness[39] and prevent development of phage resistance[40]. Other methods such as OD600 measurements also provide insights into the early stages of the infection process, but in some cases turbidity measurements are confounded by the optical density of bacterial debris and can be difficult to interpret. A one-step growth curve of the phage does provide more accurate information on the burst size, latent phase, and duration of a single infectious cycle, but this technique is not suited for testing different MOIs or different medium compositions. In addition, one-step growth curves are time-consuming, need to be repeated several times to obtain accurate representations, and can be performed for only a few phages at a time. Other plating-based methods where bacteria are evaluated after an overnight incubation, such as DLA, provide a more downstream readout than

assays performed over time. Given that most phages have a latent period of less than an hour, it is crucial to observe the initial stages of phage-bacteria interactions. Furthermore, plating-based assays make it difficult to compare multiple conditions or phages simultaneously, and require considerable time and effort. Contrary to this, our method does not require labor intensive preparations and can be performed in medium to high-throughput, while ensuring that the assay conditions remain similar for all the phages being screened.

A limitation of the fluorescent DNA dye assay is that phage preparations need to be purified to a certain extent before using them in this assay; at least treated to remove nucleic acid remains. In addition, this system might not be suited to assessing infection by temperate phages. Phages that degrade host DNA, such as T4[41], could also be difficult to assess. Nonetheless, the assay worked well for phage LUZ19, also known to partially degrade the host chromosome[29], at least in the MOIs tested here. The fact that Sytox green can stain the DNA of non-enveloped phages is also a potential limitation[22]. However, our results show that the fluorescent signal that we obtain corresponds mainly to bacterial DNA. In addition, both membrane damage and release of new phage progeny are direct results of a productive infection. Therefore, the assay successfully monitors phage-induced damage regardless of whether the staining corresponds to phage or bacterial DNA. Still, it is difficult to interpret what the height of the fluorescent signal means in this assay, as a higher signal does not indicate more damage induction. For example, a population that is killed more slowly will continue dividing, thus accumulating more DNA and leading to a higher signal ultimately than one that is killed rapidly. This makes it challenging to estimate with this assay whether a whole population of bacteria has been killed. Nonetheless, the fluorescent DNA dye method gives clear information for phage therapy screening purposes, while more specialized assays can be used complementarily to determine the effect of a given phage on an entire bacterial population. To this effect, the *lux* system presented in the study can be particularly useful. Here, a total loss of luminescence is indicative of a total reduction in the bacterial population. While this system presents the disadvantage of requiring genetically modified bacteria, its use in combination with the fluorescence DNA dye assay is valuable when more precise characterization of a particular phage is

needed.

We envision the fluorescent DNA dye assay as a tool to be used in clinical settings such as a diagnostic microbiology laboratory. Currently, there is no common accepted framework or pipeline to characterize and select phages for therapy, although some approaches have been suggested[42,43]. This, together with other factors such as stringent regulations, hampers the development of good phage therapy strategies[44]. Because of this, we believe it is worthwhile to optimize a high-throughput phage screening assay in a way that can fit into a diagnostic laboratory standard workflow. We showed that the DNA dye assay can be performed within 4 hours of incubation and thereby fits into standard diagnostic laboratory workflow. Using this method on clinical isolates revealed differences in their susceptibility profile to 5 different phages. The results obtained here were largely in agreement with plaque assays performed with the same phage-bacteria combinations. These were carried out by spotting a high number of PFU, and differences in sensitivity between strains were not quantified but rather expressed as a positive or negative result. In this analysis, plaque assays of isolates that were only partially sensitive to a certain phage were difficult to interpret, due to the appearance of ambiguous, turbid plaques, a phenomenon more often described in literature[45]. In addition, certain discrepancies were observed between both methods. In some cases, we could detect dye influx in bacteria incubated with a phage that did not form plaques on that strain, while in other cases the opposite effect was observed. This might be due to the high presence of phage defense systems in *P. aeruginosa* clinical isolates[46]. We hypothesize that certain abortive infection mechanisms might induce membrane permeabilization, potentially leading to dye influx but no plaque formation[47]. Conversely, high concentrations of phage may still form plaques on bacteria presenting other abortive infection mechanisms that would not cause a signal increase in the fluorescent dye assay[48]. In any case, further testing with a range of phage concentrations should be carried out in the cases where discrepancies are found to rule out possible false positive or false negative results. Another interesting observation is that strains sensitive to a given phage were not necessarily sensitive to another with the same receptor, further hinting at potential defense mechanisms at play. This highlights the need to focus on phage defense systems in addition to

host range when selecting phages for therapeutic use[49].

In summary, the fluorescent DNA dye assay that we present here represents a valid alternative to other existing methods and can offer additional information on how different phages behave. Moving forward, assays like this may be used in clinical settings to screen for suitable phages for personalized therapeutic approaches, although they would require significant validation before clinical use. The conditions used in this assay are easily adjustable, which would open up the possibility of including other factors in the equation, such as for instance patient serum or antibiotics. These prospects further encourage exploring new methods to develop more successful phage therapy strategies.

Materials and methods

Phages and strains

Stocks of *Pseudomonas* phages PB1, 14-1, LKD16 and LUZ19 were kindly provided by Rob Lavigne (KU Leuven). *Pseudomonas* phage PAXYB1 was obtained from the Fagenbank (Delft, Netherlands). Klebsiella phage ϕKp18 was isolated from sewage water using *K. pneumoniae* L0549, a clinical isolate from UMCU, using an enrichment procedure as previously described[50]. Phage amplification was carried out by infecting the host strain (PAO1 for the *Pseudomonas* phages, L0549 for ϕKp18) overnight at 37°C in Lysogeny Broth (LB). Bacterial debris was removed by centrifugation at 11000 RCF for 40 minutes at 4°C. Then, phages were incubated for 2 hours on ice with a solution of 10% PEG-8000 and 0.5 M NaCl and precipitated by centrifugation at 11000 RCF for 40 minutes at 4°C. The preparation was mixed with chloroform, after which the aqueous phase was sterilized using a 0.2 µm filter and incubated with DNase and RNase (5 µg/mL each) for 30 minutes at room temperature. Final purity was achieved by filtration through a Zeba Spin desalting column (40K MWCO, Thermo Fisher Scientific). PAO1 expressing GFP (PAO1 GFP+), encoded by plasmid pSMC21, was kindly provided by Jeffrey Beekman. PAO1 expressing the *lux* system (PAO1-lux) was generated by transforming PAO1 with plasmid pTNS3 together with a plasmid encoding luxABCDE (pUC18-mini-Tn7T-Gm-lux)[51]. Clinical *P. aeruginosa*

strains were obtained from the UMCU diagnostic microbiology laboratory strain collection and were isolated from patients with an invasive infection.

Transmission electron microscopy

One mL of ϕKp18 phage lysate at a concentration higher than 10^9 PFU/mL was sedimented by centrifugation at 21,000 × *g* for 1h, washed and re-suspended in 1 mL of MilliQ water. Then, 3.5 µl of the phage were deposited and incubated for 1 min on TEM grids (Carbon Type-B 400 mesh, TED PELLA). The grids were washed three times with 40 µl of MilliQ water and stained with 3.5 µl of 2% (w/v) of uranyl acetate (pH 4.0) for 30 s. Grids were imaged using a JEM-1400 plus (JEOL) TEM.

Bacteriophage host range

Ten-fold serial dilutions of the phages were spotted onto DLA plates of *K. pneumoniae* clinical strains L0506, L0549, K6310, L923, K6453, K6592, and K6500 (UMCU) and ATCC 11296. The plates were incubated overnight at 37°C and the phage plaques observed to distinguish productive infection and lysis from without[52].

Bacteriophage and bacteria genome sequencing

Bacterial DNA was extracted using the GeneJet Genomic DNA Purification kit (Thermo Fisher) and fragmented by Covaris 55 µL series Ultrasonicator. The DNA fragments were used to construct paired-end libraries with an insert size of 200-400 bp, and sequenced on the BGISEQ-500 (MGI, BGI-Shenzhen, China) platform. 1.4-2.0 Gb of sequencing data were generated for each sample with a sequencing depth of >100x. Quality control of the raw data was performed using FastP[53] and Soapnuke[54] with default parameters, and the reads were trimmed and processed using Seqtk[55]. The filtered reads were assembled into the final genomes with SPAdes v3.13.0[56]. The capsular type of the *K. pneumoniae* strains was determined using Kaptive v0.7.3[57,58] and the sequence type was determined using MLST 2.0[59]. DNA of ϕKp18 was extracted using phenol-chloroform, and fragmented by Covaris 55 µL series Ultrasonicator. The DNA fragments were used to construct DNA nanoball (DNB)-based libraries by rolling circle replication, and sequenced using the BGI MGISEQ-2000 platform (BGI Shenzhen) with paired-end 100 nt strategy. This generated 4.6-19.2 Gb of sequencing data with a sequencing depth of >10,000x.

Bacteriophage genome annotation

Open reading frames (ORFs) of the ϕKp18 genome were predicted and automatically annotated using RAST sever v2.0[60]. Additional putative functions were assigned to ORFs by BlastP v.2.10.0[61] and Hmmer v3.3.1[62]. Domains identified by Hmmer were included as 'Notes' in the annotation files. Schematics of phage genomes were built with the Linear Genomic Plot tool available at CTP Galaxy (https://cpt.tamu.edu/galaxy-pub).

Microplate reader assays

Bacteria (PAO1 or PAO1-lux) were grown to mid-log phase (OD_{600nm} ~ 0.5) in LB, pelleted and resuspended to an OD_{600nm} of 1.0 (~ 8 x 10^8 bacteria/mL) in RPMI 1640 (ThermoFisher) supplemented with 0.05% human serum albumin (HSA), then diluted 20-fold. Phages in SM buffer (100 mM NaCl, 8 mM $MgSO_4 \cdot 7H_2O$, 50 mM Tris-Cl) were incubated with 10 μM Sytox green Nucleic Acid stain (ThermoFisher) at room temperature for up to 15 minutes. Equal volumes of bacteria and phages were then mixed obtaining a final concentration of ~ 2 x 10^7 bacteria/mL bacteria and 5 μM Sytox green. Concentration of phages is dependent on the MOI, which is indicated for each experiment. Fluorescence and luminescence measurements were performed in a microplate reader (CLARIOstar, Labtech) at 37°C. Assays where only fluorescence was measured were performed in a clear, flat-bottom 96-well plate (Corning), with the following settings: $\lambda_{excitation}$ = 490 nm, bandwidth = 14 nm; $\lambda_{emission}$ = 537 nm, bandwidth 30 nm; gain = 1300. Assays were both fluorescence and luminescence were measured were performed in white opaque 96-well plates (Corning), with the following settings: $\lambda_{excitation}$ = 490 nm, bandwidth = 14 nm; $\lambda_{emission}$ = 537 nm, bandwidth 30 nm; fluorescence gain = 1000; luminescence gain = 3600. For screening clinical isolates, the protocol was slightly modified. In this case, bacteria from a fresh blood agar plate were re-suspended in saline to a McFarland standard of ~ 2, and then diluted 1:20 in RPMI in a 96-well plate. Sytox green (final concentration: 5 μM) and phages (final concentration: 2 x 10^7 PFU/mL) were added before measuring in a microplate reader as described above. The screening was performed three times; additional replicates are displayed in the supplementary materials (figure S16).

Determination of bacterial viability

Bacteria (PAO1) were grown to mid-log phase (OD_{600nm} ~ 0.5) in LB, pelleted and resuspended to an OD_{600nm} of 1.0 (~ 8 x 10^8 bacteria/mL) in RPMI 1640 with 0.05% HSA. Bacteria and phages were mixed, adjusting the final concentration of bacteria to 2 x 10^7 bacteria/mL. The phage concentration was determined according to the desired MOI. The mixture was incubated at 37°C with shaking for 120 minutes. Samples were taken at t = 0 min, t = 60 min and t = 120 min. Serial dilutions were performed in RPMI 1640 with 0.05% HSA. 5 µL of each sample was plated on LB agar and incubated overnight at 37°C. Colonies were counted, and the cfu/mL was calculated.

Flow cytometry

Bacteria (PAO1 GFP+) were grown to mid-log phase (OD_{600nm} ~ 0.5) in LB, pelleted and resuspended to an OD_{600nm} of 1.0 (~ 8 x 10^8 bacteria/mL) in RPMI 1640 with 0.05% HSA, then diluted 20-fold. Phages in SM buffer (concentration adjusted to the corresponding MOI) were mixed with bacteria in equal volumes. Sytox blue was added in a final concentration of 5 µM. The mixture was allowed to incubate at 37°C with shaking. Samples were taken at the specified time-points and diluted 10-fold in RPMI 1640 with 0.05% HSA before being analyzed. Flow cytometry was performed using the MACSQuant (Miltenyi biotech) by measuring the number of events in 10 uL of sample. Bacteria were gated based on GFP signal and forward scatters. The data were analyzed in FlowJo.

One-step growth curve

One-step growth curves were conducted according to the method described by Kropinski[18]. Briefly, bacteria (PAO1) were grown to mid-log phase (OD_{600nm} ~ 0.5) in LB. Phage was prepared at 5 x 10^6 pfu/mL in SM buffer. Phages were added 1:100 to the PAO1 culture and allowed to adsorb for 5 minutes at 37°C with constant shaking. The mixture was diluted 1:100 in LB in flask A, which was then diluted 1:10 in LB in flask B, followed by a further 1:10 dilution in LB in flask C. 500 µL from flask A were added to 25 µL of ice-cold chloroform, vortexed and kept on ice to act as the adsorption control. 100 µL samples were taken at 5-minute intervals from the appropriate flask (flask A in minutes 5-40, flask B in minutes 25-80, flask C in

minutes 65-100), added to top agar along with 2 drops of an overnight culture of PAO1 and poured onto LB agar plates. After overnight incubation at 37°C, plaques were enumerated, and pfu/mL was determined.

Plaque assay

Bacteria were grown to mid-log phase (OD_{600nm} ~ 0.5) in LB. Top agar was prepared by mixing equal parts of LB broth and melted LB agar. After adjusting the temperature to 56°C, 3 mL of top agar was mixed with 200 µL of bacterial culture and poured onto a pre-warmed LB agar plate to solidify. Phage suspensions in SM buffer (5 µL, 10^6 PFU/mL) were spotted onto the solid top agar. SM buffer (5 µL) was spotted as a negative control. Plates were incubated overnight at 37°C.

Data analysis and statistical testing

Data visualization and statistical analyses were performed in GraphPad Prism 9 and are further specified in the figure legends.

Data availability

The datasets generated and analyzed during this study are presented in the supplementary materials. Raw data and assembled genomes of the *K. pneumonia* clinical strains and phage ϕKp18 are available from Genbank, Bioproject PRJNA745534.

Additional information

Conflicts of interest

The authors declare no competing interests.

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Author contributions

J.E.E., S.H.M.R., B.W.B. and P.J.H. conceived and designed the study. J.E.E., C.T.B., A.R.C. and B.W.B. performed experiments. J.E.E., C.T.B. and A.R.C. analyzed the data and prepared the figures. A.R.C. and S.J.J.B. contributed materials. J.E.E., C.T.B., A.R.C., S.H.M.R., B.W.B. and P.J.H. wrote the manuscript. All authors discussed the results and read and approved the submitted version.

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Supplementary information

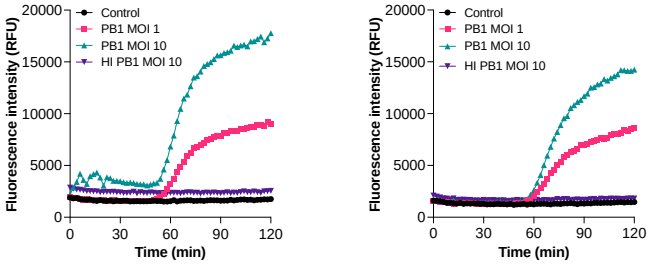


Figure S1. Fluorescence intensity over time of *P. aeruginosa* infected with phage PB1 at an MOI of 1 and 10. Control are uninfected bacteria. Bacteria infected with heat-inactivated (HI) PB1 at an MOI of 10 are shown as a further control. Two additional replicates to figure 1b are shown.

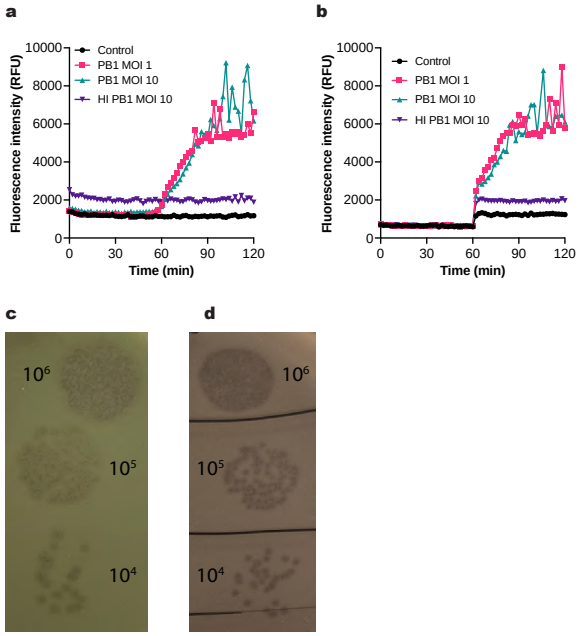


Figure S2. Infectivity of phage PB1 is not affected by Sytox green. a,b) Fluorescence intensity over time of *P. aeruginosa* infected with phage PB1 at an MOI of 1 and 10. Control are uninfected bacteria. Bacteria infected with heat-inactivated (HI) PB1 at an MOI of 10 are shown as a further control. Sytox green (5 μM) was added prior to measuring (a) or after 60 minutes (b). c,d) Spot tests performed with PB1 incubated for 2 hours in absence (c) or presence (d) of Sytox green (5 μM). Estimated phage concentrations in pfu/mL are indicated.

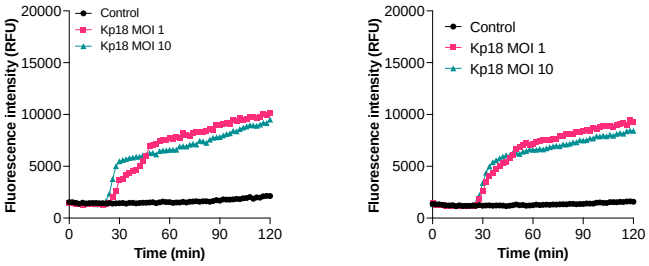


Figure S1. Fluorescence intensity over time of *P. aeruginosa* infected with phage PB1 at an MOI of 1 and 10. Control are uninfected bacteria. Bacteria infected with heat-inactivated (HI) PB1 at an MOI of 10 are shown as a further control. Two additional replicates to figure 1b are shown.

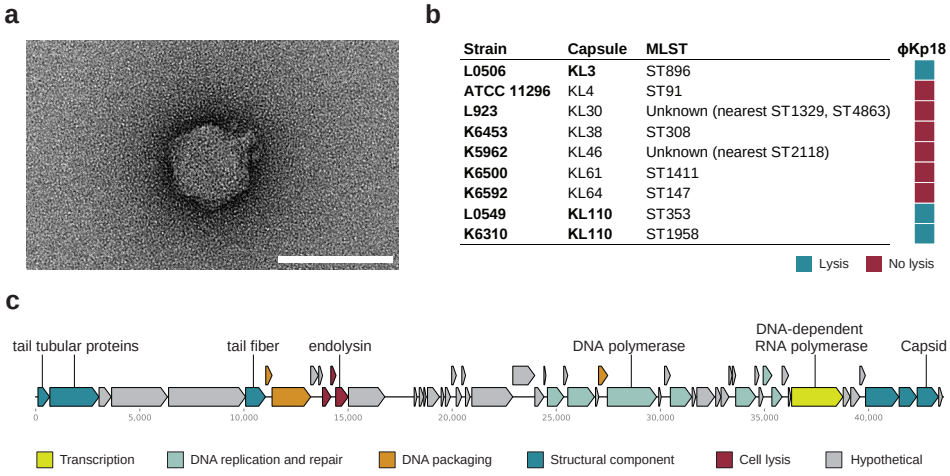


Figure S4. Characteristics of *Klebsiella* phage vB_KpP_FBKp18. a) Transmission electron microscopy image of ϕKp18 negatively stained with 2% uranyl acetate. Scale bar, 80 nm. Micrograph taken at 200,000x magnification. b) Infectivity of phage ϕKp18 against *K. pneumoniae* strains of different capsular (KL) and multiple sequence locus (MLST) types. c) Linear genome map of ϕKp18. ORFs are colored according to predicted function as shown in the key.

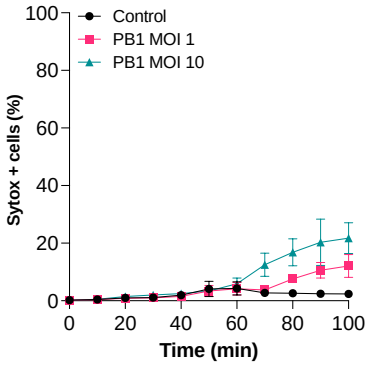
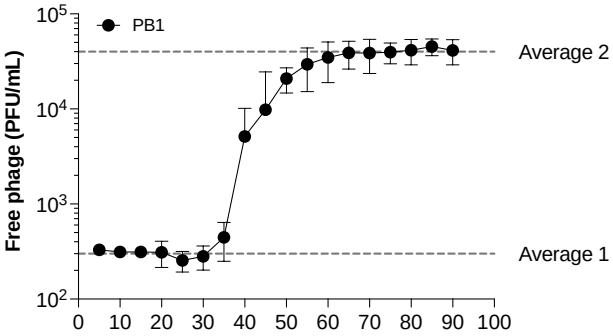


Figure S5. Part of the bacterial population can become permeable to the dye Sytox before lysing as a result of phage infection. Percentage of Sytox blue-positive cells of a GFP-positive *P. aeruginosa* population in the first 100 minutes after infection with phage PB1 at an MOI of 1 or 10, as determined by means of flow cytometry. An uninfected population is shown as control. Data represent mean ± SD of three independent experiments.



Average 1 = 300 PFU/mL

Average 2 = 40017 PFU/mL

Adsorption control (average) = 58 PFU/mL

Burst size = Average 2 / (Average 1 - Adsorption control) = 165

Figure S6. One-step growth curve of phage PB1 and calculation of its burst size, as described by Kropinski (2018)[18].

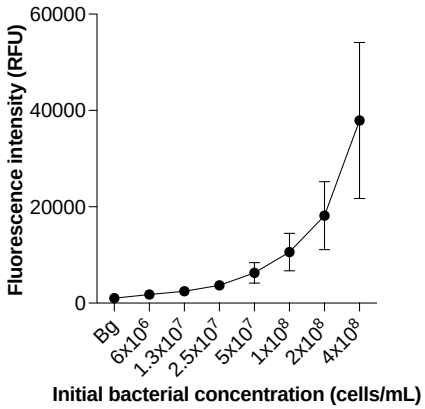


Figure S7. Fluorescence intensity of increasing concentrations of bacterial DNA stained with 5 µM Sytox green is not limited by the dye concentration. Bacteria (PAO1) in medium (RPMI 1640 with 0.05% HAS) at different concentrations were vortexed together with an equal amount of chloroform, then centrifuged shortly at high speed. The aqueous phase containing the bacterial DNA was mixed in a microplate with an equal volume of SM buffer to a final volume of 100 5 µL per well. Sytox green was added in a final concentration of 5 µM. Fluorescence intensity was measured in a microplate reader (CLARIOstar, Labtech) with the following settings: $\lambda_{excitation}$ = 490 nm, bandwidth = 14 nm; $\lambda_{emission}$ = 537 nm, bandwidth 30 nm; gain = 1300. Data represent mean ± SD of three biological replicates.

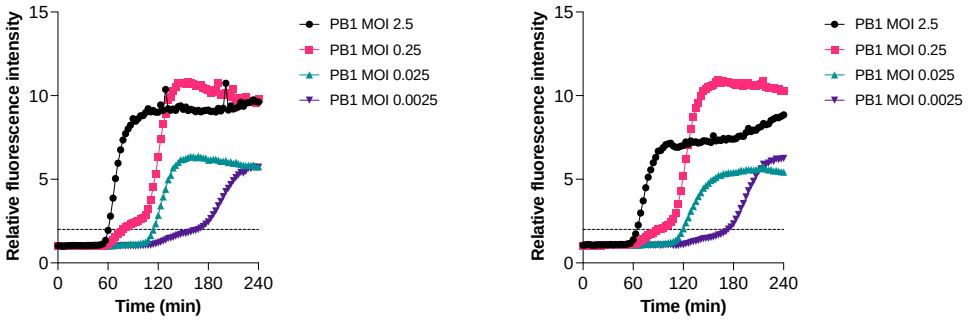


Figure S8. Relative fluorescence intensity over time of PAO1 expressing a luciferase reporter system incubated with phage PB1 (different MOIs) at 37°C in presence of Sytox green. Sytox green fluorescence intensity was measured using a fluorometer. Values were divided by the control signal (uninfected bacteria) to obtain relative fluorescence intensity. Black dotted line represents the threshold for phage-mediated damage (relative fluorescence = 2). Two additional replicates to figure 3a are shown.

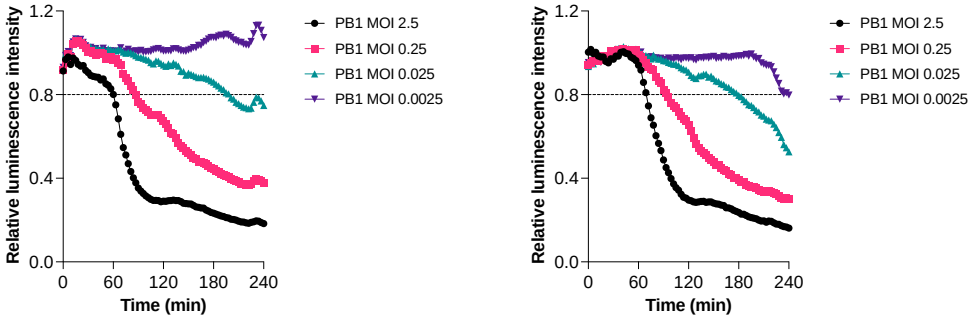


Figure S9. Relative luminescence intensity over time of PAO1 expressing a luciferase reporter system incubated with phage PB1 (different MOIs) at 37°C in presence of Sytox green. Luminescence intensity values were divided by the control signal (uninfected bacteria) to obtain relative luminescence intensity. Black dotted line represents the threshold for phage-mediated damage (relative luminescence = 0.8). Two additional replicates to figure 3a are shown.

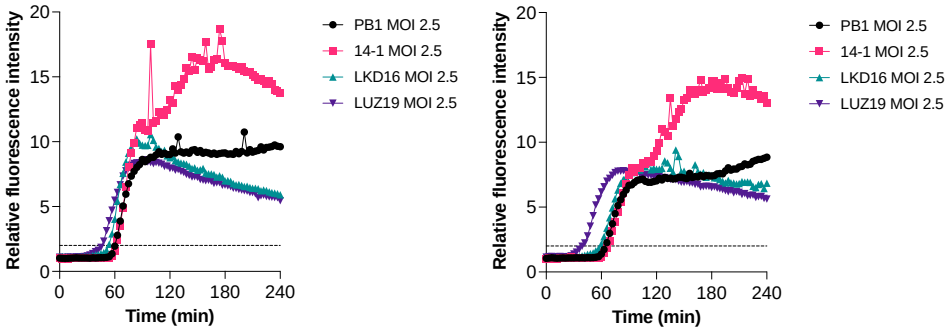


Figure S10. Relative fluorescence intensity over time of PAO1 expressing a luciferase reporter system incubated with phages (MOI = 2.5) at 37°C in presence of Sytox green. Sytox green fluorescence intensity was measured using a fluorometer. Values were divided by the control signal (uninfected bacteria) to obtain relative fluorescence intensity. Black dotted line represents the threshold for phage-mediated damage (relative fluorescence = 2). Two additional replicates to figure 4a are shown.

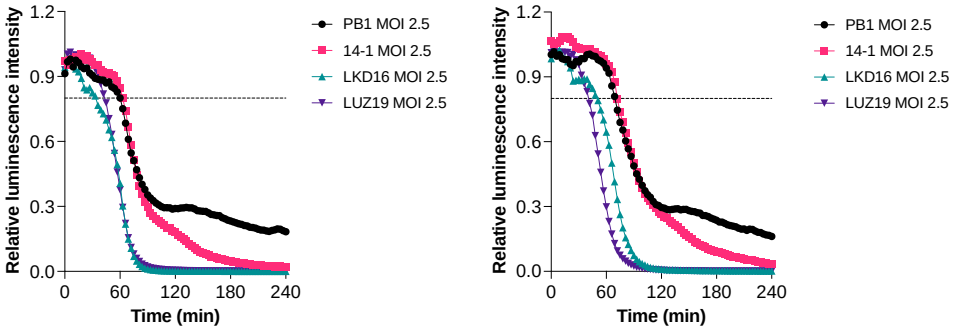


Figure S11. Relative luminescence intensity over time of PAO1 expressing a luciferase reporter system incubated with phages (MOI = 2.5) at 37°C in presence of Sytox green. Luminescence intensity values were divided by the control signal (uninfected bacteria) to obtain relative luminescence intensity. Black dotted line represents the threshold for phage-mediated damage (relative luminescence = 0.8). Two additional replicates to figure 4a are shown.

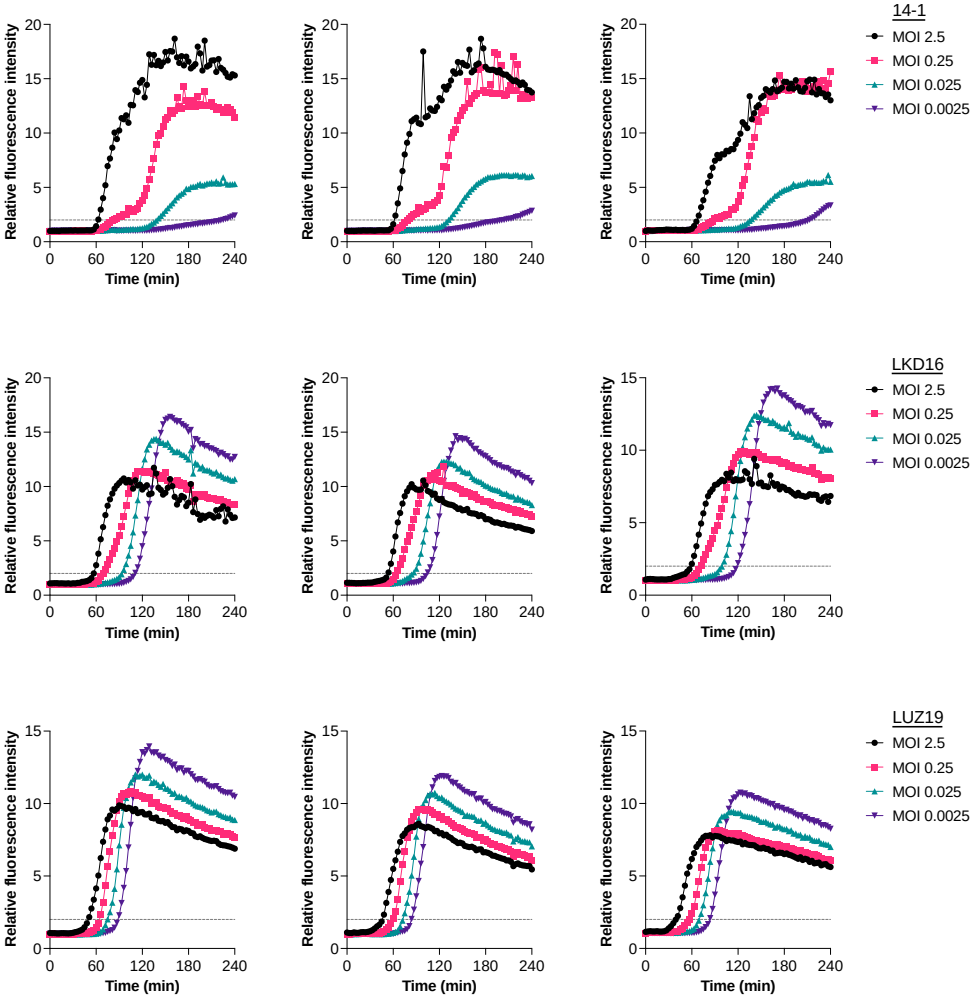


Figure S12. Relative fluorescence intensity over time of PAO1 expressing a luciferase reporter system incubated with phages (different MOIs) at 37°C in presence of Sytox green. Sytox green fluorescence intensity for phages 14-1, LKD16 and LUZ19 was measured using a fluorometer. Values were divided by control signal to obtain relative fluorescence intensity. Black dotted line represents threshold for phage-mediated damage (relative fluorescence = 2). Three independent experiments are shown.

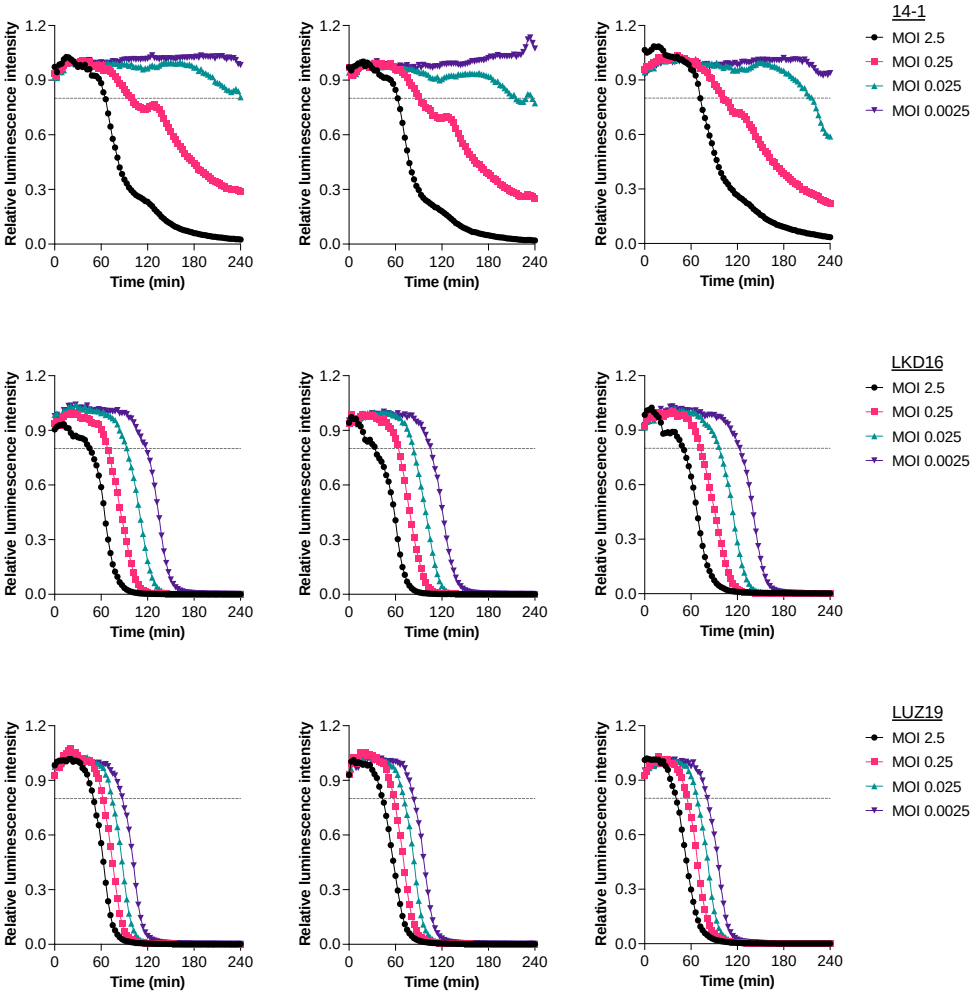
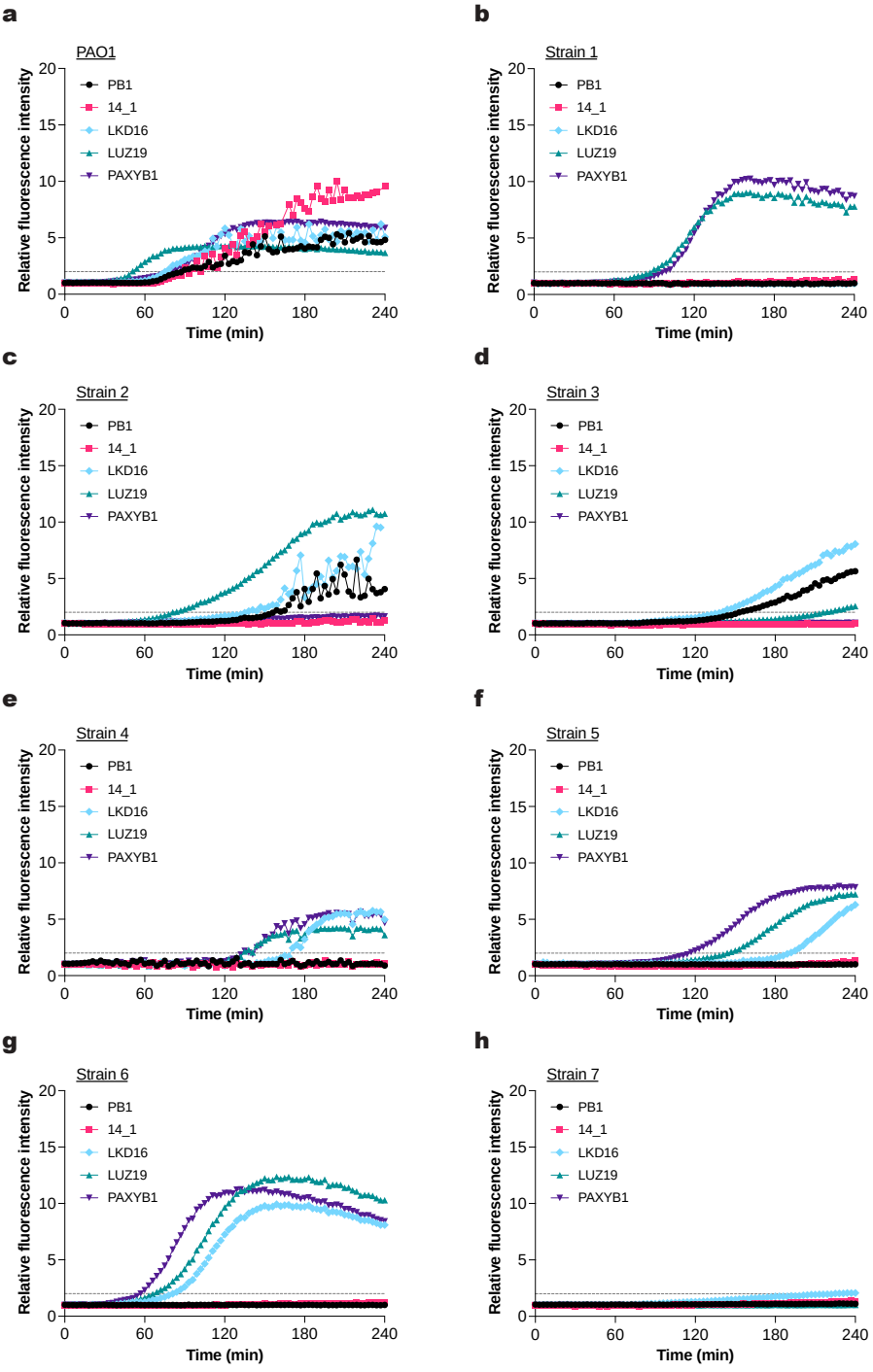


Figure S13. Relative luminescence intensity over time of PAO1 expressing a luciferase reporter system incubated with phages (different MOIs) at 37°C in presence of Sytox green. Luminescence intensity for phages 14-1, LKD16 and LUZ19 was measured using a fluorometer. Values were divided by control signal to obtain relative luminescence intensity. Black dotted line represents threshold for phage-mediated damage (relative fluorescence = 0.8). Three independent experiments are shown.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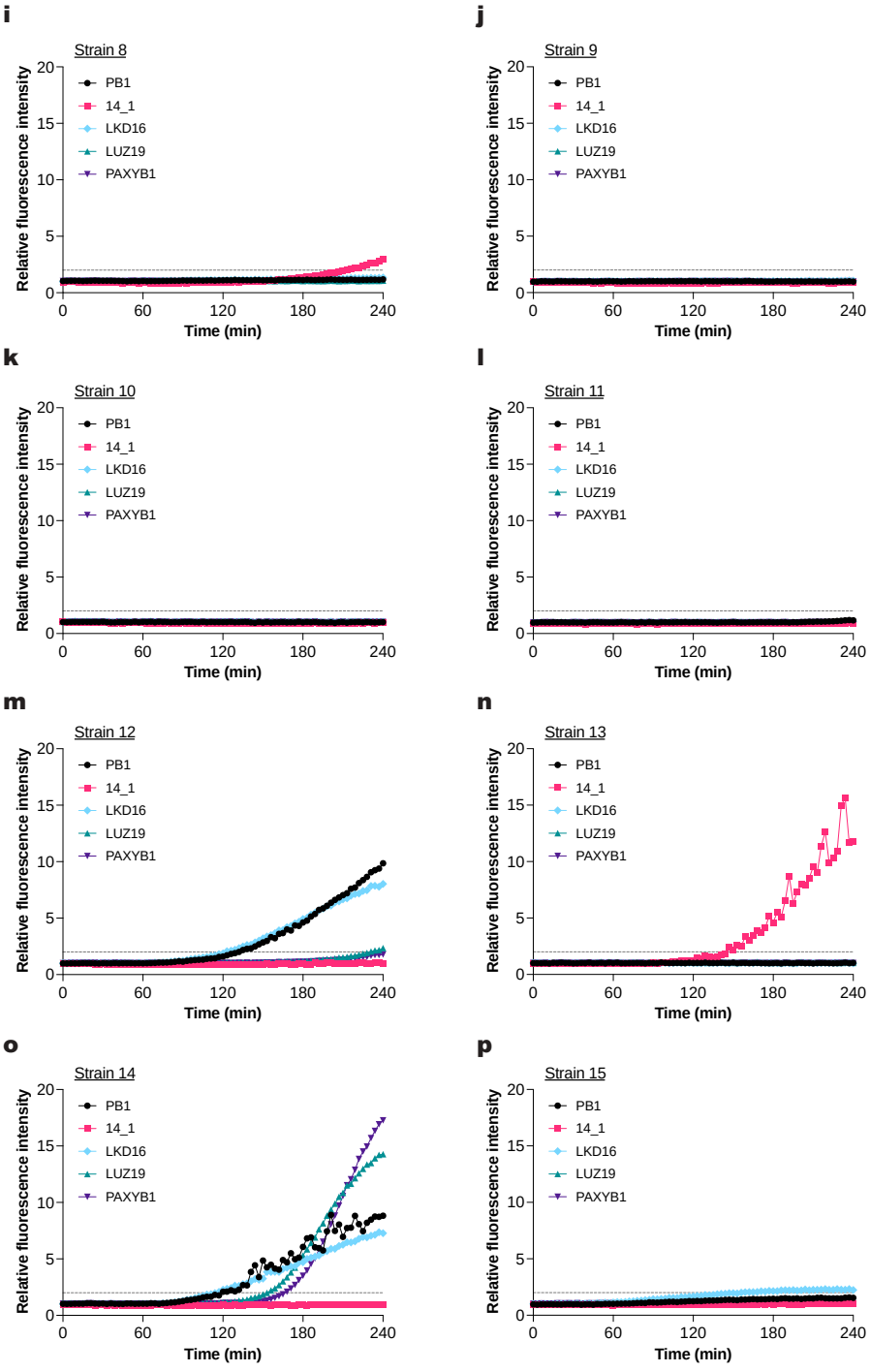
PAO1
b
Strain 1
c
Strain 2
d
Strain 3
e
Strain 4
f
Strain 5
g
Strain 6
h
Strain 7
PB1
14_1
LKD16
LUZ19
PAXYB1
Relative fluorescence intensity
Time (min)
0
5
10
15
20
0
60
120
180
240


i
Strain 8
j
Strain 9
k
Strain 10
l
Strain 11
m
Strain 12
n
Strain 13
o
Strain 14
p
Strain 15
PB1
14_1
LKD16
LUZ19
PAXYB1
Relative fluorescence intensity
Time (min)
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5
10
15
20
0
60
120
180
240

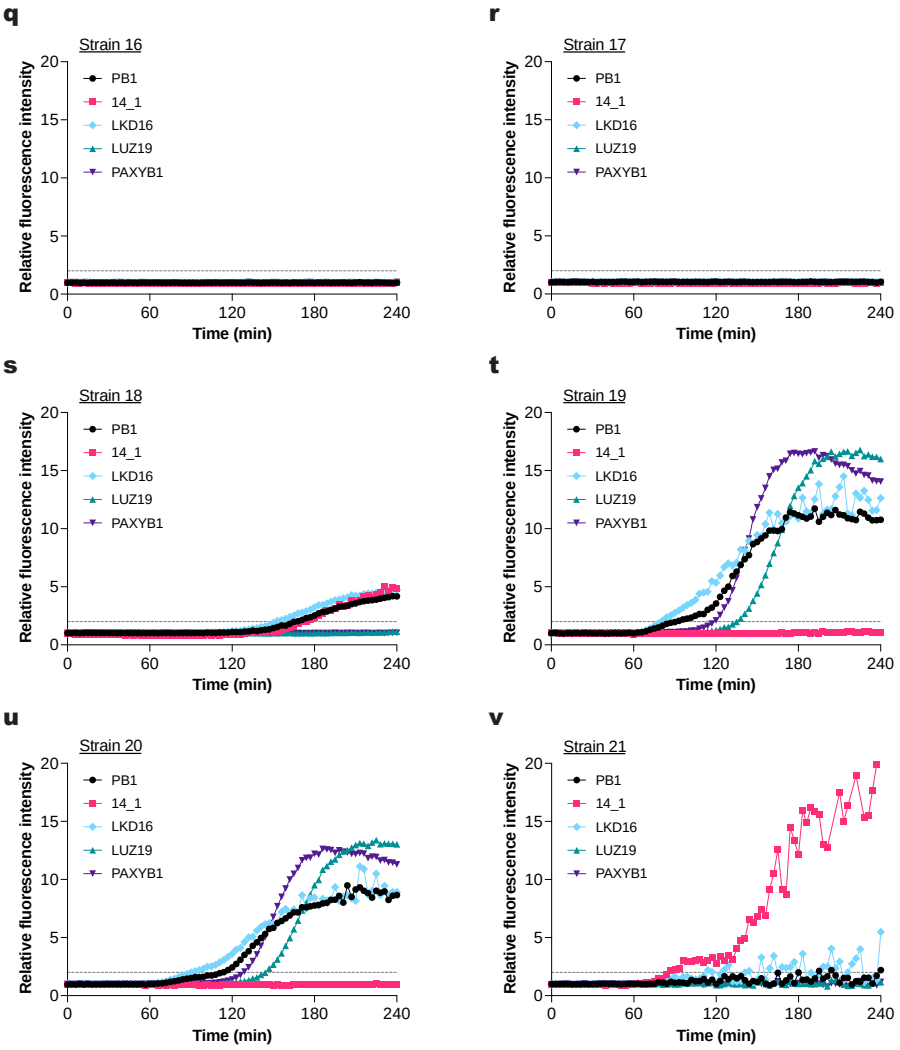
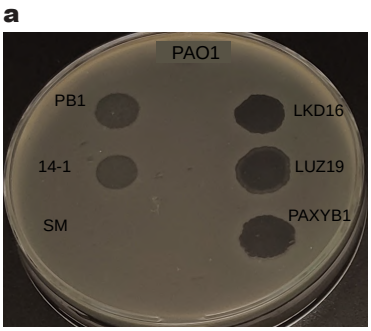


Figure S14: Phage susceptibility profiling of several *P. aeruginosa* clinical strains obtained from patients at the UMC Utrecht, as determined by fluorescent DNA dye assay. a-v) Strains were incubated with phages PB1, 14-1, LKD16, LUZ19 or PAXYB1 at an MOI of 1 at 37°C in the presence of Sytox green. Fluorescence intensity was measured using a fluorometer, and values were divided by control signal to obtain relative fluorescence intensity. Black dotted line represents threshold for phage-mediated damage (relative fluorescence = 2). A representative graph of at least three independent experiments is shown.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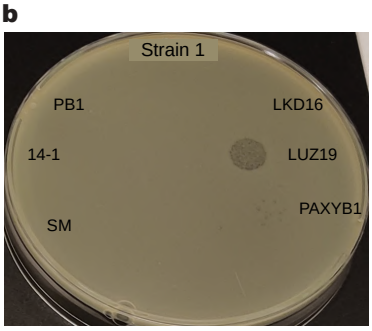
PB1
LKD16
14-1
LUZ19
SM
PAXYB1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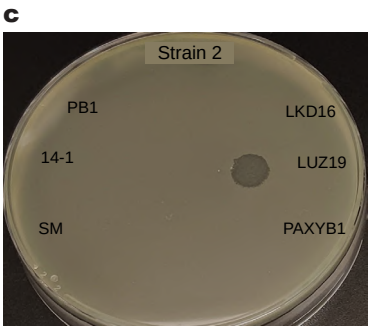
PB1
LKD16
14-1
LUZ19
SM
PAXYB1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LUZ19
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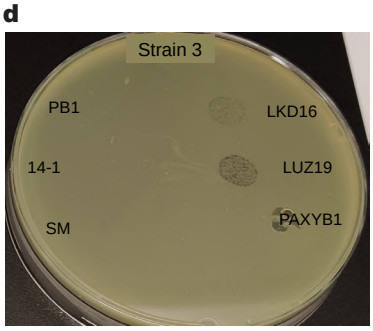
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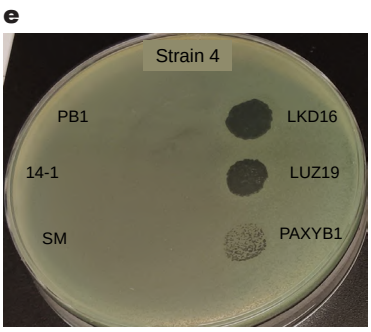
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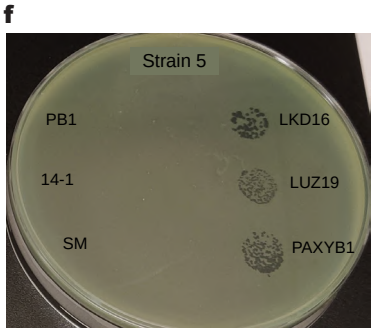
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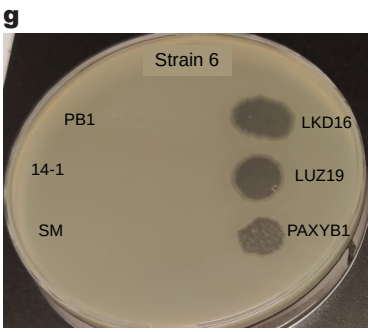
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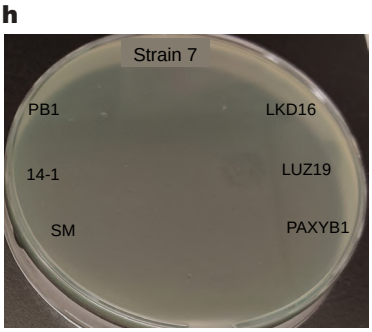
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LKD16
14-1
LUZ19
SM
PAXYB1


2

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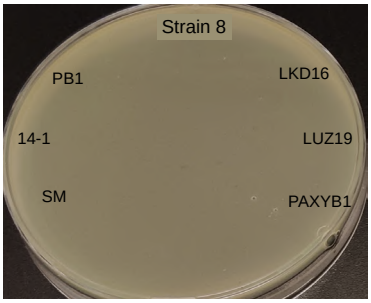
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LKD16
14-1
LUZ19
SM
PAXYB1

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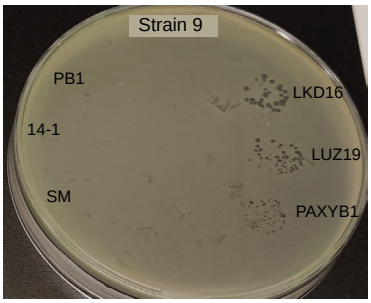
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LKD16
14-1
LUZ19
SM
PAXYB1

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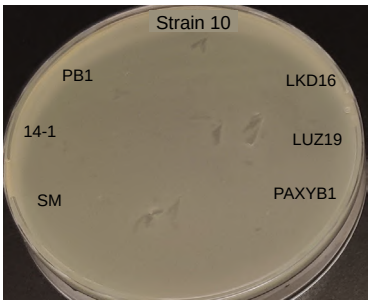
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PB1
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14-1
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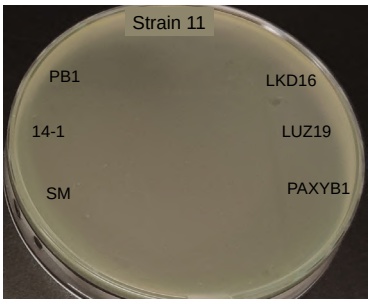
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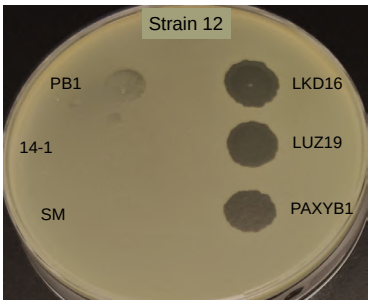
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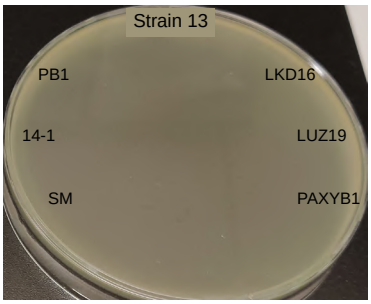
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SM
PAXYB1

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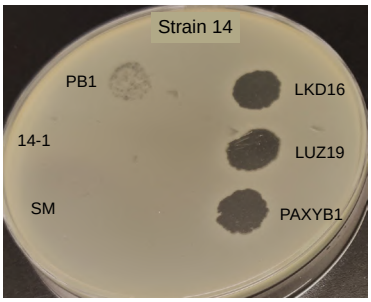
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SM
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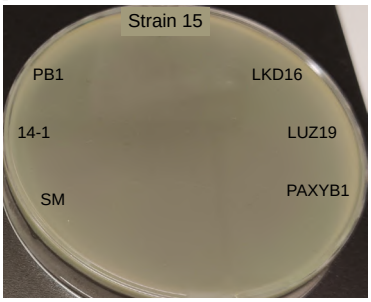
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PAXYB1

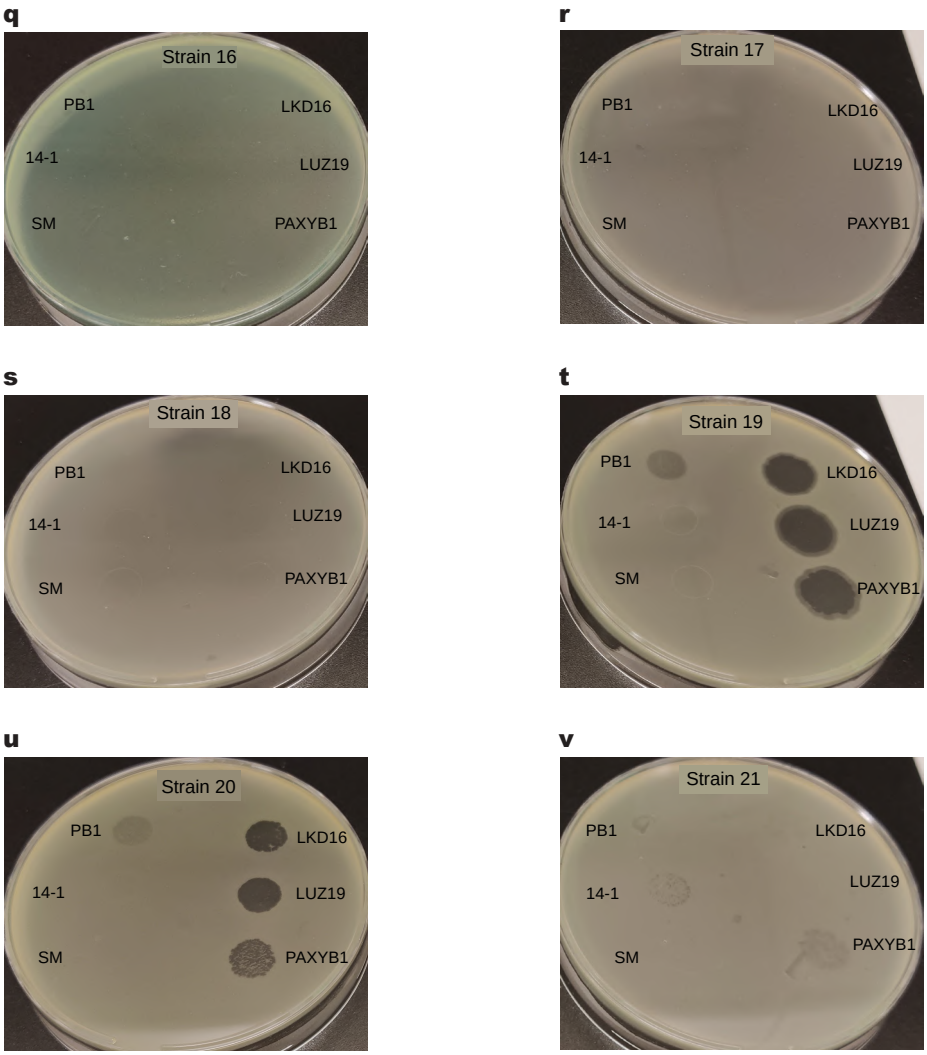
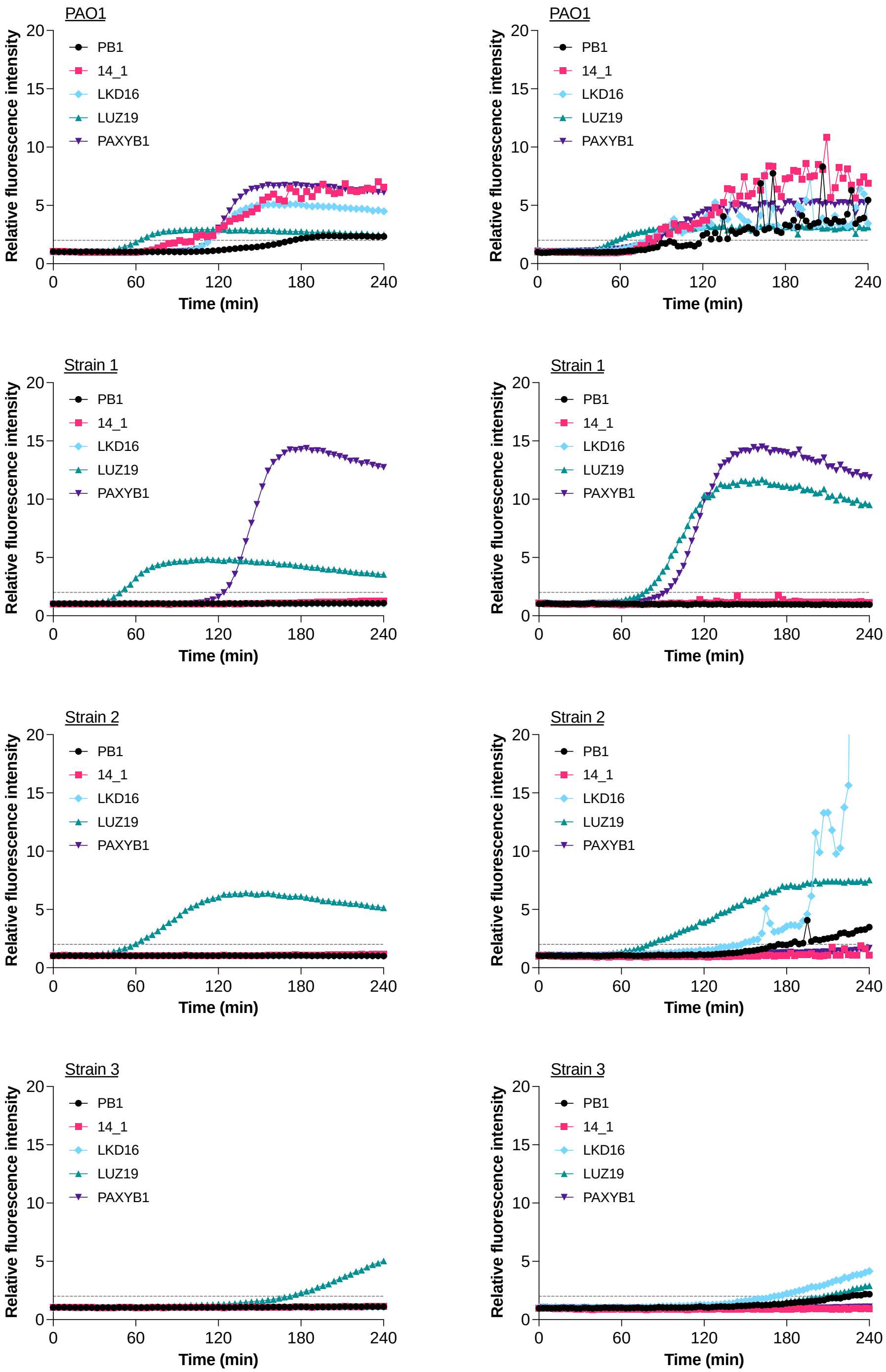
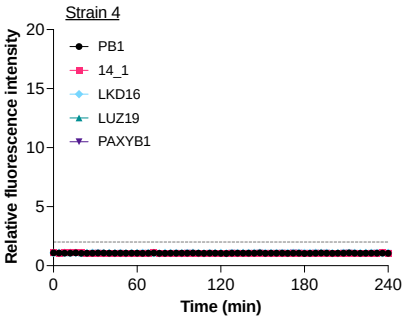
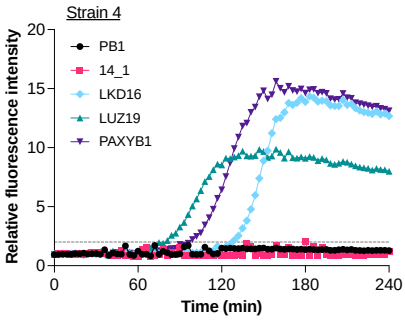
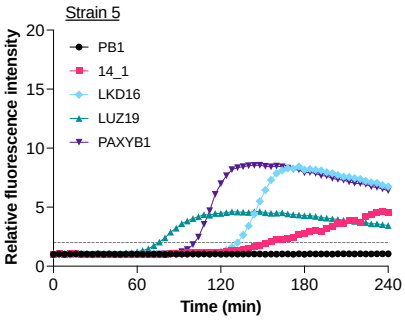


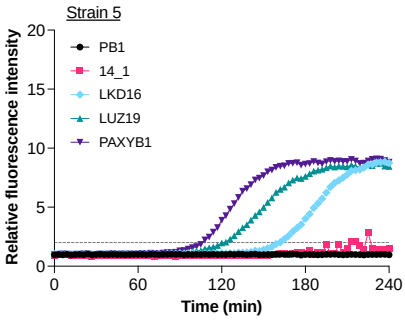
Figure S15: Phage susceptibility profiling of several *P. aeruginosa* clinical strains obtained from patients at the UMC Utrecht, as determined by plaque assays. a-v) Strains were inoculated into top agar and overlayed on a plate. Phages PB1, 14-1, LKD16, LUZ19 and PAXYB1 (10^6 pfu/mL, 5 µL) and SM buffer (5 µL) were spotted to detect plaque formation after overnight incubation at 37°C.

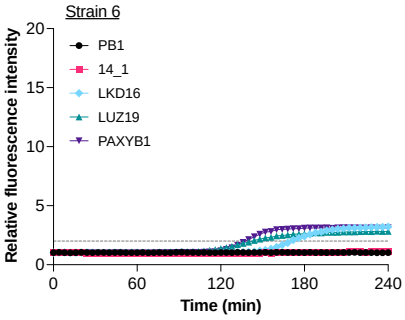

PAO1
PB1
14_1
LKD16
LUZ19
PAXYB1
Relative fluorescence intensity
0
5
10
15
20
0
60
120
180
240
Time (min)
PAO1
PB1
14_1
LKD16
LUZ19
PAXYB1
Relative fluorescence intensity
Time (min)
Strain 1
PB1
14_1
LKD16
LUZ19
PAXYB1
Relative fluorescence intensity
Time (min)
Strain 1
PB1
14_1
LKD16
LUZ19
PAXYB1
Relative fluorescence intensity
Time (min)
Strain 2
PB1
14_1
LKD16
LUZ19
PAXYB1
Relative fluorescence intensity
Time (min)
Strain 2
PB1
14_1
LKD16
LUZ19
PAXYB1
Relative fluorescence intensity
Time (min)
Strain 3
PB1
14_1
LKD16
LUZ19
PAXYB1
Relative fluorescence intensity
Time (min)
Strain 3
PB1
14_1
LKD16
LUZ19
PAXYB1
Relative fluorescence intensity
Time (min)

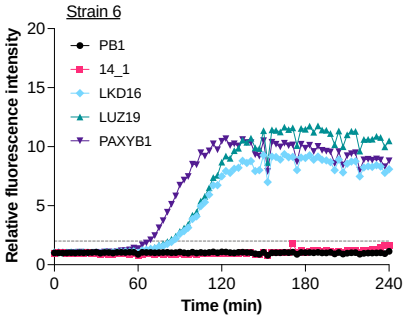

Strain 4
PB1
14_1
LKD16
LUZ19
PAXYB1
Relative fluorescence intensity
Time (min)

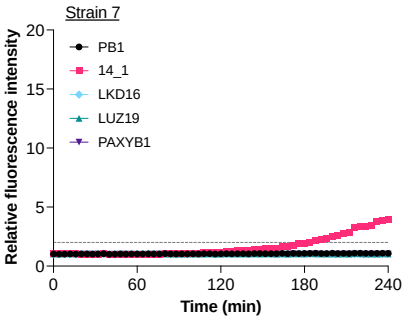

Strain 4
PB1
14_1
LKD16
LUZ19
PAXYB1
Relative fluorescence intensity
Time (min)

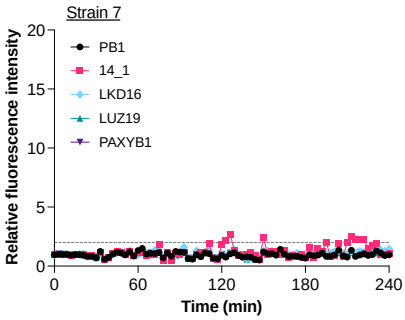

Strain 5
PB1
14_1
LKD16
LUZ19
PAXYB1
Relative fluorescence intensity
Time (min)


Strain 5
PB1
14_1
LKD16
LUZ19
PAXYB1
Relative fluorescence intensity
Time (min)

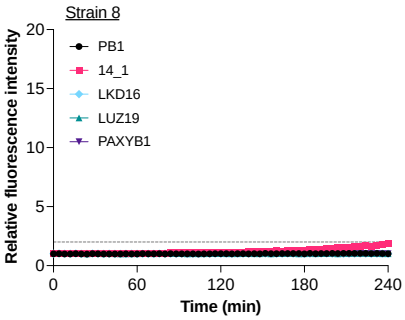

Strain 6
PB1
14_1
LKD16
LUZ19
PAXYB1
Relative fluorescence intensity
Time (min)

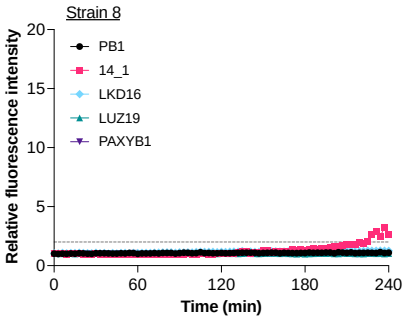

Strain 6
PB1
14_1
LKD16
LUZ19
PAXYB1
Relative fluorescence intensity
Time (min)

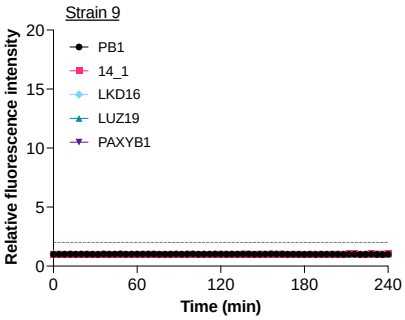

Strain 7
PB1
14_1
LKD16
LUZ19
PAXYB1
Relative fluorescence intensity
Time (min)

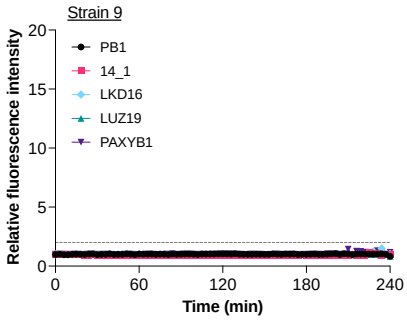

Strain 7
PB1
14_1
LKD16
LUZ19
PAXYB1
Relative fluorescence intensity
Time (min)

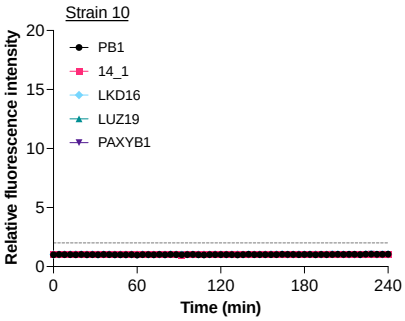

2

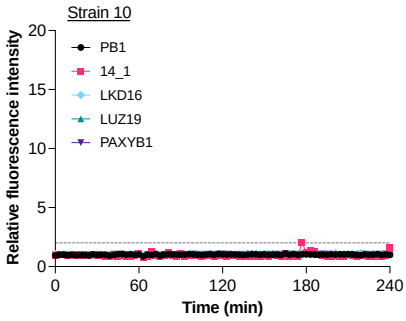

Strain 8
PB1
14_1
LKD16
LUZ19
PAXYB1
Relative fluorescence intensity
Time (min)

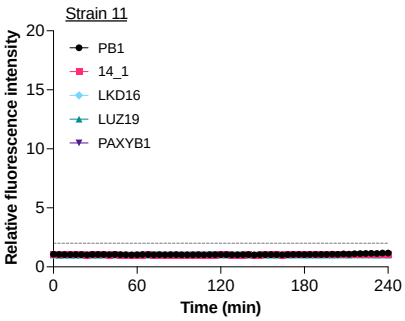

Strain 8
PB1
14_1
LKD16
LUZ19
PAXYB1
Relative fluorescence intensity
Time (min)

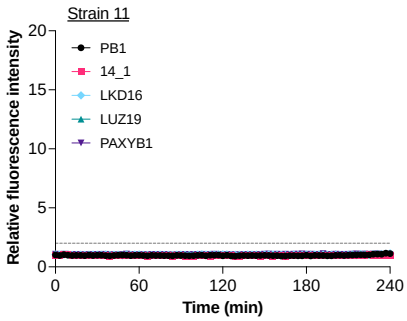

Strain 9
PB1
14_1
LKD16
LUZ19
PAXYB1
Relative fluorescence intensity
Time (min)

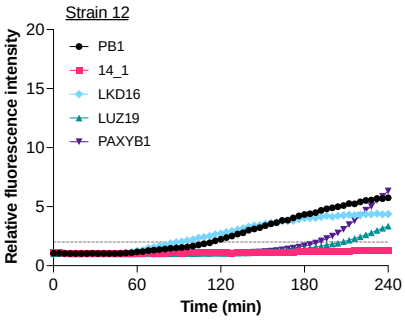

Strain 9
PB1
14_1
LKD16
LUZ19
PAXYB1
Relative fluorescence intensity
Time (min)

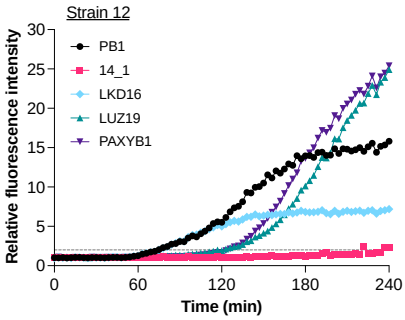

Strain 10
PB1
14_1
LKD16
LUZ19
PAXYB1
Relative fluorescence intensity
Time (min)

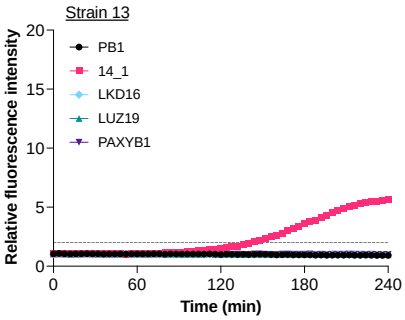

Strain 10
PB1
14_1
LKD16
LUZ19
PAXYB1
Relative fluorescence intensity
Time (min)

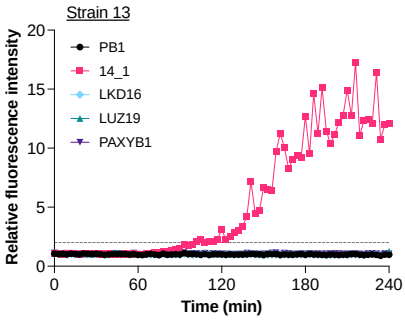

Strain 11
PB1
14_1
LKD16
LUZ19
PAXYB1
Relative fluorescence intensity
Time (min)

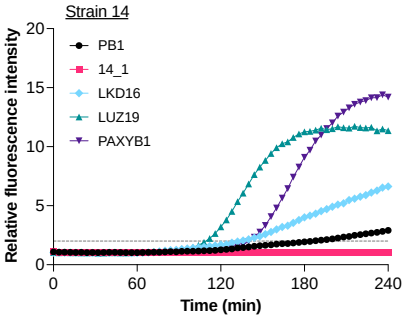

Strain 11
PB1
14_1
LKD16
LUZ19
PAXYB1
Relative fluorescence intensity
Time (min)

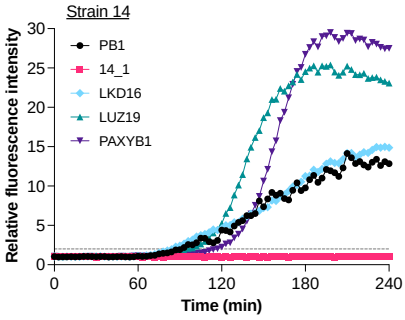

Strain 12
PB1
14_1
LKD16
LUZ19
PAXYB1
Relative fluorescence intensity
Time (min)

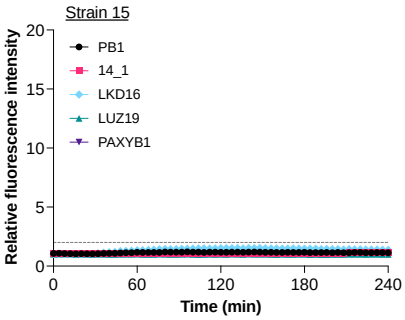

Strain 12
PB1
14_1
LKD16
LUZ19
PAXYB1
Relative fluorescence intensity
Time (min)

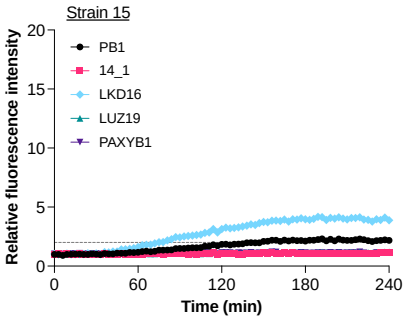

Strain 13
PB1
14_1
LKD16
LUZ19
PAXYB1
Relative fluorescence intensity
Time (min)


Strain 13
PB1
14_1
LKD16
LUZ19
PAXYB1
Relative fluorescence intensity
Time (min)

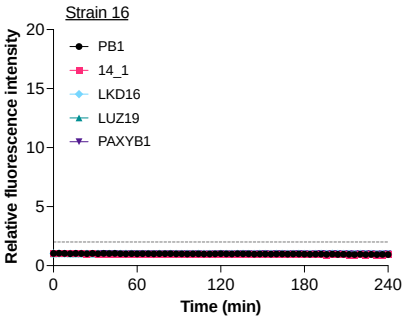

Strain 14
PB1
14_1
LKD16
LUZ19
PAXYB1
Relative fluorescence intensity
Time (min)

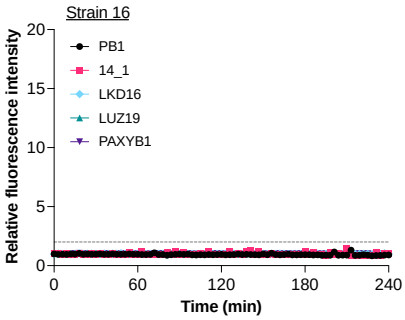

Strain 14
PB1
14_1
LKD16
LUZ19
PAXYB1
Relative fluorescence intensity
Time (min)

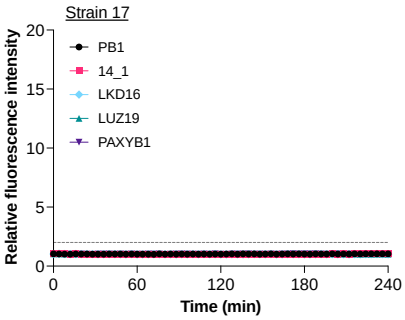

Strain 15
PB1
14_1
LKD16
LUZ19
PAXYB1
Relative fluorescence intensity
Time (min)

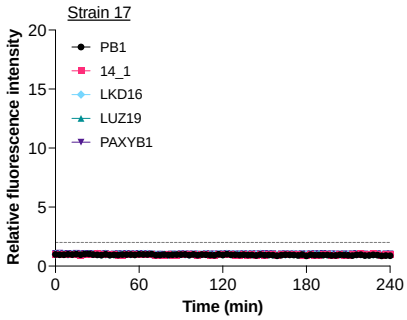

Strain 15
PB1
14_1
LKD16
LUZ19
PAXYB1
Relative fluorescence intensity
Time (min)

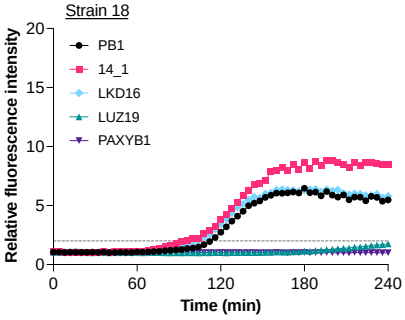

2

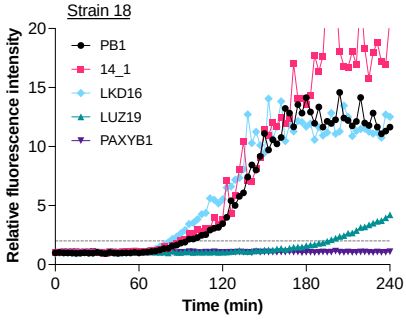

Strain 16
PB1
14_1
LKD16
LUZ19
PAXYB1
Relative fluorescence intensity
Time (min)

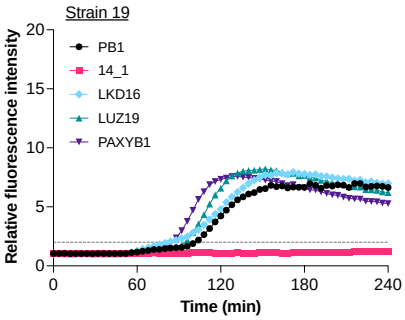

Strain 16
PB1
14_1
LKD16
LUZ19
PAXYB1
Relative fluorescence intensity
Time (min)

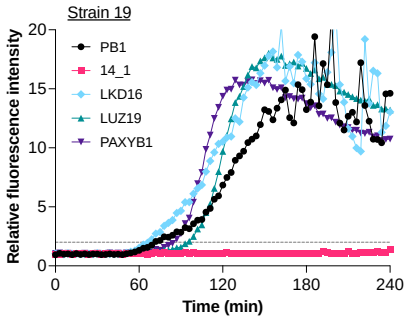

Strain 17
PB1
14_1
LKD16
LUZ19
PAXYB1
Relative fluorescence intensity
Time (min)


Strain 17
PB1
14_1
LKD16
LUZ19
PAXYB1
Relative fluorescence intensity
Time (min)


Strain 18
PB1
14_1
LKD16
LUZ19
PAXYB1
Relative fluorescence intensity
Time (min)


Strain 18
PB1
14_1
LKD16
LUZ19
PAXYB1
Relative fluorescence intensity
Time (min)


Strain 19
PB1
14_1
LKD16
LUZ19
PAXYB1
Relative fluorescence intensity
Time (min)


Strain 19
PB1
14_1
LKD16
LUZ19
PAXYB1
Relative fluorescence intensity
Time (min)

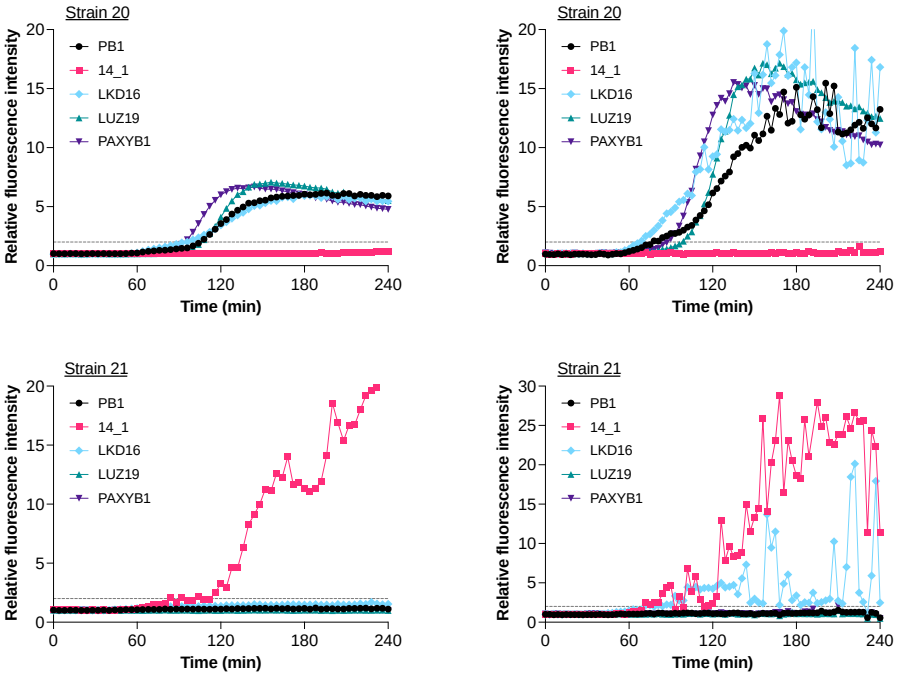


Figure S16: Phage susceptibility profiling of several *P. aeruginosa* clinical strains obtained from patients at the UMC Utrecht, as determined by fluorescent DNA dye assay. a-v) Strains were incubated with phages PB1, 14-1, LKD16, LUZ19 or PAXYB1 at an MOI of 1 at 37°C in the presence of Sytox green. Fluorescence intensity was measured using a fluorometer, and values were divided by control signal to obtain relative fluorescence intensity. Black dotted line represents threshold for phage-mediated damage (relative fluorescence = 2). A representative graph of at least three independent experiments is shown. Two additional replicates to figure S14 are shown.

Chapter 3

Human complement inhibits myophages against *Pseudomonas aeruginosa*

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Abstract

Therapeutic bacteriophages (phages) are primarily chosen based on their *in vitro* bacteriolytic activity. Although anti-phage antibodies are known to inhibit phage infection, the influence of other immune system components is less well known. An important anti-bacterial and anti-viral innate immune system that may interact with phages is the complement system, a cascade of proteases that recognizes and targets invading microorganisms. In this research, we aimed to study the effects of serum components such as complement on the infectivity of different phages targeting *Pseudomonas aeruginosa*. We used a fluorescence-based assay to monitor the killing of *P. aeruginosa* by phages of different morphotypes in the presence of human serum. Our results reveal that several myophages are inhibited by serum in a concentration-dependent way, while the activity of four podophages and one siphophage tested in this study is not affected by serum. By using specific nanobodies blocking different components of the complement cascade, we showed that activation of the classical complement pathway is a driver of phage inhibition. To determine the mechanism of inhibition, we produced bioorthogonally labeled fluorescent phages to study their binding by means of microscopy and flow cytometry. We show that phage adsorption is hampered in the presence of active complement. Our results indicate that interactions with complement may affect the *in vivo* activity of therapeutically administered phages. A better understanding of this phenomenon is essential to optimize the design and application of therapeutic phage cocktails.

Introduction

Over the last decades, the increase in multi-drug resistant bacteria has spurred a renewed interest in bacteriophages (phages) as an alternative to antibiotics[1]. Phages used in therapy predominantly follow a lytic cycle, which means that they can rapidly kill infected bacteria and propagate by spreading to neighboring cells[2]. These phages belong in the majority of cases to the order *Caudovirales*, and are morphologically characterized by an icosahedral capsid, or head, which contains the genetic material, and a tail. The tail can further determine the morphotype of the phage. Phages with contractile tails are named myophages, phages with short tails are named podophages, and phages with long, non-contractile tails are named siphophages. In contrast to most antibiotics, phages have the advantage of being very specific in the bacteria that they can target. However, this also means that phage therapy often has to be highly tailored to the needs of each patient. Therapeutic success may therefore depend on using the most optimal phages to treat each infection[3].

A pathogen for which phage therapy is particularly relevant is *Pseudomonas aeruginosa*. This species of Gram-negative bacteria is part of the ESKAPE list, a group of pathogens that are the leading cause of nosocomial infections worldwide. It is an opportunistic pathogen that causes infections ranging from burn wound complications to pneumonia and severe sepsis. It is particularly worrisome for immunocompromised patients with underlying conditions, such as cystic fibrosis or hematological malignancies. Antibiotic resistance in *P. aeruginosa* is rising rapidly, making it a prime candidate for treatment with phage therapy[4]. The potential of phage therapy against *P. aeruginosa* infections is showcased in recent case reports[5–7]. Several clinical studies have also been carried out to evaluate the safety of phage therapy in humans against this pathogen, with positive outcomes[8,9]. Efficacy is also assessed in some of these studies, although the results can be difficult to interpret because of the challenges in study design[10,11]. Nonetheless, most of the evidence comes from clinical cases and case series, with the number of randomized clinical trials still being limited.

There are several reasons why the efficacy of phage therapy is still disputed. Bacteria can evade phage infection via several defense mechanisms, and they can rapidly mutate to develop resistance to phages[12,13]. To prevent this, mixes of different phages, or cocktails, are often administered. However, finding several phages to target a pathogenic strain can be challenging, and the optimization of phage cocktail compositions is not always straightforward[14]. Another major caveat is that the patient's immune system may recognize and target phages as exogenous organisms. This has been shown to occur in different ways, such as the induction of neutralizing antibodies[15] or phagocytosis of phage virions by immune cells[16]. Depending on the patient and the route of administration, the immune response could lead to inactivation of the phages before they can reach their goal.

A major part of the immune system that phages encounter when entering the blood and tissues is the complement system. The complement system mediates innate immunity to exogenous entities, including pathogens. It consists of a cascade of proteases that can activate via different pathways: the classical pathway is activated through the action of antibodies, the alternative pathway creates an amplification loop that increases activation, and the lectin pathway is triggered by recognition of molecular patterns[17]. Following activation, the pathways converge at the formation of a C3 convertase, which cleaves C3 causing C3b to deposit on the bacterial surface. C3b can act as an opsonin, triggering and promoting phagocytosis by immune cells. The presence of C3b also causes the C3 convertase to switch specificity to recognize C5. Eventually, this leads to formation of the membrane attack complex (MAC), which can directly lyse Gram-negative bacteria[18]. Although complement could in some instances act synergistically with phage therapy[19], there is also evidence that complement can recognize viral particles[20], and even phages[21].

Our goal was to study the effect of human serum, in particular of the complement system, on lytic phage activity. To address this, we assessed different *P. aeruginosa* phages in the presence of serum. Our results reveal that serum inhibits the lytic activity of certain phages, predominantly myophages. To study the involvement of the complement system in this phenomenon, we used nanobodies inhibiting specific

complement proteins. In this way, we have demonstrated that the recognition of phages by early components of the complement cascade can impair their binding to bacteria. The fact that different phages perform differently when challenged by the innate immune system could have important repercussions in the way that we select phages for therapy.

Results

Human serum impairs activity of myophages PBJ and 14-1

We studied the effect of human serum on the activity of phages against the serum-resistant *P. aeruginosa* strain PAO1. We evaluated four different virulent phages: myophages PBJ and 14–1 and podophages LKD16 and LUZ19. PBJ is a Pbunavirus that belongs to the Pbunavirus pv141 species with 96% sequence identity over 96% of the genome of phage 14–1. Phages 14–1 (species: Pbunavirus pv141), LKD16 (species: Phikmvvirus LKD16), and LUZ19 (species: Phikmvvirus LUZ19) have been extensively characterized in the literature[22–25]. The phages were combined in different multiplicities of infection (MOIs) with PAO1 in the presence of different concentrations of human pooled serum (HPS). Phage-mediated killing was monitored using the fluorescent DNA dye assay as previously described[26]. Briefly, we used the membrane-impermeant DNA dye Sytox Green, which can only stain bacterial DNA once the cells are damaged or lysed. This assay allows us to monitor phage-mediated damage in real time, where an increase in fluorescence correlates with bacterial killing. When comparing all four phages, we found that the addition of HPS inhibited damage induction for the myophages, resulting in a much slower increase of the fluorescence signal (figure 1a,b). In contrast, no differences in activity were observed for the podophages in the presence or absence of HPS. Based on these results, we used the time of phage-mediated damage induction as a readout for phage activity to compare all conditions more easily. In this way, a shorter time indicates a more rapid or efficient phage infection. We confirmed that damage induction by phage PBJ was delayed with the addition of HPS. The effect was concentration-dependent, where a higher concentration of HPS was needed to inhibit a higher phage MOI (figure 1c). Phage 14–1 presented a

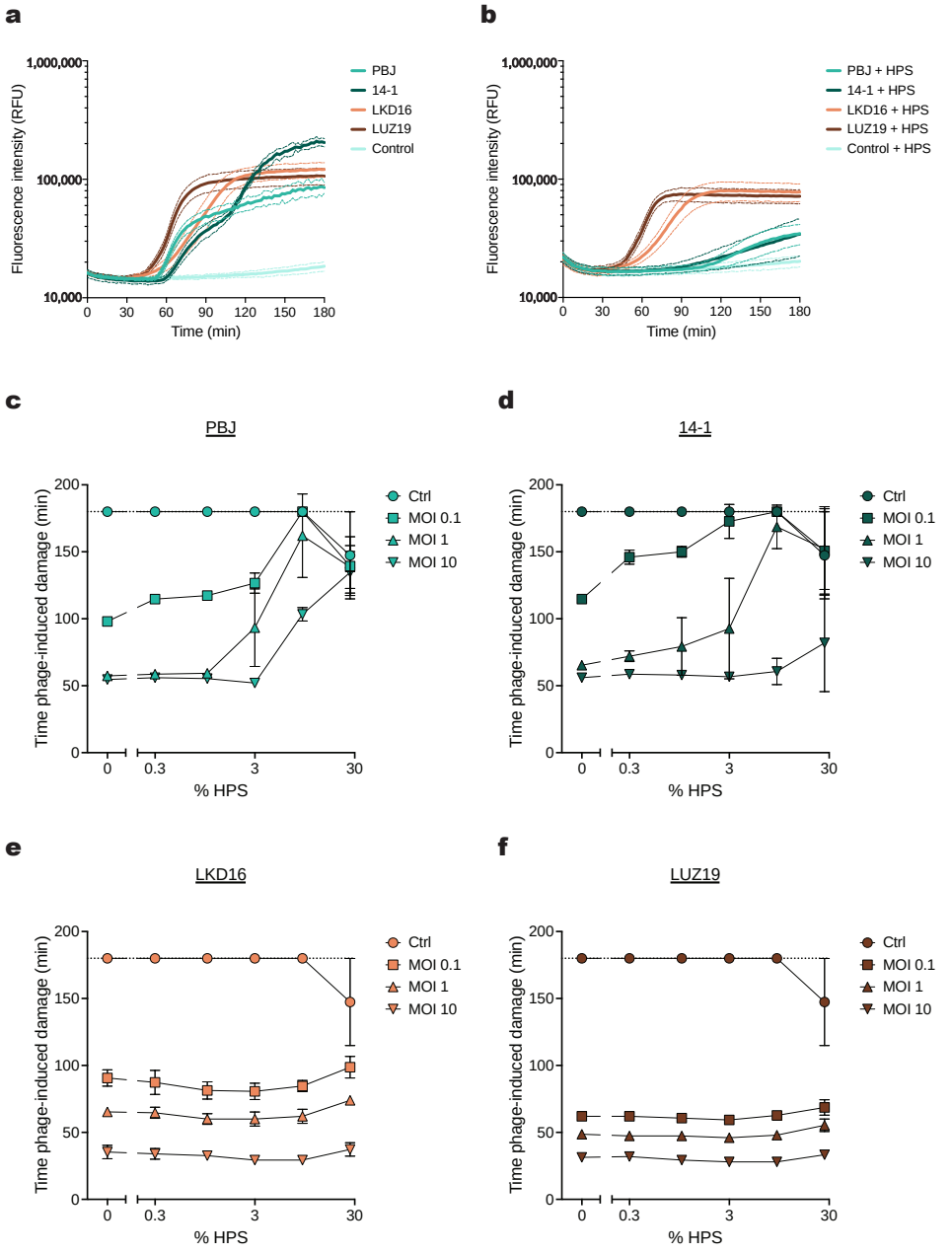

Figure 1. Human serum inhibits lytic activity of myophages. *Pseudomonas aeruginosa* strain PAO1 was incubated with bacteriophages (phages) PBJ, 14-1, LKD16, or LUZ19 at 37°C in the presence of the DNA dye Sytox Green. Phage-mediated damage is signaled by an increase in the fluorescence intensity of Sytox Green. a) Fluorescence intensity (relative fluorescence units, RFUs) over time of

PAO1 infected with different phages at a multiplicity of infection (MOI) of 1 in the absence of serum. Uninfected bacteria were used as the control. b) Fluorescence intensity (RFUs) over time of PAO1 infected with different phages at an MOI of 1 in the presence of 10% human pooled serum (HPS). The control is uninfected bacteria incubated with 10% HPS. (c–f) Time of damage induction (min) by phage c) PBJ, d) 14-1, e) LDK16, and f) LUZ19, at a range of MOIs (0.1, 1, 10) combined with HPS at different concentrations (0–30%). Time of damage induction is determined as the time after the addition of phages at which the fluorescence curve experiences a sharp and steady increase (1000 RFUs over the previous measurement). a–f) Data represent mean ± SD of three independent experiments.

similar pattern, where the lower MOIs (0.1 and 1) showed clear inhibition by HPS (figure 1d). However, this phage seemed slightly less sensitive to HPS than PBJ, as its activity was largely unaffected at the highest MOI. Strikingly, the activity of podophages LKD16 and LUZ19 did not change with the addition of HPS (figure 1e,f). These observations indicate that serum can selectively inhibit certain phages in a concentration-dependent manner, while other phages are not sensitive to inhibition by serum.

Serum-mediated inhibition is found for a broader set of myophages

To further determine whether the effect is specific to the phage morphotype, we evaluated the activity of 9 more myophages, 2 podophages, and 1 siphophage in the presence and absence of human serum. These were uncharacterized phages initially isolated from sewage water and classified according to tail morphology[27]. Phage activity was assessed using the fluorescent DNA dye assay. In the absence of serum, we observed differences in the killing kinetics of the different phages, with latent periods ranging from around 30 to over 100 min (figure S1). When serum was added, time of damage induction was greatly increased for 7 out of the 9 myophages (figures 2a and S1). In contrast, the activity of myophages Pa33 and Pa36, podophages Pa18 and Pa29, and siphophage Pa28 was not affected by the addition of serum. Notably, siphophage Pa28 and podophage Pa29 were not very active in the absence of serum, which could be due to PAO1 not being the optimal host for this phage. These observations suggest that some myophages, if not all, could be more sensitive to serum-mediated inhibition. However, given that these phages and their receptors have not been characterized, it could be that the effect is not dependent on the morphotype, but rather due to the phages being closely related or targeting the same receptor. To rule this out, we tested whether

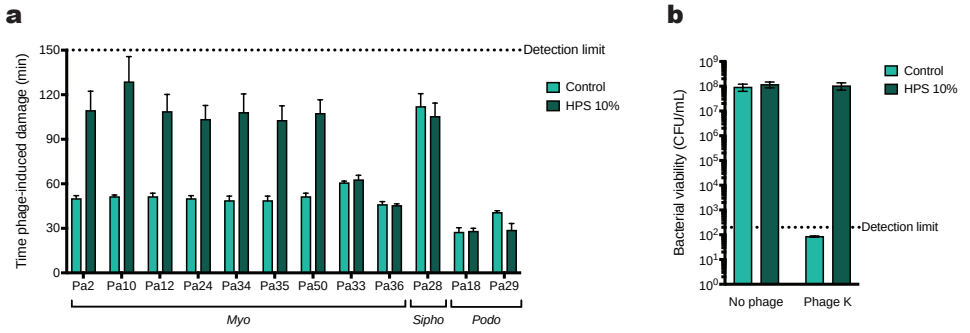

Figure 2. Human serum inhibits a variety of myophages targeting PAO1. a) Time of damage induction (min) of the different phages as determined by the fluorescent DNA dye assay. Time of damage induction is determined here as the time after the addition of phages at which the fluorescence curve experiences a sharp and steady increase (700 RFUs over the previous measurement). Phages were added at an MOI of 1 and incubated with PAO1 at 37°C in the absence of serum (control) or in the presence of 10% HPS. b) *Staphylococcus aureus* strain ATCC 19685 was incubated with phage K at an MOI of 10 in the absence (control) or presence of 10% HPS for 2 h at 37°C, after which bacteria were plated and incubated overnight. Number of recovered colonies per plated volume is expressed as colony forming units per mL (CFUs/mL). Black dotted line represents detection limit. a,b) Data represent mean ± SD of three independent experiments.

serum can also inhibit phage K, a myophage targeting *Staphylococcus aureus* that uses wall teichoic acid (WTA) as a receptor. To do so, we incubated bacteria with the phage in the presence or absence of serum and examined bacterial viability through colony counting. Indeed, no reduction in colony-forming units (CFUs) was detected when phages were added in the presence of serum (figure 2b). As phage K targets a receptor absent on *P. aeruginosa*, it seems that inhibition by serum is not dependent on the phage receptor. These results further suggest that myophages are more sensitive to inhibition by human serum.

Phage inhibition in serum is mediated by the early stages of the complement cascade

Next, we investigated the components in serum responsible for the inhibitory effect. We first aimed to rule out whether this effect was due to the potential presence of neutralizing anti-phage antibodies in our serum pool. For this, we combined phages with HPS, HPS with heat-inactivated complement (HI HPS), or IgG and

IgM purified from HPS, and studied killing kinetics with the fluorescent DNA dye assay (figure 3a). Phages performed equally well in the presence of antibodies and heat-inactivated serum as they did in the control condition. As purified antibodies and serum with inactive complement did not have an effect on phage activity, we inferred that the complement system could be driving phage inhibition. In all complement activation pathways, cleavage of C3 and subsequent deposition of the cleavage product C3b is necessary for the downstream conversion of C5. To further look into this, we examined phage activity in the presence of serum

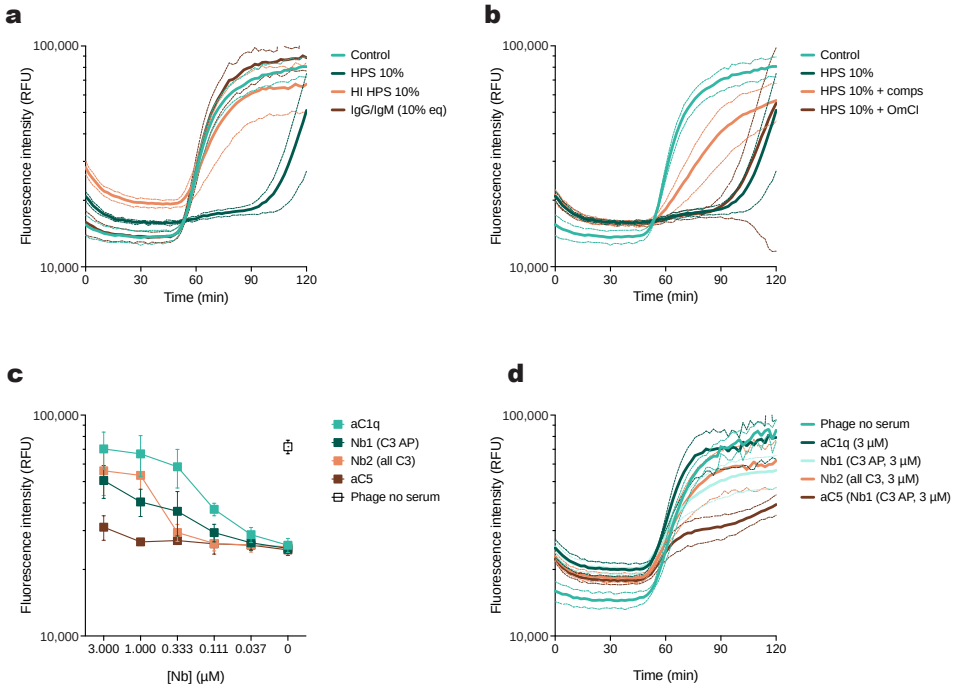

Figure 3. Inhibition of phages by serum is mediated by the complement system. PAO1 was incubated with phage PBJ at 37°C in the presence of the DNA dye Sytox Green. a) Fluorescence intensity (RFUs) over time (min) of bacteria infected with PBJ at an MOI of 10 (control), in the presence of 10% HPS, 10% HPS with heat-inactivated complement (HI HPS), or IgG and IgM purified from HPS at a concentration equivalent to 10%. b) Fluorescence intensity (RFUs) over time (min) of bacteria infected with PBJ at an MOI of 10 (control), in the presence of 10% HPS, 10% HPS with 50 µM compstatin, or 10% HPS with 20 µg/mL OmCI. c) Fluorescence intensity (RFUs) of bacteria after 90 min of infection with PBJ at an MOI of 10 in the presence of 10% HPS and nanobodies at a range of concentrations. Value for bacteria infected with phage in the absence of serum is shown as a control. d) Fluorescence intensity (RFUs) over time (min) of bacteria infected with PBJ at an MOI of 10 in the absence of serum (control) or in the presence of 10% HPS and nanobodies at a concentration of 3 µM. a–d) Data represent mean ± SD of three independent experiments.

with the addition of specific complement inhibitors: compstatin, which inhibits C3 cleavage[28,29], and OmCI, which inhibits C5 cleavage[30] (figure 3b). We observed that compstatin partially rescued phage activity in the presence of serum, while OmCI did not prevent inhibition by serum at all. Therefore, our results point to the involvement of the early stages of the complement system in the inhibitory effect of serum on phage PBJ. Notably, there was no difference in the activity of phages in the presence of heat-inactivated serum or heat-inactivated serum with complement inhibitors (figure S2). However, as the addition of compstatin only partially recovered phage activity, we tested an additional panel of complement inhibitors, in this case nanobodies. Nanobodies are single-domain antibody fragments that bind antigens, with the advantage of having a much smaller size than an antibody[31].

We used nanobody C1qNb75, which inhibits activation of the complement classical pathway by binding C1q, the first step of the cascade[32]. In addition to this, we used nanobodies hC3Nb1 and hC3Nb2, which inhibit the cleavage of C3 in the alternative pathway or all activation pathways, respectively[33,34]. Finally, we used sdAb_E4, a C5 binder, to inhibit the terminal stage of the complement cascade[35]. We assessed phage activity in serum in the presence of the nanobodies at a range of concentrations. To compare their effects, we analyzed the influx of fluorescent DNA dye after 90 min of infection (figure 3c,d). The C1q-targeting nanobody was able to recover phage activity in a concentration-dependent manner to the level of the control. Both of the C3-targeting nanobodies exhibited an intermediate effect, comparable to that of compstatin. As expected, the C5-targeting nanobody barely improved phage activity even at the highest concentration, which is in line with our observations with OmCI. Taken together, our results indicate that phage inhibition is likely caused by complement and more specifically by activation of the classical pathway.

Inhibition of phages by complement occurs at the stage of phage adsorption

One of the stages of phage infection that the complement system could be hampering is the binding of phages to their host. Phage adsorption can be measured with

assays where bacteria are incubated with phages and the remaining free plaque-forming units are quantified. However, in such an assay, both phage adsorption and phage inactivation would translate to a decrease in free plaque-forming units. To circumvent this, we assessed phage adsorption by using fluorescently tagged phages.

Producing fluorescent phages poses a challenge. Random labeling strategies may compromise the activity of the phage as the dye has to react with the capsid and tail proteins[36]. We circumvented this by producing phages tagged with a chemical handle that can be functionalized through click chemistry[37]. To do so, we used a methionine-auxotrophic strain of *P. aeruginosa*, which requires exogenous methionine to grow. Methionine can also be partially substituted by its structural homologue L-azidohomoalanine, a non-canonical amino acid that contains an azide group. In a situation of methionine scarcity, bacteria will incorporate L-azidohomoalanine into their proteins. If phages are propagated with these bacteria as hosts, their proteins will also be azide-tagged (figure 4a)[38]. The resulting virions are then available for biorthogonal labeling via click chemistry, as the azide groups can react efficiently with alkyne groups attached to a fluorophore to create a stable covalent bond. We amplified PBJ in this way and used the molecule DBCO functionalized with an Alexa-488 dye to fluorescently label our azido-tagged phages, obtaining PBJ-AF488.

We confirmed the labeling of PBJ-AF488 by means of widefield fluorescence microscopy. To do so, we incubated fluorescently labeled phages with PAO1 expressing cytoplasmic superfolder Cherry (sfCherry; bacteria: PAO1-sfCherry) to allow the phages to bind. We successfully detected bacteria with bound phages on their surface, as well as isolated phage clusters (figure 4b). The addition of serum in this setting resulted in a decrease in the number of bacteria with attached phage particles (figure 4c). Next, we used flow cytometry to confirm whether complement indeed hampers phage binding with a more quantitative approach. In this assay, we again used PAO1-sfCherry to facilitate gating (figure S3). PBJ-AF488 or heat-inactivated PBJ-AF488 was incubated with bacteria for 5 min to allow for phage adsorption in the presence of serum or serum with inhibitors. Analysis of phage

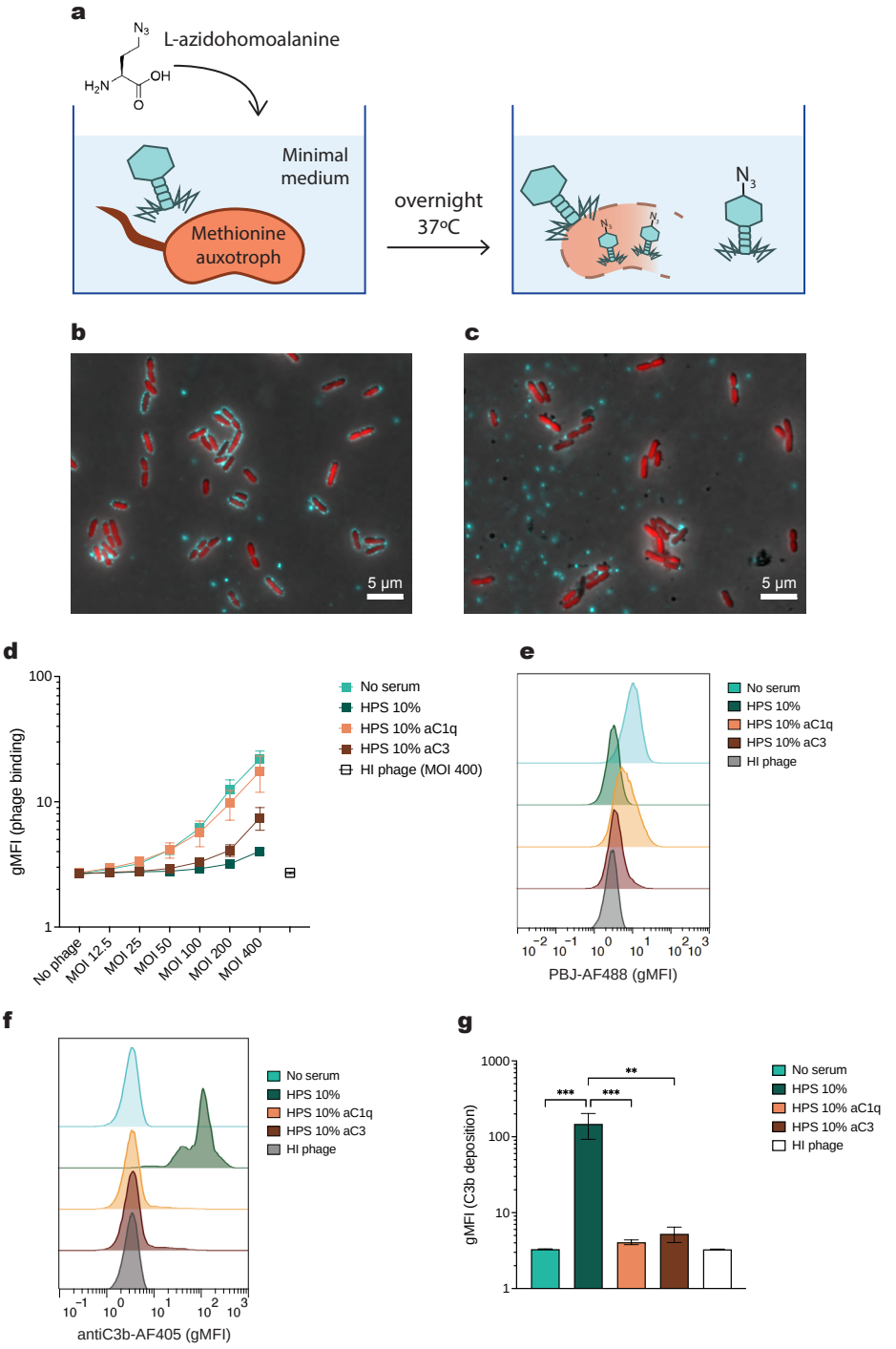
a
L-azidohomoalanine
Minimal medium
Methionine auxotroph
overnight 37°C
b
5 µm
c
5 µm
d
gMFI (phage binding)
No phage
MOI 12.5
MOI 25
MOI 50
MOI 100
MOI 200
MOI 400
No serum
HPS 10%
HPS 10% aC1q
HPS 10% aC3
HI phage (MOI 400)
e
PBJ-AF488 (gMFI)
No serum
HPS 10%
HPS 10% aC1q
HPS 10% aC3
HI phage
f
antiC3b-AF405 (gMFI)
No serum
HPS 10%
HPS 10% aC1q
HPS 10% aC3
HI phage
g
gMFI (C3b deposition)
No serum
HPS 10%
HPS 10% aC1q
HPS 10% aC3
HI phage

Figure 4. Phages tagged with an azide group and labeled with a fluorescent marker were used to study their binding to bacteria. a) Schematic view of the technique used to produce azido-tagged phages. A methionine auxotroph PAO1 mutant was cultured in minimal medium supplemented with L-azidohomoalanine. Phages were amplified overnight at 37°C using methionine auxotroph PAO1 as the host. The resulting phage progeny incorporates the non-canonical amino acid L-azidohomoalanine in place of methionine in its proteins, making them available for biorthogonal labeling via click chemistry. b,c) PAO1-sfCherry (red) was incubated with PBJ-AF488 (green, MOI 50) for 5 min at 37°C in b) buffer or c) 10% HPS, fixed in 1% PFA, washed, and immobilized on agar pads. Images were acquired with a 100X immersion objective and are overlays of the modes phase contrast and widefield fluorescence with cube filters. Images are representative of three independent experiments. d–g) Flow cytometry was performed on PAO1-sfCherry incubated with PBJ-AF488 at different MOIs. When indicated, 10% HPS, anti-C1q nanobody (1 μM), or anti-C3 nanobody (1 μM) was added for 5 min at 37°C. Samples were stained with monoclonal anti-C3-AF405 antibody and fixed in 1% PFA before measuring. Bacteria were gated on sfCherry-height and forward scatter-height signals. d) Geometric mean of the signal corresponding to bound phages. Heat-inactivated (HI) phage was used as a negative control. e) Histograms of phage fluorescent signal at an MOI of 200 or HI phage at an MOI of 400 in the various serum conditions. f) Histograms of fluorescent signal corresponding to C3b deposition (AF405) on bacteria treated with the various serum conditions. g) Geometric mean of the signal corresponding to C3b (AF405) for the conditions with PBJ-AF488 at an MOI of 200, or HI phage at an MOI of 400. Statistical analysis was performed using an ordinary one-way ANOVA with Tukey's multiple comparisons test. Significance is shown as ** $p \leq 0.01$ or *** $p \leq 0.001$. d,g) Data represent mean ± SD of three independent experiments. e,f) Signal was normalized by number of events. A representative graph of three independent experiments is shown.

binding in these conditions revealed that the phage fluorescent signal increases when increasing the MOI in the absence of serum (figure 4d). Although a relatively high MOI was necessary to obtain a sufficiently high phage-binding signal, it seems that phage binding was not yet saturated even at the highest MOI. Heat-inactivated phages did not bind to bacteria, supporting that the signal was specific for the binding of active phages. When serum was added, phage binding showed a sharp decrease (figure 4d,e). As seen before in the phage lysis assays, the inhibitory effect became less pronounced as the MOI increased. In accordance with our previous results, we could recover phage binding almost fully by inhibiting serum with the C1q-targeting nanobody (C1qNb75), while the nanobody targeting all forms of C3 (hC3Nb2) only partially recovered phage binding. In all of the conditions analyzed, the shift in the phage fluorescent signal occurred homogeneously, meaning that phage binding was evenly distributed throughout the bacterial population (figure 4e). In addition to phage binding, we stained the samples for bound C3b to confirm that there was complement activation on the bacterial surface. Very high levels of C3b were detected on bacteria incubated with active serum (figure 4f,g). Interestingly, both nanobodies could completely inhibit C3b deposition at the concentrations tested. In the conditions treated with the C3 nanobody, phage biding was still

inhibited even in the absence of C3b deposition. In conclusion, these results show that active complement, and in particular C1q, hinders the ability of phages to bind to their host.

Complement inhibitory effect is not mediated by receptor blockage

Our results from phage binding assays performed with fluorescent phages reveal that complement blocks phage binding to bacteria. This could be due to

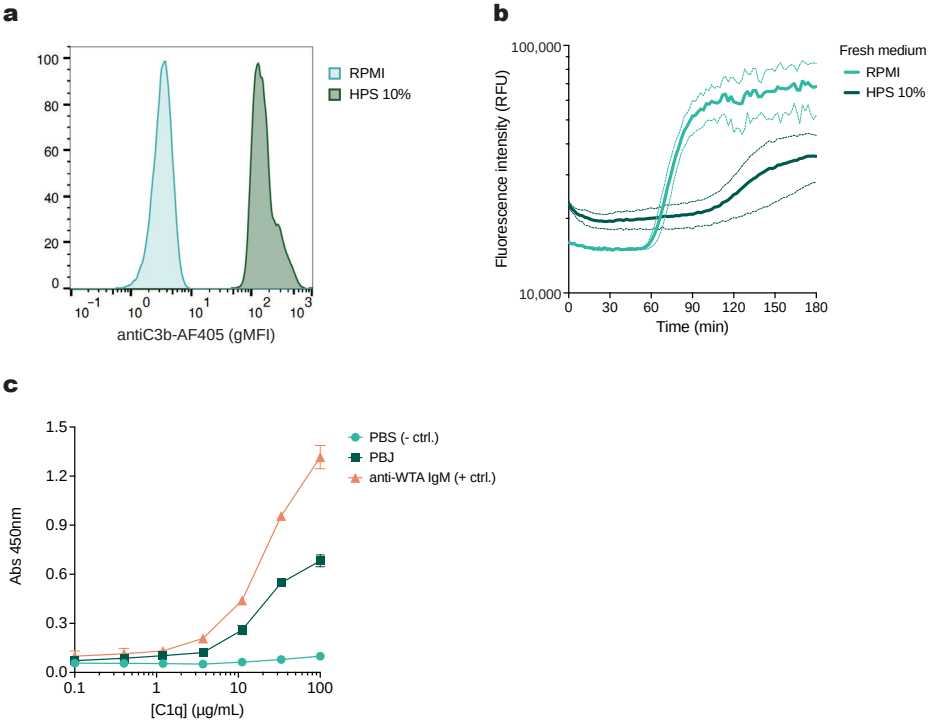

Figure 5. Inhibition of phages by the complement system is not caused by blockage of phage receptors. a) Flow cytometry histograms showing C3b deposition (AF405) on bacteria incubated with 10% HPS for 30 min at 37°C after removal of unbound serum components and staining with aC3b-AF405 nanobody. Signal was normalized by number of events. A representative graph of three independent experiments is shown. b) Fluorescence intensity (RFUs) over time of complement pre-opsonized PAO1 incubated at 37°C with phage PBJ (MOI 10) added either in buffer (RPMI) or in 10% HPS in the presence of Sytox Green. Data represent mean ± SD of three independent experiments. c) An ELISA was performed to assess the binding of C1q to phage PBJ. Wells coated with buffer were used as a negative control, while wells coated with an anti-*S. aureus* WTA human IgM were used as a positive control. Data represent mean ± SD of an experiment performed in triplicate.

complement components shielding receptors on the bacterial surface. However, it could also be caused by a direct interaction of complement with the viral particles. To discriminate which of these processes was hampering phage binding, we first studied the infectivity of phages on bacteria pre-opsonized with HPS. Opsonization was achieved by incubating bacteria with HPS for 30 min at 37 °C and washing away the unbound serum components. We confirmed that bacteria treated in this way were indeed covered with complement components C3b (figure 5a). We then used the fluorescent DNA dye assay to assess whether phages induce the lysis of pre-opsonized bacteria (figure 5b). When phages were added to the bacteria together with fresh buffer (RPMI), we still observed an increase in the fluorescence signal. In the case where phages were added together with fresh serum, phage infection was still inhibited, consistent with our previous findings. To address whether the inhibitory effect of complement is the result of an interaction with the phage, we performed an ELISA to assess the binding of purified C1q to phage particles (figure 5c). This showed that C1q can indeed bind directly to immobilized phages in a concentration-dependent manner. This interaction could be responsible for blocking the ability of phages to attach to their host. In summary, our results indicate that phage inhibition is not mediated by complement blocking phage adsorption to receptors on the bacterial surface, but rather by an interaction of complement components with the viral particles.

Discussion

The past decade has seen an unprecedented increase in interest regarding bacteriophage therapy. New efforts are being made to investigate its clinical potential, while physicians strive to work around the stringent regulations attached to its use. At the same time, numerous case reports have been published in the past few years highlighting the potential of phages as therapeutics, both to substitute and complement antibiotics. In this context, it is important to keep advancing our fundamental knowledge on how phages may behave inside the body of a patient.

The purpose of our study was to explore the interactions of phages with the human

immune system, and in particular with the complement system. To tackle this issue, we used human serum as a source of complement and characterized the performance of phages in this medium using different assays. Our results show that some phages are inhibited by serum in a concentration-dependent way, while others remain unaffected. When looking at a broader panel of phages, it was only the myophages that were challenged by this phenomenon. However, due to the limited number of podo- and siphophages available for us to test, it is difficult to fully relate the observation to the phage morphotype. Furthermore, we do not know the receptors of all the phages we tested, what their optimal host is, or how closely related they are to each other. Nonetheless, when we tested the activity of *S. aureus* myophage K, we saw that it was also inhibited by serum, further hinting at the importance of the morphotype. In any case, the examination of a more complete and well characterized set of phages, including more phages targeting other bacterial species, would be valuable in finding patterns to potentially predict sensitivity to serum.

We were also able to show that phage inhibition is mediated by the early stages of the classical complement pathway, as infective activity could be rescued by blocking C1q or C3. The action of the classical pathway, initiated by the deposition of C1q, can kickstart complement activation, while the alternative pathway can later on contribute to amplifying the reaction. This is reflected by our results with the nanobodies targeting C1q and C3. While both C3-targeting nanobodies could partially counteract the inhibitory effect of serum, it was only when C1q was also targeted that we observed a full recovery of the phage infection.

In this study, we also demonstrated a novel technique for producing fluorescently labeled phages in *P. aeruginosa*, based on incorporation of the non-natural amino acid L-azidohomolalanine and biorthogonal labeling by means of click chemistry. This method is more labor-intensive than random labeling approaches, but we found that it better preserved the activity of the phages. Furthermore, azido-chains incorporated into the phage capsid could potentially be functionalized with other molecules for a variety of applications. Using this technique, we were able to produce fluorescently labeled PBJ in high titers and study its binding to PAO1

through microscopy and flow cytometry. A high phage MOI was needed in these experiments to produce a detectable fluorescent signal. This constitutes a limitation, as the experimental conditions were not directly comparable to those of the other assays performed in this study. Nonetheless, the experiments described in Section 4 revealed that complement hampers phages by interfering with their ability to bind to the surface of the host. An intuitive explanation for this could be that C1q or C3b could shield phage receptors. However, when we assessed phage activity on bacteria opsonized with complement components using the fluorescent DNA dye assay, we observed that phages were still able to induce lysis. We hypothesize that complement activation may have a direct neutralizing effect on phages. The ability of complement to recognize and opsonize viral particles has been shown in studies with eukaryotic viruses[39], and the same could hold true for phages. Attachment of heavy proteins such as C1q or C3b to the viral particles could interfere with their infectivity. This could be particularly relevant for myophages, as they rely on the contraction of their tail to introduce their genetic material into the host[40]. Addressing these questions would be an interesting perspective for future studies.

Although we found that phage inhibition was not mediated by neutralizing antibodies in our assays, specific anti-phage antibodies in our serum pool could also be important in activating the classical complement pathway. Antibodies against head proteins of *Escherichia coli* (*E. coli*) myophage T4 have been found in individuals who have never been subjected to phage therapy[41]. The same proteins could induce the production of neutralizing antibodies and complement activation when used to immunize mice. Similarly, in a study performed on an *E. coli* podophage T7 peptide display library, recognition of certain peptides by IgM was seen to initiate a neutralizing complement response[42]. Nonetheless, our results show that C1q can bind directly to phage particles in the absence of antibodies. There is other evidence that activation of the classical pathway on viruses can occur independently of antibodies; for example, through the interaction of C1q with serum C reactive protein[43]. Additionally, direct binding of C1q to viruses such as dengue, influenza A, and SARS-CoV-2 seems to be sufficient in some cases to reduce infectivity[44–46]. Inactivation of phages by complement components independent of antibodies could explain the observations reported in several studies where phage

activity was compromised in the presence of human serum or plasma[47–49].

Taken together, our results provide evidence that certain phages may be less competent than others in targeting bacteria in certain physiological situations, like in blood or tissues where complement is present. Although it remains unclear how these results would translate to an *in vivo* situation, our observations could still have implications on how we select phages for therapy. Researchers are working on ways to optimize this process and make it faster and more efficient. To this end, phage biobanks are currently being set up in different countries, and sharing phages between different institutions is gradually becoming a more usual practice. Several phages against *P. aeruginosa* have been characterized and are being used as medicinal products in the context of magistral phage preparations[50,51]. Efforts are also being made in engineering phages to increase their host range, as well as in optimizing phage cocktails to avoid the problem of phage resistance. Additionally, bioinformatic pipelines have been developed that may allow us to predict which phages will be more effective against a certain clinical isolate[52]. However, in spite of all these important advances, the interactions of phages with the patient's innate immune system are still largely ignored in the process of therapeutic phage selection. In light of our findings, we suggest that performing screenings in the presence of serum would be valuable when designing phage cocktails.

In conclusion, this study shows that complement activation via the classical pathway can inhibit the anti-bacterial activity of certain phages, with myophages being more susceptible to this effect. We have shown that inhibition is not mediated by deposition on the bacterial surface but is rather caused by a direct effect on phages themselves. Our findings highlight the need to better understand how phages behave in physiological conditions when administered to a patient as a therapeutic. Selection of phages according to their expected performance in human blood or tissues could contribute to improving the efficacy of phage therapy.

Materials and methods

Phages and Strains

Phage PBJ was isolated in our lab after two rounds of amplification from a mixed stock using *P. aeruginosa* strain PAO1 as the host. Original stocks of *Pseudomonas* phages 14–1, LKD16, and LUZ19 were kindly provided by Rob Lavigne (KU Leuven, Belgium). *Pseudomonas* phages (Pa collection) and phage K were obtained from the Fagenbank (Delft, The Netherlands). Phage amplification was carried out by infecting the host strain PAO1 overnight at 37 °C in Lysogeny Broth (LB). Bacterial debris was removed by centrifugation at 11,000 × *g* (RCF) for 40 min at 4 °C. Phages were then incubated for 2 h on ice with a solution of 10% PEG-8000 and 0.5 M NaCl and precipitated by centrifugation at 11,000 × *g* (RCF) for 40 min at 4 °C. The preparation was mixed with chloroform, after which the aqueous phase was sterilized using a 0.2 µm filter and incubated with DNase and RNase (5 µg/mL each) for 30 min at room temperature. Final purity was achieved by filtration through a Zeba Spin desalting column (40K MWCO, Thermo Fisher Scientific, Waltham, MA, USA). Phages were recovered and stored in SM buffer (100 mM NaCl, 8 mM $MgSO_4 \cdot 7H_2O$, 50 mM Tris-Cl).

Phages labeled with an azido-handle were produced in a PAO1 metZ knockout strain, obtained from the University of Washington collection[53]. Phage amplification was performed by infecting the bacteria overnight in M9 minimal medium supplemented with methionine (5 mg/L) and the methionine homolog azido-homo-alanine (40 mg/L). After removal of bacterial remains, phages were pelleted using PEG-8000/NaCl as described above, filter-sterilized, and concentrated using an Amicon filter (100kDa cut-off) (Sigma-Aldrich, St. Louis, MO, USA). To achieve fluorescent labeling, phages were incubated with DBCO-AF488 (Jena Bioscience, Jena, Germany) for 1 h at 37 °C. Excess dye was washed off by washing with SM buffer in an Amicon filter (100 kDa cut-off).

PAO1 expressing sfCherry was obtained by transforming PAO1 with the plasmid pUCP30T modified to express sfCherry[54]. Bacteria were made competent by washing in 300 mM sucrose, after which the plasmid was introduced by electroporation.

Selection was performed on plates containing gentamycin (30 µg/mL). *S. aureus* strain ATCC 19685 was kindly provided by Ana Rita Costa (Fagenbank; TU Delft, The Netherlands).

Genome Sequencing and Bioinformatics Analysis

The genomic DNA library for phage PBJ was prepared with the Nextera Flex kit (Illumina, San Diego, CA, USA) and sequenced using the Illumina MiniSeq using a paired-end approach (2 × 150 bp). The reads were then controlled for quality using FastQC[55] and we used Trimmomatic[56] to remove adapter sequences, filter by length (>50 bp), and trim lower quality regions (Trimmomatic options: ILLUMINACLIP:NexteraPE-PE.fa:2:30:10LEADING:3 TRAILING:3 SLIDINGWINDOW:4:15 MINLEN:50). The genome was assembled de novo using the SPAdes assembler with default options, including the pipeline option "--careful"[57]. The quality of the assembly was inspected with Bandage[58] to allow us to extract the relevant circular phage contigs. The genome of phage PBJ was annotated using Pharokka[59] and compared with the closely related phage 14–1 genome (NC_011703.1).

Bacterial Growth

For all experiments, bacteria were cultured overnight in LB from a single colony at 37 °C with shaking. On the day of the experiment, bacteria were diluted 1:31 in LB and sub-cultured to mid-log phase (OD_{600nm}~0.5). Then, bacteria were washed in RPMI 1640 buffer (Thermo Fisher, Waltham, MA, USA), supplemented with 0.05% human serum albumin, Sanquin), pelleted, and resuspended to an OD_{600nm} of 1.0 (~8×10^8 bacteria/mL) in RPMI buffer. Bacteria were further diluted as specified for the different assays.

Serum Preparation and Reagents

Human pooled serum (HPS) was isolated from healthy volunteers at the UMC Utrecht (The Netherlands). Briefly, blood was drawn, allowed to clot, and centrifuged to isolate the serum. Serum from 15–20 donors was pooled, aliquoted, and stored at −80 °C. Heat-inactivated HPS (HI HPS) was prepared by treating HPS at 56 °C for 30 min to selectively inactivate complement proteins. Pooled IgG/IgM was isolated

from HPS as previously described[60]. Compstatin was kindly provided by John Lambris (University of Pennsylvania, Philadelphia, PA, USA). OMCI was produced and purified as previously described[30]. Complement-targeting nanobodies were produced as described by their developers[32–35]. Monoclonal mouse antibody bH6 recognizing human activated C3 (Hycult Biotech, Uden, The Netherlands) was produced as previously described[18].

Microplate Reader Assays

Bacteria (PAO1), phages, and Sytox Green nucleic acid stain (Thermo Fisher, Waltham, MA, USA) were mixed together to a final concentration of ~2 × 10^7 bacteria/mL and 5 µM Sytox Green. Concentration of phages is dependent on the multiplicity of infection (MOI), which is indicated for each experiment. Where indicated, human pooled serum, heat-inactivated human pooled serum, purified IgG/IgM from pooled serum, or serum with complement inhibitors was added in the concentrations specified in each of the figures. For experiments with pre-opsonized bacteria, prior to the addition of phages, bacteria were incubated with serum at 37 °C for 30 min, centrifuged at 2000–3000 × *g* (RCF) for 8 min, and resuspended in fresh medium. Fluorescence measurements were performed in a microplate reader (FLUOstar Omega, BMG Labtech, Ortenberg, Germany) at 37 °C, in a clear, flat-bottomed 96-well plate (Corning, Corning, NY, USA), with the following settings: $\lambda_{excitation}$ = 490 nm, bandwidth = 14 nm; $\lambda_{emission}$ = 537 nm, bandwidth 30 nm; gain = 1300.

Determination of Bacterial Viability

Bacteria (*S. aureus* strain ATCC 19685) were mixed with phage K to a concentration of 2 × 10^7 bacteria/mL and a phage concentration of 2 × 10^8 phages/mL (MOI of 10). The mixture was incubated at 37 °C with shaking for 120 min. Samples were taken and serial dilutions were performed in RPMI buffer. A total of 5 µL of each sample was plated on LB agar and incubated overnight at 37 °C. Colonies were counted and the CFUs/mL was calculated.

Flow Cytometry

Bacteria (PAO1-sfCherry) at a final concentration of ~2 × 10^7 bacteria/mL were mixed with PBJ-AF488 in various MOIs and, in some cases, 10% HPS. Nanobodies (anti-C1q and anti-C3) were added when indicated at 1 μM. Heat-inactivated PBJ-AF488 (heat treatment: 85 °C for 50 min) was used as a control at an MOI of 200. Samples were incubated for 5 min at 37 °C with shaking, after which bacteria were washed in cold buffer and pelleted at 4000 rpm at 4 °C for 8 min. The supernatant was removed, and bacteria were resuspended in a solution of bH6-AF405 antibody against C3b at 3 μg/mL in RPMI 1640 with 0.05% HAS. After incubation on ice for 10 min, samples were fixed using cold PFA to a final concentration of 1%. Samples were finally pelleted at 3500 rpm for 8 min, resuspended in fresh buffer to a concentration between 10^6 and 10^7 bacteria/mL, and analyzed via flow cytometry (MACS Quant, Miltenyi Biotech, Bergisch Gladbach, Germany). Per condition, 10 μL was measured. Bacteria were gated using sfCherry signal height.

Widefield Fluorescence Microscopy

Bacteria (PAO1-sfCherry) were grown to mid-log phase (OD_{600nm}~0.5) in LB, pelleted, and resuspended to an OD_{600nm} of 1.0 (~8 × 10^8 bacteria/mL) in RPMI 1640 with 0.05% HSA. Bacteria at a final concentration of 10^8 bacteria/mL were mixed with phages at an MOI of 50. HPS was added to some conditions at a concentration of 10%. The mixture was incubated for 5 min at 37 °C with shaking. Then, samples were fixed using cold PFA to a final concentration of 1%. After 15 min incubation on ice, the samples were centrifuged at 3500 rpm for 8 min. The supernatant was removed, and the samples were resuspended in fresh RPMI to an estimated concentration of 4 × 10^8 bacteria/mL. Then, the samples were immobilized on microscope slides prepared with agarose pads. To make the agarose pads, 50 μL 1% agarose gel in PBS was added to an objective slide, after which a siliconized cover slip was applied on top of the agarose gel to flatten it. Once the agarose was solid, the cover slips were removed and 10 μL of the sample was added to the pad. After the samples were dry, a cover slip was placed on the pad. The samples were imaged using a Leica TCS SP5 II microscope with an HC PL APO CS 100×/1.4 OIL objective (Leica Microsystems, Amsterdam, The Netherlands). The images were obtained by overlaying the modes phase contrast and widefield fluorescence with

cube filters for RFP (N21, dichroic mirror: 580 nm) and GFP (dichroic mirror: 500 nm), respectively.

C1q ELISA

A Nunc MaxiSorp ELISA plate (Thermo Fisher Scientific, Waltham, MA, USA) was coated overnight at 4°C with the following: 5×10^8 phage PBJ per well in PBS, 50 ng per well of an anti-*S. aureus* WTA human IgM antibody in PBS as a positive control, or PBS as negative control. To prepare the phages for this purpose, a sterile lysate of phage PBJ was concentrated by ultracentrifugation and purified of endotoxins using EndoTrap HD (Lionex GmbH, Braunschweig, Germany) according to the manufacturer's instructions. After coating, the plate was blocked with 4% skimmed milk powder (Campina, The Netherlands). Next, C1q (Complement Technology) was added in a range of concentrations. To detect C1q binding, polyclonal rabbit anti-human C1q antibody (Agilent Technologies, Santa Clara, CA, USA) was used at 1 µg/mL. This was in turn detected using goat anti-rabbit IgG antibody conjugated with horseradish peroxidase (Southern Biotech, Birmingham, AL, USA) diluted to 1:5000. Before each of the steps, the plate was washed 3 times with PBS supplemented with 0.05% Tween-20 (PBS-T). Finally, the substrate tetramethylbenzidine (TMB) was added and incubated for 3 min, after which the reaction was stopped with H_2SO_4 (0.5 M). The detection antibodies as well as C1q were diluted in PBS-T supplemented with 1% skimmed milk powder. All of the incubation steps were performed at room temperature for 1 h unless otherwise stated. Absorbance was measured at 450 nm using an iMark Microplate Reader (Bio-Rad, Hercules, CA, USA).

Data Analysis and Statistical Testing

Data visualization and statistical analyses were performed in GraphPad Prism 9 and are further specified in the figure legends. Flow cytometry data were analyzed using FlowJo™ v10.8.1 software. Widefield fluorescence microscopy images were processed using Fiji. Figures were produced using Adobe Illustrator.

Additional information

Author Contributions

Conceptualization: J.E.E., S.H.M.R., B.W.B., and P.-J.H.; methodology: J.E.E. and S.O.D.; validation: J.E.E.; formal analysis: J.E.E., C.T.-B., and C.L.; investigation: J.E.E., S.O.D., and C.T.-B.; writing—original draft preparation: J.E.E.; writing—review and editing: J.E.E., C.L., B.W.B., and P.-J.H.; visualization: J.E.E.; supervision: S.H.M.R., B.W.B., and P.-J.H.; project administration: S.H.M.R.; funding acquisition: S.H.M.R. and P.-J.H. All authors have read and agreed to the published version of the manuscript.

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Institutional Review Board Statement

Human serum from healthy volunteers was collected with informed consent in accordance with the Declaration of Helsinki through the Minidonor Service, a blood donation facility at the UMC Utrecht. Approval for this service was obtained in February 2019, with protocol number TCBio 18-774.

Informed Consent Statement

Informed consent was obtained from all donors involved in the study.

Data Availability Statement

All the datasets generated and analyzed during this study are presented in the main text or the supplementary materials. Genomic sequence of phage PBJ is available from Genbank, accession number: OR611941.

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Conflicts of Interest

The authors declare no conflicts of interest. The funders had no role in the design of the study; in the collection, analyses, or interpretation of data; in the writing of the manuscript; or in the decision to publish the results.

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Supplementary information

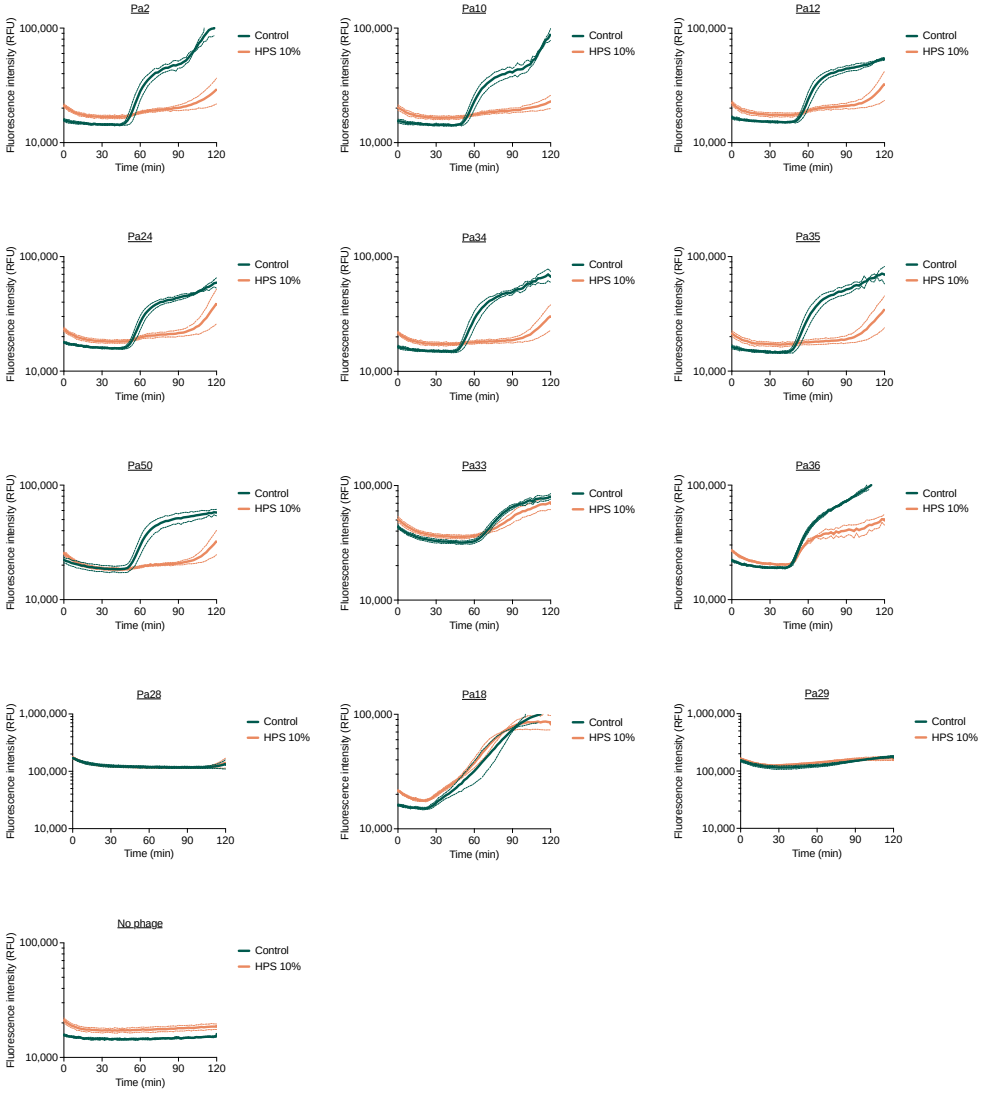

Figure S1. Human serum inhibits a variety of myophages targeting PAO1. Fluorescence intensity (relative fluorescence units, RFU) over time of PAO1 infected at 37°C in presence of the DNA dye Sytox green with different phages (MOI 1) in absence of serum (control) of in 10% HPS. Data represent mean ± SD of three independent experiments.

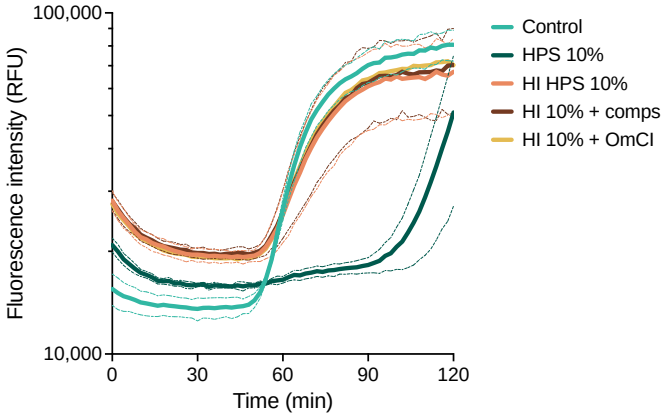

Figure S2. Inhibition of phages by serum is mediated by the complement system. PAO1 was incubated with phage PBJ at 37oC in presence of the DNA dye Sytox green. Data represents fluorescence intensity (RFU) over time (min) of bacteria infected with PBJ at an MOI of 10 (control), in presence of 10% HPS, 10% HI HPS, 10% HI HPS with 50 µM compstatin, or 10% HI HPS with 20 µg/mL OmCI. Data represent mean ± SD of three independent experiments.

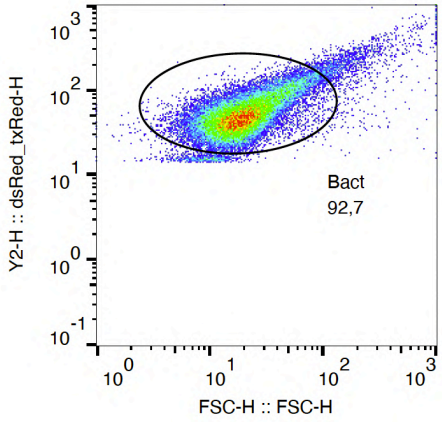

Figure S3. Example of gating strategy of PAO1-sfCherry for flow cytometry experiments. Y axis shows high of sfCherry signal, X axis shows height of forward scatter signal. Sample shown in this case are bacteria treated with 10% HPS for 5 minutes at 37°C.

Chapter 4

Finding an ally: synergy between antibiotics and phages against *Pseudomonas aeruginosa*

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Manuscript in preparation

Abstract

Bacteriophage therapy is gaining interest as an alternative form of treatment against antibiotic-resistant bacteria. Recent reports suggest that bacteriophages (phages) and antibiotics can act synergistically, achieving therapeutic success. However, it is not known which antibiotics can work best in combination with phages, or what are the mechanisms governing these interactions. In this study, we aimed to determine whether different antibiotics can act synergistically with phages targeting *Pseudomonas aeruginosa*, and to elucidate the molecular mechanisms behind synergy. To do so, we screened combinations of phages and antibiotics using a novel method based on a bioluminescence reporter for metabolic activity. Reduction in bioluminescence was monitored over time and quantified as a proxy for loss of viability. In our screening, we combined antibiotics of different classes (aminoglycosides, beta lactams and a polymyxin) with a phage targeting lipopolysaccharide (LPS) or a phage targeting type IV pili (T4P). We observed that the activity of both phages was enhanced when combined with beta lactams, as well as with aminoglycosides to a lesser extent. To study the mechanisms driving synergy, we focused on the synergistic combinations of LPS-targeting phage PBJ with the beta-lactams meropenem and ceftazidime. We used microscopy and flow cytometry to study whether these antibiotics could induce filamentation, a phenomenon previously associated with phage-antibiotic synergy. Our results showed that ceftazidime induces filamentation, while meropenem does not, indicating that this process is not required for synergy. Finally, we assessed the burst size of PBJ by performing one step growth curve assays on bacteria cultured in presence of meropenem or ceftazidime. This revealed that treatment with either of these antibiotics extends the latent period and increases the burst size of PBJ. Overall, we have identified several promising phage-antibiotic combinations, and report that meropenem and ceftazidime can boost phage anti-bacterial activity, likely due to increasing the production of phages inside infected cells. Our results may contribute to a better understanding of phage-antibiotic interactions, a key aspect of modern phage therapy.

Introduction

Bacterial antimicrobial resistance is considered a major concern in modern health care. Prolonged and extensive use of antibiotics over time has caused a decrease in their effectiveness, making the treatment of certain pathogens a real challenge[1]. *Pseudomonas aeruginosa* is one of these pathogens, as it can rapidly develop antibiotic resistance, in addition to being intrinsically insensitive to many widely used antibiotics[2]. Furthermore, the number of antibiotics in the clinical development pipeline has decreased drastically over the last decade and the ones available are not potent against multidrug resistant *P. aeruginosa*[3,4]. This demonstrates a clear need for novel therapeutics or treatment strategies against this bacterium.

One strategy that has received increasing attention is the use of bacteriophage (phage) therapy[5]. This therapy makes use of viruses that can specifically target and infect bacteria. Phages used in therapy often follow a lytic cycle, during which the bacterial machinery is hijacked to produce new viral particles, in a process which ultimately results in host lysis and spread of the new viral progeny[6]. Using phages as therapeutics comes with a number of benefits, including host-specificity, self-amplification, and self-dosing. They can target the pathogen of interest without an effect on the host microbiota, and they can amplify at the site of infection until the bacterial load is cleared. In addition, they have generally been shown to be safe and well tolerated in studies to date[7]. Although phages are often thought of as an alternative for antibiotics, recent research suggests that using them as a combination therapy together with antibiotics may yield much better results in combating bacterial infections[8]. The aim of combining phages with antibiotics is to establish synergy, where the cumulative effect of both is greater than the sum of the effects of the single agents[9].

Phage-antibiotic synergy (PAS) has been explored in various *in vitro* and *in vivo* models. Phage-antibiotic combinations could achieve bacterial reduction of biofilm or planktonic *P. aeruginosa* cultures in contrast to the individual agents[10–13]. Some studies further validate these results in animal models, demonstrating that phage-antibiotic combination therapy could increase overall survival[14,15]. Besides from *in*

vivo and *in vitro* studies, many interesting clinical case reports have been published where phage-antibiotic combination therapy eradicated *P. aeruginosa* in various types of infections, improving the patients' health[16–19]. These cases demonstrated that the combination of phages and antibiotics can inhibit biofilm production and increase antibiotic sensitivity, thereby enhancing the antimicrobial effect. However, finding an effective combination remains challenging. The molecular mechanisms that drive synergy between phages and different antibiotics have not yet been fully elucidated. As a result, clinical approaches often select phage-antibiotic combinations based on a trial-and-error method[20–22]. Reports in literature therefore describe a broad variety of combinations used to treat different bacterial infections, which makes it challenging to find potential patterns to predict synergistic phage-antibiotic combinations. Nonetheless, a few mechanisms behind PAS have been identified or proposed[23]. It seems that combining phages and antibiotics can decrease the rate at which resistance to either agent is developed[9,24]. Alternatively, when bacteria mutate to overcome a phage challenge, they may become resensitized to antibiotics or less virulent[25]. In the case of biofilm-producing *P. aeruginosa*, combining phages with antibiotics of different classes like ceftazidime, a beta-lactam, and ciprofloxacin, a fluoroquinolone, can boost bacterial reduction[26]. In addition, pre-treatment with phages may increase the effect of other antibiotics like the aminoglycosides tobramycin and gentamicin. One of the reasons for this might be that phage enzymes can degrade the extracellular matrix and increase the penetration of the antibiotics, an effect also shown for *P. aeruginosa* biofilms[27]. The stress inflicted in bacteria by sublethal antibiotic doses could also delay phage lysis as seen for the beta-lactam cefotaxime and the fluoroquinolone ciprofloxacin, potentially contributing to an increased phage production[28]. Fluoroquinolones and some beta-lactam antibiotics can induce filamentation, where bacteria become elongated and remain attached to one another due to incomplete cell division[29]. This could provide phages with an increased surface area to attach to, while also facilitating the assembly of new virions within the cell[30,31]. However, reports in literature can be contradictory, as there is also evidence that supports that antibiotics accelerate phage lysis rather than delaying it[32], and that filamentation does not have a large impact on the rate of phage adsorption[28]. Synergy may not only depend on the bacterial strain, but also on the specific phage and the class

and dose of antibiotic[9]. Overall, the mechanisms behind PAS may vary with each class of antibiotic and even each phage, so more insights are needed to fully understand this phenomenon.

In this study, we aimed to identify synergistic phage-antibiotic combinations to treat *P. aeruginosa* and unravel potential underlying mechanisms. To do so, a broad screening was used which paired antibiotics from different classes (aminoglycosides, beta lactams and a polymyxin) with phages targeting different receptors (lipopolysaccharide (LPS) or type IV pili (T4P)). These combinations were used on two laboratory *P. aeruginosa* strains and one clinical isolate. Our study demonstrates that most of the antibiotics we tested led to an increased reduction in bacterial viability when combined with phages. Synergistic interactions were quantified for the combination of phages with meropenem, ceftazidime and tobramycin. In addition, we show that meropenem and ceftazidime induced different changes in the bacterial phenotype which corresponded with enhanced phage activity. These findings may be valuable in understanding PAS and ultimately predicting beneficial phage-antibiotic combinations.

Results

Phages boost the effect of different antibiotics against *P. aeruginosa*

We investigated how different antibiotics work together in combination with phages. To do so, we performed a screening on *P. aeruginosa* laboratory strain PAO1 modified to express the *lux* system[33]. This system allows us to monitor metabolic activity through a bioluminescence readout in high throughput. In addition, the *lux* operon is inserted into the bacterial chromosome via recombination, eliminating the need for selection with antibiotics. We combined a range of concentrations of the antibiotic tobramycin with a range of multiplicities of infection (MOIs) of phage PBJ in a checkerboard assay (figure 1a). Tobramycin is an aminoglycoside that prevents protein translation by binding to the bacterial ribosome. For each of the phage-antibiotic combinations, we monitored luminescence over time for a period of 8 hours. We compared the signal obtained from an untreated growth control

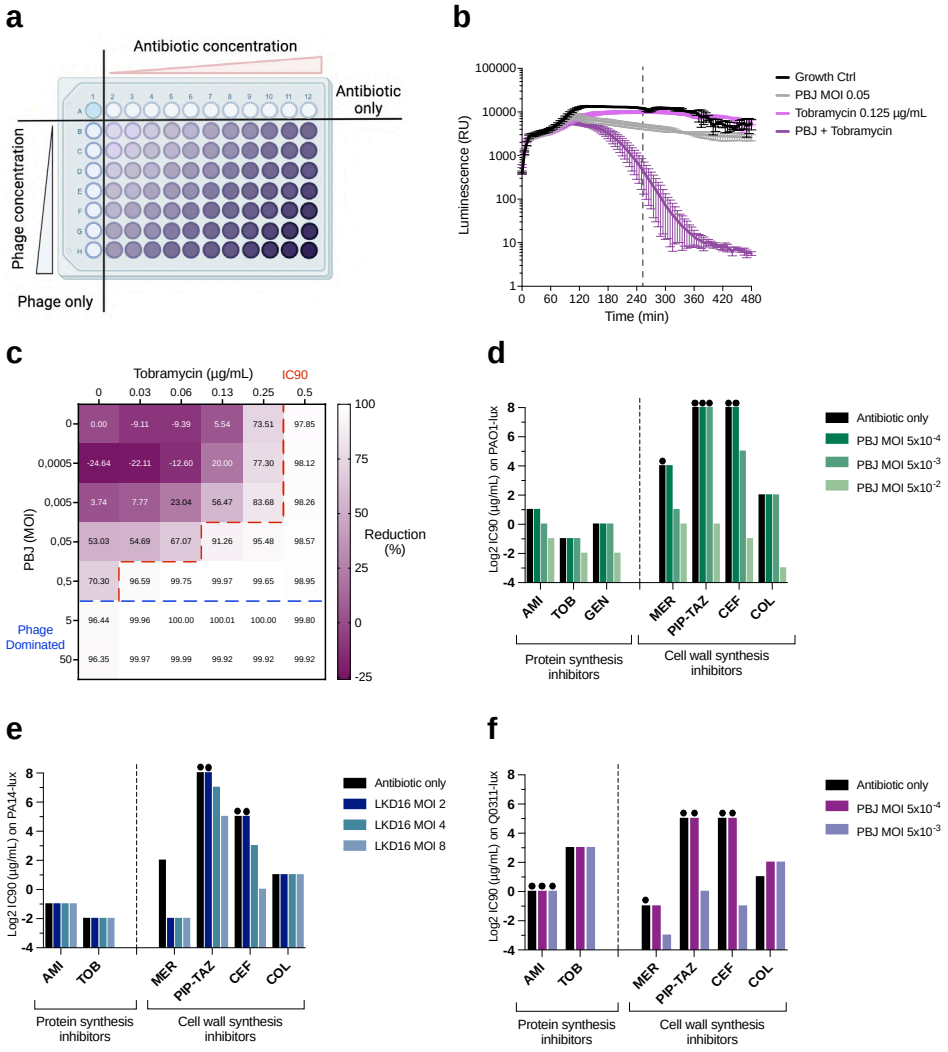

Figure 1. Combinations of phages and antibiotics achieve an enhanced reduction in *Pseudomonas aeruginosa* metabolic activity compared to the individual agents. Strains expressing a luciferase operon (*lux* system) were incubated in presence of different phages and antibiotics at 37ºC. Reduction in luminescence was monitored as a readout for bacterial metabolism. a) Combinations of phages and antibiotics were screened in a checkerboard style assay. In a 96-well plate, a range of concentrations of antibiotic (horizontal) was combined with a range of phage multiplicities of infection (MOI, vertical). b) Luminescence was measured in each well every 5 minutes over a period of 8 hours. In this example, strain PAO1 was treated with phage PBJ at an MOI of 0.05 or with tobramycin at a concentration of 0.125 µg/mL or with a combination of both agents. Luminescence values at time-point 240 minutes (dotted lined) were taken to calculate the percentage

of reduction in bacterial population. Data represent mean ± SD of three independent experiments. c) Percentage of reduction compared to the buffer control after 240 minutes was calculated for all the conditions tested in the checkerboard assay and represented as a heat map. Lighter color indicates a higher reduction in bacterial population. Dotted red line signals conditions for which more than 90% reduction was achieved. The minimum antibiotic concentration needed to achieve this is from here on referred to as IC90. In the conditions represented below the blue dotted line, bacterial reduction was predominantly phage-mediated. A representative graph of two independent experiments is shown. d,e,f) IC90 of the different antibiotics screened is represented as calculated for individual treatment or in combination with d) phage PBJ on strain PAO1, e) phage LKD16 treating strain PA14, or f) phage PBJ treating clinical isolate Q0311. Black dots indicate conditions where 90% reduction was not achieved after 240 minutes. Data were obtained from a single checkerboard experiment for each antibiotic-phage combination.

with that of bacteria treated with only antibiotic, only phage, or both (figure 1b). At low concentrations of antibiotic and phage the combination treatment achieved a much greater reduction in the luminescence signal. For instance, when combining 0.125 ug/mL of tobramycin with an MOI of PBJ of 0.005 a 1000-fold reduction was achieved compared to the growth control, while the individual treatments did not seem to affect metabolic activity at these concentrations. To visualize the whole range of phage-antibiotic combinations, we calculated the percentages of reduction in luminescence for each condition after 4 hours (figure 1c). This time point was chosen because after 4 hours fluctuations in the luminescence signal of the growth control were observed. From the percentage of reduction obtained with each combination, we determined the IC90, defined as the lowest concentration of antibiotic with which a reduction of 90% or higher was achieved after 4 hours. Combining tobramycin with phage PBJ at an MOI of 0.05 or 0.5 resulted in a lower IC90, indicating that using both agents together enhanced the reduction of bacterial metabolic activity. At an MOI of 5 or higher, the phage alone was able to achieve more than 90% reduction in metabolic activity.

We used this experimental set-up to screen other antibiotics of different classes: the aminoglycosides tobramycin and gentamicin, the beta-lactams meropenem, piperacillin-tazobactam and ceftazidime, and the polymyxin colistin (supplementary figure 1). Beta-lactams inhibit cell wall synthesis, while colistin binds to LPS and disrupts the outer cell membrane. In addition to PAO1, we also studied the effect of the different combinations on two other *P. aeruginosa* strains: PA14, a laboratory strain, and Q0311, a clinical strain isolated from a patient with an invasive infection.

As PA14 is not sensitive to PBJ, we used phage LKD16 on this strain. LKD16 differs from PBJ in both morphotype and receptor: LKD16, a Phikmvvirus, is a podophage targeting type IV pili, while PBJ, a Pbunavirus, is a myophage targeting LPS. For each of the strains, we compared the IC90 of the different antibiotics in combination with different MOIs of phage (figure 1d-f). We observed that all the antibiotics worked better in combination with phage PBJ when treating PAO1 (figure 1d). This was not seen for PA14 (figure 1e) and Q0311 (figure 1f) in the conditions tested, as the addition of phage did not lower the IC90 of the protein synthesis inhibitors or colistin. However, the range of phage MOIs tested on Q0311 was more limited than on the other two strains. Nevertheless, the beta-lactam antibiotics worked better in combination with phage on all of the strains, with meropenem and ceftazidime showing the biggest overall change in IC90. In summary, our results indicate that combining beta-lactams with phages may be advantageous when targeting different strains of *P. aeruginosa*.

Phage PBJ shows antimicrobial synergy with meropenem, tobramycin and ceftazidime

Based on the results of our screening, we decided to focus on investigating the antimicrobial activity of the combinations of phage PBJ with meropenem, ceftazidime or tobramycin on strain PAO1. To better understand the nature of the phage-antibiotic combined effects, we analyzed whether synergy was established between both therapeutic agents. Synergy can be defined as the interaction between two elements when their combined effect is greater than the sum of the effects of each individual element. To identify potential phage-antibiotic synergy (PAS) in our results, we first calculate the estimated population percentage that would remain if there was an additive interaction between the phage and the antibiotic (figure 2a). We named this parameter “calculated remaining %”. By calculating the ratio between the calculated and the actual remaining percentage that we measured, we obtain what we define as “synergy score”. If the combination of the phage with the antibiotic achieves an additive effect, the synergy score for this combination would be 1. Higher synergy scores indicate synergistic interactions, while if the score is lower than 1, the interaction between both agents would be antagonistic. We used this method to analyze the results obtained from

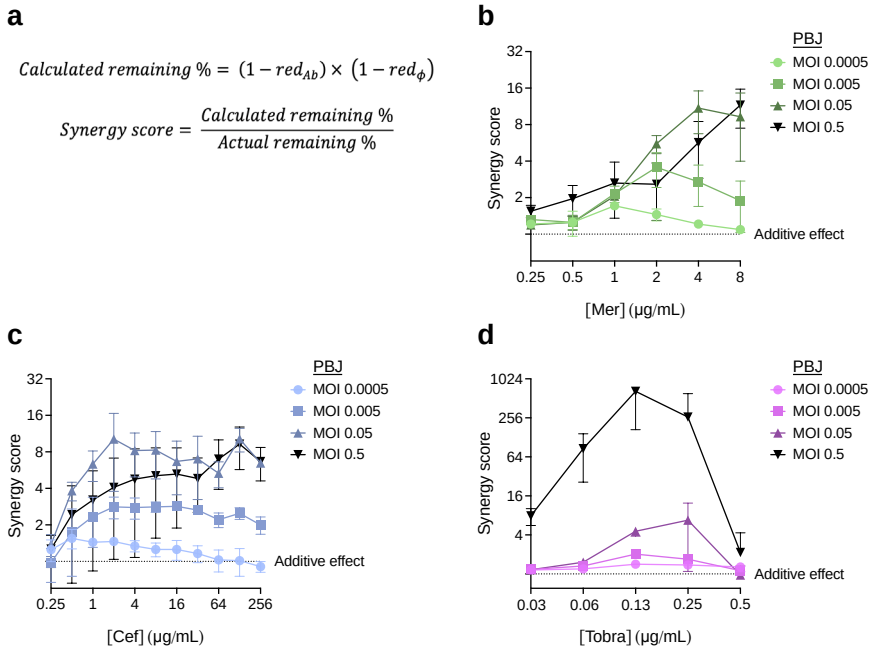

Figure 2. Checkerboard assay results obtained from *P. aeruginosa* strain PAO1 treated with phage PBJ in combination with meropenem, ceftazidime or tobramycin were further analyzed to identify synergistic phage-antibiotic combinations. a) Calculated remaining percentages reflect what would be obtained as a result of an additive effect between the reduction caused by the phage (red_{Φ}) and the reduction caused by the antibiotic (red_{Ab}). Synergy scores for each condition were obtained as the ratios between the calculated remaining percentages and the actual remaining percentages found experimentally. Synergy scores values above 1 indicate that the effect of the combination treatment was greater than the sum of the individual effects. b,c,d) Synergy score values are plotted as mean ± SD of two (c,d) or three (b) independent experiments.

checkerboard assays between PBJ and meropenem, ceftazidime or tobramycin (supplementary figures 1e, 1f and 1g, respectively). This revealed that, in these conditions, meropenem shows antimicrobial synergy with phage PBJ, specifically at antibiotic concentrations higher than 1 µg/mL and phage MOIs of 0.05 and 0.5. When combining PBJ with ceftazidime, we observed a broad synergistic pattern, where the addition of phage in MOIs ranging between 0.005 – 0.5 achieved a high synergy score for most of the tested antibiotic concentrations (figure 2c). In the case of tobramycin, the highest MOI of PBJ achieved a very high synergy score

in combination with intermediate concentrations of the antibiotic. At tobramycin concentrations higher than 0.25 µg/mL we no longer measured a synergistic effect, as the antibiotic alone already achieved a high reduction in metabolic activity (figure 2d). In conclusion, we determined that meropenem, ceftazidime and tobramycin can all work synergistically in combination with phage PBJ. However, this is antibiotic concentration dependent, with the beta lactams acting synergistically across a broader concentration range.

The beta lactams meropenem and ceftazidime induce different changes in the phenotype of *P. aeruginosa*

The biological mechanisms behind PAS are unclear, but it has been hypothesized that defects in the cell wall caused by beta lactams can lead to filamentation in bacteria. This could in turn result in a larger phage progeny per infected cell[28]. To investigate this, we evaluated how treatment with meropenem, ceftazidime, and tobramycin affects the morphology of *P. aeruginosa*. We treated bacteria expressing cytoplasmic green fluorescent protein (GFP) with antibiotics for 4 hours. Based on the synergy scores, we chose an antibiotic concentration of 2 µg/mL for meropenem and 1 µg/mL for ceftazidime, since at these concentrations synergy started to occur for most phage MOIs. After the treatment, we visualized the bacteria by means of widefield fluorescence microscopy (figure 3a-c). This revealed that bacteria treated with meropenem (figure 3b) do not appear to change in morphology, remaining similar to the control bacteria (figure 3a). In contrast, treatment with ceftazidime induced filamentation, with bacteria becoming elongated and staying attached to one another in strings (figure 3c). To quantify these changes in morphology, we moved on to flow cytometry. This technique individually analyzes each cell in a population as it passes through a laser. The way the cell makes the laser scatter provides information on the size and morphology of the cell. In addition, the technique can be used to detect fluorescence of a certain marker expressed by the cell. The signal recorded by the flow cytometer is composed of a width value and a height value. The width value reflects how long the cell is detected, and therefore its length. The height value is the intensity of the fluorescent signal of the marker of interest. We studied bacteria expressing cytosolic sfCherry and treated these for 4 hours with meropenem or ceftazidime before measuring them in the flow

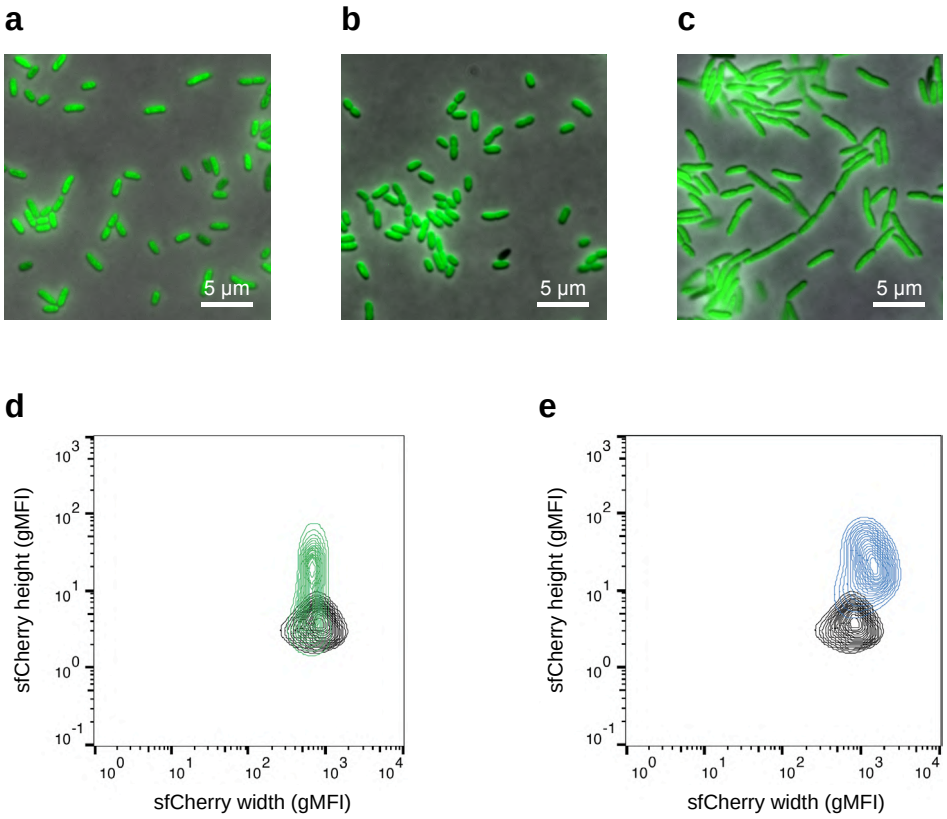

Figure 3. Antibiotic treatment induces phenotype changes in *P. aeruginosa.* a,b,c) PAO1 were incubated for 4 hours at 37ºC with a) buffer, b) meropenem (2 µg/mL), or c) ceftazidime (1 µg/mL). Widefield microscopy images were acquired with a 100X immersion objective and are an overlay of the phase contrast mode and the fluorescence mode with a cube filter for GFP (dichroic mirror: 500 nm). Images are representative of two independent experiments. d,e) PAO1 expressing cytosolic sfCherry (PAO-sfCherry) were incubated for 4 hours at 37ºC with buffer, d) meropenem (2 µg/mL), or e) ceftazidime (0.5 µg/mL) and measured by flow cytometry. Width values of sfCherry signal are plotted against height values on a contour plot. Black population represents control bacteria (untreated). Representative graphs of three independent experiments are shown.

cytometer. Here, the concentration of ceftazidime was reduced to 0.5 µg/mL, as we observed that the antibiotic had a big impact on cell viability. Bacteria were gated on forward and side scatter, and on sfCherry-height and sfCherry-width scatter to focus the analysis on intact bacteria (supplementary figure 2). Treatment with either antibiotic induced clear changes in the forward and side scatter of the bacterial

populations, with a high number of events disappearing and the remaining events shifting to higher values. We quantified the number of intact bacteria in these populations (supplementary figure 3). This revealed that, in these conditions, both ceftazidime and meropenem reduced the number of bacteria in the population by more than 10-fold, presumably both due to killing and impairing cell division, with ceftazidime achieving a bigger reduction. To analyze the morphology of the remaining bacterial population, we plotted the height of sfCherry signal against the width in contour density plots. When we compared untreated bacteria with bacteria treated with meropenem, we observed an increase in the height of the sfCherry signal, as well as a narrower distribution in the width of the population (figure 3d). In contrast, bacteria treated with ceftazidime showed an increase in both height and width of the signal, reflecting the filamentation phenotype detected previously through microscopy (figure 3e). On the whole, these results highlight that each of these antibiotics affects bacteria in a different way: filamentation could be a mechanism behind phage-ceftazidime synergy, but it does not explain PAS in the case of meropenem. However, the increase in sfCherry signal height observed with meropenem may reflect an increase in protein translation, which could have a boosting effect on phage propagation.

Burst size of phage PBJ is increased in bacteria treated with meropenem or ceftazidime

Meropenem and ceftazidime seemed to induce phenotypic changes in *P. aeruginosa*, as concluded from the microscopy and flow cytometry results. To evaluate if these changes could lead to a more productive phage infection, we performed one-step growth curve assays with phage PBJ on bacteria treated with meropenem or ceftazidime. In this assay, a single phage lytic cycle is monitored by determining the plaque forming units (PFU) in a culture of bacteria infected with the phage at a very low MOI. By representing how the number of PFU changes in the culture over time, we can identify the latent period of the phage, which corresponds to the time until the new progeny bursts out of the hosts cells. In addition, we can calculate the burst size, or number of new virions produced per infected cell. We compared the one-step growth curves of PBJ on untreated bacteria, bacteria treated with meropenem at 2 μg/mL, and bacteria treated with ceftazidime at 0.5

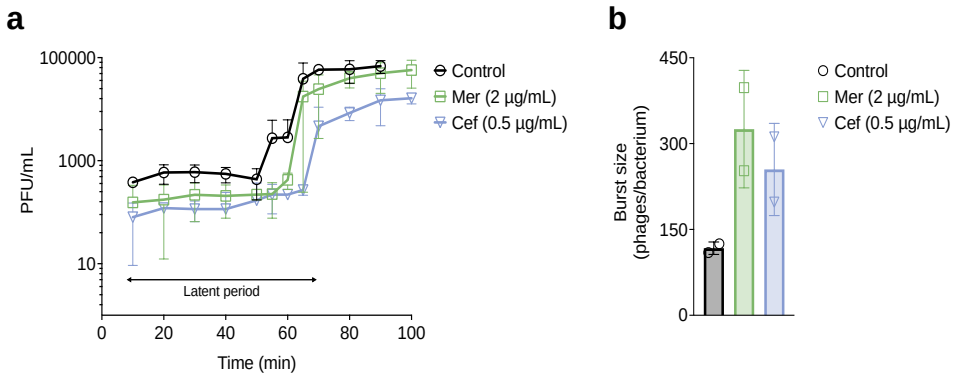

Figure 4. Antibiotic treatment increases the burst size of PBJ. a) One-step growth curve of PBJ infecting PAO1-lux cultured in buffer (control), 2 µg/mL meropenem, or 0.5 µg/mL ceftazidime. Values are normalized by subtracting the adsorption control for each curve. b) The burst size of PBJ, or number of new phages produced per infected bacterium, was calculated in the different conditions based on each of the one-step growth curves. a,b) Data are represented as mean ± SD of two independent experiments.

µg/mL (figure 4a, supplementary figure 4). In this way, we observed that the latent period in bacteria treated with either antibiotic, and especially with ceftazidime, was longer than in the untreated bacteria. Furthermore, we observed that the initial plateau of the curve was lower for both antibiotics, possibly due to fewer viable hosts being available in these conditions. Next, we calculated the burst size for each of the conditions (supplementary figure 5). This revealed that the burst size was approximately doubled in meropenem-treated bacteria (figure 4b). Ceftazidime also caused an increase in burst size, although less pronounced. Therefore, our findings suggest that treatment with meropenem or ceftazidime boosts phage production, possibly owing to an extension of the latent period.

Discussion

Phage therapy is often thought of as an alternative to antibiotics. However, there is evidence that combining both anti-microbial agents can improve their individual efficacies even against multi-drug resistant bacteria. In this study we aimed to identify patterns in phage-antibiotic combinations that can achieve a synergistic

antimicrobial effect on *P. aeruginosa*. Our results revealed that antibiotics can work better in combination with different phages even when targeting clinical strains. Aminoglycosides also seemed to have synergistic potential, although in combination with higher concentrations of phage. We used two phages with different receptors, and this did not seem to make a difference in their performance when combined with antibiotics. However, phage LKD16 was only tested on strain PA14, which was not sensitive to phage PBJ. To further establish the role of receptor specificity more phage-bacteria combinations should be tested.

Defining synergy in this kind of setting where one of the agents used is a self-amplifying entity is not straightforward. Here, we used a simple formula based on calculating the expected effect from an additive interaction between the phage and the antibiotic. The calculation we used is in agreement with the Bliss independence model, which is based on probability theory and postulates that the combined effect of two independently acting drugs can be calculated as the product of their individual effects [34,35]. Using this method, we were able to determine that meropenem, ceftazidime and tobramycin indeed could achieve synergy with phage PBJ. The outcome of combining tobramycin, an aminoglycoside, with phages is difficult to predict. While some studies show that tobramycin can achieve an improved antimicrobial activity together with phages as compared to the single treatments[26,36], other reports show that aminoglycosides can actually antagonize phage activity[37,38]. As aminoglycosides inhibit protein synthesis, it is reasonable to think that they could be detrimental to phage propagation. Nonetheless, the narrow range of concentrations at which tobramycin could synergize with PBJ might indicate that there is a “sweet spot” at which the bacteria are more vulnerable, but the translation of phage proteins is not yet blocked. In fact, the concentrations of tobramycin at which we could measure synergy were much lower than the ones tested in the studies reporting antagonism between aminoglycosides and phages. It is also worth noting that the performance of colistin did not improve with the addition of phages. This antibiotic is used in the clinic as a last resort treatment of multi-resistant Gram-negative infections[39], so it is not uncommon for phage therapy to be administered together with colistin.

In any case, the differences in methods used to assess bacterial killing complicate the comparison with other studies investigating PAS. In this research, we have used a bioluminescence reporter system to measure metabolic activity. This system enabled us to measure many different combinations in high throughput, and to accurately monitor decreases in metabolic activity compared to a healthy growth control. Based on this, we consider that reductions in luminescent signal are equivalent to reductions in the bacterial population. This constitutes a limitation of our study, as a more direct measurement could reflect the effect of the antimicrobial agents more accurately. For instance, with this system we do not account for potential differences in cellular respiration that each antibiotic could be causing. In fact, we see a disparity between the decrease that ceftazidime causes in luminescence and the noticeably larger decrease that it causes in bacterial counts as determined by flow cytometry. Still, this system allowed us to screen many different conditions in real time in a controlled medium without the need of adding any additional substrates.

Next to identifying synergistic phage-antibiotic combinations, we investigated the mechanisms behind the interactions of phage PBJ with the beta lactams meropenem and ceftazidime. Both of these antibiotics have been previously described to work synergistically in combination with phages against *P. aeruginosa*[41,42]. One of the mechanisms that can drive PAS in planktonic *Escherichia coli* is filamentation. This phenomenon can lead to an increased phage burst size because it delays the time of phage lysis[28]. Indeed, when looking at bacteria treated with ceftazidime, we could observe both filamentation and an increased burst size of phage PBJ, as well as a longer latent period. However, PBJ also presented an increased burst size and longer latent period when the host bacteria were treated with meropenem, while this antibiotic was not seen to induce filamentation. Filamentation has also been described to enhance phage infection by providing phages with a larger surface on which to adsorb, thereby effectively increasing the phage MOI[31]. Unfortunately, we were not able to assess whether this was the case in our hands, as we could not accurately measure phage adsorption in our experiments. This was because a long incubation time with antibiotics was necessary to induce filamentation, after which variations in bacterial concentrations across the different conditions made it

impossible to properly control for the phage MOI. Nonetheless, our flow cytometry results did reflect an increase in cytosolic sfCherry signal in both meropenem- and ceftazidime-treated bacteria compared to control bacteria. This could indicate that antibiotic-treated bacteria have an overall higher rate of protein expression, potentially accounting for the increase in phage burst size. A reason for this could be the induction of the SOS response, which can result in changes in gene and protein regulation and has also been linked to PAS[43,44]. On the other hand, there is also evidence that PAS can occur in mutant strains lacking an SOS response[30]. In the case of our reporter protein, it is encoded in a plasmid under the control of a strong promotor, so it is unclear whether the SOS response would influence its expression. Another explanation could be simply that proteins accumulate in the cytoplasm when division is impaired. All in all, the mechanistic explanation to why certain antibiotics are enhanced by phages remains elusive and is likely multi-faceted.

Still, it is important to keep deepening our understanding of this phenomenon. A possible future research direction could be to investigate the transcriptome of antibiotic-treated bacteria facing a phage challenge. This would provide insights into host and phage gene regulation and might explain the increase in burst size that we observed in our data. Another aspect that deserves consideration is the timing of administration of each anti-microbial agent: is it more beneficial to administer first antibiotics and then phages, or the other way around? Furthermore, the formation of biofilm is an important issue with bacteria such as *P. aeruginosa*, so it would be interesting to assess whether our observations regarding planktonic bacteria are also true for bacteria in a biofilm. Finally, there remains the issue of whether *in vitro* results can translate to a situation as complex as that of a bacterial infection in a patient treated with antibiotics and phage therapy. Here, other factors could play a role, such as the timing of administration of each therapeutic agent, the dosing at the site of infection, the local environment in which the bacteria are, or whether the bacteria are forming a biofilm.

In conclusion, our study provides additional evidence for the potential of combining phages with antibiotics as a strategy to treat *P. aeruginosa*. Particularly, we

demonstrate that certain combinations of meropenem, ceftazidime or tobramycin with LPS-targeting phage PBJ can establish synergy. We have also shown that synergy between meropenem or ceftazidime and PBJ is linked to an increase in burst size. Although this may be due to filamentation in the case of ceftazidime-treated bacteria, this effect does not explain PAS in the case of meropenem. Rather, the increases in burst size may be caused by an overall increase in protein translation as a response to treatment with beta lactams. While further research is needed to understand the mechanisms behind PAS, our findings may contribute to the understanding of this phenomenon and the future development of effective phage-antibiotic combination therapies.

Materials and Methods

Bacteriophages and bacterial strains

Phages PBJ and LKD16 were produced and purified as previously described[45]. Clinical *P. aeruginosa* strain Q0311 was obtained from the UMCU diagnostic microbiology laboratory strain collection and isolated from a patient with an invasive infection. *P. aeruginosa* strains PAO1, PA14 and Q0311 were transformed to express the *lux* system or superfolder Cherry (sfCherry) as previously described[45,46]. Excision of the gentamicin resistance cassette in PAO1-lux was performed through flippase (Flp)-mediated recombination and *sacB* negative selection as previously described[47]. PAO1 expressing GFP, encoded by plasmid pSMC21, was kindly provided by Jeffrey Beekman.

Media, reagents, and culture conditions

Bacteria were cultured on Lysogeny Broth (LB) agar plates or on LB agar plates with 30 µg/mL gentamicin in the case of PAO1-sfCherry. For experiments, bacterial cultures were started by inoculation of LB broth with a single colony (with 30 µg/mL gentamicin in the case of PAO1-sfCherry). Cultures were incubated overnight at 37°C with shaking. The next day, the bacteria were diluted 1:30 and grown to mid-log phase (OD_{600nm} ~ 0.5). Then, the bacteria were washed in RPMI buffer (RPMI 1640, Thermo Fisher, supplemented with 0.05% human serum albumin, Sanquin),

pelleted, and resuspended to an OD_{600nm} of 1.0 (~8 × 10^8 bacteria/mL) in RPMI buffer. Unless otherwise stated, incubation steps in experiments were performed at 37°C with shaking. Phages were stored and diluted in SM buffer (200 mM $NaCl_2$, 10 mM $MgSO_4$, 50 mM Tris-HCl, pH 7.5). Antibiotic stocks of tobramycin, meropenem, ceftazidime, piperacillin-tazobactam, and amikacin were obtained from the UMCU pharmacy. Gentamicin sulfate (≥590 I.U. per mg) was obtained from Merck Life Science (Amsterdam, the Netherlands). Colistin sulfate (>19000 I.U. per mg) was obtained from Duchefa Biochemie (Haarlem, the Netherlands). All antibiotics were dissolved and stored in MiliQ water.

Checkerboard assay

Phage and antibiotic dilutions were prepared in SM buffer. Bacteria (PAO1-lux, PA14-lux or Q0311-lux) in RPMI buffer (final concentration 2 x 10^7 bacteria/mL) and phages (final concentration 10^4 - 10^8 PFU/mL) and/or antibiotics (final concentration 0.03-256 µg/mL, depending on which antibiotic) were mixed in a white half-volume 96-wells plate (Greiner Bio-one) (final volume 45 µL, 1:2 SM buffer to RPMI buffer). Untreated bacteria were taken along as a growth control. Luminescence was measured every 3 minutes for 8 hours in the CLARIOstar Plus (BMG Labtech) at 37°C. The following settings were used: focal height = 13.5 mm; luminescence gain = 3600.

Widefield fluorescence microscopy

PAO1-GFP in RPMI buffer (final concentration 2 x 10^7 bacteria/mL) and antibiotics (final concentration 1 µg/mL) were mixed in a round bottom 96-wells plate (Greiner Bio-one) (final volume 45 µL). This was incubated for 4 hours. After incubation, cells were fixed by adding 6 volumes of 1% formaldehyde in RPMI buffer to the sample followed by 15 minutes incubation at 4°C. Bacteria were centrifuged at 2424 x g at 4°C for 10 minutes and resuspended in 20 µL 1% formaldehyde in RPMI+0.05% HSA. The samples were then immobilized on microscope slides prepared with agarose pads as previously described[45]. Images were taken using a Leica TCS SP5 II microscope with an HC PL APO CS 100×/1.4 OIL objective (Leica Microsystems) by overlaying the mode phase contrast with the mode widefield fluorescence with a cube filter for GFP (dichroic mirror: 500 nm).

Flow cytometry

PAO1-sfCherry at a concentration of 2 x 10^7 bacteria/mL was incubated with buffer, meropenem or ceftazidime in the concentrations indicated for 4 hours. Bacteria were diluted 1:5 and 10 µL sample was measured by flow cytometry (MACS Quant, Miltenyi Biotech). Bacteria were gated on forward and side scatter, and on sfCherry-height and sf-Cherry width scatter to remove dead bacteria or debris from the analysis.

One step growth curve

PAO1-lux was grown to mid-log phase (OD_{600nm} ~ 0.45) in absence or presence of meropenem (2 µg/mL) or ceftazidime (0.5 µg/mL). PBJ was added to the bacteria in a final concentration of 5 x 10^4 PFU/mL to allow adsorption. Next, the culture was diluted 1:100 in flask A in LB broth. Flask A culture was 1:10 diluted in flask B and this was further diluted 1:10 in flask C, both in LB broth. As adsorption control, 1 mL of flask A was added to 50 µL of cold chloroform, vortexed and kept on ice. Flask A, B and C were incubated at 37°C with shaking throughout the experiment. At different time points, 100 µL aliquots were taken from the appropriate flask: flask A in minutes 10 – 40, flask B in minutes 30 –70, and flask C in minutes 65 – 100. Aliquots were added to the soft agar overlay together with approximately 30 µL of overnight PAO1-lux culture and poured on solid agar plates. Plates were incubated overnight at 37°C and bacteriophage titer (PFU/mL) was determined for each aliquot through plaque enumeration, and normalized to the concentration in flask A. In the intervals where aliquots were taken from different flasks, an average was calculated. The burst size was calculated following the method described by Kropinski[48] using the following formula: burst size = average 2 / (average 1 - adsorption control); where average 2 is the average number of PFU after the burst, average 1 is the average number of PFU before the burst, and adsorption control is the number of PFU in the adsorption control.

Data analysis

Data visualization and statistical analyses were performed in GraphPad Prism 9 and are further specified in the figure legends. Widefield fluorescence microscopy images were processed using Fiji. Flow cytometry data were analyzed using

FlowJo™ v10.8.1 software. Figures were produced using Adobe Illustrator and BioRender.

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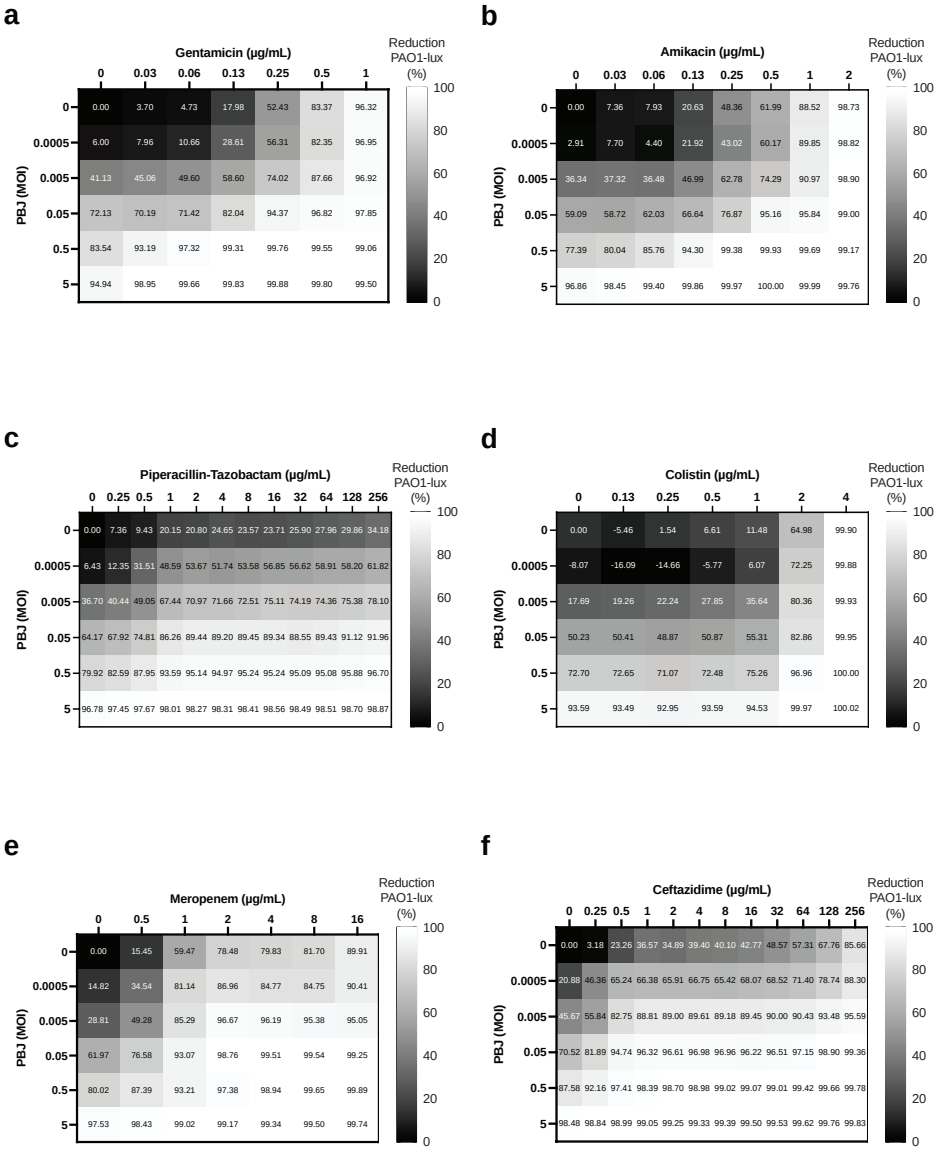
Supplementary information



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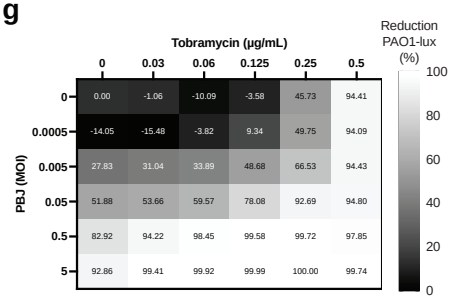
Tobramycin (µg/mL)
0 0.03 0.06 0.125 0.25 0.5
PBJ (MOI)
0 0.0005 0.005 0.05 0.5 5
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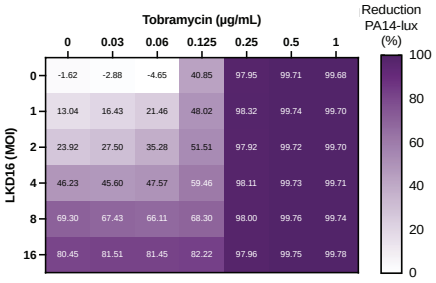
-14.05 -15.48 -3.82 9.34 49.75 94.09
27.83 31.04 33.89 48.68 66.53 94.43
51.88 53.66 59.57 78.08 92.69 94.80
82.92 94.22 98.45 99.58 99.72 97.85
92.86 99.41 99.92 99.99 100.00 99.74
Reduction PAO1-lux (%)
100 80 60 40 20 0

h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LKD16 (MOI)
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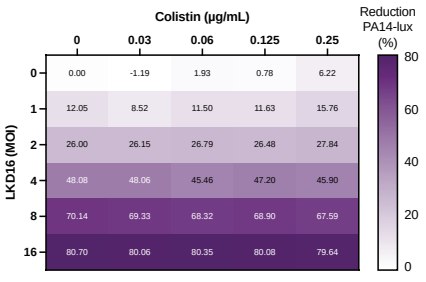
13.04 16.43 21.46 48.02 98.32 99.74 99.70
23.92 27.50 35.28 51.51 97.92 99.72 99.70
46.23 45.60 47.57 59.46 98.11 99.73 99.71
69.30 67.43 66.11 68.30 98.00 99.76 99.74
80.45 81.51 81.45 82.22 97.96 99.75 99.78
Reduction PA14-lux (%)
100 80 60 40 20 0

i


Colistin (µg/mL)
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LKD16 (MOI)
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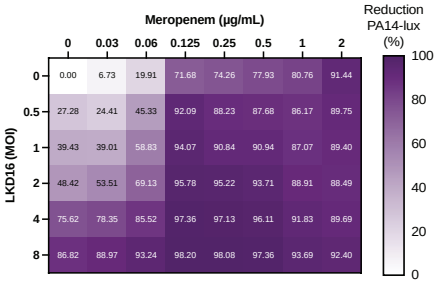
12.05 8.52 11.50 11.63 15.76
26.00 26.15 26.79 26.48 27.84
48.08 48.06 45.46 47.20 45.90
70.14 69.33 68.32 68.90 67.59
80.70 80.06 80.35 80.08 79.64
Reduction PA14-lux (%)
80 60 40 20 0

j


Meropenem (µg/mL)
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LKD16 (MOI)
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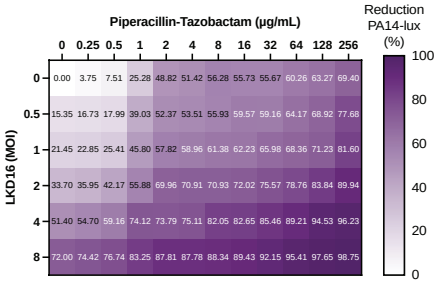
0.00 6.73 19.91 71.68 74.26 77.93 80.76 91.44
27.28 24.41 45.33 92.09 88.23 87.68 86.17 89.75
39.43 39.01 58.83 94.07 90.84 90.94 87.07 89.40
48.42 53.51 69.13 95.78 95.22 93.71 88.91 88.49
75.62 78.35 85.52 97.36 97.13 96.11 91.83 89.69
86.82 88.97 93.24 98.20 98.08 97.36 93.69 92.40
Reduction PA14-lux (%)
100 80 60 40 20 0

k


Piperacillin-Tazobactam (µg/mL)
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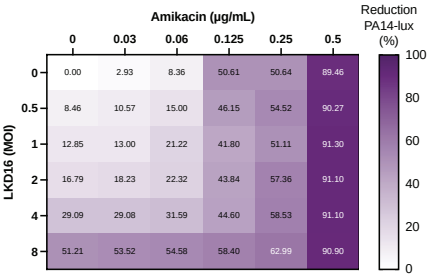
LKD16 (MOI)
0 0.5 1 2 4 8
0.00 3.75 7.51 25.28 48.82 51.42 56.28 55.73 55.67 60.26 63.27 69.40
15.35 16.73 17.99 39.03 52.37 53.51 55.93 59.57 59.16 64.17 68.92 77.68
21.45 22.85 25.41 45.80 57.82 58.96 61.38 62.23 65.98 68.36 71.23 81.60
33.70 35.95 42.17 55.88 69.96 70.91 70.93 72.02 75.57 78.76 83.84 89.94
51.40 54.70 59.16 74.12 73.79 75.11 82.05 82.65 85.46 89.21 94.53 96.23
72.00 74.42 76.74 83.25 87.81 87.78 88.34 89.43 92.15 95.41 97.65 98.75
Reduction PA14-lux (%)
100 80 60 40 20 0


4

l


Amikacin (µg/mL)
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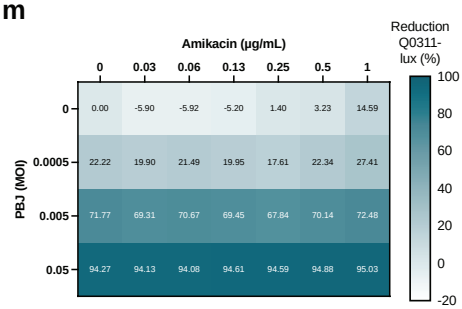
LKD16 (MOI)
0 0.00 2.93 8.36 50.61 50.64 89.46
0.5 8.46 10.57 15.00 46.15 54.52 90.27
1 12.85 13.00 21.22 41.80 51.11 91.30
2 16.79 18.23 22.32 43.84 57.36 91.10
4 29.09 29.08 31.59 44.60 58.53 91.10
8 51.21 53.52 54.58 58.40 62.99 90.90
Reduction PA14-lux (%)
100 80 60 40 20 0

m


Amikacin (µg/mL)
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PBJ (MOI)
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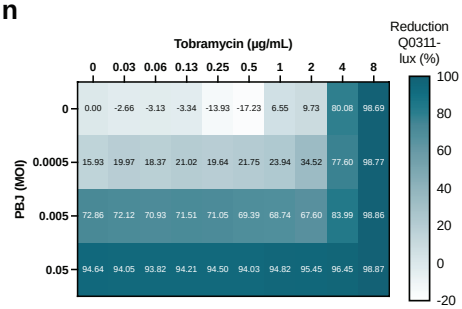
0.0005 22.22 19.90 21.49 19.95 17.61 22.34 27.41
0.005 71.77 69.31 70.67 69.45 67.84 70.14 72.48
0.05 94.27 94.13 94.08 94.61 94.59 94.88 95.03
Reduction Q0311-lux (%)
100 80 60 40 20 0 -20

n


Tobramycin (µg/mL)
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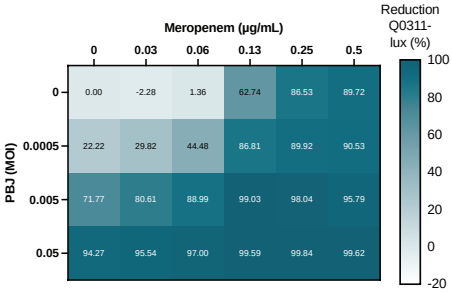
PBJ (MOI)
0 0.00 -2.66 -3.13 -3.34 -13.93 -17.23 6.55 9.73 80.08 98.69
0.0005 15.93 19.97 18.37 21.02 19.64 21.75 23.94 34.52 77.60 98.77
0.005 72.86 72.12 70.93 71.51 71.05 69.39 68.74 67.60 83.99 98.86
0.05 94.64 94.05 93.82 94.21 94.50 94.03 94.82 95.45 96.45 98.87
Reduction Q0311-lux (%)
100 80 60 40 20 0 -20

o


Meropenem (µg/mL)
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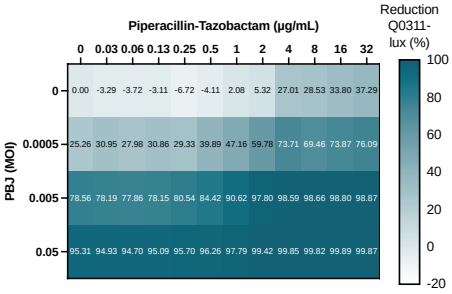
PBJ (MOI)
0 0.00 -2.28 1.36 62.74 86.53 89.72
0.0005 22.22 29.82 44.48 86.81 89.92 90.53
0.005 71.77 80.61 88.99 99.03 98.04 95.79
0.05 94.27 95.54 97.00 99.59 99.84 99.62
Reduction Q0311-lux (%)
100 80 60 40 20 0 -20

p


Piperacillin-Tazobactam (µg/mL)
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PBJ (MOI)
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0.0005 25.26 30.95 27.98 30.86 29.33 39.89 47.16 59.78 73.71 69.46 73.87 76.09
0.005 78.56 78.19 77.86 78.15 80.54 84.42 90.62 97.80 98.59 98.66 98.80 98.87
0.05 95.31 94.93 94.70 95.09 95.70 96.26 97.79 99.42 99.85 99.82 99.89 99.87
Reduction Q0311-lux (%)
100 80 60 40 20 0 -20

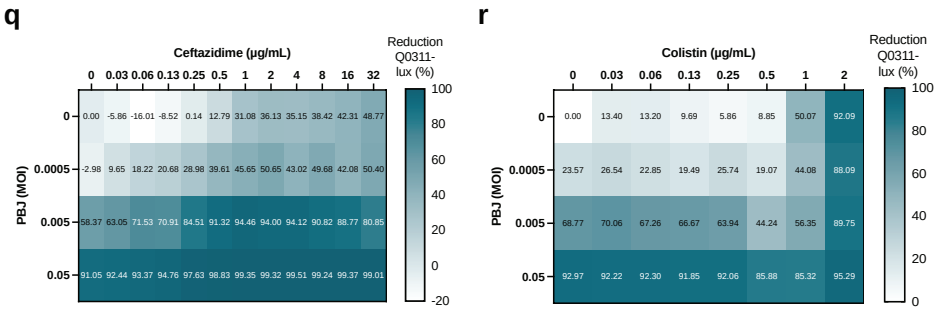


Figure S1. Effect of phage-antibiotic combinations on bacterial viability analyzed after 4 hours. PAO1 was treated with phage PBJ (MOI of 0 to 5) in combination with (a) gentamicin (0-1 μg/mL), (b) amikacin (0-2 μg/mL), (c) piperacillin-tazobactam (0-256 μg/mL), (d) colistin (0-4 μg/mL), (e) meropenem (0-16 μg/mL), (f) ceftazidime (0-256 μg/mL), (g) tobramycin (0-0.5 μg/mL). PA14 was treated with phage LKD16 (MOI of 0 to 16) in combination with (h) tobramycin (0-1 μg/mL), (i) colistin (0-0.25 μg/mL), (j) meropenem (0-2 μg/mL), (k) piperacillin-tazobactam (0-256 μg/mL), (l) amikacin (0-0.5 μg/mL). Q0311 was treated with PB1 (MOI 0 to 0.05) in combination with (m) amikacin (0-1 μg/mL), (n) tobramycin (0-8 μg/mL), (o) meropenem (0-0.5 μg/mL), (p) piperacillin-tazobactam (0-32 μg/mL), (q) ceftazidime (0-32 μg/mL), (r) colistin (0-2 μg/mL).

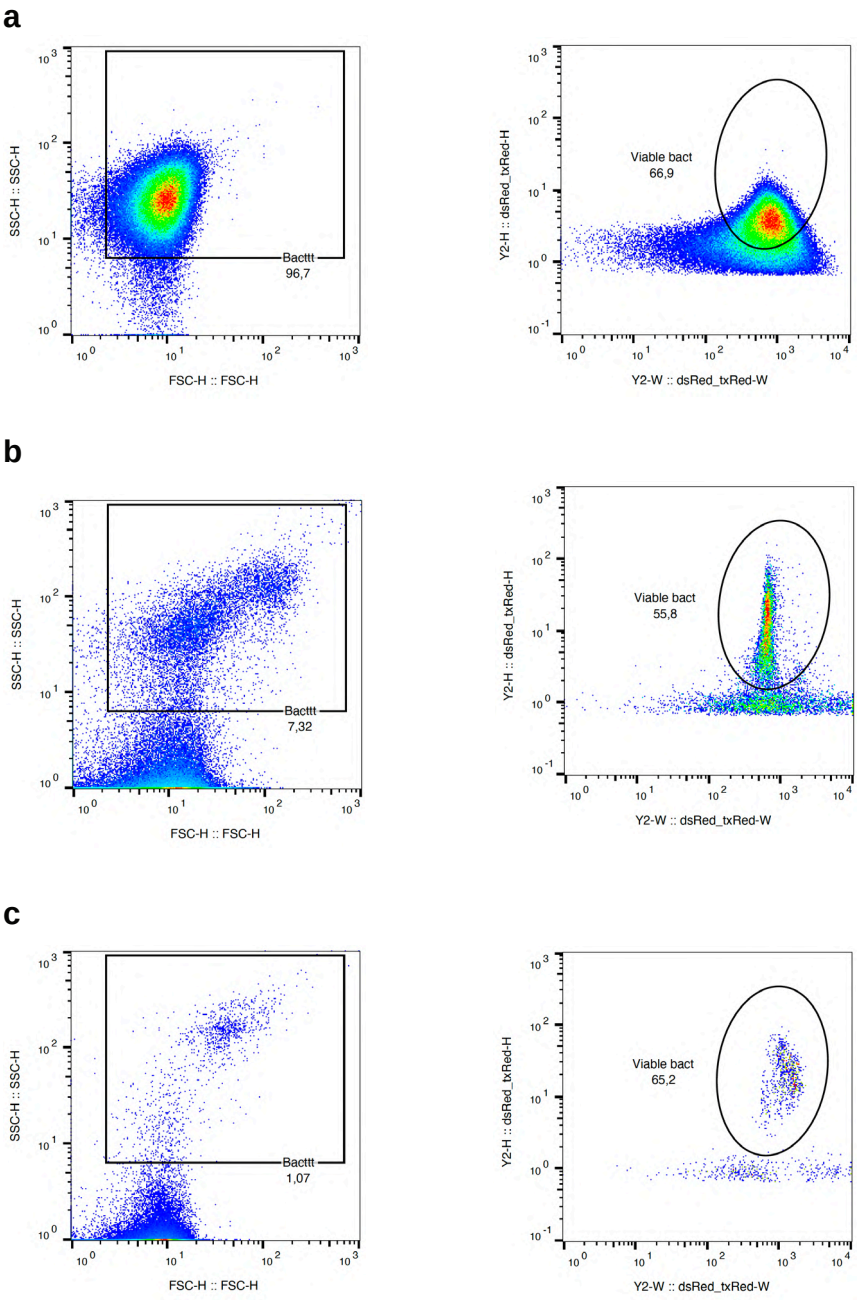


Figure S2. Flow cytometry gating strategy on a) control bacteria, b) bacteria treated with meropenem and c) bacteria treated with ceftazidime.

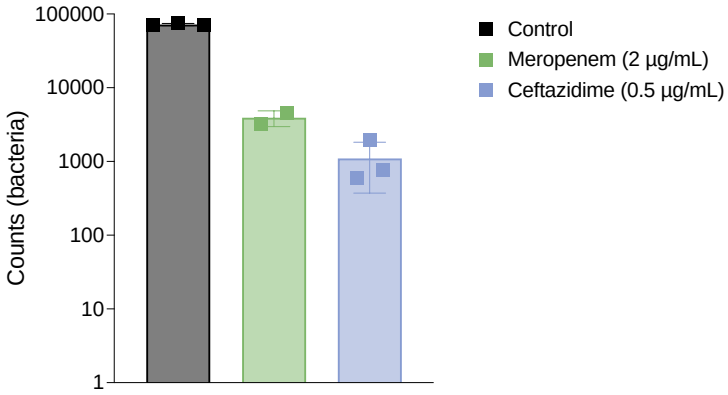


Figure S3. Counts of bacteria decrease compared to the control as a result of antibiotic treatment. Counts were measured through flow cytometry. Mean ± SD, as well as individual data points, are represented.

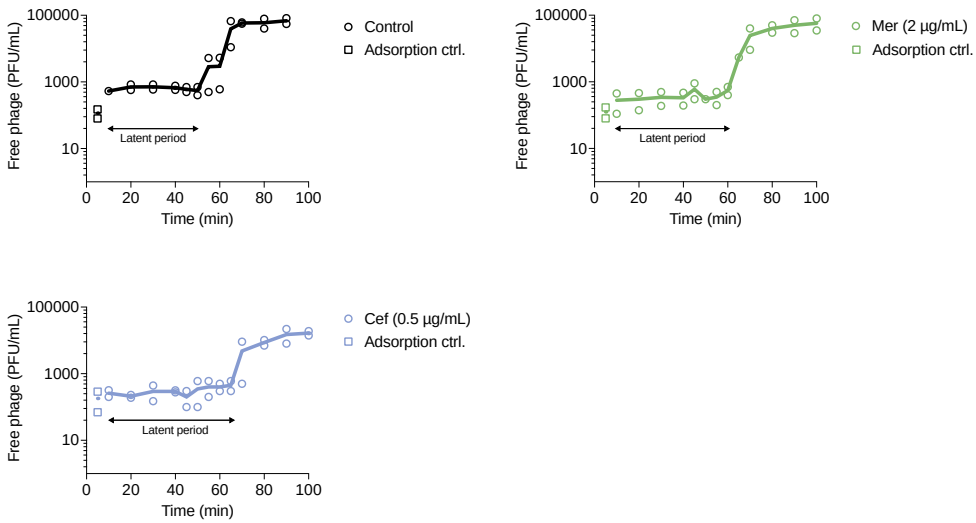
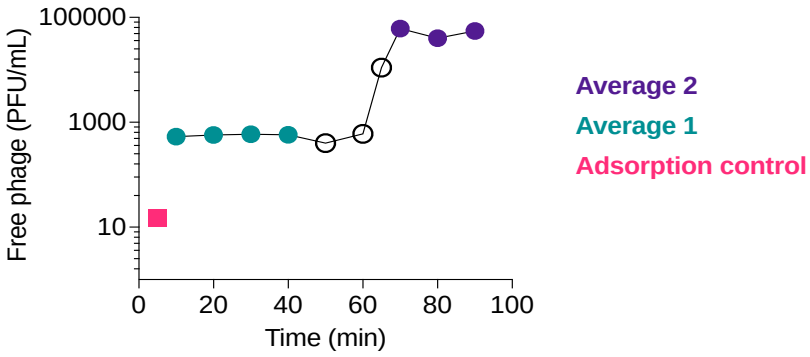


Figure S4. One step growth curves of PBJ on bacteria cultures in buffer (control, black), buffer with 2 µg/mL meropenem (green), or buffer with 0.5 µg/mL ceftazidime (blue). Data represent average and individual measurements of two independent experiments.



Burst size = Average 2 / (Average 1 – Adsorption control)

Time (min)	Ctrl 1 (PFU/mL)	Ctrl 2 (PFU/mL)	Mer 1 (PFU/mL)	Mer 2 (PFU/mL)	Cef 1 (PFU/mL)	Cef 2 (PFU/mL)
10		530	450	110	320	200
20	840	570	460	140	230	190
30	840	590	495	190	440	150
40	765	575	470	195	315	275
45	700	500	900	300	300	100
50	700	400		300	600	100
55	5200	500	500	200	600	200
60	5300	600	700	400	500	300
65	67000	11050	30000	5400	600	300
70	56000	61000	40000	9150	500	9150
80	79000	40000	50000	30000	7000	10250
90	82000	55000	71000	29000	8000	22000
100			80000	35000	14000	19000
Adsorption	80	150	170	80	290	70
Burst size	**109,5**	**124,9**	**252,7**	**397,9**	**311,8**	**197,9**

Figure S5. Burst size calculations for PBJ in bacteria grown in medium, medium with 2 µg/mL meropenem or medium with 0.5 µg/mL ceftazidime. For each one step growth curve, two independent biological replicates were obtained.

Chapter 5

Mechanisms and clinical importance of bacteriophage resistance

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Abstract

We are in the midst of a golden age of uncovering defense systems against bacteriophages. Apart from the fundamental interest in these defense systems, and revolutionary applications that have been derived from them (e.g. CRISPR-Cas9 and restriction endonucleases), it is unknown how defense systems contribute to resistance formation against bacteriophages in clinical settings. Bacteriophages are now being reconsidered as therapeutic agents against bacterial infections due the emergence of multidrug resistance. However, bacteriophage resistance through defense systems and other means could hinder the development of successful phage-based therapies. Here, we review the current state of the field of bacteriophage defense, highlight the relevance of bacteriophage defense for potential clinical use of bacteriophages as therapeutic agents and suggest new directions of research.

Introduction

The use of bacteriophages, or phages, as therapeutic agents to treat bacterial infections began immediately after phage discovery in 1917[1,2]. The initial interest in phages as antibacterial agents faded quickly following the discovery of penicillin two decades later[3], although phage therapy remained in use in former Soviet republics like Georgia and Russia[4]. In recent years, Western medicine has started to reconsider the therapeutic use of phages due to the alarming rise in infections caused by multidrug-resistant (MDR) bacteria[5]. However, the success of phage therapy might be limited by the development of phage resistance by bacteria, much akin to the resistance developed toward antibiotics. Recently, multiple mechanisms by which bacteria defend against phages have been uncovered[6,7], some specific for certain species or strains, others more widespread. Unlike antibiotics, phages can adapt and/or deploy anti-defense systems of their own to overcome the defense mechanisms of bacteria[8–14].

The evident complexity of phage–bacteria interactions needs to be considered for phage therapy to be implemented successfully[15]. It is unknown how defense systems contribute to and impact resistance formation against phages in clinical settings, and this could be a bottleneck in the development of successful phage-based therapies when left without consideration.

Here, we provide an overview of the current state of the field of natural and acquired phage resistance, highlight the relevance of phage defense for potential clinical use of phages as therapeutic agents and suggest new directions of research.

The multistep process of bacteriophage infection

There are multiple families of bacteriophages, each with specific features that influence their process of infection of a bacterial host. For the purposes of this review, we will focus on phages belonging to the order *Caudovirales*[16], which are known as tailed phages and are the most widely used in clinical applications[3].

Tailed phages have double-stranded DNA genomes and a structure made up of an icosahedral head and a tail, which usually incorporates receptor binding proteins (RBPs) such as tail spikes and tail fibers at the distal end[17]. These elements are responsible for the first step of infection, i.e. recognition of specific receptors on the surface of bacteria, and subsequent adsorption of the phage[17]. Phage receptors on the bacterial surface are typically peptide sequences or polysaccharides present on the bacterial cell wall, as well as protruding structures such as capsules, pili or flagella[18,19]. Phage attachment to the host surface often occurs first through a reversible interaction with a receptor, which is then followed by an irreversible binding event to the same or a second receptor[18]. Generally, phages recognize receptors with a great degree of specificity, meaning that the host range of a certain phage is often limited at the adsorption stage by the receptors available on the cell surface[20].

Adsorption of the phage to its native receptor on the cell triggers ejection of the genetic material of the phage into the host cytoplasm. The mechanism of this complex phenomenon is not yet completely understood for many phage types, but in *Caudovirales* it commonly involves conformational changes of the phage triggered by binding of the phage RBPs to the receptor that result in the opening of the channel required for DNA release from the capsid[21,22]. In some phages, these conformational changes lead also to the ejection of the tape measure protein that reconfigures into a channel through which the genome translocates into the cell cytoplasm[23–25]. In phages with short tails (e.g. *Podoviridae*) proteins ejected together with the genome can work to form a similar channel for genome passage[26]. The forces behind phage genome ejection into the cell cytoplasm are still unclear, with different models proposed[27]. It seems that thermodynamic and compressing pressures cause the initial release of DNA[28], with complete ejection being achieved by further hydrodynamic forces and/or bacterial proteins involved in transcription of the initial segment of the phage genome[29–31].

If the infecting phage is obligately virulent, which is preferred for phage therapy applications[32,33], the infection follows a lytic cycle once the phage genome is inside the cell. In this case, the phage hijacks the cellular machinery of the bacteria,

shutting off the expression of host genes and achieving the replication of its genome and the expression of its own genes[34]. For this purpose, some phages rely on the bacterial RNA polymerase[35], while others encode and/or co-inject their own[36]. The lytic cycle culminates with the expression of late genes, which encode structural proteins and proteins necessary for bacterial host lysis. This ultimately leads to the production of more viral particles that will in the end burst out of the host cell[37]. However, if the infecting phage is temperate, it may also follow a lysogenic cycle. In this case, the viral genome persists within the host cell, either introduced in the bacterial chromosome as a prophage or in the bacterial cytoplasm as a plasmid. The lysis–lysogeny decision may depend on peptide-based communication between the viruses[38] or on host repressor genes that form part of a quorum-sensing system[39].

Mechanisms of phage resistance

Bacteria evade phage infections in different ways. Here, we classify different resistance mechanisms in three main categories:

- Receptor adaptations: random mutations or phenotypical variations in bacteria that result in decreased phage adsorption (figure 1).
- Host defense systems: molecular pathways that have specifically evolved in bacteria to prevent or suppress phage infections (figure 2).
- Phage-derived phage defense systems: molecular pathways encoded by phages to compete with other phages to the benefit of the host (figure 3).

Receptor adaptations leading to phage resistance

In their natural environments, bacteria are subjected to constant selective pressure, which has driven bacteria and phages into an arms race to evolve defense systems and to counter them. The arms race is characterized by high mutation rates and horizontal gene transfer, and leads to rapid evolution of genetic traits and genetic diversity[40–43]. Mutations that cause cell surface alterations can result in blockage of phage adsorption, and are therefore directly beneficial to the host.

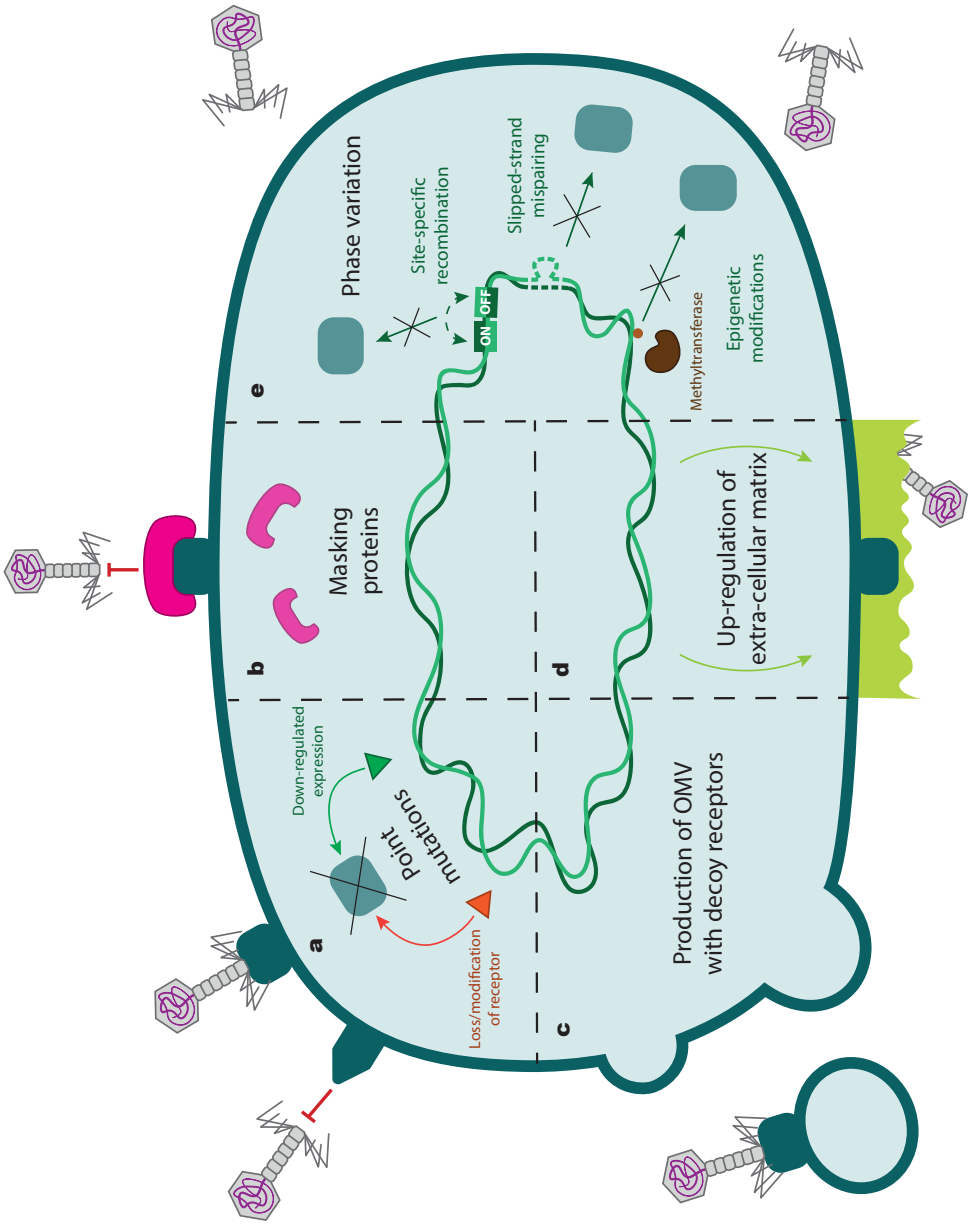

Figure 1. Host adaptations leading to phage resistance. a) Point mutations can lead to a loss or modification of the phage receptors (green rectangles), or to downregulation of their expression. b) Receptor masking proteins like TraT of *Escherichia coli* (pink) can bind to the surface-exposed regions of phage receptors, making them unavailable for the phages. c) Outer-membrane vesicles (OMVs) presenting phage receptors act as decoys to prevent the phages from encountering the bacteria.

d) An increase in the production of extracellular matrix (light green) leads to phage receptors being physically hidden. e) Phase variation occurs through three mechanisms: site-specific recombination, slipped-strand mispairing and epigenetic modifications. It can regulate the bacterial phenotype, including the expression of surface proteins like phage receptors.

Bacteria can pose a barrier to phage adsorption by decreasing the availability of the receptors to which phages bind. The acquisition of point mutations in their genome (figure 1a) is probably the simplest way by which bacteria can become fully resistant to phages. In fact, mutations in the receptor genes or their regulation have been a common way to identify the receptor of a phage[19,44]. These mutations occur often upon phage challenges, and can lead to a loss or decrease in the gene expression of certain receptors, or to modifications of their structure[45,46]. For example, *E. coli* mutates tolC and lipopolysaccharide (LPS) genes to resist infection by phage U136B[47]. Similarly, *Acinetobacter baumannii* mutates genes involved in the biosynthesis of capsular polysaccharides to avoid infection by phages ϕFG02 and ϕCO01[48]. In *Listeria monocytogenes*, loss or deficiency of wall teichoic acid rhamnosylation leads to resistance to a wide range of phages[49], and results also in serovar diversification[50]. Other proteins involved in phage adsorption and DNA injection, like the phage infection protein from *Enterococcus faecalis* (PIPEF), can also mutate as a response to phage challenges[51].

Bacteria may also block phage adhesion by producing proteins that mask or block the phage receptors on the cell surface (figure 1b). An example of this is F plasmid-encoded protein TraT, which localizes at the cell outer membrane and binds surface exposed regions of the outer membrane protein OmpA in *E. coli*[52]. This makes this common phage receptor inaccessible for phage binding. Masking molecules, such as lipoproteins, that bind phage receptors can also be produced by bacteria under stress conditions and are released during bacterial lysis[53]. Some bacteria also produce and release OMVs (figure 1c) that act as cell decoys that capture and inactivate phages[54]. Another mechanism that can prevent phages from reaching their receptors is upregulating the production of extracellular matrix typically consisting of polysaccharides, proteins, lipids and extracellular DNA (figure 1d), in a way that protects the embedded bacteria or subsequent biofilm against phage adsorption[55,56]. In *Lactococcus lactis*, plasmids encoding exopolysaccharide

biosynthesis genes can also confer protection against phages[57].

In addition to this, reversible changes in the regulation of gene expression, a phenomenon known as phase variation (figure 1e), can lead to a decrease in receptor availability[58]. These changes can be mediated by site-specific recombination[59], in which inversion of a DNA segment in the promoter or regulatory region of a gene causes its expression to be turned on or off. This is exemplified by the development of flagella in *Salmonella* spp. and fimbriae in *E. coli*[60–62]. Other receptors, such as the outer membrane protein Opc of *Neisseria meningitidis* and the subunits of *Bordetella pertussis* fimbriae[63–65], are regulated by slipped strand mispairing, i.e. programmed mutations that occur in defined regions during DNA recombination. Epigenetic modifications, such as altered methylation patterns on DNA sequences[66], also regulate expression of phage receptors, such as the O-antigen chains of LPSs in *Salmonella enterica*[67]. All of these alterations act directly on phage receptors and decrease the chances of phage adsorption. However, modifications of surface elements can come with a fitness trade-off for the host bacteria, in terms of reduced virulence or survival ability of the host[68,69], limiting the possibility to alter the receptor itself. Due to this, more specific defense systems that target phages within the host cell are also necessary, especially in the context of a complex microbial community[68,70].

Host phage defense systems

Bacteria have evolved defense systems dedicated to defense against mobile genetic elements such as phages. Many of them are clustered in regions of the genome known as defense islands[71], offering an opportunity for discovering new defense systems by analyzing the genetic regions in the proximity of other known defense systems. Such strategy has resulted in a significant and fast expansion of the known arsenal bacteria use to defend against phage infection. We will cover a number of different phage defense systems that have been identified, including those acting on viral nucleic acids and those causing abortive infection of the host.

1. Nucleic acid interference (figure *2a)*

The ability to interfere with viral nucleic acids is a common strategy that hosts

employ to limit phage invasion and propagation. One of the most widespread and longest known examples of phage defense systems are those called Restriction-Modification (R-M) systems that act on phages with DNA genomes[72,73]. In R-M systems, a methyltransferase (MTase) methylates endogenous DNA at specific sites, protecting it from cleavage by the restriction endonuclease (REase) that recognizes the foreign, unmodified DNA and cleaves it within, close to, or at a distance from the recognition site[74].

There are four classical types of R-M systems (I-IV), classified according to the characteristics of their specific components[74]. The type I R-M system consists of a protein complex of three subunits with distinct activities, the M (MTase), R (REase) and S (specificity) subunits. The S subunit dictates the target sequence specificity of both methylation and restriction by the protein complex. The abundant type II R-M system features a MTase and REase that work independently as separate proteins. These have been the major source for hundreds of commercially available restriction endonucleases used for molecular cloning. The type III R-M system also expresses the two independent MTase and REase proteins, but these exert their function as a complex. The type IV R-M system does not contain an MTase, and is thought to have evolved in response to some phages evading type I-III R-M systems by modifying their genome to evade restriction. Type IV systems overcome this counterattack by restricting the phage's modified DNA, while the bacterial DNA remains unmethylated[75,76]. It is interesting to note that MTases tend to be more conserved than REases, since the latter undergo rapid evolution to keep up with mutations in phage genomes[77].

R-M systems typically put epigenetic marks on the nucleobases. However, similar systems have been described that modify the sugar-phosphate backbone by introducing a phosphorothioate (substitution of a non-bridging oxygen with a sulfur)[78]. The Dnd system works through the doublestranded phosphorothioation of endogenous DNA by proteins DndABCDE and restriction of foreign, unmodified DNA by DndFGH[78]. Ssp proteins SspABCD also modify the host genome through phosphorothioation, but of only one of the two DNA strands[79]. This activity couples with that of SspE, which requires sensing of SspABCD to introduce nicks

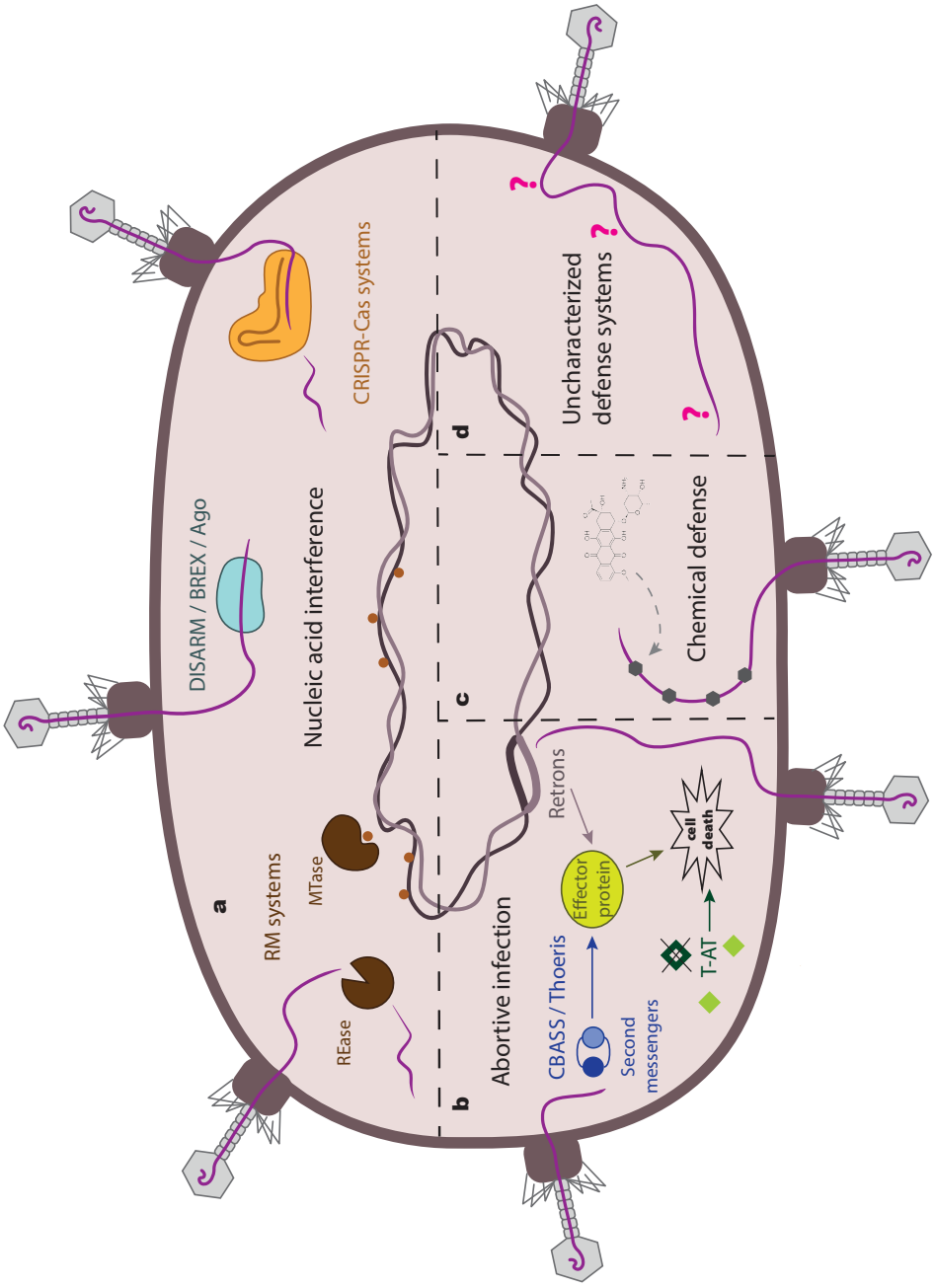

Figure 2. Host phage defense systems. a) Multiple defense systems act via nucleic acid interference. R-M systems are generally composed of an MTase that methylates endogenous DNA to distinguish it

from exogenous DNA, and of an REase that cleaves the exogenous, non-methylated DNA. DISARM interacts with phage DNA to prevent its circularization, thereby blocking its replication or lysogeny. BREX or Ago systems interact with phage DNA and prevent it from replicating without necessarily cleaving it. CRISPR-Cas systems are known as the adaptive immune system of bacteria. The CRISPR array contains sequences of foreign origin that can be transcribed and processed to act as a guide for the Cas endonuclease, which recognizes and cleaves said sequences upon reentry into the bacteria. b) Abortive infection comprises a series of mechanisms that lead to bacterial cell suicide. An example in which this can happen is through an imbalance in the concentration of toxins and antitoxins in a cell. Another example is through the action of effector proteins that might get activated directly, like in the case of retrons, or via second messengers, like in the case of CBASS or Thoeris. These effector proteins can lead to cell death in several ways, for instance through inner membrane degradation (CBASS) or through NAD depletion (Thoeris). c) Bacteria can produce secondary metabolites such as daunorubicin (depicted) that intercalate phage DNA and prevent it from circularizing and replicating. d) Analysis of genetic defense islands has recently led to the discovery of a series of defense systems that are yet to be fully characterized. These include: Hachiman, Shedu, Gabija, Septu, Lamassu, Zorya, Kiwa, Druantia, Wadjet, RADAR, DRTs, AVAST and pVips, among others.

into foreign DNA, or with that of SspFGH, which indiscriminately damages non-phosphorothioated DNA, inhibiting its replication[79,80].

Our knowledge on R-M-related defense systems is continuously expanding as more systems are being discovered through analysis of bacterial genomes. An example is the DISARM (defense island system associated with restriction– modification) systems[81], which include MTases (adenine MTase DrmMI and/or cytosine MTase DrmMII) and proteins with domains of predicted helicase (DrmA, DrmD) and phospholipase D/nuclease (DrmC) activities or of unknown function (DrmB, DrmE). Although the exact mechanism of action of DISARM is not yet understood, it is clear that it involves methylation of the host DNA to distinguish self from nonself, and that it prevents phage DNA circularization, thereby blocking DNA replication and lysogeny at an early stage of the infection. It is also postulated that DISARM might collaborate with different R-M elements, achieving a synergistic effect against phage infection[81]. The bacteriophage exclusion (BREX) defense system also targets phage DNA upon entrance in the host cell[82]. Similar to R-M systems, BREX methylates host DNA to differentiate it from exogenous DNA. However, BREX does not appear to degrade non-methylated phage DNA, and instead seems to hamper replication of the phage DNA without cleavage[82]. Methylated or glycosylated phage DNA is not sensitive to BREX, but deletion of the methylase gene of this system does not have deleterious effects on the bacteria[83].

In some bacteria, foreign DNA can also be intercepted by proteins of the Argonaute (Ago) family. These proteins are also present in eukaryotic cells, where they mediate the degradation of exogenous RNA using small interferingor microRNAs (siRNA, miRNA) as guides to recognize their targets. While this process is not as well studied in prokaryotic cells as it is in eukaryotes, prokaryotic Ago proteins (pAgo) have been found in *Thermus thermophilus* (TtAgo) and in *Rhodobacter sphaeroides* (RsAgo)[84,85]. TtAgo bases its mechanism on DNA-DNA interference rather than the RNA–RNA interference of eukaryotic Ago[86]. This protein also has an endonuclease (slicer) domain that allows it to cleave both single-stranded DNA and negatively supercoiled double-stranded DNA, normally of plasmid origin. Although it is unclear how the DNA guides used by TtAgo are formed, it appears that the activity of the protein itself is necessary for their production. RsAgo, in contrast, uses small RNA molecules as guides to target foreign DNA molecules[87]. Of note, RsAgo lacks the slicer domain, meaning that DNA interference is caused simply by binding the target rather than by cleaving it.

A particular form of nucleic acid interference, CRISPR-Cas (clustered regularly interspaced short palindromic repeats and associated proteins) systems constitute the only form of adaptive immunity described in prokaryotes so far[88,89]. They are present in many bacterial genomes, and occasionally in plasmids[90]. A CRISPR locus in a bacterial genome is composed of a CRISPR array and a Cas gene operon. The CRISPR array contains repeats and sequences of foreign origin called spacers, which form the immunological memory of the defense system. The Cas operon contains all genes coding for Cas proteins that form the machinery required for immunity. Immunity is achieved via a three-stage process that involves adaptation, expression and interference[91–93]. During the adaptation stage, parts of the foreign genetic material are captured and integrated into the CRISPR array as a new spacer[94,95]. In DNA targeting CRISPR systems, functional spacers are derived from invader sequences that are flanked by a protospacer adjacent motif (PAM), a short nucleotide sequence that ensures the targeting of foreign invaders rather than the genomic CRISPR locus[96]. At the expression stage, the CRISPR array serves as a template to transcribe a long precursor CRISPR RNA (crRNA) that is further processed into smaller mature crRNAs. Each crRNA is then loaded into

Cas proteins to form an effector complex. At the stage of interference, this effector complex patrols the cell, screening for complementary sequences that are flanked by a PAM. Upon PAM recognition, the foreign genetic material is cleaved by the Cas proteins, and the infection is contained.

While sharing the general stages described above, CRISPRCas systems are characterized by mechanistic variability and are currently classified in two classes, six types and 33 subtypes[97]. Class 1 systems, which include types I, III and IV, are characterized by the presence of a multi-subunit Cas complex that is involved in the recognition of invader DNA (type I, IV) or RNA (type III) during the interference stage. Class 2 systems, which include types II, V and VI, employ a single subunit effector protein for recognition and cleavage of the foreign DNA (types II, V) or RNA (type VI) sequence. Of the six types described so far, type II is the best-known due to its applications for genome editing technology[98]. In summary, bacteria explore a diverse set of strategies that directly block or cleave phage nucleic acids to survive phage predation.

2. Abortive infection (figure *2b)*

Abortive infection (Abi) is a commonly used phage defense strategy in which the cells sacrifice themselves before the phage completes its replication cycle to protect the rest of the population[99]. Many of the Abi systems rely on a toxin–antitoxin (T–AT) mechanism, in which the balance between a stable toxin and an unstable antitoxin determines the fate of the cell[100,101]. Infection by a phage triggers repression of the antitoxin promoter or termination of its transcription[102]. The result is that the toxin prevails, causing death of the bacterium. Some of these systems, like ToxIN of *Pectobacterium atrosepticum*, are encoded by plasmids[100].

Other common strategies that lead to abortive infection are characterized by the specific depletion of critical cellular resources upon viral infection, including enzymatic cofactors and nucleotides[103]. Examples in *E. coli* include protease Lit, which is activated by the GoI peptide of the T4 major capsid protein and cleaves translation elongation factor Tu to arrest translation[104]. Other Abi systems trigger not an individual response but a set of events. For example, exclusion

of T7 by the F plasmid-encoded PifA system occurs via reduced synthesis of macromolecules, partially impaired DNA ejection and alteration of membrane permeability[105]. In *Lactococcus* spp., Abi systems that can target phage gene replication and expression are constitutively expressed but are toxic to the cell when overexpressed[106]. Notably, most of these lactococcal defense systems are encoded by plasmids[107].

Cell suicide upon detection of invading, cytosolic DNA occurs in eukaryotic cells as well. It is mediated by the production of cyclic GMP-AMP, which activates the cGAS-STING pathway[108], and causes an upregulation of transcription of inflammatory genes. A similar pathway was found in *Vibrio cholerae* biotype El Tor, where production of cyclic GMP-AMP (cGAMP) activates a phospholipase that degrades the inner membrane leading to cell death[109]. Introduction of the operon encoding this pathway into defective *V. cholerae* and *E. coli* strains conferred resistance to a variety of phages, suggesting an important role of this system in antiphage defense. The system, called cyclic-oligonucleotide-based antiphage signaling system (CBASS), has since been found in a broad range of organisms belonging to all major bacterial phyla and at least one archaeal phylum[110]. It is thought to be an ancestor of the eukaryotic cGAS-STING pathway.

Mutations in enzymes involved in protein maturation can also be used to prevent the spread of phage infection. In *Streptococcus thermophilus*, a mutation in the methionine aminopeptidase that impairs its catalytic activity was seen to confer resistance to a broad range of phages, seemingly by hampering virion assembly[111]. While not exactly considered an Abi mechanism, this process comes at the cost of impairing bacterial growth for at least several of the strains studied.

In *V. cholerae*, a parasitic phage satellite known as phage-inducible chromosomal island-like element (PLE) defends the bacterial population from phage attack by functioning akin to an Abi system. PLE are found integrated in the *V. cholerae* chromosomes and are excised upon infection by ICP1 phages[112–114]. Using both PLEand phage-encoded products[114,115], PLE replicates and hijacks the structural components of the phage to encapsidate its own genome[113], and uses protein LidI

to disrupt the mechanism of lysis inhibition that would normally give ICP1 phages more time to produce new virions[116]. Via a combination of structural hijacking and accelerated cell bursting, PLE prevent phage spreading and efficiently protect the bacterial population while transducing their own genome to other cells.

Recently described Thoeris seems to operate via an Abi mechanism as well[117]. It presents a protein with a toll-interleukin receptor (TIR) domain which, upon phage infection, produces an isomer of cyclic ADP-ribose[118]. This molecule acts as a second messenger and activates a protein with catalytic NADase activity, leading to NAD depletion in the infected host. As a result of this, the bacterium presumably dies before phage progeny can mature. TIR domains appear to be specific toward certain phages, and multiple TIR proteins can be present within the same host.

Retrons, bacterial genetic elements composed of a reverse transcriptase (RT) and a noncoding RNA (ncRNA), have also been shown to protect against phage infection via abortive infection[7,119]. Effector proteins of multiple functions were found associated with the retrons, such as ribosyltransferases, two-transmembrane domain (2TM) genes, and genes with ATPase or HNH endonuclease domains, suggesting a diversity of mechanisms by which abortive infection may be achieved. Characterization of retron Ec48 associated with a 2TM domain gene demonstrates that it acts by sensing inhibition of DNA-repair enzyme RecBCD by proteins of the infecting phage, leading to abortive infection and cell death[119].

More recently, dCTP deaminase and dGTPase proteins have been found to protect bacterial cells from phage infection by degrading deoxynucleotides dCTP and dGTP, efficiently eliminating these from the nucleotide pool[120,121]. Depletion of these deoxynucleotides during phage infection halts phage replication and likely leads to cell death[121]. While abortive infection responses can be encoded by some of the defense systems listed above, some CRISPR-Cas systems have been found to use this strategy as well. Most well-known systems are the type III CRISPR-Cas systems that produce small signal molecules upon target RNA detection[122]. This molecule then activates unspecific nucleases and other potentially damaging activities in the cell, aborting an infection[123]. Likewise, some type I CRISPR-Cas

systems can function with an Abi mechanism. In *P. atrosepticum*, expression of a type I-F CRISPRCas system reduces phage progeny while hampering the survival of infected cells[43].

In summary, many abortive infection-like strategies have been identified in which cells typically detect infection and initiate a self-damaging response that hampers the virus in its infection process, saving the remaining population of cells.

3. Chemical defense (figure *2c)*

It is well documented that bacteria produce secondary metabolites, among which are compounds with antimicrobial activity[124]. Recently, a panel of bioactive compounds was tested to assess whether they could confer protection to *E. coli* against lysis by phage Lambda[125]. Several compounds were identified that allow bacteria to proliferate in spite of the phage challenge. Most are DNA-intercalating agents, four of which produced by *Streptomyces* spp.: daunorubicin, doxorubicin, epirubicin and idarubicin. These compounds inhibit double-stranded DNA phages targeting *Streptomyces coelicolor*, *E. coli* and *Pseudomonas aeruginosa*. DNA intercalation is thought to prevent the circularization of the phage linear DNA inside the bacterial cytoplasm, or its interaction with proteins involved in replication and transcription.

4. Uncharacterized defense systems (figure *2d)*

Bioinformatic analysis of genes in defense system clusters has led to the identification of multiple new defense systems in recent years. One such approach identified several putative defense systems based on the requirement that each putative system must contain at least one annotated protein domain enriched in defense islands. Some of these were experimentally confirmed to grant protection against at least one phage: Thoeris (now classified as an Abi system), Hachiman, Shedu, Gabija, Septu, Lamassu, Zorya, Kiwa and Druantia[6]. Zorya contains components that resemble parts of the flagellar motor, and is more abundant in Gram-negative species, especially Proteobacteria. Its proposed mechanism of action leads to cell death through membrane depolarization. Another defense system, Wadjet, does not seem to confer phage resistance but seems to target foreign plasmids by a still

unknown mechanism[6].

Additional defense system candidates were identified using an approach independent of domain annotations[7]. These candidates incorporate enzymatic activities not previously thought to be implicated in antiviral defense. Among them is the phage restriction by an adenosine deaminase acting on RNA (RADAR) system. RADAR edits RNA transcripts by catalyzing the deamination of adenosine into inosine, seemingly blocking the early stages of the phage infection cycle. Another candidate system identified in this study is the RT family defense-associated reverse transcriptases (DRT). DRTs are not linked to mobile elements, unlike most RTs found in prokaryotes, and they seem to alter phage gene expression in various ways. Antiphage activity was likewise detected in a group of nucleoside triphosphatases (NTPases) of the STAND (signal transduction ATPases with numerous associated domains) superfamily, which were given the name antiviral ATPases/NTPases of the STAND superfamily (AVAST). Members of this superfamily found in eukaryotes are often involved in programmed cell death, so it was postulated that the AVAST system may constitute an Abi mechanism. Additionally, the study found some other proteins and systems that provided protection against T7-like phages, of which the mechanisms of action need to be further investigated.

Another example of an antiphage defense mechanism that has recently started to be characterized is prokaryotic viperins (pVips)[126]. In animals, viperins are interferon-induced proteins that block the replication of several viruses[127]. In a similar way, pVips produce modified ribonucleotides that inhibit viral polymerase-dependent transcription, thereby protecting against infection by phage T7[126]. pVips with antiphage activity were identified by analyzing prokaryotic homologues of human viperin that are encoded in defense islands.

Finally, some novel defense systems that are still uncharacterized have been identified in T4- and T2-like prophages. These are briefly discussed in the following section.

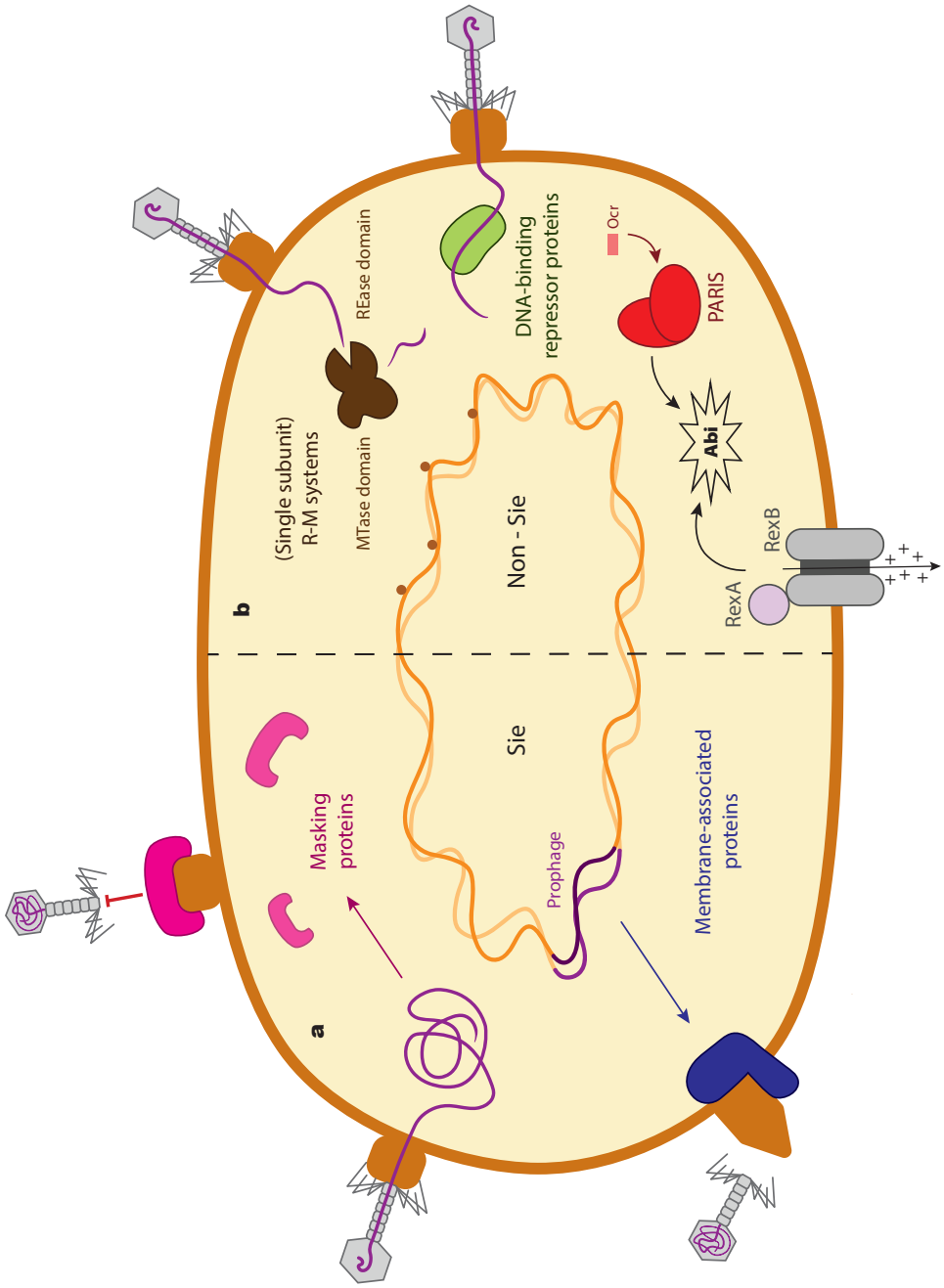

Figure 3. Phage-derived defense systems. a) Superinfection exclusion systems (Sie) are encoded

by phages to prevent other phages from infecting their host. Some phages like T5 produce proteins that mask their receptor and make it inaccessible. Other phages, especially prophages, encode membrane-associated proteins that interact with the phage receptor, blocking the DNA entry channel, triggering a conformational change or inhibiting the invading phage's enzymes. b) Prophages like Panchino of *Mycobacterium smegmatis* can confer resistance to their hosts through the expression of R-M systems or DNA-binding repressor proteins that target the DNA of newly infecting phages. Other prophage-encoded systems, like RexA-RexB or the newly characterized PARIS, can trigger an Abi response upon sensing an invasion by a new phage.

Phage-derived phage defense systems

Interestingly, phages provide bacteria with defense systems against infection by the same or closely related phage, in a phenomenon known as superinfection exclusion (Sie) (figure 3a). Some phages produce proteins to mask the cell surface receptors, blocking new infections. This strategy also protects the newly formed phages from being inactivated as a consequence of binding to receptors coming from remains of lysed bacteria. This behavior is observed for example in phage T5, which produces lipoprotein Llp that conceals its own receptor, outer membrane protein FhuA[128]. Other phages, mostly prophages[129], use membrane-anchored or membrane-associated proteins to target and block the entry of phage DNA into the bacterial cytoplasm[130]. Such proteins act by inhibiting the formation of the channel through which DNA travels across the cell membrane, by inhibiting the phage lysozyme that degrades the peptidoglycan of the bacterial cell wall, or by changing the conformation of the proteins surrounding the ejection site to prevent translocation[131].

Furthermore, prophages can mediate resistance through non-Sie-like mechanisms as well (figure 3b). The RexA-RexB system, an Abi system expressed by λ-lysogenic *E. coli*, works by reducing the membrane potential of the cell, leading to a decrease in ATP production that ultimately results in cell death[132]. Another example is the phage Panchino of *M. smegmatis*, which provides lysogens with a single subunit RM system able to recognize a broad range of phages[133]. Genes encoding repressor proteins that bind phage DNA may also be found in prophages[134]. They are thought to have a role in protecting the viability of the lysogenized bacteria, counteracting accidental prophage transcription events. Prophage-mediated phenotypic changes in bacteria are sometimes encoded in genetic elements called

morons, which are flanked by a promoter and a transcriptional terminator and can be transcribed autonomously, independent of prophage activation[135].

As with host defense systems, the discovery of phage-derived defense systems is ongoing. Analysis of Enterobacteria P4- and P2-like prophages recently led to the discovery of genetic hotspots that encode a variety of bacterial immune mechanisms[136]. Among these is the phage anti-restriction-induced system (PARIS). This system triggers an Abi response upon sensing a phage-encoded anti-restriction protein, Ocr, which inhibits R-M systems and BREX[136]. The mechanisms of action of PARIS and the other systems identified in this study remain to be further uncovered.

In summary, once inside the host, phages themselves can provide the bacteria with mechanisms of protection against further phage infection that favor both the bacteria and the phage.

Phage counterattack strategies

While the mechanisms of phage resistance exhibited by bacteria seem overwhelmingly varied, phages have also developed a broad array of opposing strategies. Just as bacterial defenses target every step in the process of phage infection, every barrier imposed by bacteria has to withstand a phage counterattack[137].

In response to variations in the bacterial cell surface receptors, phages are able to change their tropism through mutations in their RBPs. In fact, genes encoding RBPs and other proteins related to host recognition are reported to incorporate mutations at a very high frequency. This is often mediated by the activity of diversity-generating retroelements (DGRs)[138]. These are regions that are subjected to targeted mutation by means of the exchange of two variable repeats by an error-prone reverse transcriptase[139]. This type of directed mutagenesis is template dependent and affects determined adenine-specific sites, while a conserved

scaffold sequence is retained to ensure stability. This process was first described for the specificity switch of the major tropism determinant protein in *Bordetella* spp. phages. Since then, more phages have been identified that benefit from these systems[140].

To overcome the barrier imposed by capsules and extracellular layers, some phages became able to bind to these structures[18], and to degrade them using depolymerases. These enzymes may be either expressed as part of tail spike or tail fiber proteins or released in a soluble form following lysis of infected bacteria[141]. A recent review offers an overview of the diversity of phage depolymerases[142].

Phages have also developed forms of escaping targeting by RM systems. They can (i) mutate to remove restriction sites from their genome (palindrome avoidance) and therefore avoid recognition by REases[143,144]; (ii) modify the sequences recognized by REases (e.g. the glucosyl-hydroxymethylcytosine of T4 that is used instead of the regular cytosine[130]); (iii) change the distance and orientation of restriction sites to avoid restriction by REases that need to recognize two sequences at a determined distance from each other and in a specific orientation[145]; (iv) occlude the restriction sites with proteins (e.g. DarA and DarB of P1 phages) that are ejected together with the phage genome[8]; (v) sequester REases with proteins that mimic the structure of a DNA double helix (e.g. Ocr from T7)[146]; and (vi) acquire genes encoding an MTase that modifies the phage genome[147], or stimulate the activity of the host MTase for the same purpose[148].

CRISPR-Cas systems can also be evaded by phages in multiple ways[149]. Phages can acquire point mutations or deletions in the PAM sequences or in positions of the protospacer region close to the PAM sequences (i.e. the seed region of the protospacer)[150]. Alternatively, some phages use anti-CRISPR (Acr) proteins, first described in phages of *P. aeruginosa* [151]. In general, Acrs work by either preventing recruitment of the crRNA-Cas complex to the target DNA by binding the complex or occluding the PAM sequence, or by inhibiting the endonuclease domain so that cleavage cannot take place[152]. Glucosylation of phage genetic material has also been shown to protect phages against some CRISPR-Cas systems[153]. A different

strategy is employed by the jumbo *Serratia* phage PCH45 and *Pseudomonas* phage ϕKZ, which form a protein shell that encloses phage DNA in a nucleus-like compartment, physically shielding it from the CRISPR-Cas complexes[12,13]. Of note, this mechanism does not protect the phage from RNA-targeting CRISPR-Cas systems, as the transcribed mRNA is not contained within the nucleuslike compartment during translation.

Abi mechanisms can also be outsmarted by phages. Phages can avoid toxin–antitoxin mechanisms by inhibiting the protease that degrades the antitoxin, or by expressing their own antitoxin analogue[10,154,155]. Furthermore, mutations in genes involved in the metabolism of nucleic acids also prove to be effective in avoiding toxin–antitoxin systems of some bacteria like *Lactococcus* spp.[156]. Mutations in phage genes encoding peptides that activate Abi-associated enzymes, such as the Lit activator GoI peptide in the major head protein of T4, can also result in hindering of the Abi mechanism[157]. Phages ICP1 that infect *V. cholerae* overcome PLE-mediated Abi by using either a phage-encoded CRISPR-Cas system that targets the PLE genome during infection[112], or an endonuclease that binds and cleaves the PLE origins of replication[158]. It is expected that phages possess countermeasures against the more newly described defense systems as well. The Ocr protein of phage T7, known to inhibit R-M systems, was recently found to also inactivate the BREX system by binding the methyltransferase BrxX[11]. The discovery of other new phage counterattack strategies is likely just a matter of time, as interest in bacterial defense mechanisms and phage anti-defenses continues to grow.

Phage resistance mechanisms in a clinical context

The number of phage therapy case studies and clinical trials performed in humans has significantly increased in these past years, as the problem of antibiotic resistance aggravates. The efficacy of phage therapy in these studies is quite variable, ranging from negative outcomes to the resolution of severe infections in human patients (table 1, table S1). Interestingly, while phage resistance has

been shown to develop quickly *in vitro*, studies in humans have described both the presence[159] and the absence[160] of phage resistance *in vivo*. As a consequence of such variable results, there is a lack of consensus in the scientific and medical community about the potential of phages as therapeutic agents.

The human immune response to bacterial infection and the specific phage-resistance mechanisms developed by the bacteria are likely behind the distinct outcomes. The development of an immune response, particularly involving neutrophils, has been shown essential for the success of phage therapy by preventing the outgrowth of phage-resistant mutants[161]. Multiple studies have demonstrated that phage-resistant phenotypes often associate with decreased pathogenicity, with the strain becoming more susceptible to the human immune defenses[162]. Receptor adaptations such as mutations in bacterial capsule, LPS and other surface components are examples of phage-resistance mechanisms that result in increased immune susceptibility[163]. Importantly, these surface modifications also often associate with increased antibiotic susceptibility. This effect occurs, for example, in cases where the phage interacts with bacterial structures that function as drug efflux pumps[164,165]. By mutating the efflux pump to achieve phage resistance, bacteria lose the ability to pump out the antibiotics, thus gaining antibiotic susceptibility as a trade-off (for a review of mechanisms of phage-antibiotic synergism, see e.g. Tagliaferri, Jansen and Horz 2019[166]). Such interactions have been exploited in therapeutic contexts[167–170]. However, phage-resistance mutations have also been shown to pleiotropically confer increased antibiotic resistance[47], and other mechanisms of phage resistance (e.g. CRISPR-Cas, R-M systems) may lead to a phage-resistance phenotype that does not render the bacteria more susceptible to the immune system or to antibiotics. In such cases, resistance to phages may develop *in vivo* even in the presence of a strong immune response.

Unfortunately, the mechanisms underlying phage resistance are seldom, if ever, investigated in human clinical studies and trials, and represent a clear knowledge gap. Most studies look at the safety and/or clinical outcome of phage therapy, and very few have documented the development of phage resistance (Table 1),

let alone the mechanisms behind it. There are studies, however, that indicate that addressing and tackling phage resistance can lead to improved treatment outcomes. One such study found phage-resistant clones in a patient suffering from a multidrug-resistant *A. baumannii* infection after eight days of treatment with intravenous phage therapy[171]. Phage-resistance was associated with loss of bacterial capsule and increased extracellular polysaccharide production, and was overcome via an iterative process of phage cocktail formulation that resulted in the resolution of the infection. Of relevance, the phage-resistant phenotype was associated with increased antibiotic sensitivity, suggesting a fitness cost of phage-resistant mutations *in vivo*. In another study, the association between phage-resistance and increased antibiotic susceptibility was exploited to treat a patient with a chronic multidrug-resistant *P. aeruginosa* infection of an aortic graft[170]. The treatment consisted of a combination of the antibiotic ceftazidime and phage OMKO1, which binds to an outer membrane protein that is part of multidrug efflux systems of *P. aeruginosa*. This combination explored the capacity of the phage to kill the original strain and the ability of ceftazidime to kill any emerging phage-resistant variants with mutations in the multidrug efflux system, to achieve resolution of the infection.

Characterizing the mechanisms of resistance to phages that target pathogens of interest will inform about the relevance that each phage defense system has in a clinical context, in terms of frequency with which they occur in pathogens and their association with virulence and antibiotic susceptibility of the pathogen. Furthermore, certain natively present defense systems like RM may affect and reduce the choice of phages available to use in a therapeutic setting. Another issue to consider is that the development of resistance (as well as treatment efficacy) may significantly differ when using single or multi-phage treatment approaches, and may also vary with the timing and order of phage administration[172]. The more widespread use of bacteriophages for therapeutic purposes could lead to selection for phage-resistant phenotypes that arise through horizontal gene transfer of phage defense systems. Understanding the complexity of interactions and mechanisms leading to phage resistance will aid the development of phage-based treatments with better clinical outcomes and to engineered phages that may overcome host

defense systems.

Concluding remarks

In this review, we have provided an overview of the current knowledge of mechanisms behind existing and developing phage resistance, and highlighted the potential knowledge gaps and clinical importance of phage resistance for phage therapeutic strategies. While our understanding of the mechanisms behind phage resistance has expanded in recent years, many defense systems remain uncharacterized or yet undiscovered. As such, the complete picture of phage resistance development remains elusive, especially in the context of the human body.

For phage therapy to move forward, it is imperative that clinical studies and trials also assess the development of resistance in a systematic manner, in which both the emergence of phage resistance and the mechanisms behind it are included in the investigation. Sequencing technologies and genome analysis of both bacterial strains and phages may allow for the identification of defense and anti-defense systems in clinical isolates. Such data will prove invaluable for isolating and selecting candidate phages, as well as for predicting the outcome of the therapeutic intervention.

Improved understanding of how defense systems affect phage therapy, combined with an increased knowledge of the anti-defense strategies employed by phages to counteract bacterial defenses, will greatly contribute to the development of more effective phage-based therapeutic approaches.

Table 1: Case studies and clinical trials of phage therapy in humans, with associated safety, clinical, and phage-resistance outcomes.
[a] Successful: patient was healed. Clinical improvement: infection and/or associated complications were reduced but not completely resolved.
[b] Not described: resistance was not addressed. Not identified: resistance was investigated but not found.

Clinical study/case report	Safety	Clinical outcome[a]	Phage resistance[b]	Mechanism of resistance	Ref.
Tibia bone infection with MDR *A. baumannii* and *K. pneumoniae* treated with phages and antibiotics	Well tolerated	Successful	Not found in the patient; found in *in vitro* experiments with isolates	Mutations in surface adhesin and glycosyl-transferase of the EpsG family (speculated)	173
Necrotizing pancreatitis patient with an MDR *A. baumannii* infection treated with phages and antibiotics	Well tolerated	Successful	A resistant bacterial isolate was found and used to select a new phage	Loss of bacterial capsule and increased extracellular polysaccharide production	174
Cystic fibrosis patient with MDR *P. aeruginosa* infection treated with phages and antibiotics	Well tolerated	Successful	One resistant isolate was identified	Not described	175
Patient with *K. pneumoniae* urinary tract infection treated with non-active antibiotics and phages	Well tolerated	Successful	Bacteria became resistant to two rounds of phage cocktails but were ultimately sensitive to the combination of a third cocktail and previously inactive antibiotics	Not yet elucidated	176
Patient with periprosthetic joint infection and MDR *P. aeruginosa* chronic osteomyelitis treated with phages and antibiotics	Well tolerated	Successful	Not identified	Not applicable	177

Patient with MDR *P. aeruginosa* urinary tract infection treated with phages and antibiotics	Well tolerated	Successful	Not identified	Not applicable	160
Aortic graft infection with *P. aeruginosa* treated with a phage targeting an efflux pump protein and antibiotics	Well tolerated	Clinical improvement	Yes, as expected. Explored to cause sensitization of bacteria to antibiotic.	Mutations in receptor protein (M of mexAB- and mexXY- efflux system)	170
Cystic fibrosis patient with *M. abscessus* and *P. aeruginosa* infection treated with engineered phages	Well tolerated	Clinical improvement	Resistance detected *in vitro* for two of the three phages administered	Not described	178
Netherton syndrome patient with MDR *S. aureus* infection and allergy to multiple antibiotics treated with phage cocktails topically and orally	Well tolerated	Clinical improvement	Resistance to a phage cocktail was identified after 3 months of treatment	Not described	159
Thirteen patients with *S. aureus* bacteremia treated with phages and antibiotics	Well tolerated	Clinical improvement in eight of the patients	Changes in phage susceptibility were detected *in vivo* in three patients	Single nucleotide polymorphisms were detected in isolates recovered from one of the patients	179
Three lung transplant patients with MDR *P. aeruginosa* and *B. dolosa* infections treated with phages and antibiotics	Well tolerated	Clinical improvement in two of three patients	Resistant isolates were identified post-therapy in one of the patients who had a successful outcome	Not described	180
Patient with bronchiectasis and *M. abscessus* infection treated with a phage cocktail and antibiotics	Well tolerated	Failure due to antiphage immune response	Resistant isolates appeared to one out of three phages in the cocktail	Not described	181

Eight cardiothoracic surgery patients treated with antibiotics, phages and fibrin glue	Well tolerated except for an increase in inflammation	Successful in five of the patients; two patients showed improvement but died of unrelated complications; one patient did not experience sufficient bacterial clearance and died of sepsis	Bacterial isolates appeared to have mutated in one of the patients	Not described	182
Ten patients with diverse MDR bacterial infections treated with intravenous phage cocktails and antibiotics	Well tolerated except by one patient, who developed fever, wheezing and shortness of breath after first dose of the cocktail (subsequent doses were well tolerated)	Successful for seven out of ten patients	Resistance developed in three of the patients, but was overcome through administration of phages specific for the resistant isolates (two of these cases still had a successful outcome)	Not described	183
Phase I-II clinical trial to assess the safety and efficacy of a phage cocktail to treat burn wounds infected with *P. aeruginosa* in 26 patients (13 treated, 13 control)	Fewer adverse events were observed in the phage-treated group than in the group treated with the standard of care	Trial was stopped because of insufficient efficacy (patients were being exposed to 10,000-fold lower doses of phages than originally intended)	Intermediately susceptible and resistant isolates were found	Not described	184
Report of phage therapy performed on 153 patients with different infections	Diverse	Diverse	Resistance and changes in the phage typing profile were detected in multiple cases	Not described	185

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Supplementary information

Table S1: Complete list of case studies and clinical trials of phage therapy in humans, with associated safety, clinical, and phage-resistance outcomes.

[a] Successful: patient was healed. Clinical improvement: infection and/or associated complications were reduced but not completely resolved.

[b] Not described: resistance was not addressed. Not identified: resistance was investigated but not found.

Clinical study/case report	Safety	Clinical outcome[a]	Phage resistance[b]	Mechanism of resistance	Ref.
Tibia bone infection with MDR *A. baumannii* and *K. pneumoniae* treated with phages and antibiotics	Well tolerated	Successful	Not found in the patient; found in *in vitro* experiments with isolates	Mutations in surface adhesin and glycosyl-transferase of the EpsG family (speculated)	1
Necrotizing pancreatitis patient with an MDR *A. baumannii* infection treated with phages and antibiotics	Well tolerated	Successful	A resistant bacterial isolate was found and used to select a new phage	Loss of bacterial capsule and increased extracellular polysaccharide production	2
Cystic fibrosis patient with MDR *P. aeruginosa* infection treated with phages and antibiotics	Well tolerated	Successful	One resistant isolate was identified	Not described	3
Patient with *K. pneumoniae* urinary tract infection treated with non-active antibiotics and phages	Well tolerated	Successful	Bacteria became resistant to two rounds of phage cocktails but were ultimately sensitive to the combination of a third cocktail and previously inactive antibiotics	Not yet elucidated	4
Patient with periprosthetic joint infection and MDR *P. aeruginosa* chronic osteomyelitis treated with phages and antibiotics	Well tolerated	Successful	Not identified	Not applicable	5

Patient with MDR *P. aeruginosa* urinary tract infection treated with phages and antibiotics	Well tolerated	Successful	Not identified	Not applicable	6
Chronic prosthetic joint infection with *S. aureus* treated with phages	Well tolerated	Successful	Not described	Not described	7
Diabetic foot ulcer infected with *S. aureus* treated with a phage	Well tolerated	Successful	Not described	Not described	8
Patient with *S. aureus* corneal abscess and interstitial keratitis treated with a phage following antibiotic treatment	Well tolerated	Successful	Not described	Not described	9
Patient with MRSA urinary tract infection and gastrointestinal tract colonization treated with phages	Well tolerated	Successful	Not described	Not described	10
A man with burn wounds and a dog with otitis, both infected with *P. aeruginosa* and treated with phages	Well tolerated	Successful	Not described	Not described	11
Three patients with chronic bacterial prostatitis treated with phages	Well tolerated	Successful	Not described	Not described	12
Two patients with *S. aureus* infected radiation burns treated with phages and ciprofloxacin infused in a sustained release preparation	Well tolerated	Successful	Not described	Not described	13

Prosthetic joint infected with *K. pneumoniae* treated with a phage and minocycline	Well tolerated	Successful	Not described	Not described	14
Pediatric cystic fibrosis patient with an *Achromobacter* infection treated with phages and antibiotics	Well tolerated	Successful	Not described	Not described	15
Aortic graft infection with *P. aeruginosa* treated with a phage targeting an efflux pump protein and antibiotics	Well tolerated	Clinical improvement	Yes, as expected. Explored to cause sensitization of bacteria to antibiotic.	Mutations in receptor protein (M of mexAB- and mexXY- efflux system)	16
Cystic fibrosis patient with *M. abscessus* and *P. aeruginosa* infection treated with engineered phages	Well tolerated	Clinical improvement	Resistance detected *in vitro* for two of the three phages administered	Not described	17
Netherton syndrome patient with MDR *S. aureus* infection and allergy to multiple antibiotics treated with phage cocktails topically and orally	Well tolerated	Clinical improvement	Resistance to a phage cocktail was identified after 3 months of treatment	Not described	18
Cystic fibrosis patient with an *A. xylosoxidans* infection treated with phages	Well tolerated	Clinical improvement	Not described	Not described	19
Patient with colistin-only-sensitive *P. aeruginosa* septicemia	Well tolerated	Clinical improvement	Not described	Not described	20
Three patients with *S. aureus* prosthetic knee infection treated with debridement, a phage cocktail and antibiotics	Well tolerated	Clinical improvement, infection disappeared completely in two of the patients	Not described	Not described	21

Kidney transplant recipient with MDR *K. pneumoniae*	Well tolerated	Clinical improvement (not clear if due to phage therapy)	Not described	Not described	22
Thirteen patients with *S. aureus* bacteremia treated with phages and antibiotics	Well tolerated	Clinical improvement in eight of the patients	Changes in phage susceptibility were detected *in vivo* in three patients	Single nucleotide polymorphisms were detected in isolates recovered from one of the patients	23
Three lung transplant patients with MDR *P. aeruginosa* and *B. dolosa* infections treated with phages and antibiotics	Well tolerated	Clinical improvement in two of three patients	Resistant isolates were identified post-therapy in one of the patients who had a successful outcome	Not described	24
Complex bone and joint infection with *P. aeruginosa* treated with phages and antibiotics	Well tolerated	Clinical improvement; patient died because of unrelated complications	Not described	Not described	25
Phase I clinical trial to assess the safety of a phage preparation in 39 patients (18 treated, 21 control) with chronic venous leg ulcers	Well tolerated	No significant differences found between treated and control groups; trial was not designed to assess efficacy	Not described	Not described	26
Patient with bronchiectasis and *M. abscessus* infection treated with a phage cocktail and antibiotics	Well tolerated	Failure due to anti-phage immune response	Resistant isolates appeared to one out of three phages in the cocktail	Not described	27

Patient with *S. aureus* infection of a knee megaprosthesis treated with debridement, a phage cocktail prepared in a hydrogel and antibiotics	Well tolerated (adverse events described are not considered to be related to phage therapy)	Site of surgery did not heal completely and got re-infected (not with the strain targeted by phage therapy)	Not described	Not described	28
Eight cardiothoracic surgery patients treated with antibiotics, phages and fibrin glue	Well tolerated except for an increase in inflammation	Successful in five of the patients; two patients showed improvement but died of unrelated complications; one patient did not experience sufficient bacterial clearance and died of sepsis	Bacterial isolates appeared to have mutated in one of the patients	Not described	29
Phase I clinical trial to assess safety and preliminary efficacy of a phage cocktail in 9 patients with *S. aureus* chronic rhinosinusitis	Well tolerated, six patients reported mild adverse events	Preliminary efficacy results suggested clinical improvement	Not described	Not described	30
Ten patients with diverse MDR bacterial infections treated with intravenous phage cocktails and antibiotics	Well tolerated except by one patient, who developed fever, wheezing and shortness of breath after first dose of the cocktail (subsequent doses were well tolerated)	Successful for seven out of ten patients	Resistance developed in three of the patients, but was overcome through administration of phages specific for the resistant isolates (two of these cases still had a successful outcome)	Not described	31
Clinical trial to assess the safety and efficacy of a T4-like phage cocktail in pediatric patients with diarrhea	No adverse events were reported in the treated group compared to the control group	No significant differences in treatment outcome were found between the treated and the control groups (phage cocktail had not been tailored to patients' infections)	Not described	Not described	32

Phase I-II clinical trial to assess the safety and efficacy of a phage cocktail to treat burn wounds infected with *P. aeruginosa* in 26 patients (13 treated, 13 control)	Fewer adverse events were observed in the phage-treated group than in the group treated with the standard of care	Trial was stopped because of insufficient efficacy (patients were being exposed to 10,000-fold lower doses of phages than originally intended)	Intermediately susceptible and resistant isolates were found	Not described	33
Pediatric patient with *P. aeruginosa* osteoarticular infection treated with phages and antibiotics	Fever and increase in C-reactive protein associated with release of bacterial remains into the bloodstream	Clinical improvement	Not described	Not described	34
Clinical trial evaluating the safety and efficacy of a phage preparation in 24 patients (12 treated, 12 control) with chronic otitis caused by *P. aeruginosa*	Mild to moderate adverse events were reported, including discomfort, itchiness and wetness	Clinical improvement compared to the control group in all but one patient	Not described	Not described	35
Patient with chronic MRSA prosthetic joint infection treated with phages and antibiotics	Transaminitis was reported following the third intravenous dose of phages	Clinical improvement	Not described	Not described	36
Pediatric patient with congenital heart disease and P. aeruginosa bacteremia treated with phages and antibiotics	Treatment was discontinued due to cardiac decompensation with concerns for anaphylaxis (although subsequently attributed to the pre-existing disease)	Blood sterilization could be achieved; sepsis was developed again after the treatment was interrupted	Not described	Not described	37
Cerebritis patient with *A. baumannii* infection treated with phages and colistin	Brief hypotension episode	Unsuccessful; therapy was discontinued	Not described	Not described	38

Report of phage therapy performed on 153 patients with different infections	Diverse	Diverse	Resistance and changes in the phage typing profile were detected in multiple cases	Not described	39

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Chapter 6

General discussion

In this thesis, we have described several rational, *in vitro* approaches to understanding therapeutic phages. Our aim was to identify factors that can influence their antibacterial activity, and to suggest ways in which therapeutic approaches may be improved in the future. The fields of phage therapy and phage molecular biology have been growing exponentially in the past few years. In addition, the number of case reports describing successful phage therapy interventions keeps increasing. Yet in a big part of the scientific community, the concept of phage therapy is still met with skepticism. Although regulations are slowly changing in a way that will hopefully facilitate the design of clinical trials to evaluate the efficacy of phage therapy, the road ahead is still quite long in this regard. In the meantime, we are trying to bridge the gap between what we know about phages and how to use them to treat bacterial infections in the context of the human body. In this process, we have been confronted with the need to start learning from the basics, but also to develop new tools to study phage-bacteria interactions with an innovative approach.

The first research chapter of this thesis, **chapter 2**, describes how we developed a fluorescence-based assay to monitor phage-mediated killing of Gram-negative bacteria in real time. Currently, the main reference methods for testing susceptibility of clinical isolates to different phages are spot tests, double-layer agar assays, or the Appelmans method, where phage-induced lysis of a liquid bacterial culture is evaluated[1]. While these techniques are simple and provide reliable readouts, they can be quite labor-intensive, especially when dealing with a large number of strains and phages. Besides from this, they do not allow for monitoring of the phage infection in real time, a feature that may provide more detailed information on the activity of the phage on a specific isolate. Our results in this chapter show that our method, named fluorescent DNA dye assay, provides a rapid way of tracking virulent phage activity in real time in medium to high throughput. We validated the assay by comparing it with other methods such as colony plating and double-layer agar spot tests. In addition, we compare it to another novel technique for monitoring phage activity: the *lux* system. This is a previously described bioluminescence reporter gene construct that can be introduced in the bacterial chromosome to provide a luminescence signal over time reflecting the metabolic activity of the bacteria[2]. This method has been employed for other applications such as localization of bacterial

infections in an *in vivo* model but, to our knowledge, it had never been used to monitor phage infections *in vitro*. We think that assays like the fluorescent DNA dye assay could have a place in phage therapy pipelines in a more clinical setting. New methods are being explored to overcome the drawbacks of the conventional reference methods. These vary from optical density measurements to measuring respiration using a tetrazolium dye or tracking gene expression via quantitative real-time polymerase chain reaction (qPCR)[3]. Adopting such methods could speed up the process of screening for phages to target a given clinical isolate, as well as provide the possibility of testing phages in different media, for example in combination with antibiotics. We think that our method constitutes a useful addition to this repertoire. We showcased this by testing the susceptibility of a panel of clinical isolates to different phages. Here, our method yielded comparable results to conventional spot tests in 86% of 110 phage-bacteria combinations tested, with a more efficient workflow.

Besides from its potential as a phage screening tool, one of the main reasons we developed this assay was to study the interactions of phages with the innate immune system. To do so, we set out to assess the effect of the complement system in human serum on phage activity. At the start of this project, we hypothesized that phages and the complement system in serum would act synergistically in clearing bacteria. Contrary to our expectations, we saw that serum actually protected bacteria from being killed by the phages. This only happened with certain phages and not others. **Chapter 3** of this thesis describes that phages with a certain morphology, myophages, are likely more susceptible to inhibition by the complement system. To study how complement impairs phage infection, we relied on another novel method based on producing azide-tagged phages that we could then label by means of click chemistry. We showed that complement blocks phage activity at the stage of adsorption to the host. Inhibition of phages by complement could explain results seen in other studies where phage activity was impaired when phages and serum were combined[4–6]. In addition, we believe that the observation that human complement can inhibit certain phages is very important in the context of phage therapy.

Inhibition of phages by complement could have very negative consequences for therapy applications. With this in mind, it may be worthwhile to choose therapeutic phages based on whether they are sensitive to this effect. However, this may be difficult because of limitations in the host range of phages. To expand the repertoire of phages suitable for therapeutic applications, it would be interesting to perform *in vitro* evolution experiments where complement-sensitive phages are exposed to serum while infecting bacteria. In such a setting, phages might mutate to avoid recognition by C1q or other complement proteins. Analysis of these mutants would also give insights into which residues on phage capsid proteins are recognized by complement. There is already evidence that substitution of glutamic acid by lysine can help *Escherichia coli* phage Lambda escape clearance by the innate immune system in a mouse model[7]. Furthermore, exposure of arginine on the phage capsid was seen to make *E. coli* phage T4, a myophage, less sensitive to inactivation by human serum with active complement[8]. Diving deeper into how the amino acid composition of phage capsid proteins influences recognition by complement could open the door to developing engineered phages with a reduced complement sensitivity. This may fit in well with approaches where genetic engineering is used to boost the antibacterial activity and host range of phages[9].

In any case, the extent of the influence of complement on the outcome of phage therapy could also depend on the route of administration. For intra-venous applications, complement activity could be particularly detrimental, potentially neutralizing the effectiveness of the treatment or increasing the dose needed. Other tissues, nonetheless, are less rich in complement proteins[10]. However, it is also worth considering that, if recognized by complement, phages could guide the immune response to the site of infection, even at the expense of their own activity. Such an effect could be decisive in clearing bacteria, as research shows that the human innate immune system is necessary in resolving complicated infections[11,12]. On the other hand, excessive inflammation is the hallmark of many respiratory conditions. In cystic fibrosis in particular, high levels of complement activation correlate with a more severe disease[13,14]. Based on this, perhaps a better approach could be to combine phage therapy with local complement inhibitors. This would potentially reduce the levels of inflammation while preserving the activity of complement-sensitive phages.

Whether promoting or diminishing inflammation is more beneficial may depend on the type and localization of the infection. Here, too, phage engineering could offer interesting opportunities, as different immunomodulators could be expressed fused to phage capsid proteins to achieve a boosting or dampening effect on the inflammatory response.

In an effort to clarify the mechanism behind the phage-complement interactions, we found that complement C1q can bind directly to a myophage and is required for the inhibitory effect of serum. However, we did not fully unravel whether this inhibitory effect is caused purely by the interaction of C1q with the phage, or by the subsequent complement activation that C1q binding might trigger. C1q deposition on the phage could also lead to the formation of classical pathway C3 convertase on the phage capsid, made up by C4b2b[15]. In turn, these convertases would cleave C3 and cause deposition of C3b on the phage. All in all, activation of complement, and in particular of the classical pathway, on the phage could potentially lead to the deposition of large protein complexes on the viral particle. Our results show that phage activity in serum could also be partially rescued by adding a C3 cleavage inhibitor, indicating that components downstream of C1q may also play a role in interacting with phages. To determine whether phage inhibition is due to the activation of the complement cascade, or whether C1q alone can cause this effect, assays should be performed to evaluate phage activity in the presence of purified complement components[16]. For example, by performing our fluorescent DNA dye assay with purified C1q instead of whole serum we could determine if this protein alone is sufficient to inhibit phage-mediated killing.

It would also be interesting to resolve whether complement factors interact with specific targets on the phage capsid. To answer this question, one could image phages in serum by means of transmission electron microscopy coupled with immunogold staining of complement components. This technique has been used in the past, for example, to identify the location of host binding proteins on the capsid of a phage[17]. Such a strategy could also reveal why myophages are more sensitive to inhibition by complement. Deposition of complement components may cause a steric hinderance to the tail proteins, so that the tail is no longer

able to contract. Conversely, it could be that this interaction causes a premature contraction of the tail while the phage is still away from the bacterial surface. In this scenario, the virion would be unable to bind to and infect bacteria. This happens, for instance, as a result of treating phages with urea, pH changes, heat, or cationic detergents, all of which cause changes in the contact area between the sheath and the baseplate[18]. Changes in the conformation of the baseplate trigger contraction, which propagates as a wave through the sheath[19] (figure 1). Binding of complement components to the baseplate could well induce conformational changes and therefore set off this process. One the other hand, the differences observed between myophages and podophages could be due to the different sizes of these two types of viruses. If activation of the complement cascade is necessary for phage inhibition, podophages may be protected by their small size, which could prevent proper complement activation from occurring. Studying the effect of serum on a broader and more diverse sets of phages, and especially of siphophages, would give indications of whether susceptibility to complement is determined by size or by having a contractile tail.

Another factor that makes phage therapy more complex is the fact that, in practice, it is administered together with antibiotics in the majority of the cases[20]. The effects

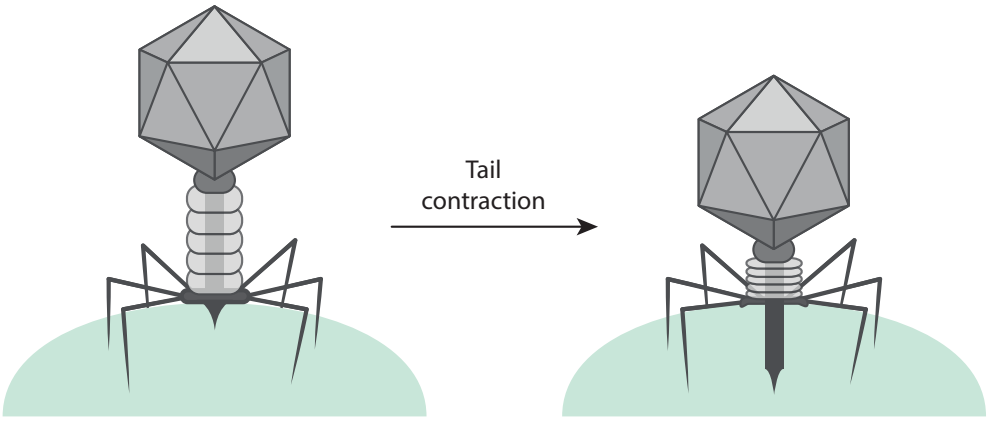

Figure 1. Myophage tail contraction is typically initiated by conformational changes in the baseplate, which propagate upwards through the sheath. As a result, the tail tube protrudes, penetrating the envelope of the host cell.

of combining phages with different antibiotics were explored in **chapter 4** of this thesis. Here, we performed a screening where we treated three *Pseudomonas aeruginosa* strains with phages and antibiotics of different classes together in different concentrations. Our results showed that beta lactams in particular, but also some aminoglycosides, can synergize with an LPS-targeting phage (PBJ). We calculated a synergy score to quantify the levels of synergy between PBJ and three clinically used antibiotics: meropenem, ceftazidime and tobramycin. Quantifying synergy, however, is not straightforward. The calculation we used here was a relatively simplistic way to determine whether the combined effect would be greater than the sum of the individual ones. This is based quite literally on the definition of synergy and fits well with the Bliss independence model, which postulates that the combined effect of two drugs acting independently is the product of their individual effects[21]. This principle is used to calculate the expected additive effect of two compounds. Based on this, "excess over Bliss" scores are calculated by subtracting the expected effect from the observed effect. These scores are used to determine if the two drugs work in a synergistic manner[22,23]. In **chapter 4**, we represented our data as the ratio between the calculated effect and the observed effect. This kind of analysis may give more weight to combinations causing an almost complete clearing of the bacterial population, even if the individual agents already achieve levels of reduction of around 90%. It can be argued that this is a misrepresentation of synergy, given that in this case the individual agents would already achieve close the maximum effect possible. In contrast, if the antibacterial effect is below the threshold we can detect, we could also be missing combinations that are also working synergistically. This is especially important to consider given that we are using an indirect method to monitor bacterial reduction. The assay that we employed to screen the different phage-antibiotic combinations is based on a bioluminescence reporter, which actually provides a readout for metabolic activity. It could be that such a method does not allow to look at small nuances in the activity of each of the agents. In addition, phages and each of the different antibiotics could be causing decreases in luminescence in different ways, for example by slowing down the bacteria metabolism without compromising viability. With this in mind, it would be worthwhile to validate our findings using different techniques, like looking at bacterial growth or with assays based on dead cell staining. In any

case, as phages are self-amplifying, it is very difficult to calculate their expected effect. Other formulas used to calculate synergy between two drugs are based on Michaelis-Menten equations, suited to predict the effect of specific enzymatic inhibitors but not of biological agents[24]. The difficulty to determine synergy where phages are involved is reflected by the lack of consensus in literature regarding this issue. Although some studies also propose a calculations based on the effects of the individual agents in checkerboard assays[25], some others just evaluate the logarithmic reduction of the population after different treatments[26], and some others simply assume that any boost in phage activity caused by adding sub-inhibitory antibiotic concentrations must necessarily be due to synergy[27]. Ultimately, it is probably not possible to determine PAS with certainty, but calculations like the one we propose can help obtain a good overview of which combinations can have the most potential together.

Our results in this chapter also raise questions about the mechanisms behind synergy in the combinations between PBJ and meropenem or ceftazidime. We observed that ceftazidime induces filamentation in the bacterial strain PAO1, while meropenem does not. Still, both treatments resulted in a bigger phage burst size. This could either indicate that filamentation is not a determining factor, or that several different cellular processes can result in synergy. The differences between meropenem and ceftazidime could be explained by the penicillin binding proteins (PBPs) that they target. While ceftazidime binds preferentially to PBP3, meropenem has higher affinity for PBP4 in *P. aeruginosa*, although it can also bind to PBP3 and other PBPs[28]. Inhibition of PBP3 is associated with filamentation, which explains the morphological changes we observe with ceftazidime[29]. In *P. aeruginosa* in particular, PBP3 has been shown to be required for cell growth, unlike other PBPs[30]. In this study, knockouts of the different PBPs were generated in *P. aeruginosa* to determine the importance of each of the proteins in the growth of this pathogen. Using such knockouts to evaluate phage-antibiotic synergy could provide definite answers of whether filamentation, or other growth alterations, are necessary for synergy to occur between phages and certain antibiotics. Another hypothesis is that induction of stress responses is a driver of PAS. While PAS can still occur in certain *E. coli* strains lacking genes involved in the SOS response[31], other stress

inducers like reactive oxygen species can also boost phage activity against this bacterium[32]. As described in **chapter 4**, we observed an increased expression of cytosolic superfolder Cherry protein (sfCherry) following antibiotic treatment. This could suggest that antibiotics induce the upregulation of protein synthesis under stress conditions, which could enhance phage production. In our system, sfCherry is encoded in a plasmid under the control of a constitutively active promoter, so it is unclear whether translation of this protein could be affected by the SOS response. To test this hypothesis, we could express a reporter protein under the control of a promoters known to be regulated by the SOS response, such as *recA*[33], and compare its expression to that of the constitutively expressed protein. In any case, it could be that the increase in fluorescence that we measure is simply due to the accumulation of proteins in the cytosol caused by an impaired cell division. A more all-encompassing approach to understand the cellular processes triggered in bacteria by a combined phage-antibiotic challenge would be to look at the transcriptome of these bacteria. Studying how the regulation of different genes is affected under these conditions could give insights into which pathways are key in the establishment of synergy.

Another of the major challenges to address in both antibiotic- and phage-based therapies is the emergence of resistance to both or either of these agents. While antibiotic resistance is not studied in detail in this thesis, resistance to phages and anti-phage defense mechanisms are reviewed in **chapter 5**. Knowledge in this field is quickly expanding as we discover more and more genes involved in the so-called bacterial immune system, as showcased in a recent review[34]. The bacterial immune system appears to be very flexible, and it has even been postulated that bacteria can exchange defense systems with one another[35]. In this way, bacteria could acquire anti-phage genes when necessary, and lose them when no longer under pressure, thus overcoming potential fitness trade-offs. Phages can also exist in bacteria in a plasmid form, known as phage-plasmids[36]. These phage-plasmids can be responsible for the spread of antibiotic resistance genes[37]. Given that antibiotic resistance genes located on mobile genetic elements, like plasmids, are also able to cross the species barrier[38], it is not far-fetched to imagine that this could also be the case with phage-defense genes. In situations such as chronic lung infections,

where multi-species colonization often occurs, this could be a big hurdle for phage therapy. Particularly, if phage cocktails targeting multiple species are used, the pressure on the different bacterial communities could trigger horizontal transfer of phage defense genes from one species to another. This is something that should be studied and considered when assembling phage cocktails.

Resistance to phages, but also to antibiotics, is another aspect in which combining both kinds of antimicrobials can be helpful. It has been shown that a combination treatment can reduce the chances of resistance emerging against either of the agents[4]. Furthermore, mutations selected to evade phage infection could re-sensitize bacteria to certain antibiotics[39]. Still, it is not clear how acquiring defense systems targeting phages intracellularly could influence the sensitivity of bacteria to antibiotics. Similarly, it is not known whether antibiotic resistance or phage resistance could have an effect on the susceptibility of Gram-negative bacteria to killing by complement. A bacterial infection in a patient treated with phages and antibiotics is a very complex situation, where an immense number of variables are at play that could influence the therapeutic outcome. To make matters worse, bacterial populations are highly dynamic, they can be formed by different strains and subpopulations, or they may be embedded in protective structures like a biofilm. Trying to encompass everything at the same time is truly overwhelming. In this thesis, we have tried to approach a few of these issues individually to understand them better. However, it is important to move towards research that addresses as many of these aspects as possible at the same time. Mathematical modeling has been used to this effect, with results that suggest that the presence of an active innate immune system is vital for the resolution of bacterial infections with phage therapy[40,41]. For a more experimental strategy, the use of animal models can be helpful, but bacteria-phage interactions cannot be monitored as closely in this type of models as they can *in vitro*. Besides from this, the immune system of other animals is not entirely comparable to that of humans[42]. Another option would be to develop more complex *in vitro* models, for example by using organoids. This may offer the possibility to mimic specific tissues, even potentially introducing complement factors and immune cells[43]. Establishing a bacterial infection in such a model could offer a good platform to explore the effects of combining phages

with antibiotics in a way that may be closer to the real situation in the human body. Nevertheless, *in vitro* results cannot fully predict clinical outcomes. For instance, in the case of phage-antibiotic therapy, biodistribution of each of the therapeutic agents will affect the dosing at the site of infection. Although *in vitro* research can offer crucial insights into the mechanisms at play in phage therapy, it should never replace clinical research.

So, can phage therapy really work? My answer is that not only can it already work, but it will only get better. The amount of research being carried out in fundamental phage biology and in clinical applications of phages keeps expanding. Still, more clinical trials are needed to prove the efficacy of phages as medicinal products. However, clinical research into phage therapy is thwarted by the fact that, in many countries, phage therapy can only be administered to patients out of compassionate use, when other treatment options fail. In these cases, it is not possible to monitor the outcome of treatment with the proper controls to account for the effect of other variables, such as the treating physicians or the formulation of the treatment. Nonetheless, more and more countries are beginning to establish phage therapy centers, while collaborating and sharing knowledge with each other. With medicine regulations also slowly changing towards accepting the use of phages and providing guidelines for it, the future of phage therapy seems bright. We can therefore have hope that phage therapy will be a valuable weapon in battling the antibiotic crisis in the coming years.

Furthermore, what will determine the outcome of therapy in the last instance is the idiosyncrasy of each patient. This is why it is extremely important to approach phage therapy as a personalized medicine. While this increases the costs of treatment, the advantages of tailoring each phage cocktail to the needs of every individual patient could well justify the downsides. The main conducting thread in this thesis is that choosing the right phages is vital if we want to achieve optimal results in clearing bacterial infections (figure 2). To really know or predict which phages these are, it is important that we keep developing tools to monitor phage infection, and that we deepen our understanding of factors that can boost or inhibit phage activity. For instance, it would be interesting to learn whether there are differences in

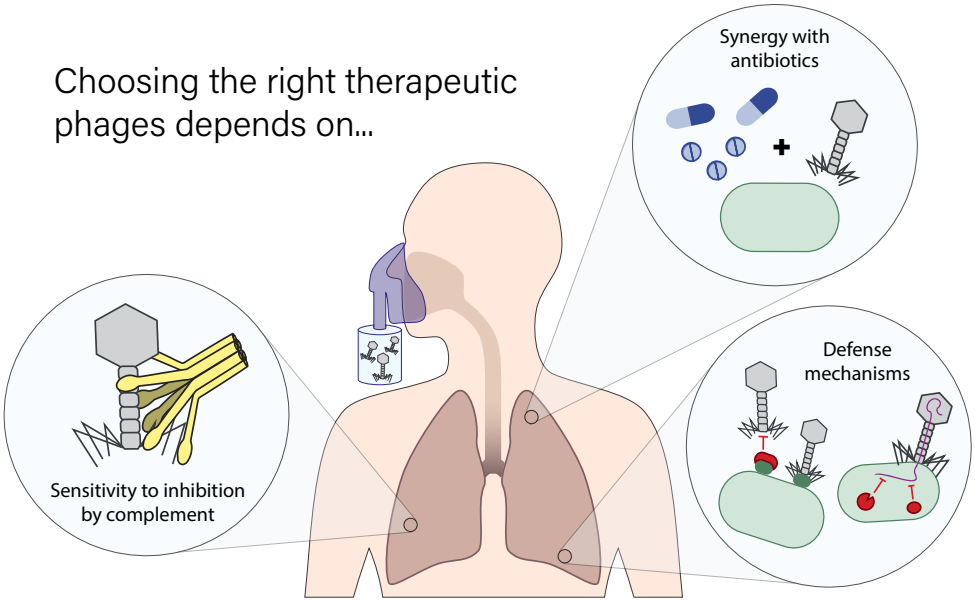

Figure 2. Based on the work presented in this thesis, we can conclude that choosing the right phages is crucial for the development of optimal phage therapy strategies. Many factors can influence phage activity in the context of the human body, such as interactions with the complement system, administration in combination with antibiotics, or defense mechanisms present in the target bacterial strains. It is important to keep developing tools to study phage biology and to account for these factors in clinical phage therapy applications.

complement activity against phages in the sera of different people. If this is the case, we could envision a future where bacterial strains isolated from a patient would be screened for phage and antibiotic susceptibility in the presence of the patient's own serum. Personalized phage therapy strategies should be adopted not only in the context of treatment with magistral preparations, but also in the design of clinical trials. The work presented in this thesis has hopefully provided some valuable tools and insights into important factors to consider in the application of phage therapy strategies. Ultimately, understanding and improving therapeutic phages may be one of our best chances in the fight against our common enemies: bacterial pathogens.

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O me! O life! of the questions of these recurring,

Of the endless trains of the faithless,
of cities fill'd with the foolish,

Of myself forever reproaching myself,
(for who more foolish than I, and who more faithless?)

Of eyes that vainly crave the light,
of the objects mean, of the struggle ever renew'd,

Of the poor results of all,
of the plodding and sordid crowds I see around me,

Of the empty and useless years of the rest,
with the rest me intertwined,

The question, O me! so sad, recurring —
What good amid these, O me, O life?

Answer.
That you are here — that life exists and identity,
That the powerful play goes on,
and you may contribute a verse.

Walt Whitman, Leaves of Grass (1892)

Inzicht in therapeutische fagen – Nederlandse samenvatting

Antibioticaresistentie is een groeiend probleem in de hedendaagse samenleving, waardoor voorheen behandelbare infecties steeds gevaarlijker worden. Het is daarom belangrijk dat er nieuwe behandelopties worden ontwikkeld. Verschillende strategieën worden momenteel onderzocht, waaronder het gebruik van bacteriofagen. Bacteriofagen (fagen) zijn virussen die specifieke bacteriën uitschakelen. Fagen dringen de bacterie binnen en gebruiken de bacteriële nutriënten om zich in de bacterie te vermenigvuldigen. De nieuwe fagen worden vervolgens uitgescheden, waarop de bacterie sterft. Deze fagen kunnen daarop weer de resterende bacteriële populatie verder infecteren. Vanwege hun specificiteit voor bacteriën, efficiëntie in het doden van bacteriën en vermogen tot vermenigvuldiging zijn bacteriofagen een interessante en potentieel zeer veilige therapeutische behandeling. Een ziekteverwekker waarvoor fagentherapie bijzonder nuttig kan zijn, is de bacterie *Pseudomonas aeruginosa*. In dit proefschrift hebben we verschillende factoren onderzocht die van invloed zijn op de effectiviteit van therapeutische fagen in het doden van deze ziekteverwekker.

Om de adaptie van gepersonaliseerde fagentherapie in de kliniek mogelijk te maken, moeten er snellere methoden worden ontwikkeld om de gevoeligheid van bacteriën voor fagen te beoordelen. De huidige methoden die fagen beoordelen voor een therapeutische behandeling zijn arbeidsintensief, tijdrovend en zijn niet geschikt voor klinisch gebruik. In **hoofdstuk 2** van dit proefschrift hebben we een snellere en minder arbeidsintensieve methode ontwikkeld die kan worden gebruikt om de bacteriedodende eigenschappen van fagen te beoordelen. Deze methode maakt gebruik van een fluorescerend signaal dat sterfte van bacteriën aantoont. De nieuwe methode werd gevalideerd door deze te vergelijken met de huidige methoden die gebruikt worden om bacteriedood aan te tonen.

De ontwikkeling van nieuwe methoden is de eerste stap om fagentherapie in praktijk te brengen. Veel andere factoren moeten echter ook worden onderzocht. Fagen kunnen een interactie aangaan met het immuunsysteem, en dit kan de efficiëntie

van fagentherapie beïnvloeden. Het menselijk immuunsysteem is normaal gesproken verantwoordelijk voor het uitschakelen van lichaamsvreemde indringers zoals virussen en bacteriën. Hoewel fagen alleen bacteriën kunnen infecteren en geen menselijke cellen, zijn ze nog steeds lichaamsvreemd. In **hoofdstuk 3** van dit proefschrift hebben we de interactie tussen het immuunsysteem en fagen onderzocht. Hierbij hebben we fagen toegevoegd aan menselijk serum, dat veel eiwitten en antilichamen bevat die een immuunrespons teweeg kunnen brengen. Onze resultaten toonden aan dat serum de activiteit van sommige fagen kan remmen, maar niet van allemaal. Waarschijnlijk heeft dit te maken met de morfologie van de fagen: fagen met een samentrekbare staart zijn gevoelig voor serum, maar fagen met een korte, rigide staart niet. We hebben ook vastgesteld dat een specifiek deel van het immuunsysteem, het complement-systeem, verantwoordelijk is voor het remmen van fagenactiviteit. Het complement-systeem is een groep eiwitten die ziekteverwekkers zeer snel herkennen, en zelfs bepaalde soorten bacteriën direct kunnen doden. Op basis van onze resultaten concluderen we dat het complement-systeem rechtstreeks kan een interactie met fagen met een samentrekbare staart aangaat, waardoor hun vermogen om aan bacteriën te binden wordt gereduceerd.

Naast het bestuderen van de rol van het immuunsysteem in fagentherapie zijn we ook geïnteresseerd in hoe het combineren van fagen met verschillende antibiotica de kans op therapeutisch succes kan vergroten. Fagentherapie wordt vaak samen met antibiotica toegediend in de kliniek, dus het is belangrijk om te begrijpen welke antibiotica beter werken in de aanwezigheid van fagen. In **hoofdstuk 4** hebbben we combinaties van fagen met antibiotica uit verschillende klassen getest. Als resultaat zagen we dat fagen synergetisch kunnen werken met bepaalde antibiotica die klinisch worden gebruikt bij de behandeling van *P. aeruginosa*-infecties, zoals meropenem en ceftazidime. Onderzoek probeert momenteel inzicht te krijgen in de mechanismen die synergie aandrijven. Een hypothese is dat antibiotica de bacteriën vergroot, waardoor er meer plek ontstaat voor fagen om zich aan te hechten, maar ook meer ruimte om zich te vermeerderen in de bacteriële cel. We hebben deze hypothese getest en gezien dat ceftazidime inderdaad bacteriën vergroot, terwijl meropenem dat niet doet. Beide antibiotica verhogen echter het aantal geproduceerde fagen per geïnfecteerde bacterie. Hoewel het niet duidelijk

is waarom dit gebeurt, kan deze toename in fagenproductie verklaren waarom er synergie is tussen fagen en deze antibiotica.

Ten slotte is er een andere factor die het succes van fagentherapie kan bepalen, namelijk de ontwikkeling van resistentie tegen fagen door bacteriën. De manieren waarop bacteriën zichzelf verdedigen tegen een fageninfectie worden besproken in **hoofdstuk 5**. Bacteriën kunnen zeer snel muteren in stresssituaties, wat ertoe kan leiden dat ze niet langer worden herkend door fagen. Ze kunnen ook de receptoren verbergen die fagen herkennen op het bacteriële oppervlak, bijvoorbeeld door maskerende eiwitten te produceren of zichzelf in een beschermende matrix te verhullen. Daarnaast hebben bacteriën ook manieren om binnendringende fagen te onderscheppen. Een daarvan is het CRISPR-Cas-systeem, waarmee bacteriën een soort immunologisch geheugen kunnen ontwikkelen waarmee ze fagen herkennen waarmee ze eerder in aanraking zijn gekomen. Andere mechanismen omvatten het opofferen van geïnfecteerde cellen om de verspreiding van fagen naar de rest van de bacteriële populatie te voorkomen. Interessant is dat sommige van deze verdedigingssystemen eigenlijk zijn gecodeerd door fagen, om te concurreren met andere fagen die dezelfde gastheer als doelwit hebben. Veel nieuwe verdedigingssystemen zijn de afgelopen jaren ontdekt, vanwege de recente beschikbaarheid van technieken om bacteriële genomen te analyseren. In dit hoofdstuk benadrukken we hoe vaak deze anti-faag mechanismen voorkomen en hoe ze in overweging moeten worden genomen in de context van fagentherapie.

Samengevat heeft het werk van dit proefschrift nieuwe technieken opgeleverd om faag-bacterie-interacties te bestuderen. Bovendien hebben we kennis vergaard over hoe de activiteit van fagen kan worden beïnvloed door factoren zoals het menselijk immuunsysteem en combinatiebehandelingen met antibiotica. Een van de belangrijkste conclusies die we uit dit onderzoek kunnen trekken, is dat het cruciaal is om de optimale fagen te kiezen om de kansen op therapeutisch succes te maximaliseren. Momenteel worden fagen voor therapie voornamelijk gekozen op basis van hun vermogen om een gegeven ziekteverwekkende stam te infecteren. We denken dat het zeer gunstig zou zijn om ook andere factoren in overweging te nemen, zoals hoe het immuunsysteem van de patiënt de fagen zal beïnvloeden,

welke antibiotica tegelijkertijd worden toegediend, en hoe resistentie tegen deze fagen zich kan ontwikkelen. Door onze kennis op deze gebieden uit te breiden, hopen we uiteindelijk te kunnen voorspellen welke fagen het meest geschikt zijn voor elke individuele patiënt, en zo bij te dragen aan de ontwikkeling van betere strategieën voor fagentherapie.

Understanding therapeutic phages – English summary

Antibiotic resistance is becoming a greater problem in today's society, leading to previously treatable infections becoming more dangerous. It is therefore important that the development of new treatment options is pursued. There are different strategies being investigated for this, and a major area of interest is the use of bacteriophages. Bacteriophages (phages) are viruses which specifically target bacteria. They bind to the bacterium and reproduce inside it. This process results in bacterial death as new phage are released. These phages can then further attack the bacterial population. This makes bacteriophages an interesting and potentially very safe therapeutic option due to their specificity towards bacteria, killing capacity and ability to self-amplify. One pathogen for which phage therapy can be particularly useful is *Pseudomonas aeruginosa*. In this thesis, we have investigated factors influencing the efficacy of therapeutic phages against this and other pathogens.

For the widespread use of personalised bacteriophage therapy, more rapid methods for assessing bacterial susceptibility to bacteriophages need to be developed. The current methods used for assessing bacteriophage characteristics are laborious and time intensive, which limits their use in a clinical setting. In **chapter 2** of this thesis, we developed a more rapid and less labour-intensive method that can be used to assess the killing characteristics of bacteriophage against Gram negative bacteria. This method provides a fluorescent signal when bacterial killing is occurring. More traditional methods for assessing bacterial killing performed in comparison to this method further confirmed its validity.

The development of new methods is one step in bridging the gap between theory and practice for phage therapy. However, many other factors also need to be investigated. Phages can interact with the immune system, and this can influence phage therapy efficacy. The human immune system is normally responsible for attacking foreign invaders such as viruses and bacteria. Although phages can only infect bacteria and not human cells, they are still foreign entities. In **chapter 3** of

this thesis, we set out to investigate how the immune system interacts with phages. To do so, we combined phages with human serum, which contains many proteins and antibodies that can mediate the immune response. Our results showed that serum can inhibit the activity of some phages, but not others. Seemingly, this has to do with the morphology of the phage: phages with a contractile tail are sensitive to serum, but phages with a short, rigid tail are not. We also determined that a specific part of the immune system, the complement system, is responsible for blocking phage activity. The complement system is a set of proteins that can recognize pathogens very rapidly, even directly killing certain types of bacteria. Based on our results, we could conclude that the complement system can interact directly with phages with a contractile tail, hampering their ability to bind to bacteria.

In addition to studying the role of the immune system in phage therapy, we were also interested in how combining phages with different antibiotics may increase the chance of therapeutic success. Phage therapy is often administered together with antibiotics in the clinic, so it is important to understand which antibiotics can work better with phages. In **chapter 4**, we performed a screening where we combined phages with antibiotics of different classes. As a result, we observed that phages can work synergistically with certain antibiotics used clinically in the treatment of *P. aeruginosa* infections, like meropenem and ceftazidime. Research is currently focusing on understanding synergy by identifying the mechanisms that drive it. One hypothesis is that antibiotics induce bacterial enlargement, creating more spaces for phages to attach but also to amplify within the bacterial cell. We tested this hypothesis and saw that ceftazidime indeed causes bacterial enlargement, while meropenem does not. However, both antibiotics can boost the number of phages produced per each individual infected bacterium. Although it is not clear why this happens, this increase in phage production can explain why there is synergy between phages and these antibiotics.

Finally, another factor that can determine the success of phage therapy is the development of resistance to phages by bacteria. The ways in which bacteria defend themselves from a phage attack are reviewed in **chapter 5**. Bacteria can mutate very rapidly in situations of stress, which can lead to them no longer being

recognized by phages. They can also hide the receptors that phages recognize on the bacterial surface, for instance by producing masking proteins or embedding themselves in a protective matrix. In addition to this, bacteria also have ways to intercept invading phages. One of these is the CRISPR-Cas system, which allows bacteria to develop a sort of immunological memory through which they recognize phages they have encountered before. Other mechanisms trigger the sacrifice of the infected cells, to prevent the spread of the infection to the rest of the bacterial population. Interestingly, some of these defense systems are actually encoded by phages, to compete with other phages targeting the same host. Many new defense systems have been discovered in recent years, due to the recent availability of tools to analyze bacterial genomes. In our review, we highlight how prevalent these anti-phage mechanisms are, and how they should be taken into consideration in the context of phage therapy.

Taken together, the work of this thesis has provided new tools to study phage-bacteria interactions. Furthermore, we have generated knowledge about how the activity of phages may be influenced by factors like the human immune system and antibiotic co-treatments. One of the main general conclusions we can draw from this research is that it is crucial to choose the optimal phages to maximize the chances of therapeutic success. Currently, phages are chosen for therapy mainly based on whether they can infect a given pathogenic strain. We think that it would be very beneficial to take other factors into account, like how the patient's immune system will affect them, what antibiotics are administered at the same time, and how resistance to these phages can develop. By expanding our knowledge on these topics, we hope to eventually be able to predict which phages are the most suited for each individual patient, thereby contributing to the design of better phage therapy strategies.

Entendiendo a los fagos terapéuticos – Resumen en español

La resistencia a antibióticos se está convirtiendo en un problema cada vez más serio en nuestra sociedad actual, haciendo que infecciones que antes eran fácilmente tratables sean ahora más peligrosas. Por esto, es importante que se investigue el desarrollo de nuevas opciones terapéuticas. Se barajan varias estrategias distintas con este fin, y entre ellas el uso de bacteriófagos está despertando gran interés. Los bacteriófagos (fagos) son virus que específicamente atacan a bacterias. Se adhieren a ellas y pueden reproducirse en su interior. Este proceso, en algunos casos, resulta en la muerte de la bacteria y la liberación de nuevos fagos al medio, tras lo que estos fagos pueden continuar atacando a la población bacteriana. Esto hace que los fagos sean una opción terapéutica potencialmente interesante y segura, debido a su especificidad contra las bacterias, su habilidad para aniquilarlas y el hecho de que se auto propagan. Uno de los patógenos contra los cuales la fagoterapia puede ser particularmente útil es *Pseudomonas aeruginosa*. En esta tesis, hemos investigado los factores que pueden influenciar la eficacia de los fagos terapéuticos contra este y otros patógenos.

Para fomentar el uso de la fagoterapia personalizada, es necesario desarrollar métodos rápidos para evaluar la susceptibilidad de las bacterias a ser atacadas por ciertos fagos. Los métodos usados hoy en día para caracterizar fagos son laboriosos y lentos, lo que limita su uso en la práctica clínica. En el **capítulo 2** de esta tesis, desarrollamos un método más rápido y eficiente para evaluar la eficacia de fagos en eliminar bacterias Gram-negativas. Este método proporciona una señal fluorescente cuando las bacterias son dañadas o mueren. La validez de esta técnica fue confirmada comparando los resultados obtenidos con ella con otros ensayos tradicionales.

El desarrollo de nuevos métodos es un paso importante para cimentar la práctica de la fagoterapia. Sin embargo, otros factores también deben ser investigados. Los fagos pueden interactuar con el sistema inmunitario, lo cual podría afectar a su eficacia. El sistema inmune humano es responsable de atacar a los organismos

invasores como virus y bacterias. Aunque los fagos no pueden infectar células humanas, siguen siendo entes extraños en nuestro organismo. En el **capítulo 3** de esta tesis investigamos cómo el sistema inmune interactúa con los fagos. Para ello, combinamos fagos con suero humano, que contiene proteínas y anticuerpos que pueden mediar la respuesta inmune. Nuestros resultados muestran que el suero puede inhibir la actividad de ciertos fagos, pero no de otros. Aparentemente, esto está relacionado con la morfología del fago: los fagos con una cola contráctil son sensibles a la inhibición por suero, y los fagos con una cola corta y rígida no lo son. También pudimos determinar que una parte concreta del sistema inmune, el sistema del complemento, es responsable de este efecto. El sistema del complemento es un conjunto de proteínas que pueden reconocer patógenos rápidamente, e incluso directamente matar a ciertas bacterias. En base a nuestros resultados, concluimos que el sistema del complemento puede interactuar directamente con fagos de cola contráctil, limitando su habilidad de unirse a las bacterias.

Además de estudiar el rol del sistema inmune en la fagoterapia, también nos interesaba cómo combinar fagos con distintos antibióticos para fomentar las posibilidades de éxito de la terapia. La fagoterapia se administra a menudo junto con antibióticos en la práctica clínica, de modo que es importante entender qué antibióticos pueden funcionar mejor en combinación con fagos. En el **capítulo 4**, evaluamos la actividad de fagos con antibióticos de diferentes clases. De esta manera, observamos que los fagos pueden funcionar de manera sinergética con ciertos antibióticos usados habitualmente en el tratamiento de infecciones por *P. aeruginosa*, como el meropenem y la ceftazidima. Investigadores en este campo están intentando dilucidar los mecanismos que gobiernan estas interacciones sinergéticas. Una hipótesis es que ciertos antibióticos inducen un alargamiento en las células bacterianas, lo cual podría crear más superficie para que los fagos se unan, además de más espacio para amplificarse en el interior de la célula. Nosotros probamos esta hipótesis y pudimos observar que la ceftazidima, en efecto, causa alargamiento en las bacterias, mientras que el meropenem no lo hace. Sin embargo, ambos antibióticos pueden incrementar el número de fagos producidos por célula bacteriana infectada. Aunque el porqué de esto no está claro, este incremento en la producción de fagos podría ser la razón de la sinergia

entre fagos y estos antibióticos.

Finalmente, otro de los factores que puede determinar si la fagoterapia es efectiva es el desarrollo de resistencia ante fagos por parte de las bacterias. En el **capítulo 5**, revisanmos las formas en que las bacterias pueden defenderse de un ataque por parte de fagos. Las bacterias pueden mutar rápidamente en situaciones de estrés, lo cual puede causar que los fagos no las reconozcan. También pueden esconder los receptores usados por los fagos para adherirse a la superficie bacteriana, por ejemplo, mediante la producción de proteínas enmascaradoras o de una matriz extracelular protectora. Además de esto, las bacterias tienen formas de interceptar a los fagos invasores una vez han sido infectadas. Una de ellas es el sistema CRISPR-Cas, que permite a las bacterias desarrollar una especie de memoria inmunológica para reconocer a fagos que las han atacado anteriormente. Otros mecanismos desencadenan el sacrificio de las células infectadas, para prevenir que la infección se propague al resto de la población. Es interesante que algunos de estos sistemas vienen codificados en el material genético de fagos, para competir con otros fagos atacando a la misma bacteria. Muchos sistemas de defensa han sido descubiertos recientemente, debido a las nuevas técnicas disponibles para analizar los genomas bacterianos. En nuestra revisión, hacemos énfasis en cuán comunes son estos mecanismos de defensa, y explicamos que deberían de ser tomados en cuenta a la hora de administrar fagoterapia.

En conjunto, el trabajo presentado en esta tesis ha proporcionado nuevas herramientas para estudiar las interacciones entre fagos y bacterias. Además, hemos generado conocimiento acerca de cómo la actividad de los fagos terapéuticos puede estar influida por factores como el sistema inmune humano y la co-administración de antibióticos. Una de nuestras conclusiones principales es que es de vital importancia elegir a los fagos óptimos para maximizar las probabilidades de éxito terapéutico. Hoy en día, los fagos terapéuticos se eligen principalmente en base a si pueden infectar a una cierta cepa patogénica. Creemos que sería beneficioso incluir otros factores en este análisis: cómo el sistema inmune del paciente va a reaccionar ante estos fagos, qué antibióticos se van a administrar a la vez, y cómo puede aparecer resistencia a estos fagos. Expandiendo nuestro

conocimiento en estas áreas, esperamos llegar a ser capaces de predecir qué fagos son los más adecuados para cada paciente, contribuyendo de esta manera al diseño de una mejor fagoterapia.

About the author

Julia Egido was born on December 28, 1995 in Zaragoza, Spain. After spending her first years in the village of Pedrola, she attended the Juan de Lanuza school in Zaragoza, from which she graduated in 2013. Following that, she started her Bacherlor's in Biotechnology at the University of Zaragoza. During her last year, she carried out her end of degree internship in the labs of Prof. José Luis Serrano and Dr. Olga Abián, where she worked on a project based on synthesizing organic nanocarriers for drugs against the Hepatitis C virus. As a result of this internship, she wrote and defended her final thesis with honors. In September 2017, Julia moved to Utrecht to start her Master's in Drug Innovation at Utrecht University. She carried out her major research internship in the Department of Pharmacology of the Utrecht Institute for Pharmaceutical Sciences, working in the lab of Prof. Roos Masereeuw on the study of a gene involved in the pathogenesis of nephropathic cystinosis. Next, she carried out her minor internship in the labs of Dr. Seino Jongkees (Chemical Biology and Drug Discovery) and Dr. Xander de Haan (Virology) at Utrecht University, working on the discovery of cyclic peptide inhibitors of the influenza virus. Still as part of her Master's, she wrote a review in collaboration with the lab of Prof. Stan Brouns, at the TU Delft, on bacterial resistance mechanisms against bacteriophages. Julia obtained her Master's title in August 2019. In October 2019 Julia started her PhD in the lab of Prof. Suzan Rooijakkers at the UMC Utrecht, under supervision of Dr. Pieter-Jan Haas and Dr. Bart Bardoel. The main focus of her work was studying therapeutic phages and factors that can influence phage therapy, particularly against *Pseudomonas aeruginosa*. This project was funded by the Netherlands Center for One Health. During her PhD, Julia participated in several national and international conferences, being awarded a poster presentation prize on two occasions (Science for Life, Utrecht 2021; European Meeting on Complement in Human Disease, Bern 2022). The results obtained from her PhD research are presented in this thesis and have been published in peer-reviewed journals.

List of publications

Related to this thesis

- Egido, J.E.; Dekker, S.O.; Toner-Bartelds, C.; Lood, C.; Rooijakkers, S.H.; Bardoel, B.W.; Haas, P. Human Complement Inhibits Myophages against *Pseudomonas aeruginosa*. *Viruses* 2023, 15(11), 2211, doi:10.3390/v15112211
- Egido, J.E.; Toner-Bartelds, C.; Costa, A.R.; Brouns, S.J.J.; Rooijakkers, S.H.M.; Bardoel, B.W.; Haas, P.-J. Monitoring Phage-Induced Lysis of Gram-Negatives in Real Time Using a Fluorescent DNA Dye. *Sci Rep* 2023, 13, 856, doi:10.1038/s41598-023-27734-w
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Other publications

- Pascha, M.N.; Thijssen, V.; Egido, J.E.; Linthorst, M.W.; van Lanen, J.H.; van Dongen, D.A.A.; Hopstaken, A.J.P.; van Kuppeveld, F.J.M.; Snijder, J.; de Haan, C.A.M.; *et al.* Inhibition of H1 and H5 Influenza A Virus Entry by Diverse Macrocyclic Peptides Targeting the Hemagglutinin Stem Region. *ACS Chem. Biol.* 2022, 17, 2425–2436, doi:10.1021/acschembio.2c00040
- San Anselmo, M.; Lancelot, A.; Egido, J.E.; Clavería-Gimeno, R.; Casanova, Á.; Serrano, J.L.; Hernández-Ainsa, S.; Abian, O.; Sierra, T. Janus Dendrimers to Assess the Anti-HCV Activity of Molecules in Cell-Assays. *Pharmaceutics* 2020, 12, 1062, doi:10.3390/pharmaceutics12111062
- Jamalpoor, A.; Eerde, A.M. van; Lilien, M.R.; Gelder, C.A. van; Zaal, E.A.; Valentijn, F.A.; Broekhuizen, R.; Zielhuis, E.; Egido, J.E.; *et al.* The Lysosomal V-ATPase B1 Subunit in Proximal Tubule Is Linked to Nephropathic Cystinosis. *bioRxiv* 2020, 2020.07.24.219808. https://doi.org/10.1101/2020.07.24.219808

PhD training certificate

Discipline-specific educational activities	ECTS
Advanced Immunology	1,5
I&I PhD retreat 2019, 2020, 2021, 2022, 2023	2,55
I&I Annual meeting 2022	0,3
I&I Summit 2023	0,3

General educational activities	
PhD Day 2021, 2022	0,4
Breaking Science	2
Writing a Scientific Paper	1,5
Introductory Biostatistics for Researchers	4,5
Introduction to R & Data	0,3
InDesign	0,6
Digital Images	1
Resin Electron Microscopy workshop	0,85
Summer Science training and pitch	0,3

Symposia/conferences	
Science for Life 2020, 2021	0,6
KNVM Spring meeting 2022, 2023	1,2
Koepel conference 2022, 2023	1,2
VoM 2022	1,5
VoM 2023	1,5
EMCHD 2022	1
EMBO Meeting Antibodies and Complement 2023	1,2

Total number of ECTS	24,3

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the best oboe buddy!

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