Reflection article

The potential of phage model systems as therapeutic agents

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Abstract

With the emergence of widespread antibiotic resistance, phages are an appealing alternative to antibiotics in the fight against multidrug-resistant bacteria. Over the past few years, many phages have been isolated from various environments to treat bacterial pathogens. While isolating novel phages for treatment has had some success, this method is very fastidious because phage isolation, characterisation, and safety assessment require considerable time investment and financial resources. Well-established phage model systems have the potential to overcome these challenges. The knowledge acquired from decades of research on their structure, life cycle, and evolution ensures safe application and efficient handling. Currently, the only downside of established model systems is their limited effectiveness against pathogenic bacteria. However, breeding model phages to infect pathogens may be possible by applying new and old experimental evolution approaches.

Infections caused by multidrug-resistant bacterial strains are one of the most pressing issues in medicine, a situation that is only expected to worsen in the coming decades (WHO 2021; Murray et al. 2022). ESKAPEE pathogens (*Enterococcus faecium*, *Staphylococcus aureus*, *Klebsiella pneumoniae*, *Acinetobacter baumannii*, *Pseudomonas aeruginosa*, *Enterobacter* spp, and *Escherichia coli*) are the principal targets for the development of novel antimicrobial strategies (Mulani et al. 2019). Among alternative treatment approaches currently under investigation (e.g., pre- and probiotics, antimicrobial peptides, antibodies, oligonucleotides for silencing resistance genes), bacteriophages (phages) are probably the most promising alternatives to treat bacterial infections (Gordillo Altamirano and Barr 2019; Maimaiti et al. 2023).

The rise and fall of phages as therapeutic agents

Independently discovered by Frederick Twort in 1915 (Twort 1915) and Félix d'Hérelle in 1917 (d'Hérelle 1917), bacteriophages (translating to "bacteria-eater") are lytic viruses that prey upon bacteria. Phages are simple entities. Their genomes, either RNA or DNA, single- or double-stranded, are protected by capsids that can take various shapes and sizes (Ackermann 2007). They are the most numerous biological entities on Earth (Brüssow and Hendrix 2002; Angly et al. 2009) and are ubiquitous in every natural, human-altered, and artificial biome (*e.g.,* wastewater treatment reservoirs, industries) (Batinovic et al. 2019).

In the early 1900s, phages had already been considered as treatments for bacterial infections in animals and humans (d'Hérelle 1918; d'Hérelle 1919; d'Hérelle 1925). However, the lack of understanding of phage biology divided the scientific community and slowly undermined clinical applications. On one side of the debate Felix d'Hérelle recognized phages as viruses and their antimicrobial action *in vitro* and *in vivo*. On the other side Jules Bordet (Nobelist and director of the Pasteur Institute in Brussels at the time), however, contested Felix d'Hérelle's work, attributing the observed bacterial lysis to the action of a "self-perpetuating lytic enzyme" (Summers 2012; Summers 2017). Furthermore, phages were lacking in standardized production and controls, and their host spectra were considered to be too narrow (Summers 2012). The association of phage therapy with German and Japanese medicine during the Second World War put an end to any further applications in the West (Summers 2012). Phages were ultimately rejected in favour of newly-discovered antibiotics (Nicolaou and Rigol 2018).

Phage comeback: an old solution for a modern problem

The overuse and misuse of antibiotics have slowly driven the emergence and spread of multidrug-resistant bacteria, creating the need for alternative or complementary solutions to classic antibiotic treatments. While many Eastern countries never ceased to use phage therapy (Villarroel et al. 2017), phages are experiencing a renaissance in Western countries (Barron 2022).

Phage therapy is the administration of one or more phages to a patient. Two distinct strategies are commonly followed: a broad or a targeted approach (Gordillo Altamirano and Barr 2019; Froissart and Brives 2021). The broad approach involves assembling a phage cocktail composed of genetically diverse phages (~10-40) with a wide host-spectrum, emulating the much broader killing spectrum of antibiotics (Villarroel et al. 2017; McCallin et al. 2018). For the targeted approach, phages are isolated from environments where bacteria are abundant (e.g., sewage or wastewater treatment plants) and tested against the target bacterium. Phages that successfully lyse the target bacterium are purified and administered to the patient (Chan et al. 2018).

A generic phage cocktail with a broad host spectrum is part of traditional, over-the-counter medicine used in Georgia, Poland, and Russia, and can be sold without a prescription to patients seeking treatment for proinflammatory or enteric diseases (Kutter et al. 2010). The EU and USA, however, have preferentially developed personalised-medicine approaches that specifically target the pathogen responsible for the bacterial infection (Froissart and Brives 2021). Nonetheless, phage therapy is considered highly experimental and can only be used in rare cases as a last resort or compassionate treatment (Chan et al. 2018; Cano et al. 2020; Dedrick et al. 2021; Dedrick et al. 2023). Because health agencies require phages to be fully characterised and produced for clinical trials under the Good Manufacturing Practices (GMPs), there is currently no broadly available phage treatment in Western countries (Rohde et al. 2018).

GMPs represent the quality, safety, and traceability standards a medicinal product or drug must meet before being authorized for clinical trials and markets (EMA 2018; Bretaudeau et al. 2020). Phages are categorised as such in the EU and the USA and are required to be produced under GMP regulations (the exception is Belgium, where phages are produced following a standardized recipe; monograph (Pirnay et al. 2018)). However, the standardisation of phage production requires considerable investment of time and money (Bretaudeau et al. 2020), is difficult to adhere to because of high phage mutation rates (Pirnay et al. 2018), and might be technologically impossible if the phage has to be trained to enhance its lytic ability or when phage cocktails are needed to make the treatment evolution-proof (Yang et al. 2020; Borin et al. 2021; Science,

Innovation, and Technology Committee 2023). In addition, the isolation, characterization, and adaptation of new phages from the environment are time-consuming mainly because of safety assessments since phages are known to carry dangerous toxins and can spread antibiotic-resistance genes *via* transduction (Krüger and Lucchesi 2015; Colavecchio et al. 2017; Jamet et al. 2017).

Phage model systems can become promising therapeutic agents

Alongside the use of newly discovered environmental phages for therapy, well-studied phage model systems should also be considered. Phage models such as λ, P22, P1, Dp-1, T4, T7, MS2 or ΦX174 have significant benefits over uncharacterized environmental phage isolates.

Phage model systems are easily obtainable, manipulatable and producible at high concentrations (Skaradzińska et al. 2020). The deep knowledge of these model systems acquired over the last ~ 100 years makes them relatively predictable and safe therapeutic agents (Wichman et al. 2005; Bull and Molineux 2008; Budynek et al. 2010; Wichman and Brown 2010; Azam and Tanji 2019). They prey on *E. coli*, *Salmonella*, and *Streptococcus* species and, therefore, could potentially be bred to (*i*) extend their host range to directly infect related but pathogenic strains belonging to these species (Van Bambeke et al. 2007; Burrowes et al. 2019; Mulani et al. 2019) and (*ii*) overcome phage resistance after being adapted in the laboratory (Bull et al. 2003; Meyer et al. 2012; Borin et al. 2021; Romeyer Dherbey et al. 2023). In our opinion, ΦX174 is a particularly interesting model system and hence, we will highlight specific advantages and features of this phage model.

ΦX174 may be a suitable candidate for phage therapy

ΦX174 is one of the oldest phage model systems (Sertic and Bulgakov 1935; Wichman and Brown 2010; Lacković and Toljan 2020). It has been used for almost 90 years to study phage, synthetic biology and evolutionary biology (Sanger et al. 1978; Smith et al. 2003; Jaschke et al. 2012; Mukherjee et al. 2015; Breitbart and Fane 2021). ΦX174 is a small (~ 30 nm), tailless coliphage belonging to the *Microviridae* family. It carries a 5,386 nucleotides ssDNA genome that contains only 11 genes (Sinsheimer 1959; Sanger et al. 1978). ΦX174 is a lytic phage that solely relies on attaching to the core oligosaccharide of the host's lipopolysaccharide (LPS) for infection. In the laboratory, ΦX174 infects – and hence is usually grown on – *E. coli* C, which produces rough type (i.e., lacking in O-antigen) LPS molecules (Feige and Stirm 1976).

ΦX174 can easily be fully synthesised (Smith et al. 2003) and manipulated in the laboratory (Christakos et al. 2016). ΦX174 is highly host-specific (Michel et al. 2010), meaning that it will be

harmless to the patient's microbiota, in contrast to phages with broader infectivity or antibiotics (Ramirez et al. 2020). Furthermore, evolution experiments can quickly extend ΦX174's host range of formerly resistant strains (Romeyer Dherbey et al. 2023), unlocking its potential as a powerful therapeutic agent. ΦX174 does not carry virulence genes, and its use is already approved *in vivo* by the FDA as a marker of immune responses in patients (Rubinstein et al. 2000; Bearden et al. 2005).

As *Microviridae* phages have been isolated from gut samples and considered part of the healthy human gut microbiome (Lim et al. 2015; Santiago-Rodriguez et al. 2016), there is a realistic possibility that the human immune system will be less prone to recognizing and degrading ΦX174 prior to successful infection (Bull et al. 2019). For all these reasons, we believe that phage ΦX174 is a promising human and animal therapeutic agent.

Current limitations

ΦX174 offers plenty of advantages, however, there are some limitations to its potential. While its small genome renders ΦX174 extremely tractable for genetic manipulation and analysis, it also means that there is very limited space to easily add extra material (such as effector genes (Du et al. 2023)) to the genome (Russell and Müller 1984). Phage model systems with bigger genomes can more easily accommodate additional genes.

As with antibiotics, ΦX174 (and most other phages) can infect slow-growing bacteria (Romeyer Dherbey et al. 2023) but cannot infect bacteria in stationary phase or dormancy (Bläsi et al. 1985). Hence, ΦX174 may be more suited to treating acute rather than persistent infections. There are phage model systems that can infect hosts in stationary phase and hence may be more appropriate therapeutic agents to treat persistent infections (Bryan et al. 2016; Tabib-Salazar et al. 2018; Kaldalu et al. 2020; La Rosa et al. 2021; Maffei et al. 2022).

ΦX174 is highly host-specific (Michel et al. 2010). To treat enterobacterial pathogens, novel ΦX174 strains must first be evolved (Romeyer Dherbey et al. 2023). Beyond enterobacterial infections, other phage model systems should be established to treat other members of the ESKAPEE group.

In addition, two major questions remain before developing ΦX174 for phage therapy. Firstly, is it possible to evolve ΦX174 to infect pathogenic strains, especially *E. coli* and *Salmonella* strains that

belong to the ESKAPEE group (Mulani et al. 2019)? Secondly, how does ΦX174 resistance affect the emergence and evolution of antibiotic resistance, and *vice versa*?

Designing an evolution-proof phage cocktail targeting a bacterial pathogen

To answer the above questions, protocols could be adapted to specifically evolve ΦX174 (or, potentially, other phage) to infect pathogenic enterobacteria (Bono et al. 2013; Burrowes et al. 2019; Kok et al. 2023; Romeyer Dherbey et al. 2023) (**Fig. 1**). Firstly, the bacterial pathogen responsible for the infection needs to be isolated and characterised (**Fig. 1A**). Then, a phage strain that can infect the pathogenic strain is evolved by serially transferring candidate phages in a mixture of permissive hosts (necessary to propagate the phage) and the targeted pathogenic strain (**Fig. 1B**). Evolving phage populations are inoculated into fresh, exponentially growing host cultures at each transfer until one or more phages are found to infect the pathogenic strain. The successful phage mutants could then be trained in a co-evolution experiment to enhance their lytic activity toward the pathogen and reduce the evolution of phage resistance (Borin et al. 2021) (**Fig. 1C**). Phage-resistant mutants will likely emerge quickly after treating the wildtype bacterium with the recently evolved phage strain (Labrie et al. 2010). These mutants may rescue the bacterial population and limit the antimicrobial action of the trained phage.

Instead of co-evolutionary phage training, a targeted approach can also be applied to reduce the probability of phage resistance evolution. For this purpose, phage resistant mutants are first generated in fluctuation experiments (Luria and Delbrück 1943; Burmeister et al. 2020; Romeyer Dherbey et al. 2023) (**Fig. 1D**). New phage strains can then be evolved to infect each resistant mutant (**Fig. 1E**). A selection of the evolved phages can then be combined to create an effective phage cocktail (Yehl et al. 2019; Yang et al. 2020; Nale et al. 2021). Phages in these cocktails cannot only infect a diverse set of resistant bacterial strains but also recombine to generate phages that can infect bacteria with novel resistance phenotypes (Romeyer Dherbey et al. 2023).

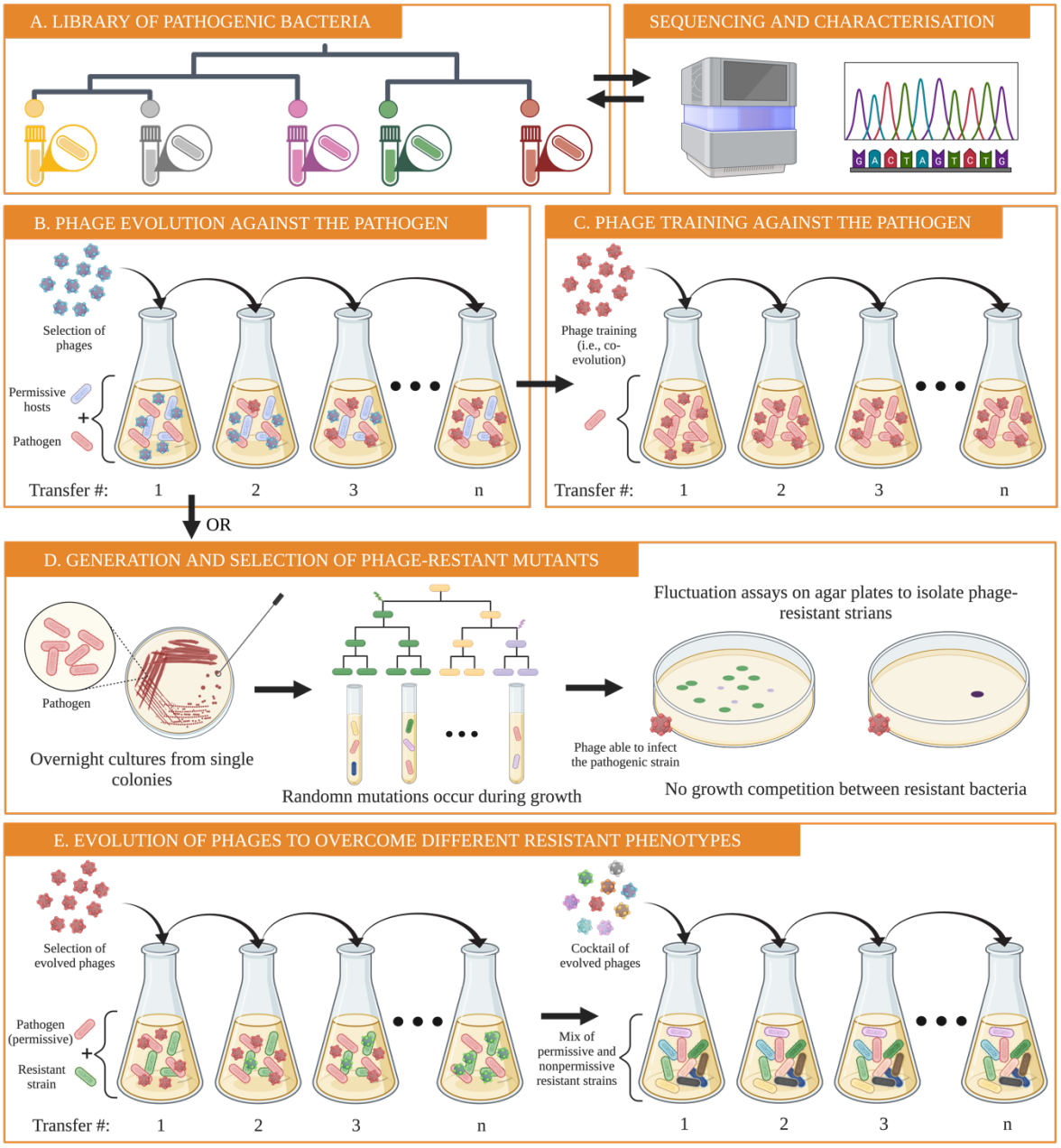

Fig. 1. Proposed procedure to develop a phage model system into a therapeutic agent. A. Bacterial pathogens are first sequenced and characterised. A phylogenetic tree can help to identify phage candidates that are most likely to be able to infect these pathogens or evolve the ability to infect during an evolution experiment. **B.** If no phage can already infect the pathogen, then a selection of phages is serially transferred daily on a host culture containing a mixture of susceptible strains and the pathogenic strain of interest. Transfers continue until a phage is found that can infect the pathogenic strain (Bono et al. 2013; Romeyer Dherbey et al. 2023). **C.** Phages capable of infecting the pathogenic strains can be further trained to enhance their lytic ability against the pathogen only, for example by phage training in a coevolution experiment (Borin et al. 2021) or through a more targeted approach (**D** and **E**) (Romeyer Dherbey et al. 2023). **D.** The emergence

of resistance to the phage can be reduced by evolving a range of phage mutants that can infect spontaneously resistant bacteria. Spontaneous phage-resistant mutants can be generated on agar plates using a fluctuation assay (Luria and Delbrück 1943). **E.** Left panel: similar to panel (**B**), phage strains are evolved to infect different phage-resistant variants without co-evolution of the bacteria (Romeyer Dherbey et al. 2023). Right panel: for resistant strains that are difficult to infect, additional evolution experiments using a cocktail of phages adapted to easier resistant phenotypes (resistant phenotypes that phages evolved to infect quickly) may speed up evolution *via* recombination. High host diversity can help maintain phage diversity in the experiment (Romeyer Dherbey et al. 2023).

Resistance phenotypes change across environments. For example, bacteria that are resistant to phages in liquid media may be susceptible to phage infection in solid media (Romeyer Dherbey 2023). More importantly, resistant phenotypes *in vitro* may not resemble resistance phenotypes *in vivo*. Future experiments should therefore explore phage resistance evolution *in vivo* (De Sordi et al. 2018; Hernandez and Koskella 2019; Hsu et al. 2019; Castledine et al. 2022).

A phage cocktail primarily aims to drive evolution toward predictable and often costly outcomes for the bacterium (Yethon et al. 2000; Matsuura 2013; Pagnout et al. 2019; Simpson and Trent 2019; Burmeister et al. 2020; Mutalik et al. 2020). Ideally, the evolved resistant bacteria will have a lower capacity to evolve new resistance types (Borin et al. 2021). Then, the immune system and/or specific antibiotics could kill the remaining mutants (Roach et al. 2017; Burmeister et al. 2020; Mangalea and Duerkop 2020). Preliminary results from our ongoing experiments show that some antibiotics can reduce the probability of phage resistance evolution significantly, even if the bacteria are already resistant to that antibiotic (Parab et al., in prep).

Phage therapy has the potential to significantly improve treatment outcomes. However, one crucial aspect that hinges on its success is often overlooked: the perception of the general public. To engage people with phage therapy, we must ensure effective communication about phage research, its current limitations, and, most importantly, its potential to save lives (Gordillo Altamirano and Barr 2019; Ji and Cheng 2021; Niang et al. 2021).

Raising phage therapy awareness with established phage model systems

Medical innovations are often met with great scepticism, especially by the general public (Johnson et al. 2020; Barrett et al. 2022). For example, the acceptance of the new mRNA COVID vaccine

has been hampered by the spread of misleading or false information (Hussain et al. 2018; Burki 2020; Longhi 2022). As phages are also viruses, their acceptance and the willingness of people to rely on them as therapeutic agents could be impeded in similar ways. Moreover, phage therapy has already had to overcome the poor reputation obtained through its association with Axis powers during the Second World War (Summers 2012). To prevent history from repeating itself, the narrative around phage therapy and its anthropological impact on modern society should be taken into consideration by scientists (biologists, anthropologists of sciences, sociologists), media, and politics.

Fortunately, we still have time to effectively and transparently communicate about the advantages and limitations of phage therapy. Phage model systems represent a convenient tool for this endeavour as we can capitalize on our profound insight into their biology and evolution (Luciano et al. 2002; Hanauer et al. 2017). The knowledge acquired about model phage systems over the last 100 years will facilitate the communication of complex concepts about phages to the general public. For example, phage T4 is already used in television reports and science cartoons (Kurzgesagt 2018) as the "default phage", thanks to its striking morphology. Similarly, other phage model systems could be exploited to communicate information on phage biology and phage therapy. Finally, integrating phage biology and phage hunt classes (i.e., phage discovery programs) may be a good way to construct collective knowledge and disseminate accurate information about phages (Elbers and Streefland 2000; Staub et al. 2016; Hanauer et al. 2017).

Conclusion

Established phage model systems are far from old-fashioned. In addition to the purely economical, biological, and medicinal advantages, they may provide non-negligible sociological benefits. These advantages could be decisive to establish phage therapy as a common, safe, and inexpensive medical practice in the West, once the required technology is readily available. Hence, in parallel with the ongoing search for novel environmental phages, we advocate investing resources into developing phage model systems for phage therapies.

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