JAC Antimicrob Resist
https://doi.org/10.1093/jacamr/dlae017

JAC-Antimicrobial Resistance

Improving phage therapy by evasion of phage resistance mechanisms

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Antibiotic failure is one of the most worrisome threats to global health. Among the new therapeutic efforts that are being explored, the use of bacteriophages (viruses that kill bacteria), also known as 'phages', is being extensively studied as a strategy to target bacterial pathogens. However, one of the main drawbacks of phage therapy is the plethora of defence mechanisms that bacteria use to defend themselves against phages. This review aims to summarize the therapeutic approaches that are being evaluated to overcome the bacterial defence systems, including the most innovative therapeutic approaches applied: circumvention of phage receptor mutations; modification of prophages; targeting of CRISPR-Cas systems and the biofilm matrix; engineering of safer and more efficacious phages; and inhibition of the anti-persister strategies used by bacteria.

Introduction

Antimicrobial resistance causes almost 5 million deaths annually worldwide and is predicted to become the leading cause of death.[1] Bacterial antibiotic resistance is driven by natural evolution, and antibiotic resistance genes are common, even in DNA isolated from ancient sediments.[1–3] However, the massive use of antibacterial agents over decades, together with their release in untreated wastewater, exerts a selective pressure that has led to a global health crisis and could lead to an era without effective antibiotics.[4,5]

Throughout history, numerous different outbreaks of severe infectious diseases have been caused by heterogeneous pathogens (belonging to different phyla) that have acquired some resistance mechanisms. Examples of such bacterial pathogens include carbapenem-resistant *Klebsiella pneumoniae*,[6,7] colistin-resistant plasmid-mediated Enterobacteriaceae,[8–10] XDR *Pseudomonas aeruginosa*,[11] carbapenem-resistant *Acinetobacter baumannii*,[12] MDR *Stenotrophomonas maltophilia*,[13–15] MDR *Burkholderia cepacia*[13] and MRSA.[16,17] The latter are some of the most common examples, but there are many more.

Although some researchers remain optimistic about a renewed antibiotic pipeline, vaccines or antibody–antibiotic conjugates,[18] every time a new antibiotic is introduced in the clinical setting, bacterial resistance arises against it as the result of accelerated evolution.[19]

One of the most recently discovered antibiotics, teixobactin, was detected in 2015 by screening uncultured soil bacteria by using iChip technology.[20]

Teixobactin is a depsipeptide synthesized by the soil bacterium *Eleftheria terrae* and has shown extremely good efficacy and toxicity profiles *in vivo* against Gram-positive bacterial infections.[21]

To date, the resistance reported against teixobactin is slow and very costly in terms of fitness;[22] however, such successes are rare. The restoration of old strategies to combat resistant pathogens (such as phage therapy), together with the use of non-antibiotic compounds [antimicrobial peptides (AMPs) and repurposed drugs], is gaining much attention.[23,24]

Bacteriophages

Bacteriophages, also called phages, are viruses that infect bacteria. They are the most abundant organisms on Earth, being found in all environments including the soil, ocean, sewage and mammalian gut. It is thought that there are around 10 phages per bacterium, yielding an estimated 10^{31} viral particles.[25] Phages are generally classified according to their life cycle into

lytic phages and lysogenic phages, although other types of phage infection are also possible, such as chronic infection or pseudolysogeny.[26,27] Lytic phages attach to the bacterial surface, inject their DNA, use bacterial machinery to replicate and subsequently lyse the cells to release multiple viral particles; by contrast, lysogenic phages inject their DNA and integrate it into the bacterial genome, so that it is passed on to progeny, where it may act as a vector for horizontal gene transfer (HGT). However, lysogenic phages can be induced and released from cells under stress situations, provoking cell lysis.[28]

Bacteria–phage interactions: an arms race

Bacteria and phages are in a permanent state of co-evolution referred to as an arms race, because when one develops a mechanism to evade the other, this causes the latter to adapt and avoid this defence system (Figure 1). Thus, bacteria have evolved defence mechanisms to protect themselves from predators, while phages, in turn, have evolved counterdefence strategies to evade these systems.[29–32] The main mechanisms of defence that bacteria have developed against phages are summarized in Table 1, and the phage resistance mechanisms are summarized in Table 2.

The use of phages for therapeutic purposes is of renewed interest in Western countries, where it fell into disuse in the 1940s with the advent of commercial antibiotics. Due to the rising antibiotic resistance rates, the use of phages in therapy has regained interest. Currently, the following minimum criteria are required for the use of phages for therapeutic purposes: (i) the phages must be strictly lytic in nature, lacking genes for lysogeny such as integrases and recombinases; (ii) they must have clear antimicrobial activity against the target pathogen; and (iii) bacterial debris and endotoxins must be removed to below predetermined thresholds of safety.[56] Although common in some eastern countries such as Georgia (Eastern Europe), phage therapy was completely abandoned with the advent of antibiotics in Western countries.[57] However, phage therapy has shown promising results, good safety profiles and efficacy in some relevant clinical cases,[58–61] as discussed below. Importantly, a recent retrospective and observational study focused on 100 consecutive cases of personalized phage therapy in Belgium revealed that more than 77% of difficult-to-treat infections experienced some clinical improvement, with bacterial eradication achieved in 61% of the total cases considered, and only 15% presenting adverse events (among which, 7% were considered mild to moderate).[62] Similarly, in 2023, Green *et al.*[63] summarized several cases of patients treated with personalized phage therapy and also concluded that there were no major adverse reactions and phage–antibiotic synergy (PAS) was frequently observed, consistent with the previously mentioned 2023 report by Pirnay *et al.*[62]

PAS is a well-known phenomenon consisting of an improved outcome after the combination of phage and antibiotics, relative to their separate effects.[64,65]

The synergism between conventional antibiotics and a pre-adapted lytic phage (*K. pneumoniae* phage M1) was made use of to resolve a severe fracture-related infection in a 30-year-old bomb victim in Belgium.[65] Pre-adapted phages were considered those mutants with broadest infectivity after 15 rounds of the Appelman protocol, which consists of growing phages iteratively on mostly refractory bacterial isolates, until the adapted phage can lyse the phage-resistant strains.[66] Although ceftazidime/avibactam reduced *K. pneumoniae* bacterial counts in mature biofilms, they did not completely eradicate them, and high doses of the lytic phage M1 alone failed. Nonetheless, combinations of phage M1 and moderate concentrations of ceftazidime/avibactam were significantly more effective, which also suggests a synergistic effect.[65] Similarly, *P. aeruginosa* phage PNM and colistin (0.5 mg/L), aztreonam (8 mg/L) and gentamicin (2 mg/L) displayed strong synergistic activity against one of the *P. aeruginosa* isolates that caused severe post-liver transplantation sepsis in a toddler.[67] A similar synergistic phenomenon was observed with another *P. aeruginosa* isolate, although the effect was slightly less intense with colistin. Finally, the treatments allowed a second liver transplantation and resulted in complete resolution of the infection.[67] The most significant case reports using phages against antibiotic-resistant isolates are summarized in Table 3.

Figure 1. Original digital illustration (made in Adobe Photoshop from a sketch) representing the bacteria–phage 'arms race', a co-evolutionary state in which one develops a mechanism to evade the other and adapt. Bacteria display defence mechanisms to protect themselves from phages, while these evolve counterdefence strategies to evade these systems.

The strategies of phage training (pre-adaptation) and PAS do not always produce the intended results, which is why it is necessary to look for methods that specifically circumvent bacterial defence mechanisms. This review summarizes some of the most relevant studies in this regard, in order to illuminate this increasingly vast field, while considering the defence mechanisms in relation to the latest advances in phage therapy.

Innovative strategies in phage therapy: counteracting bacterial defence mechanisms

One of the greatest disadvantages of phage therapy is possibly the frequent and rapid generation of phage-resistant bacterial mutants during treatment.[77] In this regard, the strategy of counteracting these phage defence systems may be a useful way of enhancing the therapeutic outcomes of phages in clinical settings (Figure 2).

Table 1. Bacterial defence mechanisms against phages

General mechanism	Specific mechanism	Description	Ref
Adsorption resistance	Modification of bacterial receptor	Either by (i) mutations of cell surface receptors; (ii) inhibitors that compete with the phage; or (iii) alteration of polysaccharide patterns that hide the receptor molecules.	29
	Biofilms	Adsorption of the phage to its bacterial receptor becomes restricted when bacteria form biofilms.	33
	Outer-membrane vesicles (OMVs)	Fragments of the outer membrane of Gram-negative bacteria containing phage receptors may be secreted and act as a bait, leading to phage adsorption and resulting in less successful infections.	34–36
Blocked uptake	Prophages	SIE mechanism: prevention of infection by other phages.	37
	Small molecules	Anthracyclines, aminoglycosides and viperins: molecules with anti-phage properties, can be secreted by bacteria.	38
Restriction	Restriction modification	Methyltransferase adds methyl groups to the host DNA to distinguish it from the foreign material (especially viral DNA), and the restriction endonuclease recognizes the viral DNA and cuts it.	39
	CRISPR-Cas	Bacteria capture short sequences (protospacers) of invading phages and integrate them in their chromosome (CRISPR array). For subsequent phage infections, the system activates and degrades the phage DNA.	40
Other mechanisms	Abortive infection systems and CBASS	Cells prevent the release of functional virions at the expense of their host cell survival (e.g. inducing programmed cell death).	41,42
	TA	The bacterial toxin induces a global metabolic latency so that phage infection does not progress (no successful phage replication or assembly).	43,44
	QS	Phage adsorption is reduced in the presence of some auto-inducers of the QS network.	45,46

Table 2. Phage evasion mechanisms against bacterial defences

Mechanism	Description	Target	References
Alternative adsorption to bacterial surface	Modification of receptor binding protein (RBP) by acquiring mutations that allow recognition of the mutated version of the receptor, or sometimes even a completely different receptor.	Receptor	47
Bacterial capsule degradation	Enzymes like depolymerases allow phages to degrade the extracellular polysaccharides present in the bacterial capsules. In some phages, the RBPs themselves have intrinsic depolymerase activity.	Capsule	48,49
Evasion of Abi	Mutation of specific genes in the phage.	Abi systems	50
Evasion of restriction modification	Reduction in the number or masking of restriction targets incorporating modified bases.	RM systems	51,52
Evasion of TA	Inhibition of a protease that would normally cleave the antitoxin or encoding own antitoxin protein.	TA systems	53,54
Evasion of CRISPR-Cas	(i) Single nucleotide substitution; (ii) complete deletion in the protospacer region or in the conserved motif adjacent to PAM[a]; (iii) anti-CRISPR systems (*acr-aca*).	CRISPR-Cas systems	55

[a]PAM, protospacer adjacent motif.

Circumventing mutation of phage receptors

Binding between a phage and its receptor is a critical point in phage infection. However, bacteria have developed defence mechanisms to block phage binding. To circumvent these mechanisms and obtain better results in phage therapy, phage cocktails can be optimized.[78] For example, phage combinations that target different receptors can be prioritized, thereby reducing the likelihood of resistance emergence. For example, Yang *et al.*[79] designed a cocktail for treating *P. aeruginosa* that contained two phages, both with a different receptor, one targeting the O antigen and the other targeting a truncated form of the O antigen that the pathogen produced when it became resistant to the initial O antigen-targeting phage. One of the phage mutants (PaoP5-m1) was found to be excellent for eliminating *P. aeruginosa* mutants with truncated O-antigen structures, as it was able to adsorb and infect all of the strains, regardless of their O-antigen structure. On the other hand, phages may encode enzymes capable of crossing barriers, such as biofilms, capsule layers or the outside of the cell, or the LPSs of the outer

membrane. These proteins are usually located in tail fibres, tail spikes or baseplates. Depolymerases, for example, are a type of enzyme used by phages to access receptors hidden by polysaccharides, or in cases where the polysaccharide chain is the receptor itself, to cleave it, thus stabilizing the binding.[80]

Prophage modification

Although phage therapy uses lytic phages, most bacteria have prophages in their genomes, often resulting in a well-documented phage resistance mechanism known as 'superinfection exclusion' (SIE).[37,81] In this situation, a prophage residing in a host cell prevents infection by other similar phage by blocking injection of DNA.[82] However, the presence of prophages in the genome is not always related to SIE defence mechanisms.[81,83,84] Many researchers have claimed that the competitive advantage of lysogens over prophage-free competitors reflects the mutualistic (rather than parasitic) relationship between prophages and bacteria.[85–87]

An interesting approach that paves the way for alternative research aimed at a better understanding of phage therapy is to target these prophages, more specifically using the gene *cI*, repressor of the lytic cycle, as a therapeutic target. Disruption of the repressor-operator *cI* by interaction with a small molecule leads to activation of the lytic phase in prophages, thus allowing them to infect their non-lysogenic counterparts and leading to their elimination.[88]

The conversion of a lysogenic phage into a lytic phage displaying activity against multiple clinical isolates of *A. baumannii* is also an example of the use of engineered phages for therapeutic purposes.[89] In this research, the authors combined the mutant phage with subinhibitory concentrations of different antibiotics and observed a decrease in the frequency of occurrence of phage-resistant bacteria; they also found that combinations including the converted lytic phage increased the survival of infected *Galleria mellonella* larvae. Along the same line, a three-phage cocktail composed of engineered phages was administered IV every 12 h for at least 32 weeks to a 15-year-old cystic fibrosis patient with a disseminated *Mycobacterium abscessus* infection.[74] Importantly, one of these phages (ZoeJ) was lysogenic and converted into a lytic phage by mutation of its repressor gene. This *M. abscessus* isolate was resistant to all antibiotics tested: clarithromycin, amikacin, tobramycin, ciprofloxacin, moxifloxacin, cefoxitin, co-trimoxazole, doxycycline and linezolid. The phage treatment resulted in a drastic clinical improvement at all levels: sternal wound closure; enhanced liver and lung functions; resolution of infected skin nodules; and weight increase. No adverse effects were observed after this first use of therapeutic phages to treat a human mycobacterial infection.[74]

Prophage induction therapy (a term coined by Lakshminarasimhan in 2022) can potentially be applied to clinical settings to target antibiotic-resistant strains of bacteria. Some researchers have investigated this phenomenon in the gut environment and concluded that medications (including non-antibiotic drugs) inhibiting bacterial growth led to an increase in phage particles due to prophage induction.[90] Importantly, the authors considered this factor an important driver of phage–bacteria dynamics in the gut. One advantage of the prophage induction therapy versus the administration of exogenous lytic phages is that the viral particles released from the pathogenic bacteria will be highly localized, with lower titres than used for phage therapy, thus minimizing activation of the immune response.[88]

Clustered regularly interspaced short palindromic repeats (CRISPR-Cas) targeting

CRISPR-Cas systems are the only adaptive immune system observed to date in the prokaryotic world. These are composed of short repeated sequences where spacers become intercalated, conforming the CRISPR-array; this is usually followed by the encoding sequences of CRISPR-associated endonucleases (Cas). Spacer sequences are phage DNA fragments that are cut and integrated into the bacterial CRISPR array, so that bacteria can recognize future infections by phages that they have previously encountered and degrade the DNA of the phages via the Cas endonucleases.[40] The main way that phages evade prokaryotic CRISPR-Cas immunity is by using anti-CRISPR proteins. These basically consist of Acr proteins (typically small proteins of 80–150 amino acids) that inhibit bacterial CRISPR-Cas activity by binding directly to the Cas protein, thereby inactivating it, so that the phages can successfully replicate in the bacterial host.[91] Acr proteins and the Aca (Acr-associated) proteins work via diverse mechanisms to inhibit critical steps of CRISPR immunity, including *cas* gene expression,[92] assembly of CRISPR ribonucleoprotein complexes,[93,94] recognition of target nucleic acids,[95,96] and recruitment of effector nucleases.[95] Therefore, the use of phages carrying specific *acr* (or other CRISPR-Cas evasion mechanisms) could be a promising therapeutic approach against MDR bacteria, and further research is needed in this regard.[97]

Biofilm targeting

As previously mentioned, biofilm populations are particularly prone to being resistant to antimicrobials and phage attack. Under most conditions, biofilms will allow phage-susceptible bacteria to be protected from phage exposure, if they are growing alongside other cells that are phage resistant; this phenomenon has implications regarding the ecology of phage–bacteria interactions, as well as in the development of phage-based antimicrobial therapeutics.[98] In turn, some phages possess genes coding for extracellular polysaccharide depolymerases that can specifically degrade the polysaccharidic components of biofilms and facilitate the access of phages to deeper layers.[99]

The interaction between phages and bacteria depends on the ability of the phage particles to diffuse through the biofilm, in which cells aggregate and adhere, sometimes hiding phage receptors.[98,100] One interesting example of how a bacteriophage can adapt to biofilm-growing bacteria is the study conducted by Blasco *et al.*[101] in 2022, in which phages of *A. baumannii* exhibited genomic rearrangement: 10 ORFs were lost and four new ORFs were produced, all of them encoding tail proteins. As a result of this recombination event, a depolymerase-expressing phenotype was visible in 81% of the strains tested. In the same study, a phage cocktail was made with this mutated and adapted phage, together with a phage known to have a depolymerase (B3), and strong activity against 24-h-old biofilms (measured by cfu—enumeration and crystal violet staining) was observed.

Tkhilaishvili *et al.*[102] tested the anti-biofilm activity of a *Staphylococcus aureus*-specific phage, Sb-1, and observed that

Sb-1 degraded the extracellular matrix formed after 24 h and also targeted persister cells. These researchers assessed the minimum biofilm eradicating concentration (MBEC) and performed confocal laser scanning microscopy (CLSM), which revealed that tail enzymes present in Sb-1 degraded the extracellular polysaccharide component of the matrix of *S. aureus* ATCC 43300. This is consistent with the findings of Son *et al.*,[103] who reported that a depolymerase present in the lytic *S. aureus* SAP-2 phage was able to disrupt 48-h-old biofilms [evaluated after safranin staining and scanning electronic microscopy (SEM)]. In another study, Gutiérrez *et al.*[104] reported that lytic phages were a promising option for fighting biofilm formation in staphylococcal infections. In fact, the results of the study confirmed that lytic phages disrupted 4-h-old biofilms, measured by quantification of cfu and crystal violet staining. With the same pathogen, Alves *et al.*[105] combined the use of the *S. aureus* phage K and a newly isolated phage, DRA88, and observed a reduction in growth of a 48-h-old *S. aureus* biofilm, as observe by crystal violet staining. Rahman *et al.*[106] combined the phage SAP-26 with erythromycin, vancomycin and rifampicin to treat 24-h-old *S. aureus* biofilms and obtained a reduction of approximately 28% in the biofilm, as measured by viable cell count, with SAP-26 alone. Erythromycin and vancomycin decreased viable cell counts by 25% and 17%, respectively. However, treatment of the biofilm with the combination of phage and rifampicin yielded a 65% reduction in growth. The combinations of phage–azithromycin and phage–vancomycin reduced the cell counts by 60% and 40%, respectively, after 24 h.[107]

Another study focusing on the eradication of *in vitro* biofilms using phages is that conducted by Akturk *et al.*,[108] in which they generated dual-species (*S. aureus* and *P. aeruginosa)* 24-h-old biofilms in an *in vitro* artificial dermis model and treated it with specific phages targeting these pathogens (SAFA and EPA1, respectively) and gentamicin. The most effective treatment concerning reduction in viable cells was obtained after multiple doses of EPA1 + SAFA + gentamicin, whereas the lowest reduction was produced by application of phages only, followed by another dose of the combined phage treatment.

Many studies have revealed the potential use of phages to eradicate biofilm formation by MDR strains.[107] For example, Khalifa *et al.*[109] evaluated the lytic activity of an isolated phage from sewage water, EFDG1, against various *Enterococcus faecalis* and *Enterococcus faecium* strains, and observed effective activity against planktonic and 2-week-old biofilms, measured by crystal violet staining and CLSM. Importantly, this phage acted on various clinical isolates regardless of their antibiotic resistance profile. In addition, the EFDG1 phage efficiently prevented *ex vivo E. faecalis* root canal infection, which may be important to prevent persistent infections associated with root canal treatment failure.

Another example is the study conducted by Lehman *et al.*,[110] who evaluated the effect of pretreating hydrogel-coated silicone catheters with mixtures of *P. aeruginosa* and *Proteus mirabilis* phages. The authors used a multiday continuous-flow *in vitro* model with artificial urine medium, in which they produced single- and dual-species biofilms during 96 h. They obtained a 2–4 log reduction in biofilm counts for both species over a period of 48 h. The results of this study suggest that pretreatment of catheters with phage cocktails can significantly reduce mixed-species biofilm formation. In a similar way, Fu *et al.*[111] showed the potential of a phage cocktail to prevent *P. aeruginosa* biofilms forming on hydrogel-coated catheters in an *in vitro* model system. The authors observed a reduction in the biofilm viable counts, determined by cfu counts and SEM. Both studies suggest the potential of applying phage cocktails to the surfaces of indwelling medical devices to minimize biofilm formation.[110,111]

Furthermore, Pallavali *et al.*[112] assessed the effect of lytic phages on 96-h-old biofilms of *P. aeruginosa*, *K. pneumoniae*, *Escherichia coli* and *S. aureus*, and observed a reduction of nearly 80% in the biomass of biofilms, quantified after cfu enumeration and crystal violet staining. Moreover, Alves *et al.*[113] showed that a novel bacteriophage cocktail reduced and dispersed *P. aeruginosa* biofilms under static and flow conditions, therefore having a therapeutic ability to control *P. aeruginosa* infections. For the static model, after contact for 4 h with the phage suspension at an moi of 10, more than 95% of 48-h-old biofilm biomass was eliminated, as measured by crystal violet staining. On the other hand, in the 48-h-old biofilm flow model, slower activity was observed by CLSM; however, 48 h after addition of phage cocktail the biofilm had dispersed. Another example is the case of the phages ϕMR299-2 and ϕNH-4, which were able to eliminate the 24-h-old biofilm of *P. aeruginosa in vitro* (using cystic fibrosis lung airway cells) and *in vivo* (using a murine lung model).[114,115] Furthermore, Lu *et al.*[116] reported enhanced dispersion of biofilms with engineered phages, designed from the T7 *E. coli* phage expressing the dispersin B (DspB). The engineered enzymatic phage reduced bacterial biofilm cell counts by 99.97%.

Other studies have demonstrated the potential of a combined therapy to eradicate biofilms. For instance, the *E. coli* phage T4 was used in combination with the antibiotic cefotaxime to eradicate 24-h-old *E. coli* biofilms. The results revealed that the addition of T4 reduced the MBEC of cefotaxime against *E. coli* biofilms by between 2- and 8-fold, indicating that the combination of T4 and cefotaxime significantly enhanced biofilm eradication.[117] In a similar way, 8-day-old *K. pneumoniae* biofilms were also eradicated using a combination of phage at an moi of 0.01 with amoxicillin (512 μg/mL). The results of this study showed a significant reduction in the bacterial counts in biofilms after application of combined therapy.[115] In addition, a 12-h-old biofilm of *K. pneumoniae* was treated by a combination of phage KPO1K2 at an moi of 1 and ciprofloxacin (1 mg/mL), and no significant differences in biofilm removal (quantified by cfu counts) were obtained. However, the combined treatment significantly prevented the emergence of resistance.[118] Other groups used the phage T4 of *E. coli* and PB-1 of *P. aeruginosa* in combination with tobramycin at different concentrations (2 and 0.5 μg/mL, respectively) to remove 48-h-old biofilms. The combined treatment led to a 99.99% decrease in the survival of *E. coli* biofilms measured by cfu/mL count relative to the use of tobramycin alone. However, the combination of tobramycin and *P. aeruginosa* phage was as effective as tobramycin alone, although the combination reduced the emergence of antibiotic- and phage-resistant cells.[119] Finally, Zhang *et al.*[120] reported that a mixture of RNA phages of *P. aeruginosa* and chloride reduced the 72-h-old biofilm growth by 94% and removed it in 88% of the cases; the biofilm growth was measured by crystal violet staining.

In general, the main two approaches implemented in regard to the administration of phages to control biofilms are IV bacteriophage therapy and the direct injection of bacteriophages to the

site of biofilm on surgical intervention. The first example is documented by Doub *et al.*,[121] but unsuccessful therapeutic outcomes were obtained since the patient continued to have culture-positive *S. aureus* knee infection, suggesting the inability of IV therapy to eradicate the biofilm infection. The second route of administration is documented by Tkhilaishvili *et al.*,[122,123] who treated a chronic relapsing periprosthetic knee infection and chronic osteomyelitis of the femur, caused by an MDR *P. aeruginosa*, with a combined treatment between antibiotics (colistin, meropenem and ceftazidime) and phages, locally applied during surgery.

A systematic review analysing 68 articles on this topic included correlation analysis that revealed some phage parameters relevant to the treatment outcome: higher phage concentrations were strongly associated with better biofilm control; and phages with higher burst sizes and shorter latent periods were the best candidates for controlling biofilms.[124]

The most significant cases of biofilm removal using phages are summarized in Table 4.

Engineered phages

Phage engineering is also a very important and powerful tool for increasing the likelihood of successful phage therapy; in particular, great effort has been made to extend the host range of phages by genetic engineering, as this is a major limitation of the application of phages in therapy. This strategy could significantly reduce the amount of work needed to search for phages to treat specific bacteria and could lead to the selection of a few well-studied phages that could become 'scaffolds' for the generation of customized phages. Furthermore, this would reduce the variability between treatments and enhance translation to clinical applications.[132]

Yehl *et al.*[133] targeted regions in the tail fibre of coliphage T3 for mutagenesis, in order to create highly diverse phage libraries that were screened to identify phages with altered host ranges. The authors showed that these engineered phages not only had a broader host range, but they were also able to suppress bacterial resistance to phage infection. They targeted naturally occurring phage-resistant bacterial mutants, which could potentially delay or even prevent the onset of phage resistance.[134] One example of this is a study in which phages containing engineered anti-CRISPR (*acr*) genes were explored by including type I anti-CRISPR genes (*acrIF1*, *acrIF2* and *acrIF3*) in the *P. aeruginosa* phage DMS3/DMS3m to obtain the potential to block bacterial replication and infection.[135] The results indicated that bacterial inhibition required the production of Acrs to be above a specific threshold, so that successful phage replication may be dependent on the competition between CRISPRs and Acrs. This work led to an innovative treatment in which anti-CRISPR phages could be used to treat intractable *Pseudomonas disease*.

Additionally, natural, engineered and chemically synthesized genomes and re-engineered functional phages that infect Gram-negative bacteria and acid-fast mycobacteria have been assembled and shown to be efficient.[136]

A plethora of studies have used genetically modified phages to enhance their potential for biofilm control.[137] Some of these are listed below. For example, expression of enzymes such as depolymerase or DspB is used to enhance phage activity.[116] Alternatively, insertion of restriction endonuclease genes or modified holin genes or deletion of export protein genes have been explored to minimize inflammatory responses and thus improve phage therapy outcomes.[138–140] One example of the latter is the endosialidases of some coliphages, specialized tail spike proteins that degrade the polysialic acid (polySia) capsule of *E. coli* K1, not only reducing the virulence of the pathogen but, importantly, limiting the tissue damage and the inflammatory processes that occur in response to bacterial invasion.[141]

Simultaneously, Park *et al.*[142] integrated a CRISPR-Cas system targeting the *nuc* gene (encoding a thermostable nuclease uniquely present in *S. aureus*, so that the microbiome will not be affected) into the temperate phage ϕSaBov. They also removed the virulence factors and infected CTH96 *S. aureus* strains and did not recover any viable cells after treatment with an moi of 100. The authors conducted *in vivo* studies with a skin infection model in C57BL/6 mice and obtained a reduction in cfu of more than two orders of magnitude after applying ϕSaBov-Cas9-nuc embedded into a hydrogel, relative to ϕSaBov-Cas9-null. To increase the host specificity, the ϕSaBov tail fibre protein was complemented with that from the phage ϕ11, which exhibited a broader spectrum, resulting in the specificity being extended to the human pathogenic clones ST1, ST5, ST8 and ST36.[142] Furthermore, this same group studied the effects of ϕSaBov-Cas9-nuc on biofilms, both *in vitro* and *in vivo*, and enhanced clearance in the biofilm using 10^8 pfu/mL of the CRISPR-Cas-transformed phage ϕSaBov-Cas9-nuc.[143]

Altogether, these findings provide evidence that engineered phages may be a viable alternative approach for use in patients with difficult-to-treat bacterial infections, including those caused by MDR bacteria that do not respond to conventional antibiotic therapy, and also to treat phage-resistant bacteria that have acquired immunity via the CRISPR-Cas system. The most relevant studies concerning engineered phages are summarized in Table 5.

Anti-persister strategies

Persister cells are a subpopulation of bacteria in a dormant state: they neither grow nor replicate and can re-establish infections once antibiotic stress has been removed.[151]

Persister cells involved in biofilm

The subpopulations of resistant phenotypes within the biofilm have been referred to as persisters.[152]

Phages have two properties that make biofilms susceptible to their action: they produce enzymes that degrade the extracellular matrix; and they can infect persister cells, remaining dormant within them, but reactivating when cells become metabolically active;[125,126] the phages themselves may be the main actors in this reactivation.[153] Furthermore, 'Trojan horse' strategies where phages are used in a first instance to destroy biofilms and activate persister cells, rendering them more susceptible to the antibiotics subsequently introduced, can be considered an elegant pathway to synergy. This strategy takes advantage of the ability of some phages to revert resistance to antibiotics, resensitizing bacteria.[65]

Table 3. Clinical case reports focusing on phage therapy

Date and country	Cause of infection	Patient(s)	Pathology/disease	Treatment	Administration route	Clinical outcome	Observation	Reference
2011, Australia	*P. aeruginosa*	67-year-old female	Urinary tract infection after intra-abdominal resections and pelvic irradiation for adenocarcinoma	6 lytic phage cocktail	Pyophage #051007 was instilled directly into the bladder every 12 h for 10 days	Resolution of infection and microbiological improvement	No antibiotic- or phage-resistant bacteria arose	[68]
2016, Bangladesh	*E. coli*	225 children	Acute bacterial diarrhoea	11 T4-like phage cocktail and Microgen ColiProteus phage cocktail	Oral, 4 days	Failure	Overgrowth of *Streptococcus gallolyticus* and *Streptococcus salivarius* in the stool	[69]
2017, USA	MDR *A. baumannii*	68-year-old	Diabetes, necrotizing pancreatitis (MDR *A. baumannii*-infected pancreatic pseudocyst)	Cocktail of 4 anti *A. baumannii* phages	Intracavitary and IV administration	Resolution of the patient's infection	Increased antibiotic sensitivity when phage and antibiotics were simultaneously administered	[70]
2018, USA	MDR *A. baumannii*	77-year-old male	Postoperative craniectomy complication: cerebritis infection	Lytic *A. baumannii* phage	IV, 98 doses	The patient died	High degree of severity of underlying assault injury	[71]
2018, Belgium	XDR *P. aeruginosa*	Male toddler	Sepsis post liver transplantation	Phage–antibiotic combination	IV therapy for 86 days	Complete resolution of all infections, second liver transplantation	Resistant phage mutants but not sufficient to re-establish proper infection	[67]
2018, USA	Bacteraemia caused by *P. aeruginosa*	2-year-old boy	DiGeorge syndrome, congenital heart disease	2 lytic phage cocktail	IV every 6 h during 36 h	Death by end-stage cardiac failure	Repeated blood sterilization after each introduction of phage therapy (therapy had to be interrupted)	[72]
2019, USA	MDR *P. aeruginosa* and MDR *Burkholderia dolosa*	67-year-old male, 57-year-old female and 28-year-old female	Three lung transplant recipients, the third one with cystic fibrosis	Cocktails of *P. aeruginosa* phages in combination with antibiotics; 1 lytic phage against *B. dolosa*	Nebulized and IV, both antibiotics and phages	Resolution of patient 1 and 2 infections; patient 3 died due to liver and kidney failure	Resistance arose after suspension of phage therapy; challenge in finding lytic phages against *B. dolosa*	[73]
2019, USA	*M. abscessus*	15-year-old	Cystic fibrosis with a disseminated *M. abscessus* infection	Three-phage cocktail	IV phage treatment, every 12 h for at least 32 weeks	Sternal wound closure, improved liver function, substantial resolution of	Genome engineering	[74]

Continued

Table 3. *Continued*

Date and country	Cause of infection	Patient(s)	Pathology/disease	Treatment	Administration route	Clinical outcome	Observation	Reference
						infected skin nodules		
2020, USA	*Mycobacterium chelonae*	56-year-old man	Refractory disseminated cutaneous *M. chelonae* infection	Muddy phage	IV	Skin lesions improved and no evidence of granulomas	No resistance observed	[75]
2022, Belgium	PDR *K. pneumoniae*	30-year-old bombing victim	Fracture-related antibiotic-resistant infection	Phage M1 and ceftazidime/avibactam, tigecycline and ciprofloxacin	Catheter in place, locally administered 3 times a day for 5 days	Skin graft vascularized, sinus tract closed and dry, no signs of infection, gain of muscle	Phage-neutralizing antibodies on Days 8–18 post-phage application (unlikely to interfere)	[65]
2023, Spain	MDR *P. aeruginosa*	58-year-old man	Infection of axillo-bifemoral bypass graft	Phages PT07, 14/01, PNM and ceftazidime/avibactam	IV 7 days	Failure	Decrease in the MIC of the antimicrobials	[76]

PDR, pandrug resistant.

Toxin–antitoxin (TA) targeting

TA modules are generally encoded by two adjacent genes: a stable toxin; and an unstable antitoxin, which is degraded under stress conditions by protease systems,[154] leading to activation of the toxin that often results in reduced bacterial metabolism.[155] One of the main functions of the TA systems is to provide bacteria with another phage resistance mechanism, using the toxin as a general metabolic switch. Therefore, these systems would be a good target for increasing the success of phage therapy. For example, genetically engineered phages that harbour genes encoding toxin inhibitors, or molecules that protect the degradation of the antitoxin by Lon proteases, could potentially be used in phage therapy.[53]

Quorum sensing (QS) targeting

QS is bacterial cell-to-cell communication mediated by the production and recognition of small molecules called autoinducers.[156] Several studies clearly show that QS also affects the susceptibility of bacteria to phage infection and the coordination of defence strategies against phages.[45,157–159] Inhibition of QS may be important to enhance phage therapy, as recently demonstrated by Høyland-Kroghsbo *et al.*,[160] who observed overexpression of QS genes in *P. aeruginosa* infected by the JBD44 phage, demonstrating that QS up-regulates the phage defence systems in the bacterial hosts. The QS network modulates several phage defence mechanisms to appropriately combat infections, shaping the outcomes of phage–host interactions and representing a crucial target to improve phage therapy. In the same line, Shah *et al.*[161] found that *Pseudomonas* lysogenic phage DMS3 encoded a QS anti-activator protein, Aqs1, which acted as an inhibitor of LasR, the master regulator of QS. The authors also found that Aqs1 protein silences multiple anti-phage defence mechanisms simultaneously, such as Abi systems. Therefore, infection studies with DMS3$^{\Delta Aqs1}$ resulted in 100-fold fewer viable cells, which proved that the presence of Aqs1 inhibited Abi-mediated resistance.

Consistently, other studies have linked the inhibition of QS and improved phage infection; for instance, Mion *et al.*[162] demonstrated the role of the disruption of QS, a strategy known as quorum quenching (QQ), to reduce bacterial virulence and increase both antibiotic and phage treatment efficiency. These researchers used the QQ enzyme *Sso*Pox-W263I, a lactonase able to degrade acyl-homoserine lactones (AHLs), to reduce the virulence and biofilm formation by clinical strains of *P. aeruginosa* from diabetic foot ulcers, thereby enhancing the susceptibility of phage and antibiotic-sensitive and phage-resistant bacteria to bacteriophages and antibiotics. The study results revealed a reduction of more than 70% in biofilm formation in 6 of 10 strains. Another example is the study by Qin *et al.*,[163] who showed the effect of a QS inhibitor, a penicillic acid, on infection of the strain PAK-AR2 and PAO1 of *P. aeruginosa*. The results show that supplementation with the QS inhibitor increased the productive infection of the cells by the *P. aeruginosa* lytic phage C11 and increased the burst size of lytic phage K5 by 45% when cells were infected at the logarithmic phase, relative to the control in the absence of penicillic acid. Finally, the study conducted by Severin *et al.*[164] demonstrated that QS also up-regulated a different phage defence system in *Vibrio cholerae*, the cyclic oligonucleotide-based antiphage signalling system (CBASS).

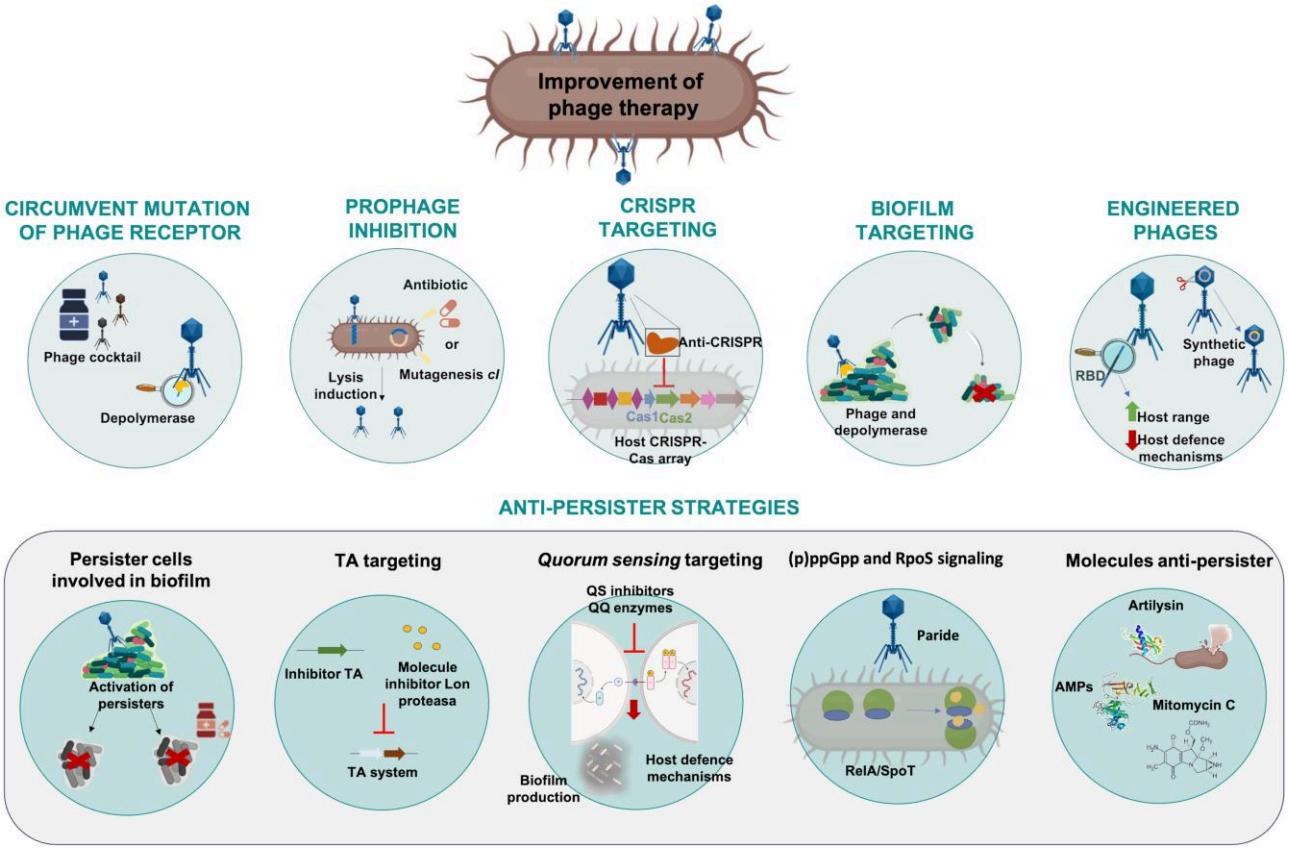

Figure 2. Strategies to improve the efficacy of phage therapy, using bacterial defence mechanisms to phage infection as a target. RBD, receptor-binding domain.

Nevertheless, if QS regulates multiple bacterial systems, inhibition of this network could in some cases lead to failure when phages are used as therapeutic agents. For example, Xuan *et al.*[165] reported that QS up-regulated the expression of phage receptor in *P. aeruginosa* PAO1 (the O antigen of the LPS), increasing phage adsorption and infection rates. Furthermore, Broniewski *et al.*[166] showed that a QS inhibitor decreased the phage adsorption rates due to down-regulation of the type IV pilus, which acted as the phage receptor. This caused delayed lysis of bacterial cultures and favoured CRISPR immunity. Therefore, the results of this group suggested that the inhibition of QS may reduce rather than improve the therapeutic efficacy of pilus-specific phages. Similarly, Ghosh *et al.*[167] demonstrated that a cocktail of different synthetic AHLs or AHL-producing strains led to induction of prophages in *E. coli*, [168] which could lead to the propagation of these and the subsequent integration in other commensal bacteria. Finally, similar situations have been observed in *E. faecalis*, *V. cholerae* and *Pseudomonas* spp., in which prophage induction was promoted after exposure to autoinducer 2 (AI-2), autoinducer 3,5-dimethylpyrazin-2-ol (DPO) and 2-heptyl-3-hydroxy-4-quinolone (PQS) respectively.[169–174]

Overall, prophage induction is a desirable feature in terms of evading phage resistance mediated by prophages (SIE) as well as provoking bacterial lysis; however, caution is required to prevent horizontal gene transfer and activation of prophage-encoded toxins, among other risks worthy of consideration.[168,175]

These studies confirm the current debate concerning the effects of the QS network on phage defence systems and phage therapy outcomes.

(p)ppGpp and RpoS signalling

Most phages enter a more or less stable state of hibernation in deep-dormant host cells (persister cells) and do not produce the lysis of the bacteria cells. However, Maffei *et al.*[172] found a newly isolated *P. aeruginosa* phage, named Paride, that can directly replicate and induce the lysis of deep-dormant hosts. Efficient replication of Paride on growth-arrested hosts specifically requires cellular stress responses in the form of (p)ppGpp and RpoS signalling that are dispensable for infections of growing hosts. Interestingly, the authors showed that a combination of Paride and meropenem could sterilize deep-dormant cultures *in vitro* and greatly reduced a resilient bacterial infection of a tissue cage implant in mice.

Anti-persister molecules

Innovative treatments to tackle dormant, persister bacterial cells, against which antibiotics are not efficient, are needed. In this context, phage therapy could be of benefit. An example

Table 4. Treatments and innovation strategies: biofilm targeting

Authors (year)	Mechanisms	Species	References
Pearl *et al.* (2008) Chegini *et al.* (2020)	Production of enzymes that degrade extracellular matrix and infect persister cell	*P. aeruginosa* *E. coli*	125,126
Eskenazi *et al.* (2022)	'Trojan horses' strategies where phage was used to destroy biofilm and activate persister cell	*K. pneumoniae*	65
Dunsing *et al.* (2019)	Phage carried depolymerase enzyme to counteract the defence of bacteria	*Pantoea stewartia*	127
Blasco *et al.* (2022)	Phage cocktail with a host-adapted phage Ab105-2ϕΔ404ad and phage vB_AbaP_B3 show strong antibiofilm activity	*A. baumannii*	101
Vidakovic *et al.* (2018) Chaudhry *et al.* (2020) Darch *et al.* (2017)	Phages are retained in biofilms; they confer protection barrier against other bacteria and phages	*E. coli* *P. aeruginosa*	128–130
Tkhilaishvili *et al.* (2018)	Anti-biofilm activity of phage Sb-1; Sb-1 degrades biofilm matrix and target persister cell	*S. aureus*	102
Son *et al.* (2010)	Phage SAP-2 carried depolymerase enzyme able to disrupt the biofilm	*S. aureus*	103
Gutiérrez *et al.* (2015)	Lytic phages can be efficient biofilm-disrupting agent	*Staphylococcus* species	104
Khalifa *et al.* (2015)	Anti-biofilm activity of phage EFDG1	*E. faecalis*, *E. faecium*	109
Lehman *et al.* (2015)	Effect of pretreating hydrogel-coated silicone catheters with phages; these phage cocktails can significantly reduce mixed-species biofilm formation	*P. aeruginosa*, *Proteus mirabilis*	110
Alves *et al.* (2014)	Phage cocktail of phages K and DRA88 reduce biofilm formation	*S. aureus*	105
Rahman *et al.* (2011)	Application of phage SAP-26 in combination with different antibiotics against 24-h-old biofilm	*S. aureus*	106
Akturk *et al.* (2023)	*In vitro* dual-species biofilm in combination with gentamicin	*P. aeruginosa*, *S. aureus*	131
Fu *et al.* (2010)	Phage cocktails prevent biofilm formation on catheters in an *in vitro* model	*P. aeruginosa*	111
Pallavali *et al.* (2021)	Lytic phages against 96-h-old multispecies biofilms	*P. aeruginosa*, *K. pneumoniae*, *E. coli* and *S. aureus*	112
Alves *et al.* (2016)	Novel phage cocktail reduces and disperses biofilm under static and flow conditions	*P. aeruginosa*	113
Alemayehu *et al.* (2012)	Phage ϕMR299-2 and ϕNH-4 eliminate *P. aeruginosa* in murine lung and on cystic fibrosis lung airway cell	*P. aeruginosa*	114
Lu *et al.* (2007)	Dispersion of biofilm with 'engineered enzymatic phages'	*E. coli*	116
Ryan *et al.* (2012)	Combined therapy to eradicate biofilm: T4 phage with cefotaxime	*E. coli*	117
Manmeet Sakshi Bedi *et al.* (2009)	Combined therapy to eradicate biofilm: phages of *K. pneumoniae* with amoxicillin	*K. pneumoniae*	115
Verma *et al.* (2009)	Combined therapy to eradicate 12-h-old biofilms: phage KP01K2 with ciprofloxacin	*K. pneumoniae*	118
Coulter *et al.* (2014)	Combined therapy to eradicate 48-h-old biofilms: phage T4 of *E. coli* and PB-1 of *P. aeruginosa* with tobramycin	*E. coli*, *P. aeruginosa*	119
Zhang *et al.* (2013)	Mixture of RNA phages and chloride to reduce biofilm	*P. aeruginosa*	120
Doub *et al.* (2022)	Recalcitrant MRSA prosthetic knee and femoral lateral plate infection	*S. aureus*	121
Tkhilaishvili *et al.* (2019)	Direct phage injection in a periprosthetic joint infection and osteomyelitis	*P. aeruginosa*	122

of the use of lytic phages against an imipenem-persister clinical isolate of *K. pneumoniae* has been reported by Pacios *et al.*[173] in a study in which a lytic phage was used in combination with the repurposed anticancer drug mitomycin C (also considered a natural antibiotic) and the conventional imipenem. Both combinations resulted in the death of persister cells and decreased the emergence of *in vitro* resistant mutants. These results were confirmed in the *in vivo G. mellonella* model, in which the combination significantly reduced the mortality rate of the larvae. Briers *et al.*[174] also described a novel artilysin (an outer membrane-penetrating, phage-derived endolysin), named Art-175, that is able to rapidly and effectively pass through the outer membrane of *P. aeruginosa* persister cells and exert its bactericidal activity within the cell, without the need for any metabolic activity. Endolysins are peptidoglycan-degrading enzymes synthesized by phages, used at the end of their replication cycle to hydrolyse the peptidoglycan from within and allow the release of newly formed virions.[33–35,175]

All of these studies provide some insights into the treatment of persister cells, prone to forming biofilms that are difficult to eradicate.

Table 5. Report cases of engineered phages

Authors (year)	Target	Methods	References
Pei and Lamas-Samanamud (2014)	T7 phage was constructed to encode a lactonase enzyme for quenching of QS	Phage-displayed vector	144
Park *et al.* (2017)	Temperate phage ϕSaBov of *S. aureus*	CRISPR-Cas9 genomic editing technique	142
Yehl *et al.* (2019)	A region in the tail fibre of coliphage T3	Mutagenesis	133
Blasco *et al.* (2019)	Repressor gene *cI* of lysogenic phage of *A. baumannii*	Knockout by homologous recombination	89
Dedrick *et al.* (2019)	Repressor gene of lysogenic phages of *M. abscessus*	Phage recombineering of electroporated DNA (BRED)	74
Pires *et al.* (2021)	Reduction of genome encoding hypothetical proteins (48%)	Yeast-based phage-engineering platform	145
Qin *et al.* (2022)	Introducing type I anti-CRISPR genes (AcrIF1, AcrIF2 and AcrIF3) into the *P. aeruginosa* phage DMS3/DMS3m	Homologous recombination	135
Mitsunaka *et al.* (2022)	Deleting the *c2* lysis-repressor gene in P22 *Salmonella* phage, host-range increase of T3 coliphage with T7 tail fibre, engineering of mycophage D29 to show luminescence (addition of the Nluc gene)	*In vitro* genome assembly and installation into appropriate host bacteria or a cell-free transcription and translation (TXTL) system	136
Nick *et al.* (2022)	Repressor gene 45 of lytic phage of *M. abscessus*	Phage recombineering of electroporated DNA (BRED)	146
Emslander *et al.* (2022)	*E. coli*, *Yersinia pestis* and *K. pneumoniae*	Cell-free method and proteomic characterization (MS)	147
Liyanagedera *et al.* (2022)	Incorporate a SpyTag moiety on the capsid head to enable rapid post-synthetic modification	Scaffold phage	148
Fa-arun *et al.* (2023)	Phage tail for the delivery of a Cas9 antimicrobial into clinically relevant human gut pathogens *Shigella flexneri* and *E. coli* O157:H7	Cosmid system	149
Prokopczuk *et al.* (2023)	Repressor and the excisionase genes of Pf4 phage in *P. aeruginosa* PAO1 strain	Mutagenesis	150

Conclusions

Because of the constant adaptation over hundreds of millions of years of co-evolution, bacteria and phages have acquired a plethora of mechanisms to defend themselves against infection and to neutralize these defence systems. This review summarizes some of the most relevant bacterial and phage defence mechanisms in order to throw some light on this increasingly vast topic, and it outlines the latest advances in phage therapy with particular focus on targeting the main bacterial defence systems (mutation of phage receptors, prophages, CRISPR-Cas, biofilm, TA systems, QS). The latter two are involved in the development of persister cells. It is therefore essential to develop more anti-persister strategies to counteract these bacterial phenotypes. In this context, phage engineering is of particular interest to improve the potential use of personalized phages in therapy.

Funding

This study was funded by projects PI19/00878 and PI22/00323 awarded to M. Tomás within the State Plan for R+D+I 2013–2016 (National Plan for Scientific Research, Technological Development and Innovation 2008–2011) and co-financed by the ISCIII—Deputy General Directorate for Evaluation and Promotion of Research—European Regional Development Fund 'A way of Making Europe' and Instituto de Salud Carlos III FEDER, the Study Group on Mechanisms of Action and Resistance to Antimicrobials, GEMARA (SEIMC, http://www.seimc.org). This research was also supported by CIBERINFEC (CIBER21/13/00095) and by a grant from the Instituto de Salud Carlos III (MePRAM Project, PMP22/00092), Instituto de Salud Carlos III, Ministerio de Ciencia e Innovación, funded by NextGeneration European Union funds that support the actions of the Resilience and Recovery Facility. O. Pacios, L. Fernández-García and M. López were financially supported by grants IN606A-2020/035, IN606B-2021/013 and IN606C-2022/002, respectively (GAIN, Xunta de Galicia). I. Bleriot was financially supported by the pFIS programme (ISCIII, FI20/00302). T. Wood was supported by the Novo Nordic Foundation Exploratory Interdisciplinary Synergy Programme (NNF19OC0058357).

Transparency declarations

None to declare.

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