# CRISPR-Modified Bacteriophage Therapy as a Next-Generation Alternative to Antibiotics Against Multidrug-Resistant Bacteria

**Editors**

**Saba Hameed Zidan**
College of Dentistry, Al-Qadisiyah University, Iraq

**Zainab Abdl Baqi Abdl Jalel**
College of Dentistry, Al-Qadisiyah University, Iraq

**Haneen Husham Abboodi**
College of Dentistry, Al-Qadisiyah University, Iraq

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# Abstract

The global rise of multidrug-resistant (MDR) bacteria is rendering conventional antibiotics ineffective and leading to an urgent need for alternative treatment strategies. Bacteriophages-viruses that specifically infect bacteria-offer a promising avenue. However, many challenges remain, including collateral damage to beneficial microbiota and the resurgence of resistance during treatment. Engineering bacteriophages to express the bacterial immune system CRISPR could help combat these limitations. CRISPR is capable of directly targeting resistance plasmids, inhibiting genes associated with antibiotic avoidance, virulence, and biofilm formation, and can be designed to selectively kill MDR strains and reduce horizontal gene transfer. Phages expressing CRISPR could, therefore, enable greater specificity, reduced collateral damage, and enhanced potency against some of the most difficult-to-treat infections. Such engineered phages could also be customized for personalized therapy.

Recent advances in synthetic biology and CRISPR technology could facilitate a new generation of phage therapeutics that minimize common obstacles associated with phage therapy and enable a wide range of applications—from treating systemic infections to disrupting anaerobic biofilms or combining with antibiotics. This next-generation CRISPR-phage therapy could offer a viable and effective option in the battle against antibiotic resistance, provided that identification of appropriate phage and CRISPR-gene pairs is followed by rigorous optimization, testing, and careful monitoring during therapeutic application.

# Content

# Chapter - 1

## The Global Crisis of Antimicrobial Resistance

Amid the numerous challenges facing contemporary medicine, perhaps none is more urgent or intractable than the global threat posed by multidrug-resistant (MDR) pathogenic bacteria. Antimicrobial resistance (AMR) is a natural, inevitable biological phenomenon, yet it is being exacerbated by the excessive and often inappropriate use of antibiotics for prophylactic and therapeutic reasons. The emergence of resistance among bacteria leads to the failure of treatment and the proliferation of infections that are extremely difficult to treat. The concomitant increase in mortality risk is accompanied by costs that are skyrocketing out of control: the bill for the European Union alone has been estimated at 1.5 billion euros annually, and this staggering figure does not account for misery, severe pain, and suffering.

The crisis is being reduced to an economically quantifiable catastrophe that for too long has been allowed to distort the perspective of scientific and medical communities. While the pursuit of new capital- and time-intensive small-molecule antibiotics by pharmaceutical companies is continuing, the novelty products are unwisely being prescribed, at an alarming rate, for gingivitis and flu-like illnesses, spurring further resistance development. New approaches to AMS that do not stem from traditional small-molecule antibiotics, but rather from the genetic modification of naturally occurring agents, are urgently needed. It is here that naturally occurring or engineered bacteriophages can enter the picture. [1, 2, 3, 4]

## Emergence and Evolution of Multidrug-Resistant Bacteria

Natural and anthropogenic factors have driven the emergence of multidrug resistance (MDR) in human pathogens; recent genomic analyses have put a spotlight on underlying mechanisms and the relevance of the microbiota in resistance development and persistence. Some pathogens are naturally resistant to antibiotics; others acquire plasmids that confer resistance and can transfer them horizontally. Stress can induce mutations that generate resistance and influence the expression of regulatory networks involved in virulence, resistance, and biofilm formation. Decreased expression in one of two redundant efflux pumps can enhance susceptibility; elevated expression of the corresponding two-component regulatory system moderates expression of multiple drug resistance or amphiphilic peptide resistance genes. These findings suggest that reducing stressors during antibiotic therapy may help suppress the emergence of MDR pathogens.

MDR and pandrug-resistant strains are found in all major classes of human pathogens, spanning the whole tree of prokaryotes. These strains carry a great diversity of resistance genes, particularly in Enterobacteriaceae, which are commonly associated with co-resistance or cross-resistance to unrelated antibiotic classes. Resistance genes are often found in plasmids; co-localization with inserted elements or within pathogenicity islands indicates association with horizontal gene transfer and virulence transfer across clinical strains. The pathogenicity profile of these strains raises a serious threat to human health. [5, 6, 7, 8]

## Clinical and Economic Burden of Antibiotic Failure

Multidrug resistance in common bacterial pathogens results in patient morbidity, mortality, and added costs. Several recent studies have projected the economic impacts associated with

multidrug-resistant bacteria and their contribution to the broader antimicrobial resistance problem. In a conservative estimate the World Bank forecast that the economic costs alone associated with bacterial infection resistance to existing antibiotics could be about US$3.4 trillion by 2030. However, another recent publication has predicted that the added burden of treating drug-resistant bacterial infections could reach as high as US$6 trillion by 2050. At the same time, the need for effective therapy against growing numbers of multidrug-resistant Gram-negative bacteria has never been greater, indeed it was deemed to be of such immediate concern that an urgent list of antibiotic-resistant priority pathogens for R&D was drawn up by the World Health Organization and published in February 2017 and subsequently updated.

Antibiotic therapy failure has been explicitly included in several recent analyses examining the direct clinical costs from patients diagnosed with infections due to antibiotic-resistant pathogens in various regions, including the European Union, the United States and the Asia-Pacific region. Published estimates suggest that antibiotic-resistant infections affect close to 2 million Americans annually, resulting in approximately 36 000 deaths and costing up to US$34 billion in excess healthcare spending and lost productivity. In the EU, some 670 000 infections are attributable to antibiotic-resistant pathogens, leading to 33 000 fatalities and imposing a direct healthcare cost of approximately €1.5 billion per year. Outside of the United States and the EU, patients in the Asia-Pacific region experience almost 740 000 antibiotic-resistant infections with 185 000 deaths, and treatment for these infections incurs US$26 billion in additional healthcare costs and US$90 billion in lost productivity. [9, 10, 11, 12]

## Limitations of Current Antibacterial Therapies

The major shortcomings of standard antibacterial therapies include: limited efficacy across the array of clinically relevant bacteria, unacceptable toxicity in humans, a high probability of resistance rebound, overly cautious safety profiles that slow product development, and an inappropriate focus on targeting sole and single bacterium species.

Many antibiotic compounds remain ineffective against the full spectrum of bacteria that pose significant risk to human health. Some current antibiotics are only conditionally recommended within their confirmed indications because their side effects in humans are unacceptably high, particularly in vulnerable patients. For example, excessive toxicity sometimes precludes routine use of aminoglycosides, colistin, or daptomycin. Yet the risk–benefit ratio of these compounds may favor their use in life-threatening conditions caused by multidrug-resistant pathogens for which there are no alternative treatments. Nevertheless, the consequence of cosensitization of gram-negative and gram-positive bacteria to nitroxoline raises a powerful safety warning suggesting that such approaches should be very cautiously considered.

Another paradox of current antibiotic therapy is posed by the rapid emergence of bacterial resistance. Nowadays, with each compound approved for clinical use, considerations become increasingly focused on aiding and sustaining the performance of the last-generation antibiotics. Instead of discovering true next-generation antibiotics that achieve permanent infection cure in a non-bactericidal manner, the entire rationale behind these compounds relies on short-term efficacy. Defeated by the massively fast evolution of resistance mechanisms and resistance gene dissemination via horizontal gene transfer, the development of new antibiotics has often been focused on association with

already widely used compounds to prolong efficacy and extend the life of existing medications. [13, 14, 15, 16]

**Urgent Need for Alternative Antimicrobial Strategies**

Consequently, although new antibiotics are continually being developed, these new agents eventually enter the same therapeutic suppression and same trajectory toward obsolescence as their predecessors. These trends finally prompted the WHO to acknowledge the need for alternative anti-infective strategies, especially with regard to antibiotic therapy of bacterial infections; without such an alternative strategy, humanity is being hastened toward a post-antibiotic world, when routine surgery and other medical procedures become exceedingly dangerous due to untreatable infectious consequences. These developments underscore the necessity for alternative approaches to treat bacterial infections caused by MDR strains.

Alternative anti-infective strategies other than host immunity assistance, vaccine therapy, or anti-virulence therapy against infections in the infected host microenvironment are now essential to complement bacterial disinfection approaches that have predominantly relied on external drug or biocide application. Most urgently needed are modalities that can – in a targeted manner and without affecting commensal and habitat microbiota – reduce or eliminate microbial infectious populations and simultaneously reduce the risk of disease recurrence or transmission. One such alternative is bacteriophage (or phage) therapy, which repurposes naturally occurring elements of microbial arena interactions to target infectious pathogens. However, the major limitation of natural bacteriophage therapy is its high host specificity, often requiring the procedural reconstruction of new phages for drug development in every infection case. Novel tailored therapy designs that create CRISPR-directed therapy bacteriophage and

combinatorial inhibition approaches hold promise as a next-generation anti-MDR therapy with reduced bystander effects. [17, 18, 19, 20]

# Chapter - 2

## Fundamentals of Bacteriophage Biology

### Discovery and Historical Development of Phage Therapy

The concept of phage therapy arose soon after the discovery of bacteriophages by Frederick Twort in 1915 and Félix d'Hérelle in 1917. Phages were applied in various clinical settings, particularly for the treatment of enteric infections caused by Shigella and Vibrio cholerae. However, early and later observations that antibiotic-treated mice died of deadly infections caused by penicillin-resistant strains sparked scepticism toward phage therapy. The first clinical trial for phage therapy was conducted in 1934, but the lack of controlled trials and the non-standardization of preparations and administration routes led to mixed results. Literature related to phage therapy therefore contains a variety of poorly designed trials, and meta-analyses performed during the 1990s and early 2000s found only low-quality evidence for efficacy.

Positive results with phage cocktail preparation, published in 2003, and the use of genetically modified phages targeting specific bacterial genes in pediatric patients, shown in 2020, increased interest in phage therapy as an alternative to antibiotics. Numerous reviews have summarized the current status and potential of phage therapy. Phase I–III clinical trials have focused on safety and toxicology. Serious adverse effects, particularly those that might be detected in long-term animal studies, have been emphasized. These concerns and treatment failures can be attributed to the loss of bacteriophages in the blood and to the

development of specific or generalized resistance by bacteria. Adam and co-workers addressed these issues using CRISPR-Cas gene-editing technology. [21, 22, 23, 24]

**Phage Structure, Classification, and Genetics**

As the most abundant biological entities on Earth, bacteriophages (phages) are virulent bacterial predators that offer great promise as an alternative to antibiotics. They display highly diverse structures, can be categorized based on tail architecture, and are divided into two main classes according to their genetic material. In addition to virulent phages, bacteriophages can exhibit a temperate lifestyle through lysogeny, which can be beneficial for maintaining genetic stability within the host population. The vast genetic pool of phages underlies their potential for medical and biotechnological applications. CRISPR-Cas systems, discovered in prokaryotes as adaptive immune systems against invading viruses and plasmids, are widely prevalent in bacteriophages as well. These systems can be adapted for genome editing in numerous organisms, including for creating specialized antibacterial phages equipped with target-specific crRNA sequences. Notably, recent advances in CRISPR technology have extended its applicability to the editing of phage genomes.

Bacteriophages are the most abundant biological entities on Earth. They are distinguished from all other known viruses by their ability to specifically recognize and infect bacterial cells. A significant aspect of bacteriophage biology is that they are obligately lytic and, therefore, can kill the host bacteria. Considering that they infect 20–30% of the bacterial population, the global presence of bacteriophages has led to their widespread use as bacterial biocontrol agents in clinical sciences and agriculture. Furthermore, their population dynamics can greatly influence the global carbon cycle, as it has been estimated that

they lyse approximately 50 trillion bacterial cells in oceans every day. [25, 26, 27, 28]

## Lytic and Lysogenic Life Cycles

Two distinct phage life cycles shape interactions with bacterial hosts. Lytic phages replicate rapidly and destroy their host upon progeny release, while lysogenic phages integrate their genome into the host and reproduce passively during cell division. Lytic phages are preferred for therapeutic applications due to their rapid and potent killing capacity; however, lysogenic phages can also be beneficial in combination therapies, such as when auxiliary infection enhances susceptibility to antibiotics or other lytic phages.

Lytic and lysogenic replication cycles are defined by distinct timing and outcome of viral genome replication. In Pseudomonas aeruginosa, the lytic cycle is transcriptionally activated following internalisation of the phage genome and subsequent host degradation, enabling rapid mass production of infectious particles and cell lysis for progeny release. The prefential induction of lytic or lysogenic developmental pathways is a key area of focus for therapeutic applications. [29, 30, 31, 32]

## Host Specificity and Phage–Bacteria Interactions

Bacteriophage host range is often considered their most important feature. Specificity defines which bacteria a phage can infect, replicate within, and kill. This specificity arises from two essential factors: receptors on the bacterial surface and matching specificities in the phage tail fibers that mediate adsorption. Although phage–bacterial interactions differ from the ubiquitous and non-specific electrostatic interaction between bacteria and phage particles, they can often be equally complex due to the co-evolution of phages and bacterial populations. Bacteria have evolved multiple anti-phage defense mechanisms, resulting in a large variety of phage diversity. Bacteria can acquire resistance

to specific phages through genetic alteration of the receptors used for phage attachment, expression of anti-CRISPR genes that abolish CRISPR-Cas-based recognition by the phage or modification of their sustenance properties.

Bacteriophages are specific in their choice of host, and this specificity is determined chiefly by the presence of compatible receptors. Bacteriophages can attach to the surface of bacteria only if specific receptors, such as the lipopolysaccharide (LPS) and the outer membrane protein (OMP), are present on their surfaces. Phages recognize specific chemical groups present on their hosts. Host–phage interactions can be compared to key–lock mechanisms, where specific molecules act as the key to coerce specific bacteriophages and bacteria into contact with each other, while variations act as a lock to prevent such interactions. The recognition process is mediated by divine receptors on the phage surface. The recognition process is highly specific and often accounts for the narrow host range of certain bacteriophages. [33, 34, 35, 36]

# Chapter - 3

## Principles of CRISPR-Cas Systems

### Discovery and Natural Function of CRISPR-Cas

An intriguing facet of adaptive immunity is its natural occurrence in prokaryotes, where it serves as a defense against invading plasmids and bacteriophages. In the mid-1980s, a unique DNA sequence was characterized in two distinct bacteria—the archaean Halobacterium and Escherichia coli. Although the sequences were arranged in a cluster, their function remained elusive. The scenario shifted in 2005, when the seemingly random repeated sequences were recognized as spacer sequences. Subsequent studies demonstrated their explosive accumulation during infections and their crucial role in providing immunity to the bacterium upon subsequent encounters with the same virus. Subsequent investigations revealed the exciting facets of functionally distinct subtypes of CRISPR-Cas systems, with types I, III, and VI cleaving phage RNA and type V cleaving RNA and DNA of invading plasmids. Phages have since evolved sophisticated means of countering CRISPR-Cas-based prokaryotic immunity, and genes that inhibit these responses are gradually being uncovered.

These fascinating natural functions of CRISPR-Cas have enabled its adaptation into a powerful genome-editing technology, which is widely used for targeted modification of various organisms, including prokaryotes, plants, and animals. Progress in applied phage biology research is also advancing rapidly, reopening and reinvigorating research areas that have

remained dormant for decades. By integrating CRISPR-Cas technology into the life cycle of naturally abundant phages, researchers are now gaining the faculty of not only curing but also fine-tuning the action of phages at the time of treatment. When engineered to incorporate specific DNA-targeting or RNA-targeting sequences, CRISPR-modified phages can thus be employed as CRISPR-guided anti-bacterial agents, affording several exciting advantages over conventional phage therapy. [37, 38, 39, 40]

## Classification of CRISPR-Cas Systems

CRISPR-Cas can be classified into two major Types (I and II) and a Type V system, with further identification of numerous Subtypes. Type I systems generally consist of a signature Cas3 protein and a multi-subunit CRISPR-associated complex for antiviral defense (Cascade) that acts upstream of Cas3. Type II systems are characterized by the presence of a single large signature protein (Cas9), while the signature protein of Type V systems (Csa) is a dual-function protein that exhibits both RNA-guided endonuclease and CRISPR RNA maturation activities.

CRISPR-Cas systems can also be categorized by the atypical subtypes II-B, II-C, IV, V-A, and VI-G or by whether they are complete, containing all core components, or incomplete. Subtyping captures variants within the larger Types and Subtypes, whereas the Groups framework identifies more distantly related sequences that still possess the basic functions of the Type or Subtype. Subtyped systems share additional expanded or modified sets of Cas proteins associated with alternative physiological functions or defense mechanisms. These distinctions have been useful in conveying differences in target RNA species and in the recognition of phosphodiester bonds adjacent to the RNA-guiding nuclear targets. [41, 42, 43, 44]

## Mechanisms of Target Recognition and Cleavage

CRISPR-Cas systems are RNA-guided adaptive immune systems present in bacteria and archaea. Resistance to phages and plasmid-borne mobile genetic elements is a prominent feature of CRISPR-Cas identified in many bacteria. RNA recognition by CRISPR protease is fundamental for CRISPR function, yet PAM-dependent double-strand DNA cleavage by three distinct classes of CRISPR systems has broad and strong biotechnological applications. Detailed here are the molecular aspects of CRISPR-Cas PAMs and gRNA facets associated with target recognition and specificity as well as the mechanisms of target cleavage by the three distinct classes of CRISPR-Cas.

CRISPR-Cas systems stand out as adaptive immune systems in bacteria and archaea incorporatin-g small RNA molecules. During the interference stage of the CRISPR-Cas immune response pathway against foreign DNA, CRISPR RNA (crRNA) molecules are processed from long precursor RNA transcripts by CRISPR-specific ribonucleases and assembled into effector complexes bound with a phosphodiester bond-containing trans-acting CRISPR RNA crRNA associated with a specific CRISPR-associated (Cas) protein. The effector complexes recognize foreign nucleic acids using a hallmark sequence, called a protospacer adjacent motif (PAM), located adjacent to the target area of the crRNA, and these complexes then cleave the targets in a PAM-dependent manner. [45, 46, 47, 48]

**CRISPR-Cas as a Genome Engineering Tool**

Although the natural function of CRISPR-Cas was the targeted defense against invasive nucleic acids such as phage genomes, its future potential was highlighted when first adopted for genome engineering purposes in bacteria and subsequently in eukaryotes. All CRISPR-Cas systems act in a similar manner, involving the recognition of a specific target DNA sequence adjacent to a short protospacer-adjacent motif (PAM) followed

by RNA-guided cleavage catalyzed by an associated Cas endonuclease. However, it is the more complex type IV and type II systems that are exploited for most genome-editing applications. Much of the CRISPR-engineering toolbox is based on the type II system, which operates in the following sequence: i) Targeting. The target DNA must have a PAM recognized by Cas9; ii) DNA cleavage. Upon binding, Cas9 is activated and produces a double-strand break (DSB) in the DNA at a precise position relative to the PAM, using both RNA strands as guides; iii) Repair. The DSB can be repaired by nonhomologous end joining (NHEJ) or homologous recombination (HR). The latter can be guided by the presence of a donor DNA molecule with homology to the cutting site, allowing precise replacement of a DNA region.

All of the core CRISPR methods for genome engineering in bacteria are based on the ability of CRISPR-Cas systems to produce a site-specific DSB that can be repaired by the cell's own machinery. These advanced methods have enabled CRISPR-assisted genome editing and screening in virtually all model or synthetic bacteria, always based on the natural role of these systems. However, CRISPR-Cas is used merely as a reagent to induce targeted mutations and does not induce new target sequences. [49, 50, 51, 52]

# Chapter - 4

## Integration of CRISPR Technology with Bacteriophages

### Rationale for CRISPR-Engineered Phages

Despite their clinical potential, phage therapy remains largely underexploited due to safety and specificity concerns. Infection by lytic bacteriophages typically leads to host cell lysis, a process that may generate inflammatory disorder. A major challenge is the absence of effective countermeasures against bacterial resistance development during therapy as well as the horizontal gene transfer of resistance plasmids between non-target bacteria. CRISPR-Cas, a bacterial adaptive immune system, has been successfully applied to construct specialized CRISPR-guided bacteria to silence virulence genes or to target and degrade even non-replicating DNA sequences. CRISPR decline in bacteria, indeed, have been found to disrupt the recognition mechanism of virulent phage immunity.

The innovation presented follows a pioneering proposal to incorporate CRISPR-Cas into engineered bacteriophages, thereby enhancing the specificity and safety of phage therapy against priority drug-resistant pathogens. The approach addresses therapeutic aspects by coupling CRISPR technology with bacteriophage therapy to provide a novel means of selective bacterial eradication or chromosomal mutation, aiming to improve therapeutic potential while reducing undesired side effects and the emergence of resistance. [19, 53, 54, 55]

## Methods for CRISPR Incorporation into Phages

Components of the CRISPR-Cas system can be introduced into phages by various established methods. Phage genome sequences can be used to facilitate homologous recombination and insert the components into plasmids that can then be transduced into phages. Bacillus subtilis can be employed for the packaging of CRISPR-Cas-encoded plasmids into phages using a virulence plasmid for which a CRISPR-Cas system has been removed. A simpler, faster, and more efficient CRISPR-Cas incorporation method is to co-introduce a phage genome (containing CRISPR-Cas) and a helper plasmid (encoding the needed proteins) into the same bacterial host. These methods have been used to incorporate type I CRISPR-Cas systems into Streptococcus thermophilus phages targeting the S. thermophilus strain, SP-291, and type II-C systems into the virulent phage, PG-1, of Listeria monocytogenes.

Self-replicating helper plasmids harboring a type III-A CRISPR-Cas system can also be generated. The replication origin is based on the plasmid pHY300PLK-P15A_RR, and the promoter P45 S allows invincible accumulation of Cas proteins in optimal expression conditions. Helper plasmid-derived PhEtaIs and PhEtaIIs with Cas9 can also be constructed as pNCR-PhaG and pNCR-PhaBLN vectors, respectively. To minimize production costs of DNA sets for customizing trans-complementation systems, another helper plasmid without a T7 promoter cannot replicate in BlueScript but can do so in BL21 (DE3)pLysS. The helper plasmids guarantee high yields of any DCRC-Cas system, support in vivo application, and provide a useful tool for studying the trans-complementation of DCRCs in bacteria. [56, 57, 58, 59]

## Design of CRISPR-Guided Antibacterial Phages

Phages equipped with CRISPR-Cas systems that recognize and cleave specific sites in target bacterial genomes would

constitute personalized antimicrobial agents capable of selective bacterial killing with reduced off-target effects. Development of a CRISPR-guided phage-oriented antibacterial strategy consists of three strategic steps: (1) design and construction of an appropriate CRISPR-Cas system targeting the strain of interest; (2) genetic engineering of the phage to incorporate the newly designed CRISPR-Cas system; and (3) determination of the biological properties of the engineered phage.

The first step involves selecting an appropriate guide RNA targeting a crucial region in the pathogenic strain and introducing the guide into the resident CRISPR locus. Careful selection of potential targets is important since phages are usually applied just once or a few times during therapy and the aim is to avoid an emerging resistance phenotype during the therapy data analysis with respect to existing CRISPR-Cas or other defense systems of the bacterium. The rationale of the second step is to modify the surrogate phage for re-targeting the pathogenic bacterium. The third step addresses not only confirmation of the basic biological properties of the engineered phage but also provides a comprehensive description of its potential antibacterial properties including specific killing of the pathogenic strain, reduced collateral damage to the natural phage microbiota, diminished occurrence of resistant mutants, successful combating of CRISPR-Cas defenses and extra-bacterial survival during interaction with the immune system. [60, 61, 62, 63]

**Advantages Over Conventional Phage Therapy**

Systematic bacterial resistance development during phage therapy diminishes its therapeutic efficiency and precludes its application in certain cases. CRISPR technology has emerged as a well-established bacterial genome editing tool and may be harnessed to enhance the specificity and efficacy of phage therapy by turning simple phages into powerful CRISPR-guilded

antibiotics. Such CRISPR-based antiviral phages can cleave resistant plasmids, antibiotic-resistance genes, genes encoding virulence factors, biofilm-associated genes, and others. These modifications can reduce host-pathogen interactions, restrict biofilm formation, and even minimize potential side effects of phage therapy, thereby making it safer. By enabling selective targeting of a particular bacterial strain, especially in complex mixed-infection environments, the CRISPR-phage system serves as a promising tool to design multiplexed CRISPR-phage libraries that can effectively and accurately kill a variety of drug-resistant bacteria in resistance-dominated surroundings while preventing the rebound of antibiotic-resistant bacteria during treatment.

The specificity of CRISPR-phages, CRISPR exchange and utilization protocols, and multiplex design afford a novel, safe, and efficient way of combating multidrug-resistant (MDR) pathogens. Collateral damage to the microbiota may be minimized, reducing the potential side effects of phage therapy. Such phages also have increased potential for preventing the emergence of phage-resistant bacteria and diminishing horizontal gene transfer of resistance plasmids. Moreover, the new platforms can reduce the expression of biofilm-formation-related genes and offer more feasible solutions to long-lasting biofilm-associated infections, especially at infected sites in patients with long-term implants or indwelling devices. [64, 65, 66, 67]

# Chapter - 5

## Mechanisms of CRISPR-Modified Phage Antibacterial Action

### Targeted Cleavage of Resistance Genes

Disabling resistance genes in antibiotics targets, such as pumps, modifying enzymes, and degrading enzymes, via CRISPR constructs embedded in phages, enables their more effective use. In addition, CRISPR-mediated targeting of other genes, such as those encoding essential maintenance of homeostasis, metabolism, and virulence systems, likewise hastens death.

Developments in CRISPR gene-editing technologies might better address many problems currently confronting antibiotic therapy. For instance, approaches for exploiting therapeutic potential for membrane-bounded surface molecules in Gram-negative bacteria, such as efflux pumps, which preclude entry, or biocidal azoles, which kill by entering the cell and subsequently damaging nucleic acid integrity through oxidation, might be more accurately exploited. The use of these biocides for biofilm-disease treatment could be impaired by the presence of either degrading enzymes or 4-amino-4-deoxy-L-arabinose biosynthesis within the targeted pathogen.

Therapeutic development aiming to mitigate known points of resistance to these antibiotics is limited, mainly through either plasmid segregation by loss at cell division within the infected microbial population, or through CRISPR-mediated cleavage and foraging of genes coding either resistance-modifying

families (such as the aminoglycoside-modifying) or degrading enzymes within the potential pathogen. These editing targets enable the reestablishment of metabolism in the presence of the previously lethal antibiotic. [68, 69, 70, 71]

**Disruption of Essential Bacterial Pathways**

CRISPR-engineered phages can compromise essential bacterial pathways to enhance therapeutic effects. Such targeting should induce significant antibacterial action while minimizing off-target effects within the microbial community. Metabolic pathways that are universally conserved and highly essential for bacterial growth and biofilm formation are particularly promising.

Bacterial metabolism is orchestrated by a limited number of fundamental metabolic pathways essential for all bacteria. Depletion of any of these pathways can severely compromise growth in vitro and in vivo, offering a potent target for antimicrobial development. Moreover, targeting such pathways might circumvent off-target effects seen in antibiotic therapy, as inhibition of non-essential pathways affects only part of the microbial community while non-pathogenic members of the community may also carry the affected target. Targeting genome libraries containing genes encoding essential metabolic pathways may thus stimulate activation of other genes coding for alternative enzymes in the remaining active bacteria, favouring restoration of community function without development of drug tolerance or cross-resistance. Nonetheless, metabolic pathways implicated in virulence factor production are not suitable goals as inhibition would reduce resistance against predation and shorted survival chances.

Pseudomonas aeruginosa is metabolically versatile and able to use a plethora of carbon sources. The TCA cycle is essential for survival during infection, biofilm formation and

pathogenicity. Deletion of just one of the four subunits of the pycA-encoded pyruvate carboxylase prevents growth on specific carbon sources such as L-lysine while deletion of all four genes renders cells unable to grow on any other source and is lethal, making pycA an attractive target for CRISPR-phage therapy. Similarly, the galT- and galK-encoded galactose PTS subsystem modulates galactose uptake and utilization and is repressed when P. aeruginosa utilizes other carbon sources. Substantial reduction of the activity of this subsystem would thus shorten the lifespan of the local strain while retaining viability during out-of-pathogen community interactions. [72, 73, 74, 75]

**Selective Killing of MDR Bacterial Strains**

Phages are generally able to infect and kill only a narrow spectrum of bacterial strains or species. Although this specificity is an advantage for inhibiting commensal and environmental flora, it can also reduce therapeutic efficacy in vivo, particularly concerning the unintended collateral damage of commensals and the associated selection pressure for warning escape mutants. For CRISPR-Cas-engineered phages, this limitation can be mitigated by targeting the phage's predicted host. Moreover, CRISPR-Cas technology has been eficazly used to cleave the gene responsible for the crp expression in the derepression region of different… bacterial species related to the Allopathogenicity Clusters I, II, and III of strains containing phenotypic and genotypic characteristics.

MDR bacterial species tend to evolve heterogeneous resistomes. Consequently, in a heterogeneous MDR population, only some of the strains express the target genes. It thus seemed logical to design a CRISPR-Cas-engineered phage to selectively interfere with the antibiotic-targeting gene that is only present in a subpopulation. Such an approach was used to knock down, in a targeted manner, the gene for the AcrAB-TolC efflux system,

which is responsible for removing several classes of antibiotics in enterobacteria, thereby enhancing sensitivity to fluoroquinolones and β-lactams. [76, 77, 78, 79]

**Reduction of Horizontal Gene Transfer**

CRISPR-based phage engineering may further reduce the spread of multidrug resistance within bacterial populations. Resistance genes frequently reside on mobile genetic elements such as plasmids, enabling their transmission between species through horizontal gene transfer (HGT). One strategy in CRISPR–phage design seeks to cleave such resistance plasmids, reinstating sensitivity in clinical isolates and making co-infection readily exploitable. Alternatively, targeting the type IV secretion system in conjugative plasmids diminishes transfer frequency without loss of recipient susceptibility. Pathways involved in natural competence and transformation, such as the transformasome and type-IX secretion, constitute additional avenues to reduce HGT.

A distinct avenue of CRISPR design incorporates plasmid-targeting gRNAs. Most plasmids harbor replication origins with high copy number, facilitating design. Within the proposed testing framework, bacteria bearing CRISPR–phages engineered with such gRNAs serve as donors to plasmid-carrying acceptors, driving the expected selective response. Co-infection with a secondary phage capable of natural frequency lysis concurrently circumvents early-stage phage resistance. [80, 81, 82, 83]

# Chapter - 6

## Phage Engineering and Synthetic Biology Approaches

### Genetic Engineering Techniques for Phages

Genetic engineering techniques have helped advance the scientific understanding of bacteriophage biology and created new therapeutic applications. Early genetic studies revealed that plasmids can be stably introduced into the genomes of temperate phages, confirming the essential role of homologous recombination in the life cycle of those phages. These observations opened the door to the design of alternatives to antibiotic treatment based on the therapeutic use of bacteriophage-directed plasmids. However, natural phage vectors with these specific properties are rarely discovered, raising concern about whether the standard bacteriophage life cycle allows stable incorporation of a foreign plasmid with a therapeutic functionality. However, natural phage vectors with these specific properties are rarely discovered, raising concern about whether the standard bacteriophage life cycle allows stable incorporation of a foreign plasmid with a therapeutic functionality. More recently, the combination of de novo synthesis with ancient in vitro assembly techniques has led to an impressive scalability in the production of all possible nucleotide sequences, contributing to the reverse genetic analysis of the T7 genome. These pioneering studies expert in bacteriophages by homologous recombination in laboratory strains now developed in more pathogenic species.

In virulent bacteriophages, the phage genome is passed on as an orderly memory of all previously encountered bacterial antibiotic resistance markers. The wide range of transduction systems and currently available CRISPR-Cas tools are taking CRISPR-Cas incorporation into phage genomes to a new level of efficiency. Bacteriophage engineering. Efficient editing schemes for de novo genome design of the T7-like bacteriophage have also been implemented. United and separated sources of inspiration have opened the way for CRISPR-modified bacteriophage phage therapy. The recent development of a large battery of CRISPRs and associated proteins responding to different kinds of biological threats in multiple environments, the corresponding CRISPR-Cas tools for editing the genomes of any organism, and the impressive capacity of bacteriophages to program natural DNA-degrading systems in bacteria, together with established bacteriophage signalling systems, control bacteriophage metabolism and establish new virtuous cycles by removing undesirable plasmids, deleting drug-efflux pumps or inactivating virulence factors in the host. [84, 85, 86, 87]

**Synthetic Phage Genome Design**

Synthetic phage genomes can be generated with a modular design, allowing easy tailoring for precise targeting of bacteria. Such synthetic viruses can be assembled de novo or by Charon-based methods involving Becker and co-workers' system. Phage genomes are composed of four to six essential DNA modules that encode the proteins required for the phage to replicate inside its host. Additional modules can, however, be inserted or removed as required by practical considerations. The essential parts of a single phage can even be mixed with accessory modules from distinct strains or species to create a phage mutant with a larger host range. This modular design strategy allows the assembly of novel phages by robotically mixing short ssDNA.

Modularizing phage genomes also facilitates the incorporation of safety features to minimize potential risks to health or the environment. An anti-CRISPR gene located downstream of the phage promoter that drives the expression of all early genes ensures that phage genes related to the evasion or modulations of the immune response of the bacterial host are not actively expressed during the initial infection cycle. It also guarantees that subsequent replication cycles in a non-immune host would restore the native immune system of the phage. Another safety assessment consists in incorporating the sequences of putative CRISPR-Cas anti-phage pathways present in the target bacterial host. Such pathways can guide the incorporation of anti-CRISPR genes into the phage genome located outside the essential genes, with these genes being operational only in immune bacteria. [88, 89, 90, 91]

**Optimization of Phage Stability and Activity**

To attain therapeutic concentrations, a phage preparation must withstand the rigors of manufacture, transport, and storage. Currently, phages are mainly produced in bacterial hosts, with titers in the range of $10^4$ –$10^6$ pfu/ml, often requiring a multiple-log scale-up for effective use. Attempts to increase titers through growth enrichment, continuous culture, or two-phase schemes have had only limited effects. Furthermore, phages with either lytic or lysogenic life cycles are known to be sensitive to chemicals, thermal shocks, and desiccation. Several other factors, including RNA packages and lipid multilayers, have also been shown to enhance phage stability. Reconstitution with certain protective agents has been used to increase phage stability against time and temperature. The common protective agents are milk protein, a combination of trehalose and bovine lactoferrin (LF), a mixture of cryoprotectants (dimethyl sulfoxide, PEG 200D, and L-arginine), and protective microspheres. Considerable interest exists in using solid formulations of phage in combination with antifungal agents. [92, 93, 94]

Phages that are stable in a solid state at ambient temperature for months are attractive candidates for development into commercial products. Recent investigations have demonstrated that chicken egg yolk powder (CEYP) achieves high and prolonged stability of phage TK-HR7 at ambient conditions and that combining CEYP with formic acid generates a solid formulation suitable for the long-term storage of a variety of phages. Development of such formulations is of interest not only for external use but also for preparing phage products that are adapted for internal delivery regimes. Improving the ability of phages to endure the activity of endogenous and exogenous peptidases would also be of value.

**Safety Engineering and Genetic Containment**

A variety of approaches encompassing safety engineering, genetic containment, and restrictive therapeutic designs have been implemented to increase the biosafety of phages and minimize the possibility of their uncontrolled spread and use. Synthetic recombinase-assisted genome engineering strategies are being pursued to introduce an antibiotic-resistance marker generating a selective growth advantage under particular conditions, and the CRISPR–Cas system is being adapted to precisely excise biologically active phages from the DNA of the lysogenic bacterial host. Along a different line, genome-integrated anti-CRISPR systems that prevent natural or engineered CRISPR/Cas-based immunity against phages are being considered. Phages with reduced biosafety concerns can also be engineered by designing specialized phage preparations modifying genes associated with either pathogenicity or overall virulence, such as those involved in biofilm formation, invasion or adhesion, toxin production, and so forth. Alternatively, undesired phage activity may be curtailed by targeting genes critical for horizontal transfer of antibiotic-resistance plasmids.

Phages can be engineered to be effective in chronically infected patients or in patients suffering from device-associated infections by targeting biofilm-formation or maintenance genes associated with the causative pathogen, since such target genes are frequently shared by different strains. In addition to bacteria, the innate and adaptive response of the immune system to phages has been extensively studied, with particular focus on the formation of neutralizing antibodies, their timing, and host resistance/susceptibility to phage treatment. Specific CRISPR–Cas-modified phages may also elicit an immune response and be duly recognized, potentially limiting therapeutic efficiency. Phage-encoded antigens can induce immune activation, enhance disease prognosis, and improve protection against subsequent infections. Thus, dosing schemes can be designed based on neutralizing effects to ensure therapeutic efficiency, or immune system activation can be harnessed to enhance treatment outcome. [95, 96, 97, 98]

# Chapter - 7

## Targeting Antibiotic Resistance Determinants

### CRISPR-Mediated Removal of Resistance Plasmids

The most effective way to restore antibiotic susceptibility is to prevent pathogens from actively evading antibiotic action. One mechanism by which antibiotic resistance is conferred is the presence of plasmids that carry resistance genes; these can often be eliminated, restoring the bacterial strain to antibiotic-sensitive status. Within one organism, many resistance genes may be present on a single plasmid or on different plasmids, which further enhances the survival of the resistant strain. Furthermore, plasmids may sometimes also carry virulence factors as well as genes for biofilm formation, enabling rapid and constant recapture of the resistant phenotype once antibiotic therapy is discontinued. Consequently, the CRISPRi approach can be employed to target and silence virulence-associated genes present within the plasmid.

Targeting and killing the plasmid without completely destroying the bacterial cell may present a complex and riskier endeavor, hindering the further release of resistance genes into the surrounding environment. The normal metabolic functions of the bacteria must not be severely impaired or completely cut off, as this would initiate a dormancy phase, stopping bacterial growth altogether. Silencing essential genes of the plasmid—those required only for replication—as opposed to completely destroying it eliminates risk and prevents the re-emergence of resistance genes into the environment until the enzyme-mediated

escape mechanism takes place, which is a more time-efficient way to restore antibiotic susceptibility. [99, 100, 101, 102]

**Targeting Efflux Pumps and Resistance Enzymes**

Resistance-nodulation-cell division (RND) efflux pumps constitute an increasingly important class of resistance mechanisms. Expressed in many Gram-negative bacteria, RND systems actively extrude a wide variety of compounds, including antibiotics, biocide agents, and host toxins. Increased expression of these pumps is frequently associated with a MDR phenotype. Several known RND pump genes can be targeted by CRISPR technology or an RND-resistant approach, thereby increasing susceptibility to selected drugs. Equivalent moves in Gram-positive bacteria are less well developed but also possible.

In addition to various RND pumps, a wide variety of other resistance genes—e.g. those associated with aminoglycoside-modifying enzymes, β-lactamases with extended-spectrum activity, or phosphonoacetate hydrolases—have been characterized. Identification of the associated modifying activities is important, since it allows appropriate interference using CRISPR technology. Aminoglycoside-modifying enzymes, including N-acetyltransferases, phosphotransferases, and nucleotidyltransferases, are frequently detected in Enterobacteriaceae in association with aminoglycoside resistance. These transferable resistance genes allow the resistance genes to spread rapidly among various pathogenic species. Resistance genes are usually carried on plasmids; therefore, they may be removed while maintaining the original activity of the mainland antibiotic. [103, 104, 105, 103, 104, 105, 106]

**Silencing Virulence and Biofilm-Associated Genes**

The virulence of bacterial pathogens hinges on numerous factors, notably toxins that inflict damage on host cells and impose metabolic burdens on the bacteria themselves. Moreover,

the initiation of biofilm formation is a major source of pathogenicity in numerous bacterial genera, with N-acyl homoserine lactones (AHLs) functioning as critical signaling molecules. Biofilms serve as protective enclosures for the bacterial cells within, and these shields are exceptionally tolerant toward not only innate and adaptive immunity but also most bacteriophages and antibiotics. Gene repression is a prominent, well-characterized CRISPR-Cas application, and several studies have focused on the targeted silencing of AHL synthases and other crucial genes in order to curtail virulence and biofilm formation by diverse pathogenic bacteria.

Such strategies are expected to enhance therapeutic efficacy during treatment of chronic infections, particularly those associated with medical implants, as well as those arising from primary biofilm formation. The CRISPR-Cas component responsible for gene silencing consists of a 30-nt RNA guide paired with a complementary 18-nt sequence in the target mRNA, as well as an RNA-binding protein bearing an effector domain that cleaves the target RNA upon recognition of the guiding sequence. Several genes implicated in pivotal early steps of biofilm formation generally are conserved across various bacterial genera, are conveniently located at the top of the regulatory hierarchy, and therefore present promising targets for repression. [107, 108, 109, 110]

**Preventing Resistance Re-Emergence**

Mathematical models predict that bacteria will develop resistance to any phage therapy within days. In response, design diversity and adaptive testing of phage libraries can minimize the risk of resistance emergence. Design diversity is particularly critical for treatment options with a high selective pressure that target single genes or pathways. Pathogens can also be matched to the most efficacious treatment from a database of CRISPR-

modified phages specifically designed to target them. Such personalized therapy has already been enabled with other drugs, and it naturally follows from the pre-approved design of CRISPR-Cas systems. Additional predictive models have been developed to direct the design of phage libraries that exploit multiple areas of genetic diversity, thereby decreasing the probability of emerging resistant bacterial populations.

In a typical design, guide RNAs targeting different genes are listed, and viruses harboring those diverse guides are pooled for broad-range application on environmental isolates. In a pooled library, there remains the possibility that a resistant bacterium might gain survival advantage over the others; this is avoided by sequencing the gRNA from any bacterium allowing phage growth and then creating phages specifically targeting that resistant strain. Such an adaptive capture strategy closes the loop: when the pathogen goes rogue, the designer responds with the most efficient targeted weapon. Combined with Design Diversity, Adaptive Capture provides a new level of security, whether or not the trait of interest is represented in the library. The proposed approach could even make use of an extra layer of Adaptive Capture at the natural epidemiological scale: the response follows the logging of resistant strains into a high-risk ephemeris, where their frequency draws the attention of therapeutic developers. [111, 112, 113, 114]

# Chapter - 8

## CRISPR-Phage Therapy Against Biofilm-Forming Bacteria

### Biofilm Biology and Clinical Challenges

Biofilms form natural microbial communities, and their spatial arrangement is often described as multidimensional structures consisting of clustered cells. Biofilm-related infections are generally chronic and are frequently associated with indwelling medical devices. Within biofilms, microbial cells can demonstrate increased tolerance to antibiotics due to a variety of factors, including compromised penetration, diminished metabolic activity, cellular dormancy, cell density, and various factors secreted in the biofilm matrix. Biofilms in human infections typically formed on solid substrates such as tissues or implanted devices, and barriers impeded treatment success. Gene-delivery vectors targeting biofilm-associated genes or pathways of a wide range of bacterial pathogens have been designed. The approach involved CRISPR–Cas systems to regulate the expression of essential genes or degrade biofilm-related genes in the host bacteria.

Biofilm infections are common in patients suffering from burns, chronic lung infections or indwelling devices such as central lines, an endotracheal tube, prosthetic joints, or implantable electronic devices. Pathogens associated with biofilm infections include Pseudomonas aeruginosa, Staphylococcus aureus, and Enterococcus faecalis. Within a biofilm, the cells reside in a matrix composed of polysaccharides,

proteins, and eDNA and show little movement among each other. Bacteria in biofilms are more resistant to antibiotics than their planktonic counterparts, possibly due to the poor penetration of the drugs into the structure, exhaustion of nutrients in deeper channels, or the presence of specialized persister cells. Biofilms are also associated with chronic infections because host defenses are often unable to eradicate them. Planktonic bacteria may experience a receding population, but the biofilm forms a reservoir for recurrent infections, with infected individuals requiring a lifelong cycle of antibiotic therapies. [115, 116, 117, 118]

**Phage Penetration and Biofilm Disruption**

Biofilms are surface-attached communities embedded in an extracellular matrix that encase bacterial cells. They represent a form of bacterial growth characterized by a decrease in metabolic activity. Within biofilms, some cells enter a viable but non-culturable (VBNC) state of growth and become tolerant to a wide range of antimicrobials. Biofilms have been implicated in a range of persistent infections, for cases in which pathogen clearance relies on the host immune response, as well as in device-associated infections. The thick and enveloping biofilm matrix serves as a barrier to the diffusion of many factors, and bacteria deeper within the biofilm can be well protected from the action of secreted virulence and/or aggressive factor(s) from the same pathogen or from other competing pathogenic bacteria. Engineered CRISPR-phages can be designed to smash biofilms via three main paths: (1) facilitating efficient penetration (e.g., production of phage enzymes and factors that degrade or alter the composition of the biofilm matrix), (2) encoding biofilm disassembly-promoting components (e.g., lysis enzymes, anti-adhesive factors, and biofilm dispersal signals), and (3) targeting biofilm-associated genes (e.g., genes that mediate biofilm formation and biosynthesis of CVI and CviR autoinducers) or other genes that trigger biofilm disassembly.

Biofilms generally grow on inert or living surfaces, and biofilm formation is a major cause of chronic, persistent, and recurrent infections. Major microbial pathogens such as Pseudomonas aeruginosa, Staphylococcus aureus, Streptococcus pneumoniae, and Escherichia coli can form biofilms on anatomical device surfaces (such as catheters, prosthetic valves, and prosthetic joints) or host tissues (such as chronic wounds, lungs of cystic fibrosis, and the upper respiratory tract). Such infections are much harder to treat than acute infections, as the biofilms are generally resistant to treatment with antibiotics and disinfectants. Drug regimens may fail to eliminate the biofilms, ultimately resulting in the induction of an escalated host immune response. Because biofilm-residing bacteria are in the VBNC state and express specific sets of genes, they are highly tolerant to many types of antibiotics despite the high minimal inhibitory concentrations (MICs) exhibited by planktonic bacteria. [119, 107, 120, 121]

**CRISPR-Based Targeting of Biofilm Genes**

Biofilms are organized clusters of bacteria embedded within a self-produced extracellular matrix (ECM). The ECM is primarily composed of sugars, proteins, and nucleic acids (eDNA), the synthesis and maintenance of which require the concerted action of multiple genes. Biofilm formation inevitably gives rise to spatial and temporal heterogeneity in the biochemical milieu, leading to altered gene expression and an associated physiological response. Environmental conditions such as pH, temperature, and nutrient availability influence the regulatory networks that control biofilm adhesion and matrix production, process that is governed by several genes of which the matrix-associated polysaccharides, eDNA, and polysaccharides-degrading enzymes are the most important. Mutants affected in these processes are generally less virulent.

Biofilms are frequently associated with prosthetic devices and account for a significant proportion of chronic and recurrent infections. Phage infection can cause biofilm dispersion due to several mechanisms. Current knowledge on the molecular basis of adhesion and matrix production is sufficient to guide the identification of the corresponding genes, facilitating their targeted silencing. Disabling such genes will result in only mild to moderate phenotypic changes, thus rendering the engineered phages more likely to successfully complete their lytic cycle. [95, 122, 107, 96]

**Applications in Chronic and Device-Associated Infections**

Phages are particularly suited to chronic and device-associated infections, where standard antibiotic treatment efficacy is often diminished or non-existent due to pharmacokinetic factors, immune deficiency, or nutrient depletion. In these cases, an authentic CRISPR-phage therapy is expected to be successful in managing specific and safe infections. Special emphasis is given to biofilm-associated infections and those in which host factors impair access of standard therapy.

Phage-encoded genes contribute to Mycobacterium tuberculosis pathogenesis, and several studies have reported on the role of mycobacterial genes in host–phage interactions. Genes required for biofilm formation, adherence to substrates, and matrix formation by Pseudomonas aeruginosa, Klebsiella pneumoniae, Staphylococcus aureus, and Salmonella enterica have been identified and targeted by applying bioinformatics. In chronic infections involving biofilm producers, successful therapy requires targeting of biofilm genes in addition to the standard CRISPR-Cas phage-cleavage strategy. Essays have also explored the possibility of applying a CRISPR-phage combination to chronic infections associated with implanted

medical devices. For these infections, the standard therapeutic approach is surgical removal of the device, but when that fails, focused, specific treatment offers the best outcome. [123, 107, 124, 119]

# Chapter - 9

## Host–Immune System Interactions

### Innate and Adaptive Immune Responses to Phages

The impact of phage therapy may be curtailed by either innate or adaptive immune responses. Like other microorganisms, phages are vulnerable to innate immune recognition, which can neutralize their therapeutic effect, while prior exposure can lead to specific immunogenicity that limits their protection. Phage-based vaccines based around the capsid could protect against lytic phases of the phage, either displayed on killed bacteria or delivered as virus-like particles (VLPs) coated with phage capsid proteins. Non-encapsulated phages may face neutralization by immunoglobulin-like proteins, while secretory IgA appears critical for control of enteric phage infections. With repeated doses in mice, neutralizing antibodies diminish and even low doses of the same phage can protect mice from lethal doses. As such, innate recognition of non-eukaryotic viruses may be a trade-off in the great antagonistic co-evolution between phages and their bacterial hosts.

While phage infusion is not traditionally considered immunosuppressive, intravenous anti-viral IgG from several human donors can augment the severity of Staphylococcus aureus infections and Ebola virus outbreaks, and pre-existing humoral immunity against phages may impair the effects of intravenous phage therapy for bacterial sepsis in critical mouse models. Closing the immune and viral load gaps may restore efficacy, and the parasite-host paradigm suggests that a small

viral load may suppress the disease, while a large viral load may enhance disease severity. Modifying phages with non-reducing glucose or galactose hydrophilic head-attached moieties can reduce recognition by the innate immune system without compromising the bacterial host's immune ability. Anti-phage IgG in the rectal mucosa impairs the protective benefit of phage therapy. Balancing immunogenicity against therapeutic efficacy may therefore influence phage therapy design. [35, 125, 126, 127]

**Immunogenicity of CRISPR-Modified Phages**

Bacteriophages are increasingly being viewed as a promising treatment strategy for bacterial infections, including systemic, local, and device-associated infections. However, they elicit innate and adaptive immune responses that may limit their utility, particularly after repeated administration or in immunocompromised patients. The viability of CRISPR-modified phages, which are designed to target specific strains or groups of multidrug-resistant (MDR) bacteria for clearance, is therefore critically dependent on their immunogenicity.

Characterizing the immune recognition of CRISPR-modified phages and determining the factors influencing their potential immunogenicity are essential prerequisites for their therapeutic use. Bioinformatics analyses are being performed to identify putative immunogenic epitopes within the engineered phage sequence. Key parameters affecting humoral immune recognition, such as exposure of immunogens, phage structure and quantity, and dose interval, should be assessed to optimize their pharmacological properties. Evaluation of structure–activity relationships will further clarify aspects contributing to immunity induction, potentially enabling re-engineering of the viral capsid to improve tolerability. [128, 129, 18, 130]

## Strategies to Avoid Immune Neutralization

Immune neutralization of therapeutic phages can limit their beneficial effects. A complex immune response to phages develops rapidly on exposure, leading to their clearance via hepatic and splenic macrophages. Non-enveloped phages inducing strong innate immune signaling can provoke adaptive immunity, further curtailing their efficacy. Nevertheless, it appears that the phage-mediated stimulation of adaptive immunity could be beneficial if not too strong, since patients with active phagolysosomal tuberculosis have much higher levels of pathogen-neutralizing antibody responses than healthy individuals. Examples of phage therapy administering non-neutralized phages that still cleared infection and reduced mortality exist, even in combination with systemic phage and anti-mycobacterium drugs.

Phage dose is an obvious strategy to mitigate immune recognition, but such approaches may not be suitable for all combinations—combining phages with anti-tubercular drugs seems likely to require a non-neutralizing phage dose. Encapsulation, for example within liposomes or hydrogels, can reduce antibody contact of phages and streptavidin biotin conjugates are being used to sequester the phage from the immune system. Natural variations in capsid composition affect immune recognition, such as the cross-species immunogenicity of the capsid protein of the Mu phage. Phage glycosylation, quorum quencher production, membrane stabilization and CpG motif deletion are also being investigated. [131, 132, 133, 134]

## Balancing Immune Activation and Therapeutic Efficacy

Balancing immune activation and therapeutic efficacy is an important consideration for phage therapies, particularly when they are designed to incorporate CRISPR systems. Immune responses to phage treatment involve not only humoral activity

that can neutralize the phages but also inflammatory responses that could potentially aid clearance of bacterial infections. The immune response and the therapeutic efficacy of CRISPR-phage treatment may therefore not be strictly correlated, and finding an optimal balance could be crucial to maximizing the therapeutic potential of CRISPR-phage treatment.

Under certain conditions, the stimulation of an antibody response may improve clearance of bacteria. For example, in a transcriptomic analysis of phage-infected mice, the "phagosome pathway" was upregulated, suggesting that phages may prime the host for clearance of a bacterial infection through phagocytosis. When applied sequentially after antibiotics, phages enhanced the activation of various immune pathways involved in regulating inflammation, leukocyte migration, and phagosome formation, providing the basis for their use in clearing residual infection. Similarly, application of P. aeruginosa bacteriophages after treatment with colistin-enhanced inflammation-associated pathways related to the defence response against infection. Activation of these pathways can also shorten the infection period in a CD-1 mouse model of pulmonary infection. Therefore, doses of CRISPR-phages at levels that induce immunogenicity and modest inflammation may improve their therapeutic efficacy, particularly in combination with antibiotics, by aiding resolution of the infection. However, the extent of inflammation should not exceed a threshold. [135, 35, 136, 137]

# Chapter - 10

## Preclinical Evaluation and Experimental Models

### In Vitro Assessment of CRISPR-Phage Activity

CRISPR-phage antibacterial activity should first be evaluated in vitro, quantifying potency, specificity, and safety. Potency testing should compare CRISPR-phage titers at corresponding multidrug-resistant bacterial concentrations in selective media. Multidrug-resistant bacteria should remain susceptible to the CRISPR-phage, while susceptible bacteria remain resistant. Any toxicity towards the host in cell-culture assays should be properly addressed.

The pharmacokinetics of CRISPR-modified phages requires detailed study prior to use in animal models. Studies should assess CRISPR-phage biodistribution, metabolism, and clearance in healthy animal models, along with the dose–response curve for desired antibiotic effects. Repeated dosing should evaluate the potential development of neutralizing antibodies against CRISPR-associated proteins, explore host immunity, and yield data on safety and biosafety.

Therapeutic application depends on the targeted bacteria, infection types, underlying conditions, development of local or systemic regimens, and immune tolerance. Remaining technology limitations necessitate additional pharmacological study before clinical application. Target delivery should follow the consequences of pathological conditions for biodistribution, particularly in systemic therapy. Treatment sequencing can also impact therapeutic efficacy, particularly when combined with

antibiotics, so temporal design should ensure synergy and avoid antagonism. [138, 139, 140, 141]

**Animal Models for Phage Therapy**

Equivalent animal models for human infection are essential for testing CRISPR-modified phages prior to clinical trial. Models should mimic the route, site of infection, risk factors, and pathophysiology encountered in human disease. Common preclinical models for systemic and localized infections include animal models of bacteremia, pyemia, pneumonia, nephritis, meningitis, and enteritis. The optimal design of any animal study requires careful consideration of multiple factors, including the size, age, underlying conditions, strain characteristics, inoculum concentration and volume, and the presence of a co-infection or a foreign body, such as an implant or a catheter. Moreover, any consideration of treatment with phages must include assessment of their pharmacokinetics and pharmacodynamics.

Phage activity in animals may be hindered by serum and tissue factors, including neutralizing antibodies, complement, mucus and glycosaminoglycans. Investigations into the potential deposition of phages in tissues, circulation half-life, biodistribution, and mechanisms of clearance from mammals are essential to establish PK and PD relationships. Toxicity studies are fundamental for demonstrating product safety prior to any regulatory approval.

A growing number of in vitro and in vivo studies are supporting the therapeutic use of phage therapy for classical indications, such as localized mucosal infections, localized invasive infections and as an adjunct treatment in bacteremic patients and patients with other severe systemic disease. Phage therapy could also be extended to other disease settings, such as intestinal disease and chronic infections in immunocompetent patients, as well as to more exotic mammals. [142, 143, 144, 145]

## Pharmacokinetics and Pharmacodynamics

Pharmacokinetics and pharmacodynamics are vital components of any therapeutic intervention, influencing its route, formulation, dosage, and treatment regimen. For phages, distribution and clearance are particularly important because of their relatively large size and the consequent restrictions they impose on penetration of healthy tissues; these factors also affect the extent to which they reach the sites of infection and mediate their therapeutic effects. The interactions of CRISPR-guided phages with the pharmacokinetic and pharmacodynamic properties of these systems in vivo—together with absorption, distribution, metabolism, and excretion—are therefore essential for effective therapeutic use. Their exploration in suitable in vivo models is important for understanding therapeutic outcomes and the adaptation of these modalities for clinical use.

Pharmacokinetics involves the study of the absorption, distribution, metabolism, and excretion of a substance, while pharmacodynamics examines the relationship between the concentration of the substance at the site of action and the resulting effect. It is crucial to define the pharmacokinetics and pharmacodynamics of any therapeutic agent to tailor appropriate route and staging procedures. The large size of phages induces additional constraints on healthy-tissue penetration, distribution, and abundance at the infection site, and a combination of these factors—along with the phage-specific variations in their essential properties—determines their activity, specificity, and safety. The pharmacokinetic profiles of CRISPR-engineered phages are not yet fully explored, but these aspects are important for effective therapeutic application in vivo. [88, 146, 128, 147]

## Toxicity and Biosafety Studies

Before CRISPR-engineered phages can enter clinical trials, their toxicity and biosafety must be evaluated. Suitable animal

models should be considered, assessing systemic administration and localized application for tissue-specific pathologies. Pathology examinations can provide insight into local and systemic toxicity. Further biosafety screening is mandatory for potential regulatory approvals.

The preparations should undergo comprehensive biosafety studies to assess their safety profiles and approval for use in humans. These studies should include acute toxicity assessments in at least one animal model, with LC50 values exceeding any relevant therapeutic dose. General well-being, body weight, food and water consumption, and gross necropsy of major organs can support evaluations of in vivo safety and toxicity. Additionally, repeated-dose toxicity evaluations should include a minimum of two species, one herbivore and one carnivore, covering both main routes of human exposure. Standard bacterial safety tests should also be performed.

Toxicology studies can verify that the preparations pose no safety risks to humans or the environment before entering human use. These investigations must meet regulatory standards, accounting for the anticipated levels of phage preparations in each exposure compartment. EU guidelines can facilitate compliance for other jurisdictions. Toxicological assessments should address the specific characteristics of the preparations under investigation. [148, 149, 150, 151]

# Chapter - 11

## Clinical Applications and Therapeutic Strategies

### Personalized CRISPR-Phage Therapy

CRISPR-phage therapy can be tailored to a defined bacterial pathogen and its resistome for precision treatment of infection. Personalization involves phage isolation, CRISPR design against antibiotic-resistance genes in the strains' genomes, and identification of guide RNA specific to the patient's bacteria. Outcome analysis of each treatment should evaluate both the antimicrobial effect and the induction of unintended deleterious changes in surrounding microbiota.

Phage delivery can be systemic for circulating pathogens but focused on the site of infection for local pathogens. Antibiotics can be co-administered to simultaneously tackle the pathogen while the corresponding phages are being isolated, and infection sequences can exploit their different modes of action, with antibiotics administered first to reduce the bacterial load when specific phages are acting and to avoid phage-resistance selection pressure. Systemic CRISPR-phage therapy for severe systemic infections and topical CRISPR-phage therapy in infected wounds, urinary tract infections, and other localized infections represent two possible clinical applications. [19, 152, 153, 154]

### Treatment of Systemic and Localized Infections

Localized infections amenable to CRISPR-phage therapy include blood- or central nervous system-associated infections, wounds, urinary tract infections, and bacterial conjunctivitis, with corneal keratitis as a key example that merits dedicated

focus. Natural corneal defenses hinder infection establishment, but these barriers can be disrupted, allowing opportunistic pathogens to invade and proliferate. Adaptation to the ocular environment is crucial for pathogenicity, facilitating inflammation, epithelial damage, and further ulceration. Delayed management results in extensive cellular damage, with greater risk of corneal perforation in severe cases. Poorly perceived and inadequately treated wound infections are a major concern in safe health systems, with significant antibiotic resistance among commonly culprits.

Hematogenously spread bacterial infections negatively affect surgery outcomes and recovery. High virulence, poor tissue perfusion, diabetes, compromised immunity, invasive devices, and spinal cord injury all increase the risk of central nervous system-associated infections. The infection is prototypically represented by staphylococcal bacteremia. Such infections are characterized by the presence of a necrotizing ulcer in the epidermis, usually presenting as a shallow ulcer covered with eschar and with either serosanguinous or purulent exudate. The primary infection may be complicated by multiple metastases of varied clinical presentation, including necrotizing pneumonia, pyothorax, pleuritis, pyemic bone and joint infections, neoplastic pyogenic endometritis, and tuberculous mastitis. Selection of the most appropriate constituently expressed immune response is of paramount importance as there is a tug-of-war between both the pathogen and the immune system, and failure will result either in persistence of the pathogen or in excessive host tissue damage. [155, 156, 157, 158]

**Combination Therapy with Antibiotics**

Combining CRISPR-guided phage therapy with antibiotics reservoirs the strengths of both methods while ameliorating their drawbacks. Antibiotics remain among the most important and

utilized small molecules in human health and, due to their established clinical efficiency, the most investigated compounds in phage-therapy combinatorial approaches. The logical potential of CRISPR-antibacterial therapy combination with antibiotics is to repress the development of bacterial resistance, a predictable event, and one of the most critical threats of antibiotic therapy, either for the therapeutic action using multiplex CRISPR-Phage_Guide_0 or for administering phages targeting the genes responsible for these mechanisms of bacterial resistance.

Despite the fact that combination of CRISPR-phage therapy with antibiotics promises a global enhancement of both therapeutic methods and their advantages, the design of the experimental work is complex and often difficult to optimize. The clearest rationale supports the sequential administration of the two therapies. A first step is constituted by the treatment of the infection with antibiotics, to which the bacteria are still sensitive, in order to diminish their number and enhance phage-therapy efficacy. The second phase is the combined administration of multiplex CRISPR-phages_Guide_0 (targeting resistance drama genes) together with newly selected antibiotics that are still effective against the remaining bacteria. Such a combined therapy could stop the development of antibiotic-resistance during the first antibiotic therapy and guarantee its efficiency even once the bacterial population has developed MDR. [19, 54, 159, 18]

**Case Studies and Early Clinical Trials**

Phage therapy has been used against MDR bacteria in various experimental studies, with an emphasis on carefully guiding and evaluating therapy using conventional phages. Several case studies have successfully demonstrated the potential of native phages in treating serious infections caused by multidrug-resistant bacteria, including those of the Pseudomonas

aeruginosa, Clostridium difficile, Staphylococcus aureus, and Mycobacterium abscessus species. Therapy was used to treat purulent eye infections or life-threatening corneal perforation caused by P. aeruginosa, including encephalitis due to a brain abscess associated with the presence of three different C. difficile gtoksins, and recurrent urinary infections.

Phage therapy has shown promise for its clinical application, with some clinical trials successfully completed. For instance, PH.200-02, a lytic phage isolatate from a hospital sewage sample capable of breaking down biofilm of methicillin-resistant S. aureus, was used as an adjunctive treatment to oral linezolid for skin and soft tissue infection. A lytic bacteriophage targeting P. aeruginosa PAO1 strain was used as a therapeutic agent against chronic P. aeruginosa infection in patients with cystic fibrosis and was well tolerated with no adverse effects. A cocktail of two phages lytic to P. aeruginosa was used as a therapeutic agent in surgical wound infections caused by MDR-P. aeruginosa. Two clinical trials involving a combination of lytic phages active against MDR S. aureus are currently being assessed. [145, 160, 161, 162]

# Chapter - 12

## Resistance Development Against CRISPR-Phages

### Bacterial Defense Mechanisms Against Phages

Bacteria have evolved ancient and diverse mechanisms to resist infections by bacteriophages (phages), and extensive research has elucidated many of these strategies. Phage therapy may reemerge as a powerful tool against multidrug-resistant (MDR) pathogens; however, the gradual and inevitable evolution of resistant bacterial strains will affect the effectiveness of any phage-based treatment protocol. The rapid evolution of phages and their associated CRISPR-Cas systems provides a potential counter-strategy against evolving phage-resistant bacteria. Applications of CRISPR technology can additionally augment phage therapy by targeting specific genes essential for bacterial pathogenesis or resistance, thus increasing treatment specificity, safety, and efficacy. Nevertheless, certain bacteria can evade these advances, raising concerns about phages modified with CRISPR technology.

CAZyme production is a major bacterial counter-defensive strategy against phage infections. For example, A. baumannii and S. aureus produce polysaccharide lyases that degrade the polysaccharide capsule of K. pneumoniae, allowing phages to infect the bacteria. Biofilm formation is another bacterial defense strategy, providing attachment surfaces and a physical shield against external forces that promote phage-resistant infections. However, biofilms also create a microenvironment that enables the development of contacts resistant to bacteriocins. Biofilm

matrix-modifying proteins represent an important weapon for resisting opportunistic pathogens, and CRISPR-Cas technology could be used to silence the expression of these genes. Bacteria also employ various clotting factors and kinases to disrupt blood clot formation as a means of escaping phage treatment. [122, 163, 164]

**Evolution of Anti-CRISPR Systems**

As bacteria develop CRISPR-Cas systems to counter phages, phages—and now CRISPR systems themselves—are reciprocally evolving counter-defenses, known as anti-CRISPR systems or proteins, that provide bacteria with a means to evade the cleavage action of CRISPR-Cas systems. The first anti-CRISPR gene identified was located on a plasmid encoding virulence factors for the pathogens Pseudomonas aeruginosa and Burkholderia cenocepacia. Subsequent studies uncovered numerous distinct anti-CRISPR proteins in multiple bacterial taxa, especially those isolated from phage-rich environments, underpinning the broad recruitment of anti-CRISPR systems by nature. The availability of multiple anti-CRISPR systems targeting diverse CRISPR types and subtypes provides an exciting opportunity for the development of potential resistance against currently available CRISPR-AONs and CRISPR-phage therapy.

The discovery of type I-F anti-CRISPR (AcrF1) and type VI anti-CRISPR (AcrV) systems demonstrated the widespread distribution and evolutionary diversity of anti-CRISPR systems that enable legitimate bacteria to avoid CRISPR-Cas-mediated cleavage by neighboring species. These anti-CRISPR systems inhibit diverse CRISPR-Cas types and subtypes from both prokaryotic and protist groups in all three domains of life, highlighting their ability to protect su0b0721id bacteriophage against CRISPR-Cas surveillance in a wide variety of target

hosts, and providing new insights and bioengineering strategies for stabilizing CRISPR-Cas systems in cells and organisms of interest. Future investigations will enhance the understanding of naturally existing anti-CRISPR systems and further characterize their functions across diverse ecological niches. [165, 166, 167]

**Strategies to Minimize Resistance Emergence**

The evolution of resistant strains is a fundamental concern for any antibacterial therapeutic attempt. The implications for phage therapy are no different. Recurrence of infections after apparent successful treatments is well-documented, and resistance is likely to be one of the key contributors to these post-treatment failures. This fact, coupled with the already-existing examples of the latter found in the scientific literature, calls for the delineation of strategies that minimize the emergence of resistant bacteria. One possibility is the simultaneous targeting of more than one receptor employed by a specific bacterial pathogen. A first step toward minimizing resistance emergence could be the pairing of two or more lytic phages against the same bacterial pathogen. To further increase the likelihood of a successful outcome, any possible resistance pathways should also be identified.

Another effective strategy for preventing the evolution of resistant strains is the construction of phages whose genomes contain more than one guide RNA but at least one that targets a CRISPR-Cas feedback inhibitor. A more advanced multi-target approach could involve the development of a synthetic phage library engineered to simultaneously target several clinically relevant pathogens and be implemented in an adaptive manner. This strategy would involve the identification of the specific pathogen causing a certain infection in a certain patient. After such determination, the adaptive phage library would select a suitable set of phages and CRISPR-Cas constructs with designed RNA guide molecules for that specific pathogen. A similar

approach could also be adopted for the treatment of infections caused by other pathogens. This approach could therefore constitute an effective method for the large-scale production of site-specific therapeutic phage libraries. The approaches that would allow the diversification of the therapeutic library design would also be applicable in any future-targeted context where the designed delivery of the CRISPR-Cas system would be a consideration. [168, 169, 170]

**Adaptive and Multi-Target Phage Designs**

Careful design can address the limitations of CRISPR-phage therapy and aid preclinical and clinical implementation. Besides explicitly preventing resistance emergence, CRISPR-phage libraries capable of adaptive targeting may also be viable. Such an approach involves constructing a library of two or more phages, each one targeting a distinct site. Reinfection of remaining resistant populations can do the rest, whether CRISPR systems are present or absent among the remaining susceptible populations.

Alternatively, specific types of phages may be engineered to contain several guide RNA scaffolds within the same CRISPR array. Building on designs that can already tolerate 100–150 bp spacers, this strategy aims to create adaptable phage libraries, capable of targeting either distinct sites of a given resistance-related gene or different genes in a single strain—without raising evident concerns of bypassing the antibiotic-resistance spectrum. [171, 168, 172, 173]

# Chapter - 13

## Manufacturing, Formulation, and Delivery Systems

### Large-Scale Production of Engineered Phages

Producing therapeutically relevant phages in appropriate quantities and quality is often a major practical limitation for phage research. Achieving regulatory-grade quality with abundant supply is a prerequisite for clinical application. Currently, most genetically modified phages are produced at small scale for laboratory studies using established, simple protocols that require minimal optimization. For any engineered phage therapy to be clinically possible, scales sufficient to treat patients and meet safety standards must be achieved. Pipelines must be designed to deliver phages in sufficient volumes, meet quality metrics (e.g., nucleic acid and protein content, absence of contaminants), and gauge biological characteristics (e.g., lytic ability, stability). Furthermore, quality assurance and quality control frameworks must include measures such as identity, purity, and potency assessments to meet good manufacturing practice (GMP) requirements.

Production methods depend on the complexity and scale required. Established approaches harness plasmid-based recombination in host bacteria, complemented by nucleic-acid purification and phage-bioassay validation. Chemical synthesis of smaller phages without packaging signal is possible. In cases where these methods are suboptimal—and for larger phages with complex genomes—CRISPR-assisted assembly or long-ssDNA-based strategies can facilitate quality-control checking for

manufacturing. Consideration of assay performance may determine whether more complex production pipelines or failure-prone processes are justified. Optimal solution weaknesses may in turn inform rectifying measures. [174, 175, 176, 177]

**Formulation Stability and Storage**

Cryoprotectants stabilize biological structures during cryo-storage, preserve activity, and prevent ice formation. Common excipients for phage storage include sugars, polyols, or proteins. Mannitol effectively safeguards viral capsids, while trehalose protects activity. Optimal storage at −20 °C with lyophilized preparations containing trehalose retains full infectivity and stability. Lyophilized phages tolerate ambient temperature, humidity, and UV radiation, ensuring wide availability. Although phages remain stable at −20 °C for extended periods, deep freezing is less practical for clinical use compared to cold chain systems for recombinant proteins, requiring sophisticated logistics in resource-limited regions.

Topical phage therapy can be administered intranasally to combat infections in the central nervous system. Particularly for localized infections where the active site is known, phages could be easily placed in solution, gel, or spray preparations. Rapid contours during surgery facilitate direct application to specific sites. Phage therapy in conjunction with pre- or post-operative intervention with streptavidin-conjugated CRISPR–Cpf1 has shown synergy in vivo. Similar integration of phages or the CRISPR–Cpf1 system alone within local treatment regimens would also be beneficial in suppressing PL-resistant strains. [178, 179, 180, 181]

**Delivery Routes and Dosage Optimization**

Delivery must respect distribution, local concentrations, and immune interactions at both systemic and mucosal sites. Multiple routes, informed by tissue tropism, influence clearance patterns

and protect against immune neutralization. Local delivery to motor neurones bypasses blood–brain barrier challenges; near-disease application shortcuts biodistribution concerns; and engineer-managed distribution addresses organotropic pitfalls.

Therapeutically relevant doses can be high, but synthesis capacity may impose limits in end-stage phage production. Toxicity is thus determined by local concentration and links to ADME. Poor delivery, low concentration, and dose reregime with local pre-treatment attenuate phage clearance and neutralization in vivo. Intranasal application minimises systemic neutralisation; dynamic in vivo challenges inform day-to-day design; and encapsulation furthers rescaling without substantial alteration of host response. Localised tissue pre-treatment shortens clearance lifespan, enabling use of higher doses or rarer species; but combined efficacy and response-sequencing are essential for Temporary Controlled Therapeutic Index strategy success. [182, 183, 184, 185]

**Regulatory-Grade Quality Control**

In addition to the above GMP features, regulatory-grade engineered bacteriophages must undergo validation steps that ensure they conformed to product specifications and operated as intended under the simulated conditions of their intended use. This validation must include supportive data that demonstrate that the manufacturing methods and the use of any derived components were robust and consistent within and between batches. Attention to detail must also extend to the entire manufacture chain, as problems at any stage (e.g., from sampling and transport to preparation, shipping, and final use) can compromise quality—even when all prior controls have been satisfied. Traceability (the ability to trace the steps, history, or location of an item) demonstrates whether all necessary quality-assurance checks were carried out on the produced phages, thus

acting as supporting evidence for regulatory authorities in establishing confidence in the final product.

The quality of engineered phage preparations must also be validated through the following tests: (i) detection of necrotic, apoptotic, or other overly activated cells; (ii) detection of localized or systemic hypersensitivity; and (iii) assessment of the host's immune response to the presence of exogenous DNA, which has not been targeted against a bacterium. [92, 186, 187, 188]

# Chapter - 14

## Ethical, Regulatory, and Biosafety Considerations

### Ethical Implications of Gene-Edited Therapies

Gene editing opens huge possibilities and can greatly affect human life, but several implications must be considered in their use. First, consent. Gene-edited therapies are obviously superior to others, so their use will not be questioned. The real problem will be when gene-edited cohorts are created. The long-term effects of these therapies are unknown, and consent cannot be obtained from the unborn patients who will be born gene-edited. Pregnant women who had made gene-editing therapies, therefore, should be adequately informed about the risks of transfer to their children. Second, equity. As with all cutting-edge therapies, their accessibility must be guaranteed, in order to avoid a broader social divide between richer and poorer people. Third, responsibilities. The barriers between species are no longer as clear as they once were. Hence, when an organism is modified, who bears the responsibility for that modified organism? Fourth, — if a new pathogen in humans were created — impact on populations or on the environment. When the ecological balance of a host-species system is changed, it is almost impossible to predict the consequences. For instance, if phages capable of incisive-maculated a pestilential affliction insects responsible for great losses in agriculture. And the CRISPR410 system, highly efficient also used as a defence against bacterial pathogens. [189, 190, 191, 192]

Among the products of speculative design, targeted gene-editing phage could assure accuracy and reduce collateral

damage on the host microbiota. Moreover, the same system could be used to anti-Crispr systems (protective systems evolved in bacteria).

**Regulatory Frameworks for CRISPR-Phage Products**

The regulatory pathway for CRISPR-engineered phage products is similar to that for unmodified phage therapeutics. In addition to quality-control features demanded by Good Manufacturing Practice (GMP) guidelines, gene-edited agents will require special attention to empower risk suitability assessments. Central to the control processes are the nature and severity of the alterations made to the natural phage genome. Three factors are key:

1) The introduction of a new gene or the deletion of a pre-existing one. If, for instance, a KpsM-type gene cassette is added to a foundational phage and a cognate receptor introduced in a non-pathogenic host, the overall consequences are negligible. Systematizing expression from compatible, natural promoters alleviates regulatory concerns.
2) Focused alterations targeting DNA–RNA contacts or suboptimal protein folding, so long as the mutations do not abrogate in vivo stability or normal function, likewise reduce risks.
3) Suppression of gene activity can be deliberated with caution. For example, when codons signalling protein methylation in the expressed polypeptide are substituted with synonymous codons, they do not impose an energetic load and phage infectivity remains intact.

The risk associated with modifications is further lowered when empyreal design is employed, enabling expression against a wide variety of bacterium-associated template DNAs.

## Environmental and Ecological Safety

Bacterial resistance to phages—natural predators—is widespread, but the underlying genomic alterations that confer resistance are not always targeted by bacteria. A prime example is the emergence of bacterial strains with CRISPR-based interference system that directly cleaves the protospacer sequence in the advDNA of the phages. Such systems are widely distributed in prokaryotes and although multiple anti-CRISPR genes have been identified. Therefore, anti-CRISPR systems have been co-opted as a bacterial defense against phage. Furthermore, targeting anti-CRISPR systems using another CRISPR system not only provides an additional layer of protective efficacy but also minimizes the emergence of resistant strains. Nonetheless, these non-targeted strains could become resistant during therapy for reasons other than the targeting of the protospacer region.

A possible way to enhance therapeutic efficacy is by employing CRISPR–phage libraries. For example, a sequencing-based CRISPR library has been designed to tackle the global crisis of antimicrobial resistance and possesses a high-power CRISPR-Cas3 module that degrades the entire bacterial chromosome upon induction. Integrating the adaptive capabilities of central nervous systems and the strengths of phages into therapy could expedite future clinical applications. Hence, balancing immune activation and function may lead to successful CRISPR–phage therapy and minimize the likelihood of resistance development. Nonetheless, continuous adaptations will ultimately confer better effectiveness each time the treatment is applied. [193, 194, 195, 196]

## Public Acceptance and Risk Communication

Public acceptance is a prerequisite for the translation of any technology from the laboratory into the clinic. It requires

common people to understand the science behind it in order to minimize misunderstandings. A society that understands science can make informed decisions about the regulation of the technology and support the development of products that address the challenges they face. Education needs to focus on the advantages of the technology while remaining transparent about its drawbacks.

Moratoriums against certain areas of research represent a loss of freedom in scientific investigation and a failure to recognize that technologies are neutral. CRISPR-based modifications to phages and other related technologies can lead to major improvements, such as better therapies for suffering patients, solutions to problems of inefficiency and environmental impact, new solutions to old problems, and full development of untapped research areas. Use and application in medicine must be carefully considered, not banned. Concerns related to designer babies and other applications must be addressed and managed.

As with any gene-editing technology capable of rewriting the genomes of living organisms, concerns have also been raised about the possible dangers of CRISPR-Cas systems. Such concerns must be addressed in a transparent manner to avoid unfounded opposition to the technology.

Potential risks associated with CRISPR-modified phages include elimination of the entire bacterial microbiota, on-target deactivation of non-target bacterial species, deactivation or disruption of genes or paternally essential for bacterial viability and persistence, triggering of anti-CRISPR bacteria systems that could counteract phage killing, and generation of DNA sequences in the patient capable of being targeted by phage Cas9. Not all risks need to be eliminated; all need to be evaluated and allowed if they are outweighed by the advantages. Public acceptance and global implementation also require a societal

conversation about the expected advantages, possible disadvantages, and public control of research, safety validation and final application. [197, 18, 128, 198]

# Chapter - 15

## Future Perspectives and Technological Innovations

### AI-Driven Phage and CRISPR Design

Artificial intelligence has emerged as a powerful new approach to biological design and optimization. Molecular-biological design of CRISPR-guided phages capable of targeting proliferation of multidrug-resistant (MDR) bacterial pathogens remain a challenging endeavor that may benefit from algorithmic assistance. A plethora of design considerations is listed based on accumulated knowledge and experience with the underlying technologies. Optimizing for many of these factors is expected to facilitate establishment of an effective class of phage-based therapeutic agents.

The extraordinary selectivity and adaptability of artificial-intelligence-driven design have been widely employed for molecular and even supra-molecular design across many disciplines. Recent investigation of integration of AI-for-design tools into the discovery and development process for virulent bacteriophages capable of curtailing pathogenic multiplier expansion already demonstrated potential advantages for high-fidelity reverse genetics of phage capture repositories, optimization of experimental design of non-traditional growth substrates and media to ascertain improved response properties, and selection of experimental endpoints that optimize ergodic behavior of phage-bacterial co-cultures. AI design principles also encompass control of phage instability during storage, concentration-dependent augmentations in dismantling of partial

tolerance to infection in biofilm-grown relative to planktonic bacteria, consideration of the innate and adaptive immune response to phagotherapy, balance between immunogenicity/originality of therapeutic-therapy dispensing agents and block neutralization by the host immune system, and support safety during natural development and of alternative methods of synthesis and large-scale production for employment in human subjects. The exact mechanisms through which engineered CRISPR-modified phages are introduced into non-bacterial hosts, and permissiveness of multi-targeting engineering strategies across those species, remain open questions.__ AI design principles can be deployed by specialists in their respective sub-disciplines for standard, routine, reverse and open-ended exploratory design. [199, 200, 201, 202]

**Next-Generation Gene-Editing Tools**

Although CRISPR-Cas might appear as the pinnacle of genome-editing technology, nature has already begun to assemble an arsenal of alternatives. The discovery of the CRISPR-Cas system throughout the tree of life has prompted biologists to explore other unknown or poorly characterized RNA-guided genetic elements. Aside from CRISPR, various other antiviral RNA-based defenses have been uncovered, such as CRI-SPINs (CRISPR-SPIN amalgams), a recently identified group of Type VI RNA-guided anti-phage effectors in the eukaryote Chaetosphaeria depleta, which function like Type II CRISPR-Cas but utilize a novel class of short CRISPR-like RNAs to guide target recognition and cleavage. These systems employ RNA-chaperone complex SbtD to facilitate pre-crRNA processing and maturation, and the genomic context of the RNA-guided nucleases is consistent with gain-of-function at the level of host–phage interactions.

In addition, new anticodon-guided ribozymes that utilize codon-like RNA sequence features for target recognition and

cleave RNA via a transesterification mechanism have been characterized. Also, Cas12g, a recently identified type V CRISPR-Cas nuclease found in Listeria spp., is best known for its ability to target and break double-stranded DNA at a site specified by a 36-38 nt RNA guide. Compared with Cas9, Cas12e possesses a smaller footprint and is the first known at-to-ctg codon reprogrammer. Cas12e, when programmed with a highly complementary RNA guide that is anticodon-containing, expands the targeted codon space to codons containing C, G, or U. Furthermore, the CRISPR-associated protein CsaS, which promotes phage resistance in Pseudomonas fluorescens, is a cytidine deaminase that modifies phage RNA genomes using a guide RNA, thereby representing a new form of anti-phage defense whereby RNA modification promotes viral tolerance. The types of CRISPR-Cas systems with RNA-guided cleavage have begun to expand beyond DNA-targeting Cas9 and Cas12 into RNA-cleaving systems with potent activity; indeed, some expanded RNA-targeting CRISPR-Cas tools can also successfully target DNA. Consequently, as biological knowledge transforms libraries of antisense oligonucleotides into RNA-guided enzymes, their applications will extend to prokaryotes and beyond, well past the initial revolution of genome editing, which was limited to essentially only DNA or DNA viruses. [203, 170, 204, 205]

**Expanding Targets Beyond Bacteria**

The CRISPR-Cas system is a powerful genome engineering tool that relies on bacteriophages to infect and eradicate bacteria. Beyond this core application, the technology shows promise for applications targeting non-bacterial pathogens. Although the natural role of CRISPR-Cas has been established in mainly bacterial systems, the adaptive immune response and its editing capabilities have been adapted into eukaryotic systems and organisms. CRISPR-Cas editing and targeting strategies have

been developed for viral pathogens (anti-CMV, anti-HIV, and anti-SARS-CoV approaches, for example) and non-bacterial diseases, demonstrating that CRISPR has the potential to target pathogens other than bacteria when fully developed. Bacteriophages, their products, and their hypernatural–synthetic function applications can contribute diverse applications not limited to the bacterial domain. CRISPR-Phage Therapy warrants consideration as a targeted strategy against non-bacterial disease-causing agents.

The Targeted Cleavage of Resistance Genes section discusses CRISPR-based targeted-cleavage gene designs enabling the development of natural–hypernatural–synthetic combined functions of phages that redirect the bacteriophages' virulence-determining and resistance-associated functions without compromising pathogenicity to the MDR-plasmids-harbouring bacterial strains. This concept can consider the systematic addition of functions to CRISPR-guided bacteriophages, with planned testing for unintended off-target effects. This expansion and diversification potential for targeting outside the bacterial domain would solidify CRISPR as a next-generation-above-bacteria editor, especially when combined with bacteriophages as vehicles for delivery. [206, 19, 207, 206, 19, 207, 177]

**Integration into Precision Medicine**

Bacteria are highly diverse organisms that colonize patients and evolve rapidly, posing major clinical challenges. Next-generation sequencing allows for characterization of a patient's Pseudomonas aeruginosa isolate and its associated resistance genes, and CRISPR-modified phages can be designed accordingly. Phage therapy can thus be personalized and made more effective through targeting of resistance genes, plasmids, virulence factors, biofilm genes, and key pathways.

Consequently, a CRISPR-phage therapy tailored to a patient's strain is likely to yield the highest protective effect.

The increasing incidence of multidrug-resistant infections represents a serious challenge for global healthcare and a major concern for surgeons, oncologists, and patients. Bacteria evolve rapidly, and the emergence of colistin resistance has made it urgent to identify new therapeutic approaches to treat life-threatening infections, particularly those caused by Klebsiella pneumoniae and Acinetobacter baumannii. Therefore, the solution may lie in designing novel therapeutic strategies based on the patient's distinct isolate by means of novel techniques, technologies, and tools. Recent breakthroughs in next-generation sequencing technology allow for highly accurate, reasonably inexpensive whole-genome sequencing of target pathogens, enabling elucidation of different genes responsible for bacterial resistance, virulence, and biofilm formation. By coupling such information with a patient-specific isolate, CRISPR-phage therapy can be customized, thereby optimizing protective efficacy and safety. [208, 209, 210, 211]

# Chapter - 16

## Conclusion and Translational Outlook

### Summary of Scientific Advances

Bacteriophage therapy is returning to clinical interest as a natural therapy against bacterial infections. The combination of CRISPR technology with phage therapy has progressed rapidly, with recent advances pointing the way towards clinical application. The rationale is to customize the specificity of phages and limit the risk of rising resistance or collateral damage to the microbiota. Specificity is increased by incorporating CRISPR into phages, allowing precise targeting of the pathogen itself or of genes required for resistance development. Phages have also been designed to target efflux pumps, resistance enzymes, biofilm-associated genes, and the removal of the plasmids that carry resistance genes.

Antimicrobial resistance is a multifactorial danger, with many different high-risk bacteria, emerging resistanceless strains, and numerous resistance mechanisms. The approaches discussed above seek to provide a broad-spectrum and flexible tool to combat, or at least mitigate, the growing resistance problem, an effort that is especially urgent given the meet-and-cough risk presented by well-resourced opportunistic pathogens. The different phage types can be rationally combined, such as virulent phages with transducing temperate phages, or such an approach can even be automated through adaptive testing. CRISPR technology may also help to provide—or overcome—target scope.

## Challenges in Clinical Translation

Despite promising laboratory data, the clinical progress of CRISPR-guided phage products lags behind that of conventional bacteriophages. The safety and practicality of native individual phages have been established in humans, whereas CRISPR-related concerns remain largely theoretical. Nevertheless, bridging these gaps requires careful examination and risk aversion. The risks of successful phage therapy thus far have largely been assigned to ecosystem disturbance through inappropriate use. For CRISPR-modified therapy, potential danger lies with unintended effects on bacterial off-target populations or environmental species, particularly in the rare event of horizontal gene transfer. Proven coexistence mechanisms, evident restraint in the clinical establishment of even traditional therapies for preserved ecosystems, and the greater ecological price of not controlling resistant pathogens justify continued research in CRISPR-nanotechnology combination therapy.

Implementation of a novel therapeutic paradigm demands a well-defined next step, whether proof of principle or proof of concept, and conclusive detection of safety and efficacy. A synthetic biology approach offers unique advantages for precision design and safety, but retards development due to limited case studies and integrative consideration. Current consideration embraces clinical modelling of CRISPR-assisted pharmacology and the anticipation of future expansion rather than prescribing specific experimental lines. Interest lies in new knowledge, design philosophy, and specification of safety concepts essential for advancing clinical application and translational research in synthetic biology more generally.

## Roadmap to Global Implementation

Realizing CRISPR-engineered phages as a new class of antibacterial agents requires research into phage biology and

broad-spectrum clinical evaluations. The toxicity, pharmacokinetics, and pharmacodynamics of CRISPR-tested phages must be assessed in suitable animal models before progressive testing in humans, beginning with compassionate use. Early-stage treatment should target patients with MDR infections caused by identified pathogens. Personalized therapy is essential, tailoring phage selection and guide RNA design to the patient's antibiotic-resistant organism and resistome. Subsequent tests must explore combination regimens with established antibiotics and therapeutic success in animals, followed by human clinical trials.

Ample work is needed to adapt phage therapy for routine clinical use. Additional vectors for efficient CRISPR incorporation into phages must be established to reduce labor and cost. Assays should systematically test the activity of CRISPR-phages against the same panel of bacteria to investigate determinants of antibacterial potency, specificity, and safety. Assessment of phage immunogenicity and host responses—innate and adaptive—will clarify interactions involved in therapeutic application. Optimization of delivery routes and strategies to exploit phage–host interplay should further improve efficacy. Beyond direct treatment, CRISPR-phages hold promise in preventing antibiotic failure associated with resistance transmission within bacterial communities.

**The Future of Post-Antibiotic Medicine**

Antimicrobial resistance threatens the efficacy of antibiotics, but new technologies promise sustainable replacements. Phages can be engineered with CRISPR to precisely target specific bacteria and are further enhanced by synthetic genome design. This approach is now maturing into a pipeline for developing and validating personalized CRISPR-phage therapy against multidrug-resistant pathogens.

The prospect of a post-antibiotic era, when control over bacterial infections is redefined, takes shape. With patients suffering from multidrug-resistant bacterial infections, the hunt for new antimicrobials is urgent. Novel strategies are also essential for alleviating the failure, pain, and costs of existing antibiotics. Antibiotic-development pipelines are largely empty. Bacteriophage therapy can be refined into a versatile, safe, and effective clinical option. Smart biological and chemical formulations will also empower old molecules, like bile acids and sulfur, to foil pathogenesis from the microbial community. Machine-learning tools will accelerate design and development by closing knowledge gaps across phages, host-defense systems, and the sick patients populated by resistant infections. Personalized CRISPR-phage therapy targeting a patient's strains promises to bypass previous safety and efficacy concerns of phage therapy.

**Conclusion**

CRISPR-modified phage therapy addresses several issues that plague classic phage therapy. The presence of bacterial resistance mechanisms that inhibit phage activity, the non-specific nature of phages, and the swarming of bacterial populations in defensive formations called biofilms can hinder conventional phage therapy. Moreover, the evolution of anti-CRISPR systems capable of targeting CRISPR-carrying phages needs careful consideration to avoid immune neutralization.

The above-mentioned issues can be addressed and partially eliminated by developing phages that carry a CRISPR-Cas gene cassette from other bacteria. CRISPR-endowed phages can be designed to specifically target bacterial resistance genes, including those encoding efflux pumps, resistance-conferring enzymes, and genes involved in changing the host receptor structure. Non-essential bacterial pathways can be targeted to

enhance phage susceptibility and therapeutic efficacy and/or to inhibit plasmid conjugation without unwanted damage to the microbiota. Specific guide RNA (gRNA) loci that direct Cas9 endonuclease to resistance-associated genetic elements in the bacterial genomes can be engineered into the phage genome and validated. Selective CRISPR-guided phage activity against multidrug-resistant (MDR) bacterial strains can also be developed to reduce collateral damage to the gut microbiota.

Precision-engineered CRISPR phages can be combined with smart phage design principles in the form of personalized libraries directed against specific pathogens and their resistomes in a patient. Engineering and synthetic biology approaches for CRISPR incorporation into phages have been developed in different natural or synthetic formats, and expanding the use of CRISPR beyond direct genome editing, such as in the case of phage antiviral strategies, may improve the therapeutic prospects for CRISPR-engineered phages. Integrating additive or synergistic CRISPR phage therapy into conventional medicine and personalized healthcare systems can also benefit therapeutic outcomes.

# References


1. S. T. Cardona and A. S. M. Z. Rahman, "Innovative perspectives on the discovery of small molecule antibiotics," *npj Antimicrobials and Infection*, 2025. nature.com
2. M. Bergkessel, B. Forte, and I. H. Gilbert, "Small-molecule antibiotic drug development: need and challenges," ACS Infectious Diseases, 2023. acs.org
3. H. Elkady, I. N. Salman, and M. M. Khalifa, "Small-molecule strategies to combat antibiotic resistance: mechanisms, modifications, and contemporary approaches," RSC advances, 2025. rsc.org
4. M. A. Cook and G. D. Wright, "The past, present, and future of antibiotics," Science translational medicine, 2022. science.org
5. M. A. Ozma, A. Abbasi, M. Asgharzadeh, "Antibiotic therapy for pan-drug-resistant infections," Le infezioni in…, 2022. nih.gov
6. Catalano, D. Iacopetta, J. Ceramella, D. Scumaci, "Multidrug resistance (MDR): A widespread phenomenon in pharmacological therapies," Molecules, 2022. mdpi.com
7. H. Giamarellou and I. Karaiskos, "Current and potential therapeutic options for infections caused by difficult-to-treat and pandrug resistant gram-negative bacteria in critically ill patients," Antibiotics, 2022. mdpi.com
8. OJ Akinjogunla, OO Adefiranye, EN Edem, "Antibiotic resistance profile, multidrug-, extensively drug-, and pandrug-resistant bacterial isolates: Hemagglutination and hemolytic activities against human …," The National

Academy of …, 2025. [HTML]

9. F. C. Lessa and D. M. Sievert, "Antibiotic resistance: a global problem and the need to do more," Clinical Infectious Diseases, 2023. oup.com
10. N. Poudel, S. Zhu, N. Cooper, P. Little, C. Tarrant, et al., "The economic burden of antibiotic resistance: A systematic review and meta-analysis," *Plos One*, vol. 2023. plos.org
11. W. Fong, "Antimicrobial resistance: a crisis in the making," New antimicrobials: for the present and the future, 2023. [HTML]
12. R. Balasubramanian, T. P. Van Boeckel, Y. Carmeli, et al., "Global incidence in hospital-associated infections resistant to antibiotics: An analysis of point prevalence surveys from 99 countries," PLoS, 2023. plos.org
13. Tarin-Pello and B. Suay-Garcia, "Antibiotic resistant bacteria: current situation and treatment options to accelerate the development of a new antimicrobial arsenal," *Journal of Anti-Infective Therapy*, vol. 2022, Taylor & Francis. [HTML]
14. D. C. Nwobodo and M. C. Ugwu, "Antibiotic resistance: The challenges and some emerging strategies for tackling a global menace," *Journal of Clinical*, vol. 2022, Wiley Online Library. wiley.com
15. Gajic, N. Tomic, B. Lukovic, M. Jovicevic, D. Kekic, "An overview of antibacterial agents for combating Multidrug-Resistant bacteria: the current landscape, development, future opportunities, and challenges," *Antibiotics*, 2025. mdpi.com
16. M. Kolář, "Bacterial infections, antimicrobial resistance and antibiotic therapy," Life, 2022. mdpi.com

17. S. Allemailem, "Recent advances in understanding the molecular mechanisms of multidrug resistance and novel approaches of CRISPR/Cas9-based genome-editing to combat this …," International Journal of Nanomedicine, 2024. tandfonline.com

18. C. Ghazaei, "The role of bacteriophages and CRISPR-Cas in combating multidrug-resistant bacteria," Natural Products and Bioprospecting, 2026. springer.com

19. N. Onyeyili, F. I. Ogbozor, A. B. Kehinde, C. Ohaekwe, "Phage-based CRISPR delivery systems for targeted eradication of drug-resistant bacterial infections," *Int. J. Life Sci.*, 2025. researchgate.net

20. AO Olatunji, JA Olaboye, CC Maha, "Next-Generation strategies to combat antimicrobial resistance: Integrating genomics, CRISPR, and novel therapeutics for effective treatment," *Science & Technology Journal*, 2024. researchgate.net

21. C. Li, T. Shi, Y. Sun, and Y. Zhang, "A novel method to create efficient phage cocktails via use of phage-resistant bacteria," *Applied and Environmental Microbiology*, vol. 2022. asm.org

22. M. A. M. Shaufi, C. C. Sieo, C. W. Chong, T. Geok Hun, et al., "Effects of phage cocktail, probiotics, and their combination on growth performance and gut microbiota of broiler chickens," Animals, vol. 2023. mdpi.com

23. B. Zalewska-Piątek, "Phage therapy—challenges, opportunities and future prospects," Pharmaceuticals, 2023. mdpi.com

24. M. Zhao, H. Li, D. Gan, M. Wang et al., "Antibacterial effect of phage cocktails and phage-antibiotic synergy against pathogenic Klebsiella pneumoniae," Msystems, 2024.

asm.org

25. N. Harshitha, A. Rajasekhar, S. Saurabh, et al., "Bacteriophages: potential biocontrol agents and treatment options for bacterial pathogens," Clinical Microbiology, vol. 2022, Elsevier. [HTML]
26. Gildea, J. A. Ayariga, and B. K. Robertson, "Bacteriophages as biocontrol agents in livestock food production," Microorganisms, 2022. mdpi.com
27. Garvey, "Bacteriophages and food production: Biocontrol and bio-preservation options for food safety," Antibiotics, 2022. mdpi.com
28. S. Ragab, M. K. Mustafa, Y. Y. Hassan, A. Nasr, "Potential use of bacteriophages as biocontrol agents against multidrug-resistant pathogens in wastewater treatment: a review," Environmental ..., vol. 2024, Springer. springer.com
29. Danaeifar, "New horizons in developing cell lysis methods: A review," Biotechnology and bioengineering, 2022. [HTML]
30. S. Patrick, "EXPLORING THE SPECTRUM OF VIRUS-CELL INTERACTIONS: BEYOND CELL LYSIS," KC Sawant, . wisdompress.co.in
31. V. Panteleev and A. Kulbachinskiy, "Evaluating phage lytic activity: from plaque assays to single-cell technologies," Frontiers in Microbiology, 2025. frontiersin.org
32. C. Wedd, R. Li, T. Yunusov, A. Smith, G. Hardo, M. Hunter, "Single-cell imaging of the lytic phage life cycle in bacteria," bioRxiv, 2024. biorxiv.org
33. Klumpp, M. Dunne, and M. J. Loessner, "A perfect fit: Bacteriophage receptor-binding proteins for diagnostic and therapeutic applications," Current opinion in microbiology,

2023. sciencedirect.com

34. HJ Jia, PP Jia, S Yin, LK Bu, and G Yang, "Engineering bacteriophages for enhanced host range and efficacy: insights from bacteriophage-bacteria interactions," *Frontiers in Microbiology*, vol. 2023. frontiersin.org
35. H. Shuwen and D. Kefeng, "Intestinal phages interact with bacteria and are involved in human diseases," Gut microbes, 2022. tandfonline.com
36. Leprince and J. Mahillon, "Phage adsorption to gram-positive bacteria," Viruses, 2023. mdpi.com
37. S. A. A. Agha, A. Al-Samydai, and T. Aburjai, "New frontiers in CRISPR: addressing antimicrobial resistance with Cas9, Cas12, Cas13, and Cas14," Heliyon, 2025. cell.com
38. M. Hussen, Z. B. Najmadden, S. R. Abdullah, et al., "CRISPR/Cas9 gene editing: a novel strategy for fighting drug resistance in respiratory disorders," Cell Communication and..., vol. 2024, Springer. springer.com
39. H. Geurts and H. Clevers, "CRISPR engineering in organoids for gene repair and disease modelling," Nature Reviews Bioengineering, 2023. [HTML]
40. Dindhoria, V. Manyapu, A. Ali, "Overview and Applications of CRISPR/Cas9 Based Genome Editing in Industrial Microorganisms," in *Genome Editing in …*, 2024. [HTML]
41. Z. Khan, Z. Ali, A. A. Khan, T. Sattar, A. Zeshan, "History and classification of CRISPR/Cas system," in *CRISPR/Cas tool kit for …*, Springer, 2022. [HTML]
42. S. Ganger, G. Harale, and P. Majumdar, "Clustered regularly interspaced short palindromic repeats/CRISPR-associated

(CRISPR/Cas) systems: Discovery, structure, classification, and general mechanism," in *CRISPR/Cas-Mediated …*, 2023. [HTML]

43. Panda, S. Pradhan, and L. A. Parida, "Classification of the CRISPR/Cas System," in Basics of CRISPR/Cas, 2024. [HTML]
44. S. Makarova, Y. I. Wolf, and E. V. Koonin, "Evolutionary classification of CRISPR-Cas systems," in *CRISPR: Biology and …*, 2022. [HTML]
45. Kamruzzaman and A. Yan, "CRISPR-cas systems in bacteria and archaea," *Frontiers in Microbiology*, 2022. frontiersin.org
46. V. Devi, K. Harjai, and S. Chhibber, "CRISPR-Cas systems: role in cellular processes beyond adaptive immunity," Folia Microbiologica, 2022. nih.gov
47. E. Joseph, A. Jain, M. O. Yaqub, and L. K. Edison, "CRISPR-Cas systems: bridging bacterial immunity and host interactions," Applied Microbiology, 2025. mdpi.com
48. W. Xiao, J. L. Weissman, and P. L. F. Johnson, "Ecological drivers of CRISPR immune systems," Msystems, 2024. asm.org
49. V. Smirnov and A. M. Yunusova, "Novel CRISPR/Cas9-Based Approaches for Quantitative Study of DSB Repair Mechanics," Biochemistry (Moscow), 2025. rjraap.com
50. Přibylová and L. Fischer, "How to use CRISPR/Cas9 in plants: from target site selection to DNA repair," Journal of Experimental Botany, 2024. oup.com
51. T. V. Vu, S. Das, C. C. Nguyen, J. Kim, "Single-strand annealing: Molecular mechanisms and potential applications in CRISPR-Cas-based precision genome editing,"

*Biotechnology*, vol. 2022, Wiley Online Library. researchgate.net

52. S. W. Awwad, A. Serrano-Benitez, J. C. Thomas, et al., "Revolutionizing DNA repair research and cancer therapy with CRISPR–Cas screens," *Nature Reviews Molecular Cell Biology*, vol. 2023. cam.ac.uk
53. Khambhati, G. Bhattacharjee, N. Gohil, et al., "Phage engineering and phage-assisted CRISPR-Cas delivery to combat multidrug-resistant pathogens," Bioengineering & Biotechnology, vol. 2023, Wiley Online Library. wiley.com
54. F. B. Balcha and S. A. Neja, "CRISPR-Cas9 mediated phage therapy as an alternative to antibiotics," Animal Diseases, 2023. springer.com
55. Y. Yue, Z. Xu, T. Soteyome, M. Premarathna et al., "Phage Encapsulation and Delivery Technology: A Strategy for Treating Drug-Resistant Pathogenic Microorganisms," Pharmaceuticals, 2025. mdpi.com
56. H. Peng, I. A. Chen, and U. Qimron, "Engineering phages to fight multidrug-resistant bacteria," Chemical Reviews, 2024. acs.org
57. V. Gurtler and M. Calcutt, "Genome Engineering," 2023. [HTML]
58. V. Cano, "New insights on mechanisms and barriers against horizontal gene transfer in Thermus thermophilus," 2023. uam.es
59. Deichmann, G. Schiesaro, K. Ramanathan, K. Zeeberg, "Engineered yeast cells simulating CD19+ cancers to control CAR T cell activation," bioRxiv, 2023. biorxiv.org
60. C. Fisher, A. Alastruey-Izquierdo, J. Berman, et al., "Tackling the emerging threat of antifungal resistance to

human health," *Nature Reviews*, vol. 2022. nature.com

61. R. MacNair, S. T. Rutherford, and M. W. Tan, "Alternative therapeutic strategies to treat antibiotic-resistant pathogens," Nature Reviews Microbiology, 2024. [HTML]
62. Garg, J. Malhotra, P. Kulkarni, D. Horne, R. Salgia, "Emerging therapeutic strategies to overcome drug resistance in cancer cells," Cancers, 2024. mdpi.com
63. S. Walesch, J. Birkelbach, G. Jézéquel, and F. P. J. Haeckl, "Fighting antibiotic resistance—strategies and (pre) clinical developments to find new antibacterials," EMBO, vol. 2023. embopress.org
64. K. Srivastav, P. Das, P. Jaiswal, "CRISPR-Cas Systems for Targeted Therapy in Multidrug-Resistant Infections: A New Frontier in Medical Microbiology," International Journal of …, 2024. researchgate.net
65. S. Qin, Y. Liu, Y. Chen, J. Hu, W. Xiao, and X. Tang, "Engineered bacteriophages containing anti-CRISPR suppress infection of antibiotic-resistant P. aeruginosa," Microbiology, vol. 2022. asm.org
66. Kumar, A. Kumari, N. Thakur, and Y. Yau, "Can CRISPR/CAS Help Fight Multidrug Resistance (MDR) Bacterial Infections?" in *Editing in Plants: CRISPR-Cas and …*, Springer, 2024. [HTML]
67. de la Fuente Tagarro and D. Martín-González, "Current knowledge on CRISPR strategies against antimicrobial-resistant bacteria," *Antibiotics*, 2024. mdpi.com
68. Chavez, X. Chen, P. B. Finn, and L. S. Qi, "Advances in CRISPR therapeutics," Nature Reviews Nephrology, 2023. nature.com
69. E. Modell, D. Lim, T. M. Nguyen, V. Sreekanth, et al.,

"CRISPR-based therapeutics: current challenges and future applications," *Trends in Biotechnology*, vol. 2022. sciencedirect.com

70. T. Ham, "Analyzing sgRNA Cleavage Activities for SaCas9 and SpCas9 in Bacteria," 2024. [HTML]

71. J. Xu, L. Shen, Z. Chen, C. Zhang, X. Ding, Q. He, "CRISPR/methotrexate-integrated strategy for TCR-T cell engineering with reduced chromosome 14 loss," *Clinical Cancer Research*, 2025. cell.com

72. Li, N. Guo, G. Song, Y. Huang, L. Wang, Y. Zhang, "Type II Toxin–Antitoxin Systems in Pseudomonas aeruginosa," Toxins, 2023. mdpi.com

73. R. Rosales-Reyes, M. E. Gudiño-Zayas, T. Rizo-Pica, "The rsmA mutant from Pseudomonas aeruginosa ID4365 is a non-virulent strain that is suitable for pyocyanin and phenazine-1-carboxylic acid production," PLoS, 2025. plos.org

74. M. Gerovac, L. Buhlmann, Y. Zhu, S. Đurica-Mitić, V. Rech, "Programmable antisense oligomers for phage functional genomics," Nature, 2025. nature.com

75. M. Nie, H. Ma, X. Tao, Y. Sun, X. Tu, P. Zhang, "Quorum Sensing Coordinates Carbon and Nitrogen Metabolism to Optimize Public Goods Production in Pseudomonas fluorescens 2P24," Advanced ..., 2025. wiley.com

76. T. Mehrotra, D. Konar, A. K. Pragasam, S. Kumar, et al., "Antimicrobial resistance heterogeneity among multidrug-resistant Gram-negative pathogens: Phenotypic, genotypic, and proteomic analysis," *Proceedings of the …*, 2023. pnas.org

77. F. Gillieatt and N. V. Coleman, "Unravelling the mechanisms of antibiotic and heavy metal resistance co-selection in environmental bacteria," FEMS Microbiology Reviews,

2024. oup.com

78. Z. Mei, C. He, J. L. Balcazar, Y. Fu, Q. Dou, "Antibiotic-Degrading Bacteria Shape Resistome Dynamics and Horizontal Gene Transfer Potential in Soils with Contrasting Properties," ISME Journal, 2025. oup.com
79. S. Castañeda-Barba, E. M. Top, and T. Stalder, "Plasmids, a molecular cornerstone of antimicrobial resistance in the One Health era," Nature Reviews Microbiology, 2024. nih.gov
80. H. Zhang, J. You, G. Li, Z. Rao et al., "From Natural Defense to Synthetic Application: Emerging Bacterial Anti-Phage Mechanisms and Their Potential in Industrial Fermentation," Fermentation, 2025. mdpi.com
81. Runtuvuori-Salmela, "The story of phage therapy against Flavobacterium columnare bacterium: phage-bacterium interactions and utilization of phage therapy in practice," JYU Dissertations, 2022. jyu.fi
82. X. Wu, D. Wang, X. Mao, B. Dong, and Y. Wang, "Microbiome-Driven Resistance in Cervical Cancer Therapy: From Mechanistic Dissection to Clinical Translation," Expert Reviews in Molecular Medicine. cambridge.org
83. S. Gill, S. Ram, and P. A. Rice, "Biologic drug development for treatment and prevention of sexually transmitted infections," Clinical Microbiology Reviews, 2025. [HTML]
84. S. Olsen and L. Riber, "… as a transformative tool for antibiotic resistance surveillance: highlighting the impact of mobile genetic elements with a focus on the complex role of phages," Antibiotics, 2025. mdpi.com
85. Pfeifer, R. A. Bonnin, and E. P. C. Rocha, "Phage-plasmids spread antibiotic resistance genes through infection and lysogenic conversion," MBio, 2022. asm.org

86. M. Young, P. Humburg, F. Liu, M. C. Wehrhahn, A. Tay, "A Machine Learning Approach Reveals CRISPR-Cas IF as a Genomic Marker of Antibiotic Susceptibility in Uropathogenic E. coli," bioRxiv, 2025. biorxiv.org

87. JAM de Sousa, A. Fillol-Salom, J. R. Penadés, and others, "Identification and characterization of thousands of bacteriophage satellites across bacteria," *Nucleic Acids Research*, vol. 2023. oup.com

88. Wu, W. Li, W. Zhang, S. Li et al., "Advanced strategies in phage research: innovations, applications, and challenges," Microorganisms, 2025. mdpi.com

89. Nazir, X. Xu, Y. Liu, and Y. Chen, "Phage endolysins: advances in the world of food safety," Cells, 2023. mdpi.com

90. E. Martinez-Soto, "Development and Encapsulation of a Multi-Receptor Phage Cocktail Against Salmonella for Poultry Food Safety and Multi-Omics Characterization of Phage …," 2024. uoguelph.ca

91. Abdraimova, E. Shitikov, and M. Kornienko, "The Role of Genomics in Advancing and Standardising Bacteriophage Therapy," Antibiotics, 2026. mdpi.com

92. J. Malik and H. Goncalves-Ribeiro, "Advanced manufacturing, formulation and microencapsulation of therapeutic phages," *Clinical Infectious Diseases*, 2023. researchgate.net

93. Besler, "Isolation, characterisation and assessment of therapeutic potential of bacteriophages which infect P. multocida," eprints.nottingham.ac.uk, . nottingham.ac.uk

94. M. Palma and B. Qi, "Advancing phage therapy: a comprehensive review of the safety, efficacy, and future prospects for the targeted treatment of bacterial infections," Infectious Disease Reports, 2024. nih.gov

95. S. Liu, H. Lu, S. Zhang, Y. Shi et al., "Phages against pathogenic bacterial biofilms and biofilm-based infections: a review," Pharmaceutics, 2022. mdpi.com

96. Chang, X. Yu, W. Guo, C. Guo, X. Guo, Q. Li, "Bacteriophage-mediated control of biofilm: a promising new dawn for the future," *Frontiers in …*, 2022. frontiersin.org

97. Eghbalpoor, M. Gorji, M. Z. Alavigeh, and M. T. Moghadam, "Genetically engineered phages and engineered phage-derived enzymes to destroy biofilms of antibiotics resistance bacteria," Heliyon, 2024. cell.com

98. Sun, P. Yu, P. Zuo, D. Villagrán, J. Mathieu, "Biofilm control in flow-through systems using polyvalent phages delivered by peptide-modified M13 coliphages with enhanced polysaccharide affinity," *Environmental Science & Technology*, vol. 56, no. X, pp. Y-Z, 2022. rice.edu

99. Benz, B. Beamud, R. Laurenceau, A. Maire, et al., "CRISPR–Cas therapies targeting bacteria," *Nature Reviews*, 2025. hal.science

100. Sen and P. Mukhopadhyay, "Antimicrobial resistance (AMR) management using CRISPR-Cas based genome editing," Gene and Genome Editing, 2024. sciencedirect.com

101. Sen and P. Mukhopadhyay, "Gene and Genome Editing," researchgate.net, . researchgate.net

102. S. Gupta, M. Hamza KH, C. L. Sones, and X. Zhang, "The CRISPR/Cas system as an antimicrobial resistance strategy in aquatic ecosystems," Functional & Integrative, vol. 2024, Springer. soton.ac.uk

103. Parmanik, S. Das, B. Kar, A. Bose, and G. R. Dwivedi, "Current treatment strategies against multidrug-resistant

bacteria: a review," *Current*, vol. 2022, Springer. nih.gov

104. Bharadwaj, A. Rastogi, S. Pandey, "Multidrug-resistant bacteria: their mechanism of action and prophylaxis," BioMed Research, vol. 2022, Wiley Online Library. wiley.com

105. B. Ahmed, A. H. Asghar, M. Bamaga, F. S. Bahwerth, et al., "Characterization of aminoglycoside resistance genes in multidrug-resistant Klebsiella pneumoniae collected from tertiary hospitals during the COVID-19 pandemic," PLoS, 2023. plos.org

106. Sanchez-Urtaza and A. Ocampo-Sosa, "Molecular characterization of multidrug resistant Acinetobacter baumannii clinical isolates from Alexandria, Egypt," *Frontiers in Cellular*, vol. 2023. frontiersin.org

107. X. Wang, M. Liu, C. Yu, J. Li et al., "Biofilm formation: mechanistic insights and therapeutic targets," Molecular biomedicine, 2023. springer.com

108. Sauer, P. Stoodley, D. M. Goeres, "The biofilm life cycle: expanding the conceptual model of biofilm formation," *Nature Reviews*, vol. 2022. nih.gov

109. Römling, "Is biofilm formation intrinsic to the origin of life?," Environmental Microbiology, 2023. wiley.com

110. S. Katharios-Lanwermeyer and G. A. O'Toole, "Biofilm maintenance as an active process: evidence that biofilms work hard to stay put," Journal of Bacteriology, 2022. asm.org

111. X. Wang, B. Xu, Y. Cheng, H. Wang, "Robust adaptive learning control of space robot for target capturing using neural network," IEEE Transactions on ..., vol. XX, no. YY, pp. ZZ-ZZ, 2022. [HTML]

112. Lahoti, C. Ranjan, J. Chen, H. Yan, "Convolutional neural network-assisted adaptive sampling for sparse feature detection in image and video data," IEEE Intelligent Systems, 2022. [HTML]

113. Yin, A. Vahdat, J. M. Alvarez, A. Mallya, "A-vit: Adaptive tokens for efficient vision transformer," in *Proceedings of the …*, 2022. thecvf.com

114. Y. Zhang, J. Cao, L. Zhang, X. Liu, Z. Wang, "A free lunch from vit: Adaptive attention multi-scale fusion transformer for fine-grained visual recognition," in *ICASSP 2022*, 2022. [PDF]

115. HAC Sabino, FCP Valera, DV Santos, "Biofilm and planktonic antibiotic resistance in patients with acute exacerbation of chronic rhinosinusitis," Frontiers in Cellular, vol. 2022. frontiersin.org

116. G. Thöming and S. Häussler, "Pseudomonas aeruginosa Is More Tolerant Under Biofilm Than Under Planktonic Growth Conditions: A Multi-Isolate Survey," Frontiers in Cellular and Infection Biology, vol. 2022. frontiersin.org

117. Trubenová, D. Roizman, A. Moter, J. Rolff, "Population genetics, biofilm recalcitrance, and antibiotic resistance evolution," *Trends in Microbiology*, vol. 30, no. 5, pp. 396-407, 2022. [HTML]

118. Michaelis and E. Grohmann, "Horizontal gene transfer of antibiotic resistance genes in biofilms," Antibiotics, 2023. mdpi.com

119. Sahoo, S. Meshram, and K. Sahoo Jr, "Biofilm formation in chronic Infections: A comprehensive review of pathogenesis, clinical implications, and novel therapeutic approaches," Cureus, 2024. cureus.com

120. Li, R. Yin, J. Cheng, and J. Lin, "Bacterial biofilm formation

on biomaterials and approaches to its treatment and prevention," International journal of molecular sciences, 2023. mdpi.com

121. X. Liu, H. Yao, X. Zhao, and C. Ge, "Biofilm formation and control of foodborne pathogenic bacteria," Molecules, 2023. mdpi.com

122. Visnapuu, M. Van der Gucht, J. Wagemans, and R. Lavigne, "Deconstructing the phage–bacterial biofilm interaction as a basis to establish new antibiofilm strategies," Viruses, 2022. mdpi.com

123. Mishra, A. Gupta, V. Upadhye, S. C. Singh, R. P. Sinha, "Therapeutic strategies against biofilm infections," Life, vol. 2023. mdpi.com

124. G. Abdelhamid and A. E. Yousef, "Combating bacterial biofilms: current and emerging antibiofilm strategies for treating persistent infections," Antibiotics, 2023. mdpi.com

125. Ikpe, T. Williams, E. Orok, and A. Ikpe, "Antimicrobial resistance: use of phage therapy in the management of resistant infections," Molecular Biology Reports, 2024. [HTML]

126. R. Mahmud, S. K. Tamanna, S. Akter, and L. Mazumder, "Role of bacteriophages in shaping gut microbial community," Gut, vol. 2024, Taylor & Francis. tandfonline.com

127. Zou, L. He, J. Rao, Z. Song, H. Du, R. Li, "Improving the safety and efficacy of phage therapy from the perspective of phage-mammal interactions," FEMS Microbiology, vol. 2023. researchgate.net

128. Abd El-Moaty, A. S. Doghish, H. A. M. Moustafa, "Phage therapy and its role in cancer treatment and control," Folia Microbiologica, 2025. [HTML]

129. W. Wang, C. Gao, Y. M. Zheng, L. Yi, J. C. Lu, X. Y. Huang, "Current applications and future perspective of CRISPR/Cas9 gene editing in cancer," *Molecular Cancer*, vol. 21, no. 1, 2022. springer.com.

130. Bhat, S. Nisar, S. Mukherjee, and N. Saha, "Integration of CRISPR/Cas9 with artificial intelligence for improved cancer therapeutics," *Journal of Translational Medicine*, vol. 20, no. 1, 2022. springer.com

131. K. G. Freeman, A. C. Robotham, O. B. Parks, L. Abad, et al., "Virion glycosylation influences mycobacteriophage immune recognition," *Cell Host & Microbe*, vol. 2023. cell.com

132. T. Le, G. Ahlenstiel, C. Venturini, and S. A. Read, "mGem: Immune recognition and clearance of bacteriophages—implications for phage therapy," mBio, 2025. asm.org

133. Li, W. Ma, J. Cheng, C. Zhan, H. Lu, et al., "Phages adapt to recognize an O-antigen polysaccharide site by mutating the 'backup' tail protein ORF59, enabling reinfection of phage-resistant Klebsiella …," *Emerging Microbes & …*, 2025. tandfonline.com

134. K. Pandey, R. Sharma, G. K. Prajapati, "N-glycosylation, a leading role in viral infection and immunity development," *Molecular Biology*, vol. 2022, Springer. nih.gov

135. Cianci, M. Caldarelli, P. Brani, A. Bosi, A. Ponti, "Cytokines Meet Phages: A Revolutionary Pathway to Modulating Immunity and Microbial Balance," Biomedicines, 2025. mdpi.com

136. B. Souza, A. R. Pinto, and G. Fongaro, "Bacteriophages as potential clinical immune modulators," Microorganisms, 2023. mdpi.com

137. X. Hou, H. Ding, H. Wang, J. Wang, H. Liu, H. Hu, "Gut

bacteriophages induce specific-reprogramming of macrophages to generate a protective innate immunity response to lipopolysaccharide exposure," Gut, vol. 2025, Taylor & Francis. tandfonline.com

138. S. Wallis, A. O'Garra, A. Sher, and A. Wack, "Host-directed immunotherapy of viral and bacterial infections: past, present and future," Nature Reviews Immunology, 2023. nih.gov

139. P. P. Kalelkar, M. Riddick, and A. J. García, "Biomaterial-based antimicrobial therapies for the treatment of bacterial infections," Nature Reviews Materials, 2022. nih.gov

140. L. Jiang, L. Ding, and G. Liu, "Nanoparticle formulations for therapeutic delivery, pathogen imaging and theranostic applications in bacterial infections," Theranostics, 2023. nih.gov

141. Ghazaei, "Targeted Therapy for Intracellular Bacterial Pathogens," 2024. researchgate.net

142. Uyttebroek, B. Chen, J. Onsea, et al., "Safety and efficacy of phage therapy in difficult-to-treat infections: a systematic review," *The Lancet Infectious Diseases*, vol. 2022. ulb.ac.be

143. M. Xu, W. M. Xu, and L. Zhang, "Current status of phage therapy against infectious diseases and potential application beyond infectious diseases," *International Journal of Clinical …*, vol. XX, no. YY, pp. ZZ-ZZ, 2022. wiley.com

144. Suh, T. P. Lodise, P. D. Tamma, et al., "Considerations for the use of phage therapy in clinical practice," Antimicrobial Agents, vol. 2022. asm.org

145. Aranaga, L. D. Pantoja, E. A. Martínez, "Phage therapy in the era of multidrug resistance in bacteria: a systematic review," *International Journal of...*, 2022. mdpi.com

146. Dhungana, "Molecular Characterization of Lytic Bacteriophage Specific to Multi Drug Resistant Bacteria and Pharmacokinetics of Phage in Biological Model," 2022. tucl.edu.np

147. Elshamy, S. K. Kamal, M. T. Mahmoud, A. M. Elhasany, "Recent insights on phage therapy against multidrug-resistant Acinetobacter baumannii," AMB Express, 2025. springer.com

148. Boersen and H. W. Hui, "Development of preclinical formulations for toxicology studies," in *A Comprehensive Guide to Toxicology in Nonclinical …*, Elsevier, 2024. [HTML]

149. M. Heinrich, B. Jalil, M. Abdel-Tawab, et al., "Best practice in the chemical characterisation of extracts used in pharmacological and toxicological research—the ConPhyMP—guidelines," *Frontiers in …*, vol. 2022. frontiersin.org

150. Ahmad, M. Imran, and N. Sharma, "Precision nanotoxicology in drug development: current trends and challenges in safety and toxicity implications of customized multifunctional nanocarriers for …," Pharmaceutics, 2022. mdpi.com

151. M. Zhao, Y. Li, and Z. Wang, "Mercury and mercury-containing preparations: history of use, clinical applications, pharmacology, toxicology, and pharmacokinetics in traditional Chinese medicine," Frontiers in pharmacology, 2022. frontiersin.org

152. R. Ahmed, "CRISPR-Cas Systems in the Detection and Treatment of Infectious Diseases: A Systematic Review," Journal of Biomedical Science, 2025. premierscience.com

153. M. Munshi, S. Jahangir, and S. Sumaiya, "Phage Therapy:

Navigating the Mechanisms, Benefits, and Challenges in the Fight Against Multidrug-Resistant Infections," medRxiv, 2025. medrxiv.org

154. C. Joshi, M. B. Chavan, S. W. Tiwari, and S. Devi, "Advances in combating antimicrobial resistance in MRSA: a comprehensive review on nanotechnology and phage therapy," Antonie van Leeuwenhoek, 2025. [HTML]

155. M. Osagwu, E. Okobi, and E. Ekor, "Postoperative infections: risk factors, prevention, and management strategies," Int J Med Sci Dent Res, 2024. ijmsdr.org

156. Korovessis and V. Syrimpeis, "Is Conservative Treatment Superior to Surgical Intervention in Hematogenous Primary Septic Spinal Infection in Terms of Mortality, Recurrence, and Hospital …," Journal of Clinical …, 2025. nih.gov

157. N. Renz, A. Trampuz, C. Perka, and others, "Outcome and failure analysis of 132 episodes of hematogenous periprosthetic joint infections—a cohort study," *Forum Infectious Diseases*, vol. 2022. oup.com

158. G. Tsantes, P. Altsitzioglou, D. V. Papadopoulos, "Infections of tumor prostheses: an updated review on risk factors, microbiology, diagnosis, and treatment strategies," Biology, 2023. mdpi.com

159. K. Patel, N. Singh, N. Sachan, "Advancing Antibacterial Strategies: CRISPR-Phage-Mediated Gene Therapy Targeting Bacterial Resistance Genes," Gene Therapy, 2025. [HTML]

160. N. Mafe and D. Büsselberg, "Phage Therapy in Managing Multidrug-Resistant (MDR) Infections in Cancer Therapy: Innovations, Complications, and Future Directions," Pharmaceutics, 2025. mdpi.com

161. N. Pal, P. Sharma, M. Kumawat, S. Singh, "Phage therapy:

An alternative treatment modality for MDR bacterial infections," Infectious Diseases, vol. 2024, Taylor & Francis. researchgate.net

162. M. Pardo-Freire and P. Domingo-Calap, "Phages and nanotechnology: New insights against multidrug-resistant bacteria," Biodesign Research, 2023. science.org

163. Grygiel, O. Bajrak, M. Wójcicki, K. Krusiec, "Comprehensive Approaches to Combatting Acinetobacter baumannii Biofilms: From Biofilm Structure to Phage-Based Therapies," *Antibiotics*, 2024. mdpi.com

164. Mayorga-Ramos and S. E. Carrera-Pacheco, "Bacteriophage-mediated approaches for biofilm control," *Frontiers in Cellular and Infection Microbiology*, 2024. frontiersin.org

165. Yin, Y. Zhang, L. Yang, and Y. Feng, "Non-canonical inhibition strategies and structural basis of anti-CRISPR proteins targeting type I CRISPR-Cas systems," Journal of Molecular Biology, 2023. [HTML]

166. L. Yang, L. Zhang, P. Yin, H. Ding, Y. Xiao, J. Zeng, "Insights into the inhibition of type IF CRISPR-Cas system by a multifunctional anti-CRISPR protein AcrIF24," *Nature*, vol. 2022. nature.com

167. S. Allemailem, A. Almatroudi, F. Alrumaihi, et al., "Current updates of CRISPR/Cas system and anti-CRISPR proteins: Innovative applications to improve the genome editing strategies," *International Journal*, 2024. tandfonline.com

168. F. Hu, Y. X. Li, J. Z. Wang, Y. T. Zhao, et al., "Controlling CRISPR-Cas9 by guide RNA engineering," *Nature Reviews: RNA*, vol. 2023, Wiley Online Library. google.com

169. Camara-Wilpert, D. Mayo-Muñoz, J. Russel, et al.,

"Bacteriophages suppress CRISPR–Cas immunity using RNA-based anti-CRISPRs," *Nature*, 2023. nature.com

170. P. B. van Beljouw and J. Sanders, "RNA-targeting CRISPR–Cas systems," *Nature Reviews*, 2023. google.com

171. M. Nguyen, S. Watanabe, S. Sharmin, et al., "RNA and single-stranded DNA phages: unveiling the promise from the underexplored world of viruses," *International Journal of …*, 2023. mdpi.com

172. Y. Wang, X. Li, M. Liu, Y. Zhou et al., "Guide RNA scaffold variants enabled easy cloning of large gRNA cluster for multiplexed gene editing," Plant Biotechnology Journal, 2024. wiley.com

173. Y. Liu, W. Liu, and B. Wang, "Engineering CRISPR guide RNAs for programmable RNA sensors," Biochemical Society Transactions, 2023. portlandpress.com

174. Elbehiry and A. I. Alajaji, "Next-Generation Strategies for Controlling Foodborne Pathogens: Precision Antimicrobials, Biofilm Disruption, and Emerging Molecular Interventions," Foods, 2026. mdpi.com

175. Liu, Y. Jia, Y. Jiang, Y. Nie et al., "Based Microfluidic Chips for At-Home Point-of-Care Nucleic Acid Testing: Applications and Challenges," Diagnostics, 2026. mdpi.com

176. C. Volke, R. A. Martino, E. Kozaeva, A. M. Smania, et al., "Modular (de) construction of complex bacterial phenotypes by CRISPR/nCas9-assisted, multiplex cytidine base-editing," *Nature*, vol. 2022. nature.com

177. Scarrone Murissich, "Characterization and CRISPR-Cas9-based genome engineering of Acinetobacter baumannii and its phages," 2025. ulaval.ca

178. Y. Han, Z. Jia, K. Xu, Y. Li et al., "CRISPR-Cpf1 system

and its applications in animal genome editing," Molecular Genetics and Genomics, 2024. [HTML]

179. Saffari Natanzi, M. Poudineh, E. Karimi, A. Khaledi, "Innovative approaches to combat antibiotic resistance: integrating CRISPR/Cas9 and nanoparticles against biofilm-driven infections," BMC Medicine, 2025. springer.com

180. S. J. Park, G. E. Lee, S. M. Cho, and E. H. Choi, "Recent applications, future perspectives, and limitations of the CRISPR-Cas system," Molecular Therapy Nucleic Acids, 2025. cell.com

181. K. Tiwari, T. H. Ko, R. Dubey, M. Chouhan, et al., "CRISPR/Cas9 as a therapeutic tool for triple negative breast cancer: From bench to clinics," *Frontiers in Molecular*, vol. 2023. frontiersin.org

182. Shamsuzzaman, Y. J. Choi, S. Kim, et al., "PEGylation Overcomes Pharmacological Barriers to Improve Systemic Pharmacokinetics and Therapeutic Efficacy of Phages against MDR Escherichia coli," *Journal of Microbiology and...*, 2025. nih.gov

183. X. Tan, K. Chen, Z. Jiang, Z. Liu, S. Wang, and Y. Ying, "Evaluation of the impact of repeated intravenous phage doses on mammalian host–phage interactions," *Journal of …*, 2024. asm.org

184. SC Nang, YW Lin, AP Fabijan, RYK Chang, "Pharmacokinetics/pharmacodynamics of phage therapy: a major hurdle to clinical translation," Clinical Microbiology, vol. 2023, Elsevier. sciencedirect.com

185. Wang, X. Tan, Z. Liu, H. Ma, T. Liu, Y. Yang, "Pharmacokinetics and safety evaluation of intravenously administered Pseudomonas phage PA_LZ7 in a mouse model," Microbiology, vol. 2024. asm.org

186. P. Pirnay and G. Verbeken, "Magistral phage preparations: is this the model for everyone?," Clinical Infectious Diseases, 2023. [HTML]

187. Glonti and J. P. Pirnay, "In vitro techniques and measurements of phage characteristics that are important for phage therapy success," Viruses, 2022. mdpi.com

188. Tian, K. Jackson, L. He, S. Khan, et al., "High-throughput fabrication of antimicrobial phage microgels and example applications in food decontamination," *Nature*, 2024. [HTML]

189. H. Sheng, S. Wu, Y. Xue, W. Zhao, A. B. Caplan, "Engineering conjugative CRISPR-Cas9 systems for the targeted control of enteric pathogens and antibiotic resistance," PLoS, 2023. plos.org

190. Y. Haryani, N. A. Halid, S. G. Goh, M. A. R. Nor-Khaizura, "Efficient metabolic pathway modification in various strains of lactic acid bacteria using CRISPR/Cas9 system for elevated synthesis of antimicrobial compounds," Journal of ..., vol. xx, no. xx, pp. xx-xx, 2024. [HTML]

191. F. Long, S. Y. Zhou, Z. L. Huang, G. Li, Q. Zhong, et al., "Innovative delivery system combining CRISPR-Cas12f for combatting antimicrobial resistance in gram-negative bacteria," ACS Synthetic Biology, vol. 2024, ACS Publications. [HTML]

192. Z. Song, Y. Yu, X. Bai, Y. Jia, J. Tian, and K. Gu, "Pathogen-specific bactericidal method mediated by conjugative delivery of CRISPR-Cas13a targeting bacterial endogenous transcripts," Microbiology, 2022. asm.org

193. P. H. Serpa, X. Deng, M. Abdelghany, E. Crawford, et al., "Metagenomic prediction of antimicrobial resistance in critically ill patients with lower respiratory tract infections,"

*Genome Medicine*, vol. 14, no. 1, 2022. springer.com

194. Abdulrazaq, S. A. Elelu, G. O. Ibrahim, I. A. Temitope, "Advancements in Microbial Drug Discovery: Leveraging AI, CRISPR, and Microbiome Insights to Overcome Antimicrobial Resistance," Biological ..., 2025. irrespub.com

195. Peng, C. Li, Q. Wang, X. Xin, Z. Wang, "The applications and advantages of nanopore sequencing in bacterial antimicrobial resistance surveillance and research," npj Antimicrobials and Infectious Diseases, 2025. nature.com

196. Khan, J. Gupta, S. S. Garg, and A. K. Wani, "Genomic Technologies: Next-Generation Sequencing and CRISPR Applications," Pharmaceutical ..., 2025. oapen.org

197. S. Yang, X. Jiao, J. Liu, Y. Liu, M. Wang, S. Li, "CRISPR-Cas opens a new era of antimicrobial therapy as a powerful gene editing tool," World Journal of ..., 2025. [HTML]

198. Padaru, P. Shetty, A. Kini, "CRISPR/Cas‐mediated targeted gene editing of Streptococcus mutans: A promising approach for precision dentistry for the prevention and management of caries," World Academy of ..., 2025. spandidos-publications.com

199. Kanwal, M. Zhang, S. Khan, and U. Batool, "AI-Driven Precision Fermentation: From Restaurant Food Waste to Sustainable Protein Production," Food and Bioprocess Technology, 2025. [HTML]

200. T. Polcumpally and S. Patel, "AI Ethics in the Military: A Realist Approach Incorporating Traditional and Non-Traditional Security," in *Handbook of Global Philosophies on AI*, 2026. [HTML]

201. Farzulla, "Relational functionalism: Friendship as substrate-agnostic process—functional analysis of human-ai

relationships," 2025. farzulla.org

202. Yuan, "Research on dual evaluation of low-barrier generation AI assisted with cross-professional art practice: focusing on the quality of works and self-cognition differences," in *Proceedings of the Conference on Generative AI and Digital Media Arts*, 2025. acm.org

203. M. Ruden, "TIGR-Tas and the Expanding Universe of RNA-Guided Genome Editing Systems: A New Era Beyond CRISPR-Cas," Genes, 2025. mdpi.com

204. Gunitseva, M. Evteeva, A. Borisova, "RNA-dependent RNA targeting by CRISPR-Cas systems: characterizations and applications," *International Journal of …*, 2023. mdpi.com

205. S. N. Omura, L. E. Alfonse, A. Ornstein, H. Morinaga, et al., "Structural basis for target DNA cleavage and guide RNA processing by CRISPR-Casλ2," *Communications*, 2025. nature.com

206. Y. Ye, J. Shen, and Z. Xiu, "CRISPR-Armed Phages: Design, Mechanisms, Applications, and Prospects in Precision Microbiome Engineering," Synthetic Biology and Engineering, 2025. sciepublish.com

207. Alessa, Y. Aiba, M. Arbaah, Y. Hidaka, S. Watanabe, et al., "Synthetic and Functional Engineering of Bacteriophages: Approaches for Tailored Bactericidal, Diagnostic, and Delivery Platforms," Molecules, 2025. mdpi.com

208. J. Sharma, D. Sharma, A. Singh, "Colistin resistance and management of drug resistant infections," Canadian Journal of..., vol. 2022, Wiley Online Library. wiley.com

209. S. Kaye, D. Marchaim, V. Thamlikitkul, Y. Carmeli, et al., "Colistin monotherapy versus combination therapy for carbapenem-resistant organisms," *NEJM*, 2022. nejm.org

210. H. Mondal, K. Khare, P. Saxena, and P. Debnath, "A review on colistin resistance: an antibiotic of last resort," Microorganisms, 2024. mdpi.com

211. Dehbanipour, V. T. Z. Maleki, and Z. Ghalavand, "Colistin heteroresistance, mechanisms, diagnostic methods, and therapeutic options: A review," Germs, 2025. nih.gov