Chapter

The War between Bacteria and Bacteriophages

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Abstract

The rapid emergence and dissemination of multidrug-resistant (MDR) bacteria represents a worldwide crisis concerning that humankind is re-entering the 'pre-antibiotics' era. Before the discovery of antibiotics, bacteriophage therapy was widely enforced to combat bacterial infections. However, the discovery of penicillin in 1940 and other novel antibiotics replaced phage therapy, and they are being used as the first line of defence against pathogenic bacterial infections. Factors such as selective pressure resulted in bacteria becoming insensitive to one or multiple antibiotics, frequently leading to limited treatment options. This prompted a renewal of interest to the phage therapy that remains dubious due to its disadvantages such as host specificity and the development of bacterial resistance against phages. Evolution of bacterial genomes allowed bacteria to acquire vast mechanisms interfering with phage infection such as inhibition of phage adsorption, prevention of phage entry, superinfection exclusion, restriction-modification and abortive infection. Interestingly, phages have developed diverse counterstrategies to circumvent bacterial anti-phage mechanisms including digging for receptors, adapting to new receptors and masking and modifying restriction sites. Understanding the complex dynamics of bacteria-phage interaction is a preliminary step towards designing synthetic phages that can overcome limitations of phage therapy and potentially lead to defeating MDR bacteria.

Keywords: bacteria-phage arms race, CRISPR system, anti-CRISPR system, superinfection exclusion (Sie), restriction-modification, abortive infection (Abi)

1. Introduction

Antimicrobial resistance is a global public health crisis. According to Public Health England [1], each year approximately 25,000 people die across Europe due to hospital-acquired infections caused by antibiotic-resistant and MDR bacteria such as *Mycobacterium tuberculosis*, Methicillin-resistant *Staphylococcus aureus* and multiresistant Gram-negative bacteria. Gram-negative infections include those caused by *Escherichia coli*, *Klebsiella pneumoniae* and *Pseudomonas aeruginosa* [2]. Nevertheless, it is estimated that by 2050, the global yearly death toll will increase to 10 million. Accelerating emerge of antimicrobial resistance seriously threatens the effectiveness of treatments for pneumonia, meningitis and tuberculosis, in addition to diminishing prevention of infections acquired during surgeries and chemotherapies. The crisis of the antibiotic resistance requires urgent, coordinated action.

Misuse and overuse of antibiotics must be controlled, implementation of new policies regarding prescriptions has to be internationally addressed; and development of new therapeutics is urgently required [1].

Félix d'Herelle, known as the father of bacteriophage (or phage) therapy [3], brought an evolutionary discovery of phages as therapeutics for various infections and conditions. Phage therapy was widely enforced in the 1920s and 1930s to combat the bacterial infections. However, in the 1940s, the newly discovered antibiotics replaced the phage therapy (except Russia, Georgia and Poland) [4].

The emergence of MDR bacteria prompted a renewal of the interest to the phage therapy as an alternative treatment to overcome a broad spectrum of resistant bacterial infections. Phage therapy and phage cocktails that contain a mixture of different bacteria-specific phages, drawn interest within molecular biology and modern medical research as potential antimicrobials that could tackle the crisis of antimicrobial resistance. Nonetheless, the phage therapy remains controversial due to its disadvantages such as bacteriophage resistance: bacteria-phage evolutionary arms race that could put a burden on a long-time application of phage therapy as an anti-infectious agent [5].

Phage therapy has many advantages, primary because phages are very specific (generally limited to one species) and easy to obtain as they are widely distributed in locations populated by bacterial hosts including soil and seawater, and they do not have any known chemical side effects like antimicrobials [6].

Understanding host-phage interactions and 'the war between bacteria and phages' are steps towards designing engineering 'broad-spectrum phage' that can overcome the limitations of phage therapy and potentially overcome a wide range of resistant bacterial infections [6].

2. The evolutionary phage-host arms race

Phages are obligate intracellular parasites that distinctively infect bacterial cells. Although phages are very specific to their host, generally limited to one species, they pose an enormous threat to bacteria as in some habitats they outnumber their hosts by nearly 10-fold number [7]. Phages are the most abundant, ubiquitous and diversified organisms in the biosphere [8, 9]. Phage-host interaction and fight for the survival led to the evolution of bacterial and viral genomes and, therefore, to the evolution of resistance mechanisms. Bacteria, continuously, evolve many molecular mechanisms, driven by gene expression to prevent phage infection. These evolving phage-resistance mechanisms in bacteria induce the parallel co-evolution of phage diversity and adaptability [10, 11]. The co-evolving genetic variations and counteradaptations, in bacteria and phages, drive the evolutionary phage-host arm race [11, 12].

Leigh Van Valen, an evolutionary biologist, metaphorised the co-evolutionary arm race and proposed the Red Queen hypothesis [13].

'It takes all the running you can do, to stay in the same place' the Red Queen says to Alice in *Through the Looking-Glass*.

The Red Queen hypothesis proposes that to survive, microorganisms must constantly adapt, evolve and thrive against ever-evolving antagonistic microorganisms within the same ecological niche [14].

Bacteria have developed various anti-phage mechanisms including non-adaptive defences (non-specific) and adaptive defences associated with Clustered Regularly Interspaced Short Palindromic Repeats (CRISPR) along with CRISPR-associated (Cas) proteins [7, 15–18].

The non-specific adaptations (analogues to innate immunity in multicellular organisms) act as primary mechanisms to evade viral infection, and they include mechanisms that inhibit phage adsorption and prevent nucleic acid entry, superinfection exclusion systems, restriction-modification systems and abortive infection [7, 19].

On the other hand, the adaptive resistance (analogues to the acquired immunity in multicellular organisms) serves as a second line of defence, which is very efficient and phage-specific.

Interestingly, it was observed that the bacterial anti-phage mechanisms are generally present in a genomic array, known as 'defence islands' [20]. The 'defence islands' are enriched in putative operons and contain numerous overrepresented genes encoding diverged variants of antiviral defence systems. Moreover, scientific evidence and characteristic operonic organisation of 'defence islands' show that many more anti-phage mechanisms are yet to be discovered [21–24].

Although bacteria have developed several resistance mechanisms against phages, phages can circumvent bacterial anti-phage mechanisms on the grounds of their genomic plasticity and rapid replication rates. These counterstrategies include point mutations in specific genes and genome rearrangements that allow phages to evade bacterial antiviral systems such as CRISPR/Cas arrays by using anti-CRISPR proteins and abortive infection by hijacking bacterial antitoxins, as well as escaping from adsorption inhibition and restriction-modification mechanisms [15–18].

This chapter will comment on the genetic basis of bacterial resistance to phages and different strategies used by phages to evade bacterial resistance mechanisms.

3. Preventing phage adsorption and phage's counterstrategy

Phage adsorption to host-specific receptors on the cell surface is the initial step of the infection and host-phage interaction. Depending on the nature of bacteria, whether it is Gram-positive or Gram-negative proteins, lipopolysaccharides, teichoic acids and other cell surface structures can serve as irreversible phage-binding receptors [19]. These receptors might be present in the cell wall, bacterial capsules, slime layers, pili or flagella [25].

Bacteria have acquired various barriers to inhibit phage adsorption, such as blocking of phage receptors, production of extracellular matrix (e.g. capsule, slime layers) and production of competitive inhibitors [26–31]. The diversity of phage receptors in the host is influenced by co-evolutionary adaptations of phages to overcome these barriers [32]. This includes diversity-generating retroelements (DGRs) and phase variation mechanisms causing phenotypical differences within the bacterial colony [7, 33, 34].

Phase variation is a heritable, yet reversible process regulating gene expression in bacteria; genes can switch between a functional (expression) and a non-functional state leading to phenotypical variations within the bacterial population even when strains have identical genotype. Sørensen et al. [35] investigated the underlying resistance mechanism of *Campylobacter jejuni* (NCTC11168) to phage F336. They have discovered that phage F336 relies on the hypervariable O-methyl phosphoramidate (MeOPN) modification of capsular polysaccharides (CPS) for successful adsorption to the bacterial surface. Nevertheless, loss of MeOPN receptor on the bacterial cell surface due to phase variation in the *cj1421* gene encoding the MeOPN-GalfNAc transferase (MeOPN transferase attaches MeOPN to GalfNAc and Hep side chains of CPS) results in phage resistance [35, 36].

DGRs are genetic elements diversifying DNA sequences and the proteins they encode ultimately mediating the evolution of ligand-receptor interactions. Error-prone DGRs and random mutations in the bacterial genes encoding cell surface receptors lead to the alternation and change in the structural composition of the phage receptors, making them non-complementary to the phage's anti-receptors, known as receptor-binding proteins (RBP) [34] (**Figure 1(1)**).

Yet, phage's replication is exceedingly error-prone, therefore causing many random mutations in the genes encoding the RBP or tail fibres. Phages also possess DGRs that mediate phage's tropism by accelerating the variability in the receptor-coding genes through reverse transcription process [37]. The changes in the nucleotide sequence in the RBP-coding gene may ultimately lead to the adaptation to the modified receptor (**Figure 1(2)**), thus the ability to adsorb and infect the bacterial cell.

Unsurprisingly, bacteria also exhibit different strategies to block their receptors [28–31].

Figure 1(4) demonstrates the findings from studies conducted on *Staphylococcus aureus* by Nordstrom and Forsgren [38]. Mutants of *Staphylococcus aureus* producing higher anticomplementary protein A were found to adsorb fewer phages than *Staphylococcus aureus mutants with scarce of protein A, which had an apparent increased ability to adsorb phages* [38]. These findings indicate that some bacteria, including *Staphylococcus aureus, are capable of production of surface proteins* that mask the phage receptors making them inaccessible for phage recognition and attachment (**Figure 1(3)**).

Receptors located on bacterial cell surface serve a vital role in bacterial metabolism; they may function as membrane porins, adhesions or chemical receptors [19]. Therefore, mutation or complete loss of the receptor might be lethal for bacteria. To inhibit phage adsorption, bacteria can produce surface molecules, such as exopolysaccharides.

Exopolysaccharides are extracellular polysaccharides acting as a physical barrier, composing slime or capsules surrounding bacterial cells that lead to inaccessible host receptors for efficient phage adsorption [39] (**Figure 2**). Studies conducted by Looijesteijn et al. [40] shown that exopolysaccharides *produced by Lactococcus lactis function as external protection from phages and the* cell wall destructing lysozyme, due to masked cell surface receptors [40].

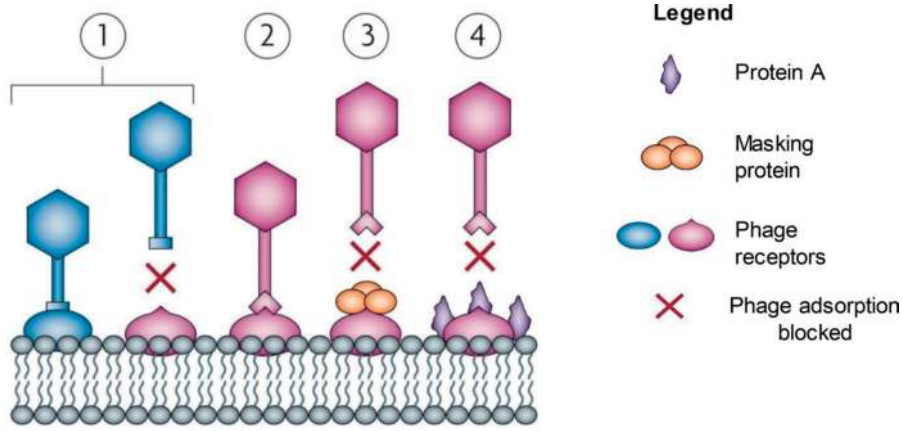

Figure 1.
Bacterial defence mechanisms preventing phage adsorption and phage's counteradaptations. (1) Phage adsorption to a host-specific receptor site on a host cell surface. Bacterium evolves phage resistance by the modification of these cell surface receptors; phage is incapable of binding to the altered receptor. (2) Phage's adaptation to these modifications through mutations in receptor-binding protein gene that leads to the co-evolution of bacterial genetic variation. Bacteria are also capable of producing proteins that mask the phage recognition site receptors (3 and 4), thus making the receptor inaccessible for phage adsorption [28–31]. Image courtesy of springer nature: https://www.ncbi.nlm.nih.gov/pubmed/20348932.

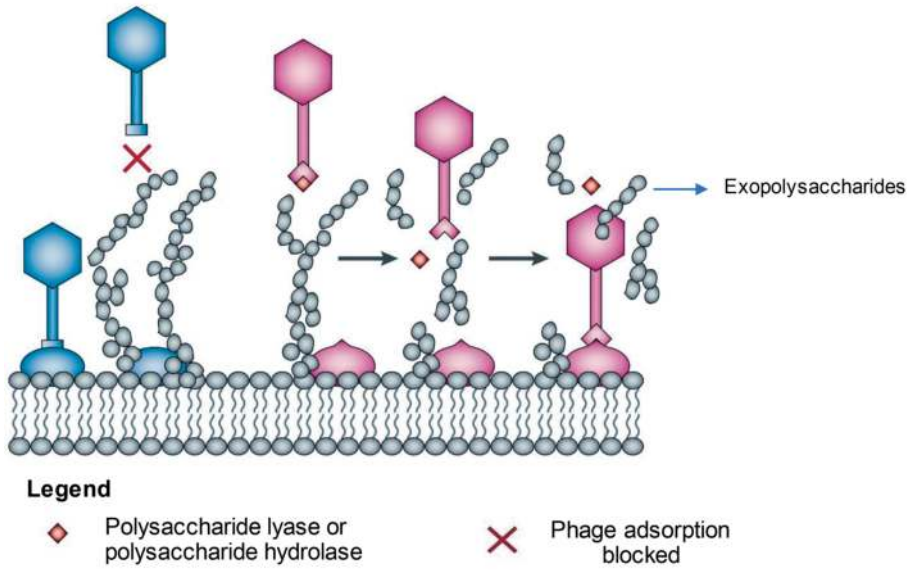

Figure 2.
Bacterial strategies to inhibit phage adsorption and phage strategies to access host receptors. Some bacteria are capable of the production of exopolysaccharides, which act as an outer shield, protecting a cell from the phage infection [28–31]. If the phage does not possess any polysaccharide-degrading enzymes, it cannot access the host cell membrane receptor. However, some phages evolved mechanisms allowing them to recognise these extracellular matrixes and degrade them by the means of hydrolases and lyases [15–18]. Image courtesy of Springer Nature: https://www.ncbi.nlm.nih.gov/pubmed/20348932.

Nevertheless, some phages evolved mechanisms allowing them to recognise these extracellular matrixes and degrade them by utilising hydrolases and lyases (**Figure 2**) [15–18]. The polysaccharide-degrading enzymes allow phages to gain access to the receptor that may lead to the viral propagation. They are commonly present bound to the RBPs or exist as free soluble enzymes from previously lysed bacterial cells [41].

4. Preventing phage DNA entry and phage's counteradaptations

If phage bypasses primary antiviral strategies, it is now able to initiate infection by adsorption to a specific receptor site on a host cell surface through phage RBP [42, 43]. Upon interaction with the cell receptors, the phage injects its genetic material (single or double-stranded DNA or RNA) into the cytoplasm of the host. Depending on the nature of the phage and growth conditions of the host cell, it follows one of the two life cycles: lytic or lysogenic (**Figure 3**).

In the lytic cycle, virulent phages degrade host's genome leading to the biosynthesis of viral proteins and nucleic acids for the assembly of phage progeny. Eventually, the bacterial cell lysis, releasing a multitude of newly assembled phages, is ready to infect a new host cell [46].

In contrast, temperate phages might enter the lytic or lysogenic cycle, if the host cell exists in adverse environmental conditions that could potentially limit the number of produced progeny (**Figure 3** demonstrates typical lifecycle of temperate phage using coliphage λ as an example) [44, 45]. In the lysogenic phase, repressed phage genome integrates into the bacterial chromosome as a prophage. This process causes the proliferation of prophage during replication and binary fission of bacterial DNA.

Prophage only expresses a repressor protein-coding gene. The repressor protein binds to the operator sites of the other genes and ultimately inhibits synthesis of phage enzymes and proteins required for the lytic cycle.

When the synthesis of the repressor protein stops or if it becomes inactivated, a prophage may excise from the bacterial chromosome, initiating a lytic cycle (induction) which leads to the multiplication and release of virulent phages and lysis of a host cell [44, 45].

If the phage remains in the nearly dormant state (prophage), the lysogenic bacterium is immune to subsequent infection by other phages that are the same or closely analogous to the integrated prophage by means of Superinfection exclusion (Sie) systems [47].

Sie systems are membrane-associated proteins, generally, phage or prophage encoded, that prevent phage genome entry into a host cell [47]. **Figure 4** shows the role of Sie system (proteins Imm and Sp) in blocking phage T4 DNA entry into Gram-negative *Escherichia coli*. Despite successful attachment to the phage-specific receptor, phage DNA is directly blocked by Imm protein from translocating into the cytoplasm of the cell. Sp system, on the other hand, prevents the degradation of the peptidoglycan layer by inhibiting the activity of T4 lysozyme [26–31, 48].

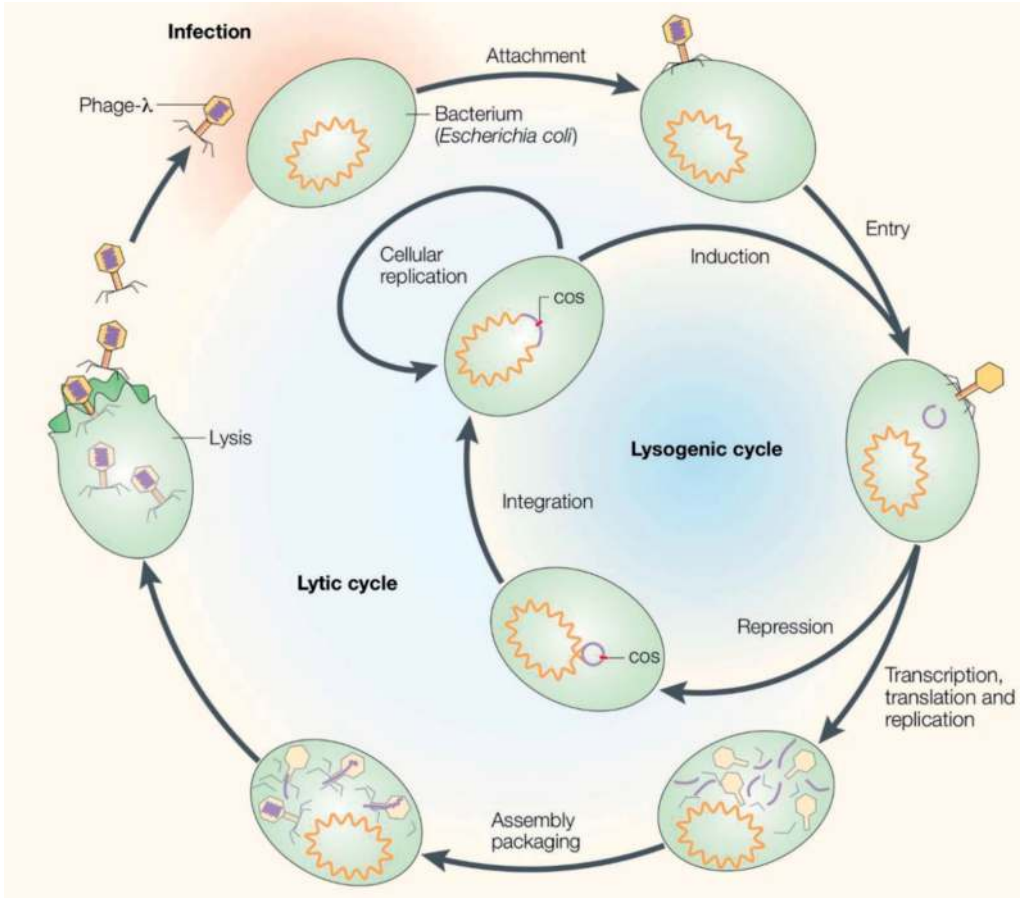

Figure 3.
Lytic and lysogenic life cycles of a temperate coliphage λ that infects Escherichia coli [44, 45]. cos—cohesive sites: the joining ends that circularise the linear phage λ DNA. Image courtesy of Springer Nature: https://www.nature.com/articles/nrg1089.

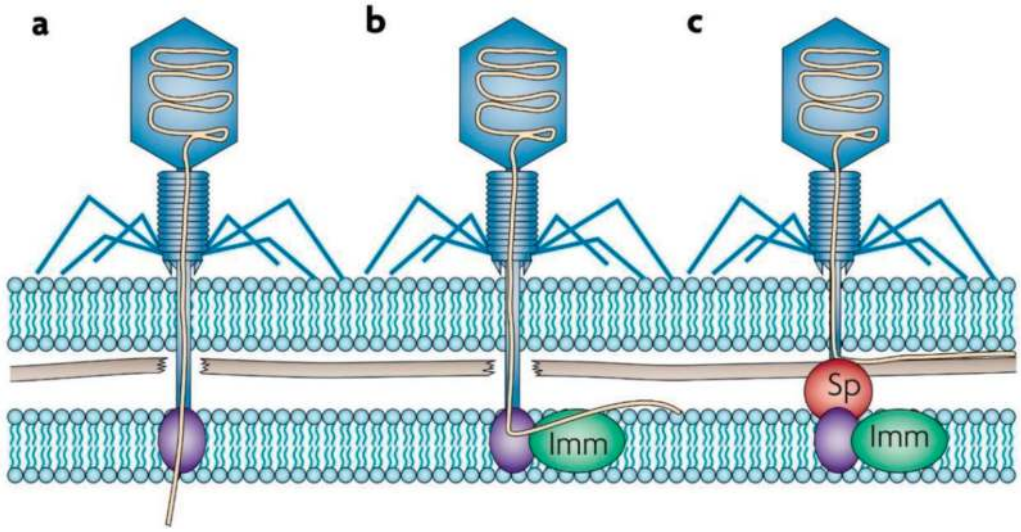

Figure 4.
Superinfection exclusion systems preventing phage DNA entry in Gram-negative Escherichia coli. (a). Standard T4 phage: upon attachment to phage-receptor on the surface of the host cell, an inner-membrane protein aids the translocation of phage DNA into the cell's cytoplasm. (b) Imm encoding phage T4: Imm protein directly blocks the translocation of the phage DNA into the cytoplasm of the cell. (c) Imm and Sp encoding phage T4: phage DNA is prevented from entering the cell's cytoplasm by Imm; and Sp protein prevents degradation of the peptidoglycan layer by inhibiting the activity of T4 lysozyme [28–31]. Image courtesy of Springer Nature: https://www.ncbi.nlm.nih.gov/pubmed/20348932.

5. Host strategies to cleave invading genomes and evolutionary tactics employed by phages to bypass these antiviral mechanisms

The evolution of bacterial genomes allowed bacteria to acquire vast mechanisms interfering with every step of phage infection. In a case where a phage succeeded to inject its viral nucleic acid into a host cell, bacteria possess a variety of nucleic acid degrading systems such as restriction-modification (R-M) systems and CRISPR/Cas that protect bacteria from the phage invasion.

5.1 Restriction-modification systems

It has been reported that R-M systems can significantly contribute to bacterial resistance to phages [49].

R-M systems incorporate activities of methyltransferases (MTases) that catalyse the transfer of a methyl group to DNA to protect self-genome from a restriction endonuclease (REase) cleavage and REases, which recognise and cut foreign unmethylated double-stranded DNA at specific recognition sites, commonly palindromic. To protect self-DNA from the degradation, methylases tag sequences recognised by the endonucleases with the methyl groups, whereas unmethylated phage (nonself) DNA is cleaved and degraded (**Figure 5**) [26, 27, 50–52].

R-M systems are diverse and ubiquitous among bacteria. There are four known types of R-M within bacterial genomes (**Figure 6**). Their classification is mainly based on R-M system subunit composition, sequence recognition, cleavage position, cofactor requirements and substrate specificity [26, 27, 50, 51].

Due to the diversity of R-M systems, phages acquired several active and passive strategies to bypass cleavage by REases. Passive mechanisms include reduction in restriction sites, modification and change of the orientation of restriction sites, whereas more specific, active mechanisms include masking of restriction sites,

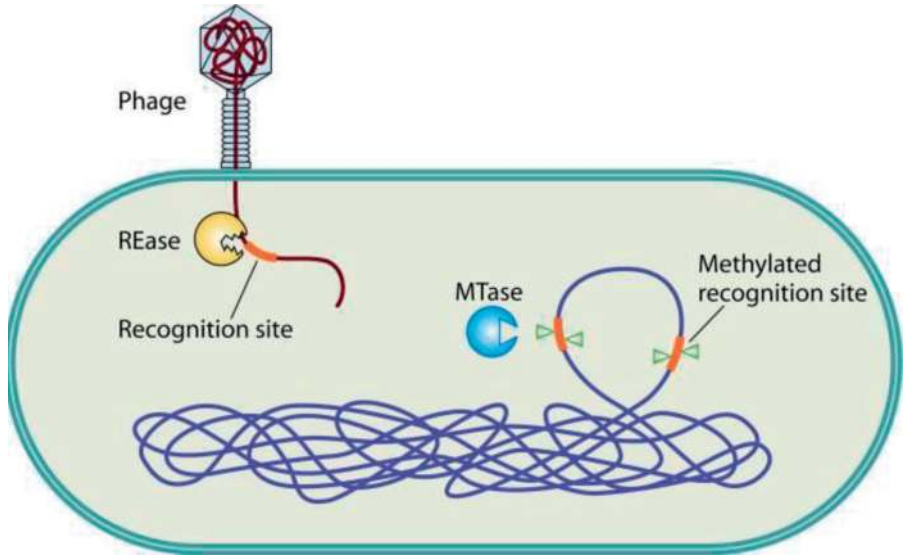

Figure 5.
General representation of the bacterial restriction-modification (R-M) systems providing a defence against invading phage genomes. R-M systems consist of two contrasting enzymatic activities: a restriction endonuclease (REase) and a methyltransferase. REase recognises and cuts nonself unmethylated double-stranded DNA at specific recognition sites, whereas MTase adds methyl groups to the same genomic recognition sites on the bacterial DNA to protect self-genome from REase cleavage [50, 51]. Image courtesy of: https://www.ncbi.nlm.nih.gov/pmc/articles/PMC3591985/.

stimulation of MTase activity on phage genome or degradation of an R-M system cofactor (**Figure 7**) [15–18].

Fewer restriction sites in the evading genome lead to the selective advantage of this phage as its DNA is less prone to cleavage and degradation by the host REase (**Figure 7a**). Also, some phages incorporate modified bases in their genomes that may lead to successful infection of the host cell as REase may not recognise the new sequences in the restriction sites. A decrease in the effective number of palindromic sites in DNA or change in the orientation of restriction-recognition sites can affect R-M targeting. Alternatively, the recognition sites within the viral genome can be too distant from each other to be recognised and cleaved by the REase [15–18, 53].

Interestingly, phage genome might be methylated by bacterial MTase upon successful injection into a host cell. Methylated recognition sites on viral genomes are therefore being protected from the cleavage and degradation by REase, leading to the initiation of the phage's lytic cycle. Viral progeny remains insensitive to this specific bacterial REase until it infects a bacterium that possesses a different type of REase, in which case the new progeny will become unmethylated again and will, therefore, be sensitive to the R-M system of the cognate bacterium [28–31].

The fate of the host cell chiefly confides in the levels of R-M gene expression and ultimate proportion of the R-M enzymes and their competition for the sites in the invading phage genome [52].

Furthermore, some phages encode their own MTase that is cooperative with the host REase, and thereby viral DNA cannot be recognised as nonself. Phages can also stimulate the activity of host modification enzymes that can rapidly methylate viral DNA, thus protecting it from the activity of REase.

Alternatively, phages can bypass R-M systems by masking restriction sites. For example (**Figure 7c**), coliphage P1, while injecting its DNA into a host cell, it also co-injects host-genome-binding proteins (DarA and DarB) that mask R-M recognition sites [53, 54].

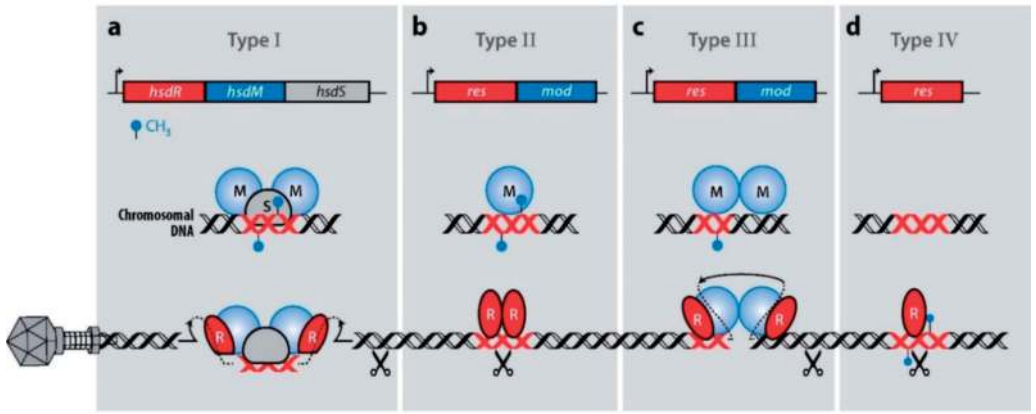

Figure 6.
Four distinct types of restriction-modification (R-M) systems. (a) Type I R-M system is composed of three subunits forming a complex: hsdR (restriction), hsdM (modification) and hsdS (specificity subunit that binds to an asymmetrical DNA sequence and determines the specificity of restriction and methylation). Two hsdM subunits and one hsdS subunit are involved in methylation of self-DNA. On the other hand, two complexes of hsdR, hsdM and hsdS (where each complex consists of two hsdR, two hsdM and one hsdS subunit) bind to the unmethylated recognition sites on phage DNA and cleave the DNA at random, far from their recognition sequences. Both reactions—methylation and cleavage—require ATP. (b) Type II R-M system is composed of two distinct enzymes: palindromic sequence methylating methyltransferase (mod) and endonuclease (res) that cleave unmethylated palindromic sequences close to or within the recognition sequence. (c) Type III R-M system is formed of methyltransferase (mod) and endonuclease (res) that form a complex. Methyltransferase transfers methyl group to one strand on the DNA, whereas two methyltransferases (endonuclease complexes) act together to bind to the complementary unmethylated recognition sites to cleave the DNA 24–26 bp away from the recognition site. (d) Type IV R-M system contains only endonuclease (res) that recognises methylated or modified DNA. Cleavage occurs within or away from the recognition sequences [26, 27, 50, 51]. Image courtesy of: https://www.annualreviews.org/doi/abs/10.1146/annurev-virology-031413-085500?journalCode=virology.

As shown on an example of a Coliphage T7 (**Figure 7d**), some phages code for proteins that directly inhibit REase. Coliphage T7 possesses proteins that can mimic the DNA backbone. Ocr, a protein expressed by Coliphage T7, directly blocks the active site of some REases by mimicking 24 bp of bent B-form DNA, and it has a high affinity for the EcoKI REase component, thereby interfering with R-M system [53].

Lastly, phage-bacteria arm race allowed phages to gain capabilities of degrading necessary cofactors of R-M systems. For instance, coliphage T3 encodes S-adenosyl-l-methionine hydrolase that destroys an essential host R-M cofactor (the S-adenosyl-l-methionine). The removal of this necessary co-factor will lead to the inhibition of the REase, thereby successfully infecting the host cell [15–18].

5.2 CRISPR/Cas system

CRISPR along with CRISPR-associated (Cas) proteins is the type of adaptive heritable 'immunity' of bacteria, thus very specific and effective; and it is prevalent within the bacterial domain [55]. The CRISPR are DNA loci consisting of short palindromic repeats (identical in length and sequence), interspaced by segments of DNA sequences (spacer DNA) derived from previous exposures to phages. The spacer DNA sequences act as a 'memory', allowing bacteria to recognise and destroy specific phages in a subsequent infection. Genes encoding Cas proteins are adjacent to CRISPR loci [56].

Although some studies have suggested that CRISPRs can be used for pathogen subtyping [57], it has been found that CRISPR typing is not useful for the epidemiological surveillance and outbreak investigation of *Salmonella typhimurium* [58].

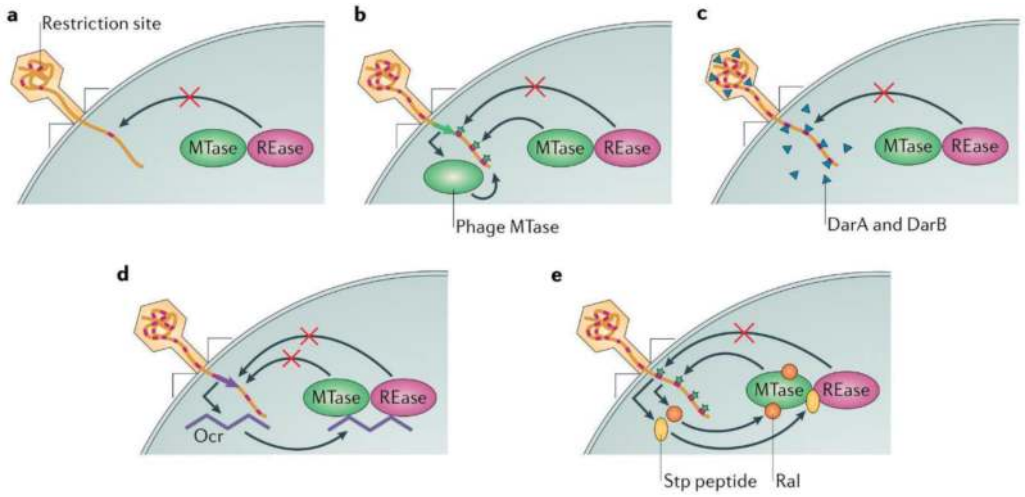

Figure 7.
Phage's passive and active strategies to bypass restriction-modification (R-M) systems. (a) Phages that possess fewer restriction sites in their genome are less prone to DNA cleavage by the host restriction endonuclease (REase). (b) Occasionally phage DNA might be modified by bacterial methyltransferase (MTase) upon successful injection into a host cell. Methylated recognition sites on viral DNA are, therefore, being protected from the cleavage and degradation by REase, leading to the initiation of the phage's lytic cycle. In addition, some phages encode their own MTase that is cooperative with the host REase; thus viral DNA cannot be recognised as nonself. (c) Some phages, for example, coliphage P1, while injecting its DNA into a host cell, it also co-injects host-genome-binding proteins (DarA and DarB) that mask R-M recognition sites. (d) Phages such as Coliphage T7 possess proteins that can mimic the DNA backbone. Ocr, a protein expressed by Coliphage T7, mimics the DNA phosphate backbone and has a high affinity for the EcoKI REase component, thereby interfering with R-M system. (e) In addition, some phages (e.g. Ral protein of Coliphage λ) can also stimulate activity of the bacterial modification enzyme in order to protect own DNA from the recognition by the bacterial REase as nonself. The peptide Stp encoded by Coliphage T4 can as well disrupt the structural conformation of the REase-MTase complex [15–18]. Image courtesy of: https://www.nature.com/articles/nrmicro3096.

The CRISPR/Cas phage resistance is mediated in three-step stages: adaptation (acquisition), where spacer phage-derived DNA sequences are incorporated into the CRISPR/Cas system; expression, where *cas* gene expression and CRISPR transcription lead to pre-CRISPR RNA (pre-crRNA) that is then processed into CRISPR RNA (crRNA); and interference, during which the crRNA guides Cas proteins to the target (subsequently invading DNA) for the degradation. The cleavage of the target (proto-spacer) depends on the recognition of complementary sequences in spacer and protospacer [59, 60].

CRISPR/Cas systems have been classified into three major types: Types I, II and III, which are further divided into subtypes that require different types of Cas proteins. Although the CRISPR/Cas array is diverse among the bacteria and it is continuously co-evolving in response to the host-phage interactions, the defence activity in all three types of the CRISPR is comparable [21–23] **Figure 8** illustrates the defence mechanisms in three distinct CRISPR/Cas arrays.

The Type II, CRISPR/Cas9, which was first identified in *Streptococcus pyogenes*, *gained considerable interest within scientific studies as a precise genome editing tool. CRISPR/Cas9 system is unique; a single Cas 9 protein (in addition to prevalent Cas 1 and Cas 2) is involved in the processing of* crRNA and destruction of the target viral DNA [56, 61].

In the adaptation stage, phage-derived protospacer (snippet of DNA from the invading phage) is incorporated into the bacterial genome at the leader end of the CRISPR loci. In expression phase, the *Cas9* gene expresses Cas9 protein possessing DNA cleaving HNH and RuvC-like nuclease domains; CRISPR locus is then transcribed and processed into mature crRNA. Finally, in interference step, the complex

consisting of Cas9, crRNA and separate trans-activating crRNA (tracrRNA) cleave 20 base pairs crRNA-complementary target sequence that is adjacent to the protospacer adjacent motif (PAM) [62].

To bypass CRISPR/Cas that has an incredibly dynamic rate of evolution, phages acquired array of strategies to succeed in propagation; this includes mutations in the protospacers or in the PAM sequences and expression of anti-CRISPR proteins, and even some phages encode their own functional CRISPR/Cas systems [15–18, 63].

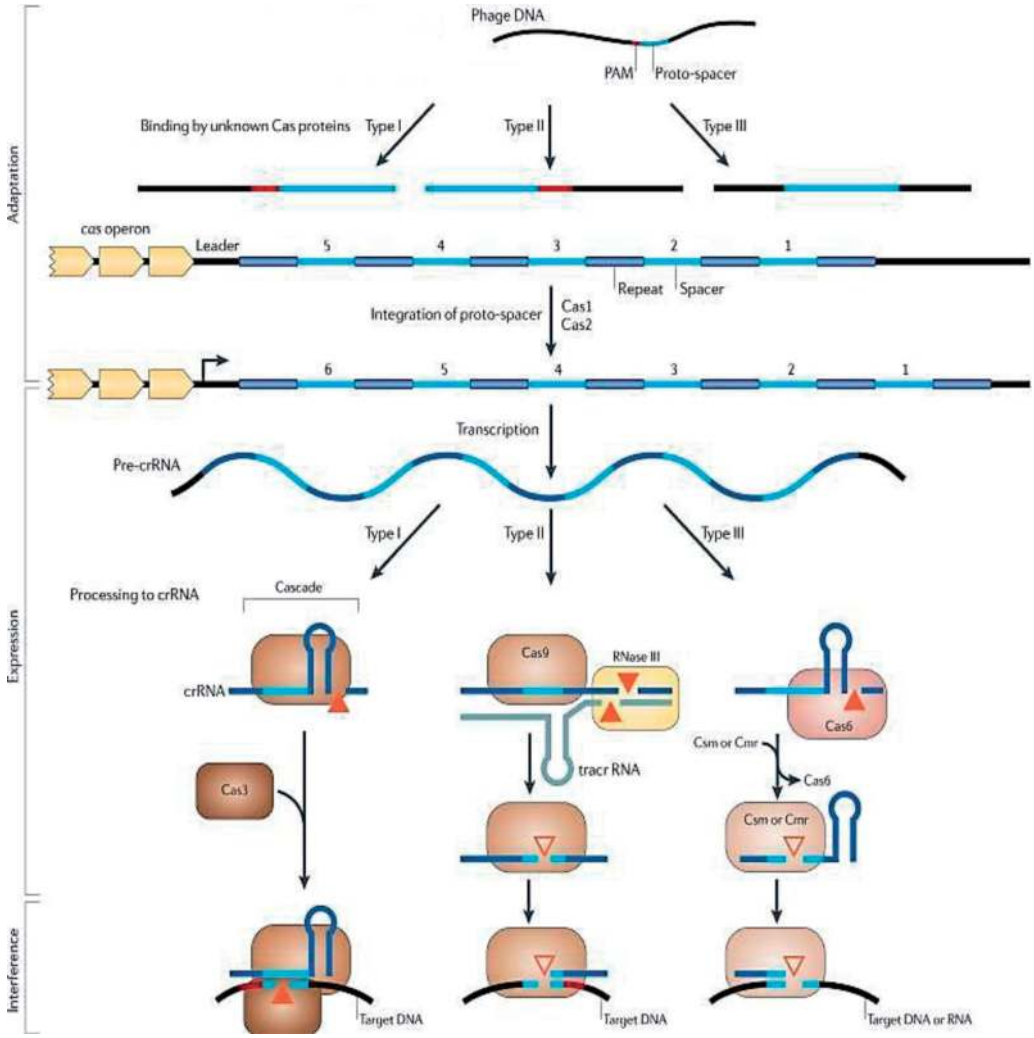

Figure 8.
Image showing mechanisms of adaptation, expression and interference in three different types of CRISPR/Cas arrays. Type I and Type II CRISPR/Cas arrays rely on the protospacer adjacent motif (PAM), contained within phage nucleic acid, to 'select' the phage-derived protospacer. Next steps in the adaptation stage are similar in all three types; protospacer is incorporated by Cas 1 and Cas2 proteins into the bacterial genome at the leader end of the CRISPR loci to form a new spacer. In expression step, CRISPR loci are transcribed into pre-crRNA. The crRNA processing and interference stage is distinct in each type of the CRISPR/Cas system. In Type I, the multisubunit CRISPR-associated complex for antiviral defence (CASCADE) binds crRNA to locate the target, and with the presence of Cas3 protein, the invading target genome is degraded whereas in Type II, Cas9 protein is essential in the processing of the crRNA. TracrRNA recognises and attaches to the complementary sequences on the repeat region that is then cut by RNase III in the presence of Cas9. Lastly, in Type III, processing of pre-crRNA into crRNA is dependent upon the activity of Cas6. Mature crRNA associated with Csm/Cmr complex targets foreign DNA or RNA for the degradation [21–23]. Image courtesy of: https://www.nature.com/articles/nrmicro2577.

Phages can evade interference step of Type I and Type II CRISPR/Cas system by a single point mutation or deletion in their protospacer region or in the PAM sequence (**Figure 9**). Phages with single-nucleotide substitutions or deletions positioned close to PAM sequence can bypass the CRISPR/Cas activity and complete their lytic cycles; in contrast, phages with multiple mutations at PAM-distal protospacer positions do not [15–18, 28–31].

In some circumstances, however, although the phage successfully evades CRISPR/Cas interference, the host cell may survive by the acquisition of new spacer sequences (derived from invading phage) into their own CRISPR/Cas system. This new spacer provides the bacterium with an accelerated spectrum of phage resistance [15–18].

Prophages integrated within *Pseudomonas aeruginosa* possess genes that encode anti-CRISPR proteins directly suppressing CRISPR/Cas-mediated degradation of the phage genome (**Figure 10**). According to Wiedenheft [64], these proteins might interrupt CRISPR RNA processing by preventing mature crRNA from binging to the crRNA-guide complex or by preventing the assembled crRNA-guided complex from interacting with target substrates through binding to it [64].

Prophages do not only contribute to bacterial resistance to invading phages, they can also encode proteins that contribute to bacterial virulence and antimicrobial resistance [58, 66].

Bacteria can also resist phages by possessing phage-inducible chromosomal islands (PICI) which prevent phage replication. Nevertheless, phages evolved their genomes to overcome this very specific antiviral strategy. For example, *Vibrio cholerae* ICP1 phages possess their own CRISPR/Cas systems that inactivate PICI-like elements (PLE) in *Vibrio cholerae* (**Figure 11**). Studies conducted by Naser et al. [67] have shown that phage CRISPR arrays have evolved by the acquisition of new spacers targeting diverse regions of PLEs carried by *Vibrio cholerae* strains. Furthermore, the addition of the new spacers within phage CRISPR/Cas loci enables the phages to expand their ability to counter PLE-mediated phage defence of diverse *Vibrio cholerae* strains [67].

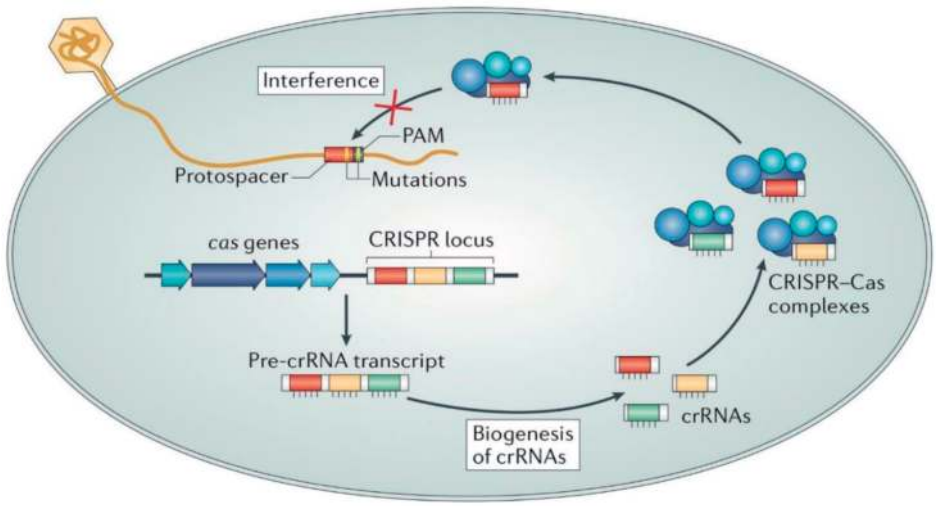

Figure 9.
Evasion by mutation. Mutations in the phage protospacers or in the PAM sequences allow the phage to escape interference step of the CRISPR/Cas system that would lead to the degradation of the phage genome [15–18]. Adapted image courtesy of: https://www.nature.com/articles/nrmicro3096.

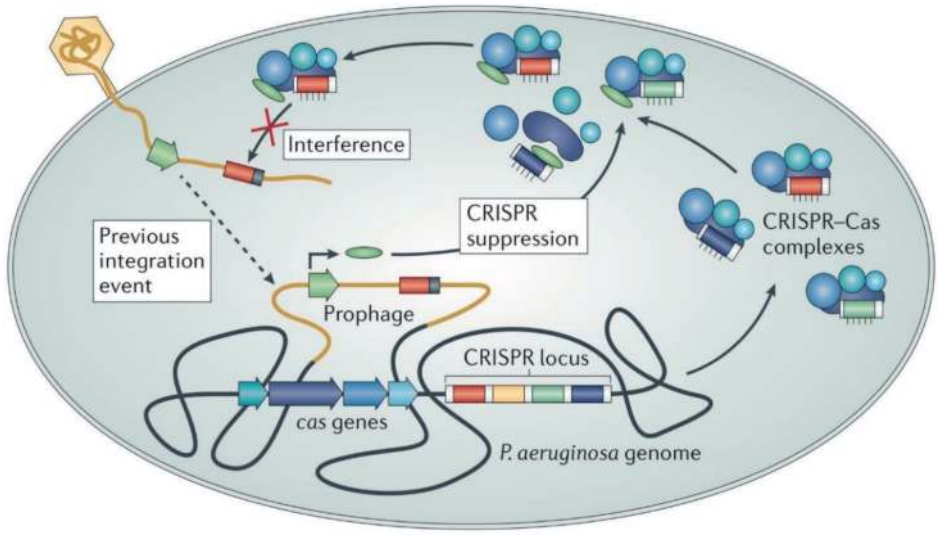

Figure 10.
Anti-CRISPR proteins expressed against CRISPR subtype I-F systems. Temperate phages such as Pseudomonas aeruginosa possess genes encoding anti-CRISPR proteins that directly interfere with the bacterial CRISPR/Cas system [15–18]. Adapted image courtesy of: https://www.nature.com/articles/nrmicro3096.

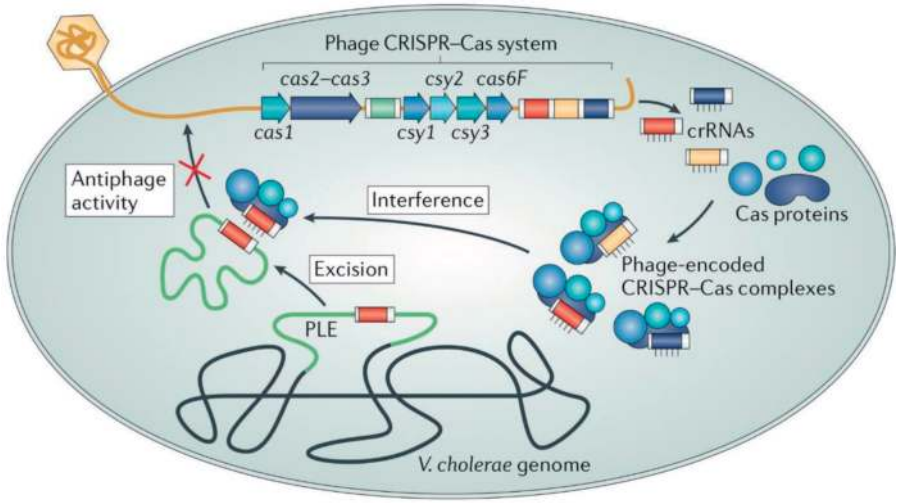

Figure 11.
Phage-encoded CRISPR/Cas systems in Vibrio cholerae ICP1 phages. Upon adsorption and injection of viral genome into a host cell, phage crRNAs and CRISPR/Cas complexes are expressed and target phage-inducible chromosomal island (PICI) in the host genome; in the Vibrio cholerae, they are termed as PICI-like elements (PLE). If the spacers within phage CRISPR locus are complementary to the bacterial PLE, the CRISPR machinery is then able to specifically target this genetic element and inactivate it, leading to the viral propagation. However, in the absence of such targeting, phage CRISPR/Cas system can acquire new spacers to evolve rapidly and ensure effective targeting of the PLE to restore phage replication [15–18, 65]. Adapted image courtesy of: https://www.nature.com/articles/nrmicro3096.

6. Overcoming host abortive infection systems: toxin-antitoxin

Abortive infection (Abi) systems promote cell death of the phage-infected bacteria, inhibiting phage replication and providing protection for bacterial populations [68].

Abi systems require both toxins and antagonistic antitoxins. Antitoxins are proteins or RNAs that protect bacterial cell from the activity of toxins in a typical cell life cycle, whereas toxins are the proteins encoded in toxin-antitoxin locus that

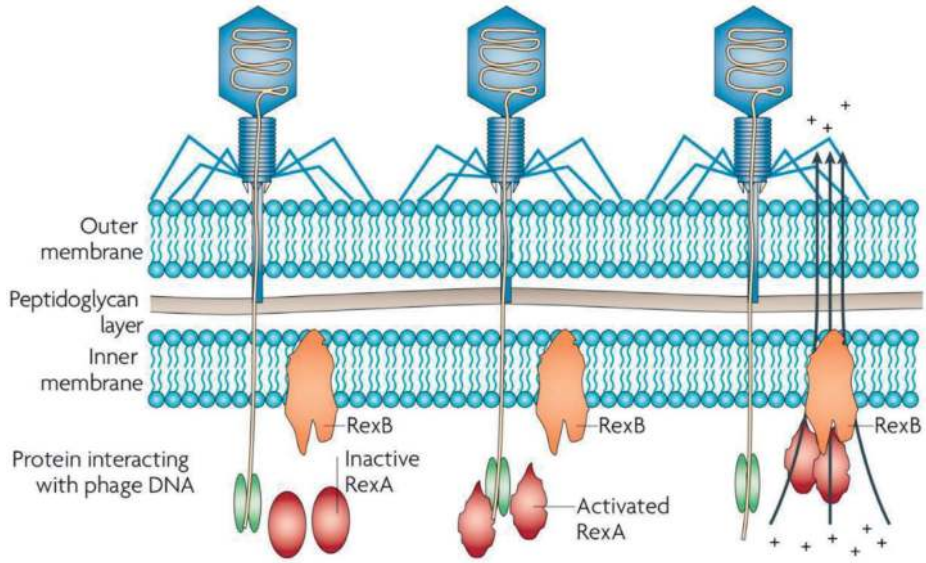

Figure 12.
Abortive infection (Abi) systems in Escherichia coli. The Rex system is a two-component Abi system. A phage protein-DNA complex (formed during phage replication) activates the sensor protein RexA, which in turn activates RexB. RexB is an ion channel that causes depolarisation of the bacterial membrane leading to cell death [28–31]. Image courtesy of Springer Nature: https://www.ncbi.nlm.nih.gov/pubmed/20348932.

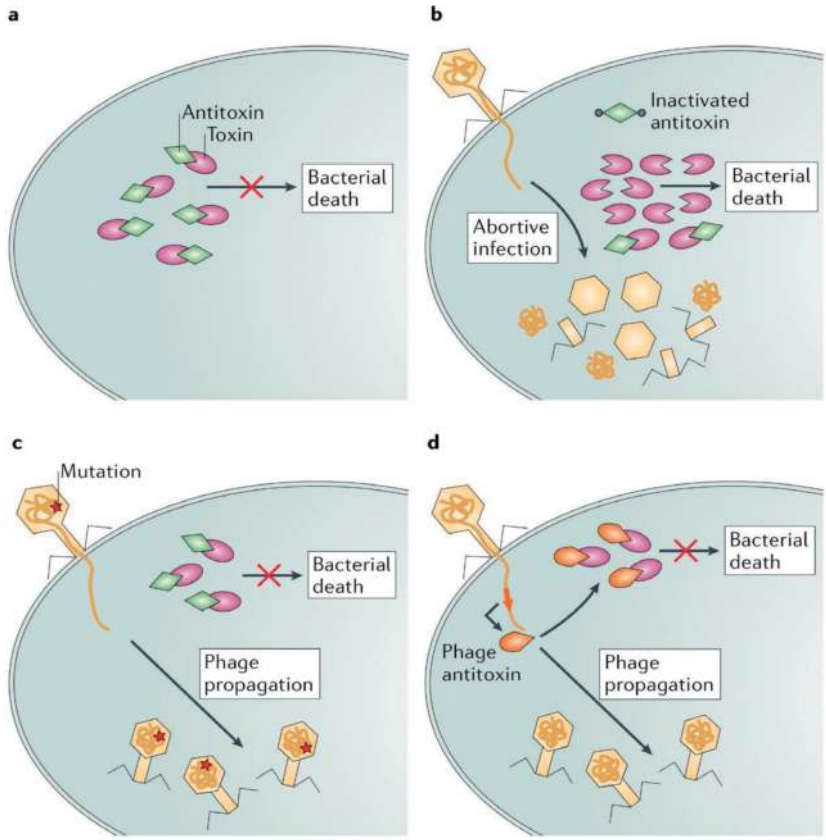

Figure 13.
Escaping abortive infection mechanisms. (a) In a typical cell life cycle, antitoxins protect bacterial cell from the activity of toxins. (b) During phage infection, the expression of antitoxin encoding gene is suppressed, leading to the lethal activation of the toxin. (c) Mutations in certain phage genes can lead to escaping Abi systems activity, thereby a successful viral propagation without killing the host cell. (d) Some phages encode molecules that functionally replace the bacterial antitoxins, thus suppressing toxin activity and avoiding host cell death [15–18]. Image courtesy of: https://www.nature.com/articles/nrmicro3096.

disrupt cellular metabolism (translation, replication and cell wall formation), causing cell death. During an infection, the expression of the antitoxin encoding gene is suppressed, leading to the lethal activation of the toxin [69]. **Figure 12** illustrates the mechanism of Abi systems in *Escherichia coli* [70].

Interestingly, phages evolved an array of tactics to circumvent Abi systems. This includes mutations in specific phage genes and encoding own antitoxin molecules that suppresses bacterial toxin [15–18]. **Figure 13** provides a broad overview of the strategies employed by the phages to by-pass Abi systems.

Bacteria-phage interaction is therefore very complex, and it is crucial to understand the molecular basis of this interaction and how bacteria and phages 'fight' each other. It has been reported that Anderson Phage Typing System of *Salmonella* Typhimurium can provide a valuable model system for study of phage-host interaction [71].

7. The potential application of phages as antibacterial therapeutics

The rapid emergence and dissemination of MDR bacteria seriously threaten global public health, as, without effective antibiotics, prevention and treatment of both community- and hospital-acquired infections may become unsuccessful and lead to widespread outbreaks.

Carbapenems and colistin are antibiotics of last resort, generally reserved to treat bacteria which are resistant to all other antibiotics. Until not long ago, colistin resistance was only described as chromosomal, however, in 2016 Liu et al. reported the emergence of the first plasmid-mediated colistin resistance mechanism, MCR-1, in Enterobacteriaceae [72]. Furthermore, the increasing occurrence of colistin resistance among carbapenem-resistant Enterobacteriaceae has also been reported [73]. This is of significant concern as infections caused by colistin and carbapenem-resistant bacteria are very challenging to treat and control, as the treatment options are greatly limited or non-existent. Thus, the discovery and development of alternative antimicrobial therapeutics are the highest priorities of modern medicine and biotechnology.

Phages should be considered as great potential tools in MDR pathogens as they are species-specific (specificity prevents damage of normal microbiota), thus harmless to human; they have fast replication rate at the site of infection, and their short genomes can allow to further understand various molecular mechanisms implied to 'fight' bacteria. In addition, this understanding can enable scientists to 'manipulate' viral genomes and engineer a synthetic phage that combines the antibacterial characteristics of multiple phages into a single genome.

The escalating need for new antimicrobial agents attracted new attention in modern medicine, proposing several potential applications of phages as antibacterial therapeutics including phage therapy, phage lysins and genetically-engineered phages.

7.1 Phage therapy

Phage therapy utilises strictly lytic phages that have bactericidal effect. As phages are host-specific, 'phage cocktails' containing multiple phages can broaden range of target cells. Nevertheless, selection of suitable phages is at the paramount to the successful elimination of clinically important pathogens, and it includes avoidance of adverse effects, such as anaphylaxis (adverse immune reaction) [74].

7.2 Phage-derived enzymes: lysins

In order to hydrolyse and degrade the bacterial cell wall, phages possess lysins.

The spectrum of efficiency of natural lysins (derived from naturally occurring phages) is generally limited to Gram-positive bacteria; however, recombinant lysins have shown an ability to destabilise the outer membrane of Gram-negative bacteria and ultimately lead to rapid death of the target bacteria [74].

7.3 Bioengineered phages

Bioengineered phages have the potential to solve inherent limitations of natural phages such as narrow host range and evolution of resistance. Various genetic engineering methods have been proposed to design phages with extended antimicrobial properties such as homologous recombination, phage recombineering of electroporated DNA, yeast-based platform, Gibson assembly and CRISPR/Cas genome editing [75].

Engineering of synthetic phages could be tailored to enhance the antibiotic activity, to reverse antibiotic resistance or to create sequence-specific antimicrobials [74].

8. Conclusions

The antagonistic host-phage relationship has led to the evolution of exceptionally disperse phage-resistance mechanisms in the bacterial domain, including inhibition of phage adsorption, prevention of nucleic acid entry, Superinfection exclusion, cutting phage nucleic acids via restriction-modification systems and CRISPR, as well as abortive infection.

Evolvement of these mechanisms has been induced by constant parallel co-evolution of phages as they attempt to coexist. To survive, phages acquired diverse counterstrategies to circumvent bacterial anti-phage mechanisms such as adaptations to new receptors, digging for receptors and masking and modification of restriction sites and point mutations in specific genes and genome rearrangements that allow phages to evade bacterial antiviral systems such as CRISPR/Cas arrays, as well as mutations in specific genes to bypass abortive infection system. Conclusively, the co-evolving genetic variations and counter-adaptations, in both bacteria and phages, drive the evolutionary bacteria-host arm race.

Besides, accumulating evidence shows that phages contribute to the antimicrobial resistance through horizontal gene transfer mechanisms. Indeed, many bacterial strains have become insensitive to the conventional antibiotics, posing a growing threat to human; and although in the past, western counties withdrew phage therapy in response to the discovery of therapeutic antibiotics, now, phage therapy regains an interest within the research community. There are apparent advantages of phage therapy, such as specificity, meaning only target bacteria would encounter lysis, but not healthy microbiota inhabiting human's system. Additionally, 'phage cocktails', containing multiple bacteria-specific phages, could overcome the issue of phage-resistance as phages do adapt to these resistance mechanisms. However, 'phage cocktails' would require large numbers of phages that would have to be grown inside pathogenic bacteria in the laboratory, putting laboratory staff and the environment at risk.

Alternatively, building up the understanding of host-phage interactions and 'the war between bacteria and phages' could potentially lead to defeating antimicrobial resistance by designing synthetic phages that can overcome the limitations of phage therapy.

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Abbreviations

Abi	abortive infection
CPS	capsular polysaccharides
CRISPR	clustered regularly interspaced short palindromic repeats
crRNA	crispr RNA
DGR	diversity-generating retroelement
DNA	deoxyribonucleic acid
MDR	multidrug-resistant
MeOPN	O-methyl phosphoramidate
MTase	methyltransferase
PAM	protospacer adjacent motif
PICI	phage-inducible chromosomal island
PLE	PICI-like element
RBP	receptor-binding protein
REase	restriction endonuclease
R-M	restriction-modification
RNA	ribonucleic acid
Sie	superinfection exclusion
tracrRNA	trans-activating crRNA

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