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(Ph)ighting phages – how bacteria resist their parasites

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Abstract

Bacteria are under constant attack from bacteriophages (phages), bacterial parasites that are the most abundant biological entity on earth. To resist phage infection, bacteria have evolved an impressive arsenal of anti-phage systems. Recent advances have significantly broadened and deepened our understanding of how bacteria battle phages, spearheaded by new systems like CRISPR-Cas. This review aims to summarise bacterial anti-phage mechanisms, with an emphasis on the most recent developments in the field.

Introduction

Throughout evolution, bacteria have been preyed upon by parasitic bacteriophages (phages). Everywhere bacteria are found, they coexist with their respective phages, undergoing continuous cycles of infection. As a consequence, in order to survive and thrive, bacteria have developed an arsenal of anti-phage mechanisms. Due to the immense evolutionary pressure imposed by phages, the diversity and sophistication of bacteria's anti-phage mechanisms are astounding, and we are only now beginning to appreciate the complexity of the interactions between bacteria and these parasites. In addition, the study of anti-phage mechanisms has resulted in invaluable tools, such as restriction enzymes and CRISPR-based gene editing techniques. This review summarises the mechanisms employed by bacteria to resist their phages, with an emphasis of novel developments in the field.

Bacteriophage-host interactions

The red queen hypothesis states that an organism must constantly evolve to maintain their relative fitness in the face of a predator (McLaughlin and Malik, 2017). In the context of the bacteria-phage relationship, this means that bacteria continuously evolve and update anti-phage mechanisms, while phages adapt to overcome these mechanisms. Competitive bacteria-phage coevolution, often referred to as an "evolutionary arms race", has produced a

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multitude of bacterial defence mechanisms that act to inhibit every stage of the phage life cycle (Figure 1). Although not discussed extensively in this review, phages have developed as many means to circumvent these defence strategies. For example, a metagenomic study in this issue (Uribe et al., 2019) identified phylogenetically widespread phage-encoded anti-CRISPR genes, encoding proteins that neutralise CRISPR immunity.

As a result of this arms race, bacteria and phages coevolve, and seem to exist in stable equilibria without dramatic fluctuations or extinction events in natural environments (Fernandez et al., 2018). Key to this arms race is the propensity of bacterial defence systems to spread through horizontal gene transfer (Stern and Sorek, 2011). Whereas this in principle could lead to an extensive proliferation of defence mechanisms to provide more protection to the host population, bacteria only tend to have a subset of the available diversity of anti-phage mechanisms. This in part due to fitness costs associated with carrying defence systems. Therefore bacteria, even in the context of a race for survival against their parasites, must tune the trade-off between the cost of carrying anti-phage systems and the benefit of resisting phage infection (van Houte et al., 2016a).

Preventing phage entry

Infection begins with the binding to specific surface proteins or cell wall components of the host cell, an event that is followed by the injection of the phage's genome. Consequently, bacteria use both broad and phage-specific mechanisms to prevent phage adsorption and injection (Figure 2).

Preventing phage adsorption

Many bacteria spend much of their life cycle embedded in biofilms, an extracellular matrix made up of polymers where bacteria live in close proximity, often on surfaces. Biofilms protect bacteria in various ways, but how these structures affect phage-bacteria interactions remains incompletely understood. Computational modelling suggests that biofilms can conditionally survive and grow in the presence of phage (Simmons et al., 2018). This was also shown experimentally in *Escherichia coli* with a virulent mutant of phage P1 where, depending on nutrient availability and phage infectivity, the bacterial colony size reaches an equilibrium (Eriksen et al., 2018). In this scenario, cells inside the colony divide, and are shielded by peripheral cells that get infected. Another study showed that while early biofilms were quickly eradicated, mature *E. coli* biofilms avoided clearance by T7 phage (Vidakovic et al., 2018). Fluorescent labelling of cells and phages revealed that the biofilm structure prevents phage access to the biofilm interior. This depended on the presence of the host protein curli, which forms amyloid fibres that promote the formation of an extracellular matrix and a dense cell packing.

In addition to the protective shield provided by biofilms, Gram-negative bacteria can secrete outer membrane vesicles (OMVs), spherical structures made up of outer membrane components and periplasmic cargo which pinch off the cell (Schwechheimer and Kuehn, 2015). Since they contain exposed outer membrane proteins that can act as phage receptors, OMVs can act as decoys, sequestering extracellular phage. One report showed that pre-incubation with OMVs reduced T4 infectivity in *E. coli*, and phage-bound OMV complexes

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could be visualized with electron microscopy (Manning and Kuehn, 2011). Similarly, it was shown recently that *Vibrio cholerae* OMVs could bind three different phages, an interaction that was dependent on the presence of phage receptor in the OMVs (Reyes-Robles et al., 2018).

Bacteria can also prevent adsorption by hiding or masking surface receptors. For example, in *Pseudomonas aeruginosa*, type IV pili can be glycosylated to prevent the binding of several pilus-specific phages (Harvey et al., 2018). Receptors can also be blocked by polysaccharide capsules, which shield the whole bacterial surface. The polysialic acid capsule of *E. coli* K1 prevents phage T7 attachment to its receptor, lipopolysaccharide (LPS), thereby reducing infectivity (Scholl et al., 2005). In response, phages can have enzymes in their tails that degrade various capsules, giving rise to an evolutionary arms race that results in the extreme diversification of capsule synthesis and hydrolysing enzyme genes of the host and phage, respectively (Fernandes and Sao-Jose, 2018). Finally, surface proteins can also hide phage receptors. *E. coli* lytic phage T5 uses the outer membrane iron uptake protein FhuA as its receptor and expresses the lipoprotein Llp to mask it. This prevents additional T5 particles, and possibly other phages that use FhuA as receptor, such as T1 and phi80, from entering and disturbing T5's infection cycle (Pedruzzi et al., 1998). This phenomenon is an example of superinfection exclusion (Sie), a process where intracellular phages, including prophages, block the infection of the same (homotypic Sie) or a different (heterotypic Sie) phage.

Another mechanism to prevent adsorption is the introduction of mutations within receptor genes that affect the protein or its expression. This is a common mode of resistance that is perhaps best exemplified by the identification of mutations in LamB, the phage lambda receptor, in *E. coli* resistant cells (Clement et al., 1983). More recently it was found that receptor expression can be modulated by lysogenic phages via Sie. The *P. aeruginosa* prophage D3112 expresses the protein Tip, which interacts with the ATPase PilB to prevent type IV pili extension. D3112, as well as other phages that use these pili as receptors, are therefore unable to infect D3112 lysogens (Chung et al., 2014). Indeed, a systematic screen of *P. aeruginosa* Sie mechanisms identified many prophages interfering with either type IV pilus function, or with the O-antigen, another typical *P. aeruginosa* phage receptor in the surface polysaccharide (Bondy-Denomy et al., 2016).

Preventing DNA injection

Blocking the entry of phage DNA into the cytoplasm is another mechanism of preventing phage infections. The *E. coli* prophage HK97 confers both homotypic as well as heterotypic (against the closely related phage HK75) Sie thorough the expression of gp15 (Cumby et al., 2012). This is an inner membrane (transmembrane) protein that interacts with the host glucose transporter PtsG, and most likely disrupts its association with phage components required for translocating the viral genome across the inner membrane, thereby preventing the transfer of DNA into the cytoplasm (Cumby et al., 2015). Another recent example of a heterotypic Sie mechanism preventing DNA injection is found in the mycobacteriophage Fruitloop. During the lytic cycle, Fruitloop gp52 inactivates Wag31, an essential mycobacterial protein involved in cell wall synthesis at the cell poles (Ko and Hatfull, 2018).

This prevents DNA injection by an unrelated group of mycobacteriophages that rely on Wag31, including the phages Hedgerow and Rosebush.

Targeting bacteriophage nucleic acids

Once the phage genome is injected into the host cell, it will initiate the lytic cycle, a genetic programme to achieve viral propagation. Temperate phages have the choice of entering the lysogenic cycle, which requires the repression of the lytic genes by a transcription factor. Possibly the most widespread homotypic Sie mechanism that targets the phage nucleic acid is the use of this repressor, expressed by prophages to maintain lysogeny, to abort the lytic programme of other closely related phages that infect the lysogen (Johnson et al., 1981). However, the most direct mechanism to target the viral nucleic acids is the employment of nucleases that degrade the injected genome.

Restriction-modification systems

Restriction-modification (RM) systems (see (Tock and Dryden, 2005) for a more detailed review) are an ubiquitous and extremely diverse mode of anti-phage defence. They are normally made up of two activities; a restriction endonuclease and a methyltransferase (the modification component). The restriction endonuclease recognises short DNA motifs, usually 4–8 base-pairs long, and cuts the phage DNA. These DNA motifs exist in both the bacterial host and invading phage, but the host protects its genome by using the methyltransferase to modify its own DNA to avoid recognition by the restriction enzyme. An invading phage is usually not methylated, and will therefore be cut upon injection. RM systems are classified into four major types, based their mechanism of action and subunit composition (Tock and Dryden, 2005). Both type I and III systems translocate along DNA and cleave away from the recognition sites. Type II, known for their use in molecular cloning, cleave within or near the recognition site. Type IV systems lack a methylase and only contain a restriction endonuclease which only cleaves modified DNA. Finally, there are examples of “inverted” RM systems that do not belong to any of these types. The phage ϕC31 can propagate in *Streptomyces coelicolor* A2(3) harbouring the four-gene “phage growth limiting” (*pgl*) locus, but only mounts one cycle of infection. The released phages are unable to reinfect Pgl^+ hosts (Chinenova et al., 1982), presumably due to the action of the methyltransferase *pglX* (Sumby and Smith, 2002), which modifies new phage DNA to make it susceptible for restriction in the next Pgl+ host by an unknown mechanism.

RM systems and DNA modfications exemplify an elaborate “arms race” between *E. coli* and phage T4. T4 contains hydroxymethylcytosine (HMC) instead of cytosine in its DNA, inhibiting all type I-III RM systems that recognise sites containing cytosine. To counter this, *E. coli* uses McrBC, a type IV system specific for HMC-containing DNA (Raleigh and Wilson, 1986). In response, T4 can glycosylate its DNA, which impairs McrBC activity. Against this, *E. coli* has evolved an additional type IV system, the GmrSGmrD system, that can cleave glycosylated DNA (Bair and Black, 2007).

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CRISPR-Cas systems

One of the most significant scientific advances in the last decades was the discovery of CRISPR-Cas bacterial immune systems [for a recent review, see (Hille et al., 2018)]. These systems are present in approximately 50% of sequenced bacteria and 90% of sequenced archaea (Makarova et al., 2015), and provide resistance against invading phages (Barrangou et al., 2007) and plasmids (Marraffini and Sontheimer, 2008). Uniquely, CRISPR systems are adaptive rather than innate immune systems, where exposure to a previous infection is memorised. The molecular basis for immunological memory are short (30–40 base pairs) "spacer" sequences acquired from invader genomes, flanked by similarly short semi-palindromic repeats. Repeats and spacers are transcribed and processed into small CRISPR RNA (crRNA) guides. CRISPR associated (*cas*) genes, usually adjacent to the CRISPR locus, encode the protein machinery required for the acquisition of new spacer sequences upon infection (the adaptation phase) and for the sequence-specific elimination of the invader (the targeting phase). During the latter, RNA-guided Cas nucleases use the crRNAs to recognise and cleave the invader's nucleic acids via complementary base pairing. Based on the composition of *cas* genes, CRISPR systems can be classified into two classes, six types, and multiple subtypes (Koonin et al., 2017), with diverse mechanisms of action (Figure 3).

CRISPR targeting – destroying the invader

Types I, II, and V use the crRNA guide to recognise the complementary target sequence in the DNA of the invader, known as the protospacer. In addition to this complementarity, cleavage requires the presence of a conserved protospacer adjacent motif (PAM) in one flank of the target (Gasiunas et al., 2012; Jinek et al., 2012; Sashital et al., 2012; Zetsche et al., 2015). As a consequence of this targeting requirements, phages harbouring mutations that eliminate the PAM or the complementarity between the protospacer and the crRNA, can escape targeting (Deveau et al., 2008; Semenova et al., 2011). Whereas type II and V systems employ a single-subunit RNA-guided nuclease (Cas9 and Cas12, respectively) (Sapranauskas et al., 2011; Zetsche et al., 2015), type I systems use a multi-subunit crRNA Cas complex known as Cascade to locate the protospacer and a second nuclease, Cas3, recruited by Cascade to the target, to cleave its DNA (Brouns et al., 2008; Sinkunas et al., 2013).

Type III CRISPR systems are composed of the main effector complex (Csm or Cmr for III-A or III-B, respectively), and of an accessory RNase (Csm6 or Csx1 for III-A or III-B, respectively). Transcription across the target is an absolute requirement for immunity (Goldberg et al., 2014) since the effector complex binds the protospacer within the nascent RNA through complementary RNA-RNA base-pairing (rather than recognising target DNA). This binding unleashes the non-specific single-stranded DNase activity of Cas10 (the main subunit of both the Csm and Cmr complexes), which cuts the non-template strand of the transcribed viral DNA (Kazlauskiene et al., 2016; Samai et al., 2015). In addition, target recognition results in the synthesis of cyclic oligo-adenylates (cOA) by another domain of Cas10, a ligand that activates the Csm6/Csx1 non-specific RNase (Kazlauskiene et al., 2017; Niewoehner et al., 2017). This accessory RNase is required for targets that are poorly recognised by the crRNA guide and thus provide inefficient activation of the Cas10

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ssDNase, such as weakly transcribed (Rostøl and Marraffini, 2019) or mutated targets (Jiang et al., 2016). Finally, the Csm3/Cmr4 subunit of the effector complex cleaves the protospacer RNA (Hale et al., 2009; Tamulaitis et al., 2014), an event that neutralizes both activities of the Cas10 subunit. Type III-A immunity does not require the recognition of a PAM on the RNA target and can tolerate between 6–8 mismatches between the protospacer and the crRNA (Pyenson et al., 2017). Consequently, type III-A CRISPR systems offer more robust defence against rapidly mutating phage invaders than a type II (Pyenson et al., 2017) and Type I (Silas et al., 2017) systems.

Type VI CRISPR systems are characterised by the effector protein Cas13 which, uniquely, only targets RNA (Abudayyeh et al., 2016). Like in type III CRISPR systems, transcription across the target is required for interference, and there is no PAM sequence requirement (Meeske and Marraffini, 2018). Binding to a target RNA results in the cleavage of both target and non-target transcripts, the latter causing a growth delay observed in cells undergoing type VI CRISPR immunity (Meeske and Marraffini, 2018; Abudayyeh et al., 2016). Although *Leptotrichia shahii* Cas13a with a reprogrammed spacer was able to confer protection against the single-stranded RNA virus MS2 in an *E. coli* heterologous host, none of the spacers found so far (neither on type VI nor in any other CRISPR loci) match RNA viral genomes, and therefore the role of Cas13 in anti-phage immunity still remains unclear.

CRISPR adaptation – remembering the invader

Adaptation is the process where a short sequence from an invader is incorporated into the CRISPR array as a new spacer, offering protection against future invaders containing the same or very similar protospacer sequences [for a recent review, see (McGinn and Marraffini, 2018)]. Because different bacteria in the population acquire different new spacers from the invader, the result of CRISPR adaptation is the extreme diversification of spacer repertoire of the population, which in turn is fundamental to prevent the emergence of escape phages with mutated target sequences (van Houte et al., 2016b). CRISPR adaptation has two steps; the selection of functional sequences from the invader's genome (known as prespacers) and their insertion into the leader end of the CRISPR array.

A functional spacer must (i) not target the host chromosome (i.e. avoid autoimmunity), and (ii) be flanked by the correct PAM in type I, II or V systems, or produce a crRNA complementary to a transcript in type III and VI systems. It has been found that both type I and II systems prefer the acquisition from DNA molecules with free DNA ends such as DNA breaks or phage *cos* sites (the phage DNA end that is injected first) (Levy et al., 2015; Modell et al., 2017). These observations suggest a model where phages (and plasmids), which have relatively small genomes and therefore replicate more frequently than the host chromosome, are more prone to breaks and stalls at replication forks, increasing the probability of prespacer acquisition from their genomes. In addition, DNA injection during phage infection or plasmid conjugation offers a free DNA end for the CRISPR acquisition machinery. The selection of invader sequences that are flanked by a functional PAM can be performed by either the nuclease that recognizes the motif during targeting, Cas9, for type II systems (Heler et al., 2015), or by Cas proteins dedicated to this task, such as Cas4 for some type I systems (Lee et al., 2018; Kieper et al., 2018; Shiimori et al., 2018). Interestingly, a

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study published in this issue of *Cell Host & Microbe* suggests that the spacer repertoire of the surviving population, at least in type II systems, is determined by the rate of acquisition of each sequence, not so much by the potency of the DNA cleavage it mediates (Heler et al., 2019). Very little is known about spacer acquisition in type III, V and VI systems. Adaptation has only been observed in the *Marinomonas mediterranea* III-B system, which harbours a relatively rare reverse transcriptase-Cas1 (RT-Cas1) fusion. In this marine bacterium, integration of DNA spacers derived from cellular RNA is achieved via a reverse transcription reaction mediated by this fusion protein (Silas et al., 2016). This is thus an elegant way for the transcription-dependent type III systems to sample from well-transcribed regions and ensure functional spacers.

Once selected, functional prespacers are integrated into the CRISPR array by the Cas1-Cas2 complex via a reaction mechanism similar to that of retroviral integrases and transposases (Nunez et al., 2015). Spacers are added to the leader end of the CRISPR array, and in type II systems this is achieved by the recognition of a ‘leader-anchoring sequence’ directly upstream of the first repeat (McGinn and Marraffini, 2016; Wright and Doudna, 2016). Significantly, leader-proximal spacers provide better immunity, and integrating the spacers from the most recent infection in this polarized manner ensures protection against the most pressing viral threat (McGinn and Marraffini, 2016).

A unique aspect of type I adaptation is the presence of priming, where pre-existing spacers against an invader enhances further adaptation against the same threat (Datsenko et al., 2012). This allows immunity to keep up with rapidly mutating phages that might have altered their target sequence and thus escaped CRISPR targeting. Primed adaptation requires the binding by Cascade to mutated protospacers, harbouring a non-functional PAM or seed sequence mismatch with the crRNA. Moreover, a study in this issue (Jackson et al., 2019) revealed that “slipped” (imprecisely acquired) spacers, although providing less efficient interference, enhance primed acquisition. After binding the DNA, Cascade recruits Cas1-Cas2 along with Cas3 (Redding et al., 2015) to capture prespacers from the target.

Prokaryotic Argonautes

Prokaryotic argonautes (pAgos) represent a recently discovered bacterial innate defence mechanism found in approximately 9% of bacterial genomes and 32% of archaeal genomes [for a recent review, see (Hegge et al., 2018)]. They are often encoded within defence islands, regions enriched for phage resistance systems, and have undergone extensive horizontal gene transfer (Makarova et al., 2009), two factors which suggest a defensive role. So far, several mechanisms have been demonstrated, including DNA-guided DNA silencing and RNA-guided DNA silencing. For the former mechanism, it was shown in two systems that the apo form of pAgo can first degrade invader DNA sequence non-specifically. Degradation products from this DNA are used as guide DNAs, which allows sequence-specific interference against the same target (Swarts et al., 2017; Zander et al., 2017). Some pAgos are also predicted to be catalytically inactive, but are encoded near other nuclease genes that might be guided by pAgo to the invader. So far, however, only *in vivo* immunity against invasive plasmids has been shown; the role of pAgo in defence against phages, if any, remains elusive.

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Abortive infection and toxin-antitoxin systems

Abortive infection (Abi) and toxin-antitoxin (TA) systems are widespread, albeit poorly understood, stress systems. They can work as antiviral systems by stressing the infecting cells to disturb the phage life cycle and prevent virion release. Abi systems are phenotypically, rather than genetically, defined, and are always involved in disrupting phage infection. TAs, on the other hand, usually comprise a genetically well-defined TA gene pair, with the toxin causing the stress and the antitoxin inhibiting the toxin's catalytic activity. The line between Abis and TAs is blurred and they share some overlap, with some previously identified Abi mechanisms employing TA genes to create the antiviral cell stress, for example the lactococcal Abi systems AbiQ (Samson et al., 2013) and AbiE (Dy et al., 2014). A central outstanding question is to what extent activation of these systems upon infection leads to permanent cell death as opposed to temporary growth arrest and dormancy.

Abortive infection

Abi is a process by which cells prevent release of functional phage virions at the expense of host cell survival/fitness. It is considered an altruistic action; a "programmed cell death" that prevents the spread of the phage to the surrounding clonal bacterial population. This is achieved through the perturbation of essential cellular processes such as translation, transcription, and replication, or by inducing membrane leakage. How phage infection is recognised to trigger the Abi response is often unknown.

Although Abi systems are widespread, most have been characterised in *E. coli* and *Lactococcus lactis*, a Gram-positive bacterium used in dairy production. The Lit and PrrC systems of *E. coli* are activated by phage T4 and disrupt translation. The Lit protease of *E. coli* K12 is activated by the Gol peptide of the T4 major capsid protein, a gene that is transcribed late in the phage infection cycle (Bingham et al., 2000) and cleaves the ribosomal elongation factor EF-Tu, thereby arresting translation for both the phage and its host. PrrC of *E. coli* CT196 cleaves the $tRNA^{Lys}$ in the anticodon loop; this depletes the $tRNA^{Lys}$ pool and inhibits global translation (Kaufmann, 2000). In *L. lactis*, more than 20 Abi systems have been identified. The single-gene *abiK* system is able to reduce infectivity of many prevalent lactococcal phage groups 10^6-fold. AbiK possesses polymerase activity, synthesising long DNA molecules with random sequences *in vitro* (Wang et al., 2011). Since phage mutants that escape AbiK have mutations in phage-encoded recombinases, it was hypothesized that AbiK-synthesised DNA interferes with phage recombination, preventing phage replication and maturation. How this activity is also detrimental to the host, and what triggers AbiK during infection, remains unclear. Another lactococcal Abi system, AbiZ, reduces the burst size of phage Φ31 by 100-fold. AbiZ seems to act cooperatively with the phage pore-forming protein holin to induce premature lysis and the release of immature, non-infectious phage particles (Durmaz and Klaenhammer, 2007).

Recently, a kinase-mediated Abi mechanism protecting against Siphoviridae phages was uncovered in the common skin bacterium *Staphylococcus epidermidis* (Depardieu et al., 2016) (Figure 4). Upon infection, the eukaryotic-like Serine/Threonine kinase Stk2 was found to phosphorylate a range of targets in diverse cellular pathways, including transcription, translation, replication, and metabolism. This widespread phosphorylation

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presumably disrupts these pathways to result in host death. Phages able to escape Stk2 activation carry mutations in the *pacK* gene, suggesting that PacK induces Stk2 auto-phosphorylation to initiate this defence pathway.

Toxin-antitoxin systems

A fundamental feature of TA systems is the instability of the antitoxin: it is labile and must be continuously expressed to remain at appropriate stoichiometric ratios with and neutralize the toxin [for a recent review, see (Harms et al., 2018)]. Toxins can possess various catalytic activities, including DNase and RNase, or can inhibit DNA replication, ATP synthesis, or the cell division machinery. There are at least six TA types, categorised based on the nature of the toxin and antitoxin (protein or RNA), and the mechanism of toxin neutralisation, with many bacteria harbouring dozens of TA gene pairs (*E. coli* K-12 has more than 35 TA pairs (Harms et al., 2018)). This high genetic diversity reflects the many functions found for TA systems: in addition to phage defence, they have been implicated in stress responses, plasmid maintenance, and persister cell formation.

Some TA systems can directly inhibit the phage life cycle. The plant pathogen *Pectobacterium atrosepticum* possesses the ToxN/ToxI TA pair, where the endoribonuclease ToxN is sequestered by binding the noncoding RNA antitoxin ToxI (Fineran et al., 2009). Upon phage infection, the RNase activity of ToxN is unleashed to destroy both host and phage transcripts (Blower et al., 2011), arresting the infection. Similarly, the MazF/MazE TA system of *E. coli* can suppress phage T4 infection by activating MazF's ribonuclease activity (Alawneh et al., 2016). To counter this, phage T4 carries the ADP-ribosyltransferase Alt, which modifies and inhibits MazF (Alawneh et al., 2016). The finding that phages have evolved mechanisms that inactivate TA systems strongly indicates that they are part of the host-phage arms race. For example, in addition to Alt, the T4 *dmd* gene directly binds and suppresses two other toxins, LsoA and RnlA (Otsuka and Yonesaki, 2012).

As mentioned above, it is not clear whether TA induction causes altruistic suicide of the infected cell or only a temporary growth arrest. The activation of toxins can in some cases be reversible. With the MazF/MazE and ToxN/ToxI systems, cells where the toxin MazF or ToxN was expressed could be rescued and were viable upon induction of the expression of the antitoxin (MazE or ToxI, respectively) after a delay (Fineran et al., 2009; Pedersen et al., 2002). Recently, a comprehensive analysis of MazF cleavage sites revealed that most mRNAs, as well as rRNA precursors, are cleaved at multiple sites (Culviner and Laub, 2018). Presumably, upon toxin neutralization, cells can replenish their RNA pool and resume growth. Given the extensive diversity of TA systems, it is probable that they can work both as dormancy induction and programmed cell death (Abi) systems, and the outcome will depend on a range of factors including the toxin mechanism of action, the duration of the toxin's activity, and the life cycle of the phage.

Bacteriophage assembly interference

Phage-Inducible Chromosomal Islands (PICIs) form a group of genetic elements that parasitise phages for replication and transmission [for a recent review, see (Penades and Christie, 2015)]. PICIs are integrated into a bacterial chromosome and excise in the presence

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of a specific “helper phage” (by infection or lysogen induction). Although the main role of PICIs seems to be the dissemination of the genetic material they harbour (in many cases bacterial virulence determinants), they interfere with the phage life cycle, and can therefore be classified as an anti-phage mechanism.

PICI genomes are often small (~15 kb), encoding genes required for excision and integration, factors that promote PICI packaging and dissemination, and a repressor that inhibits their expression in the absence of the helper phage. PICIs are best characterized in *Staphylococcus aureus*, where they are named “SaPIs” (*S. aureus* Pathogenicity Islands). SaPIs are induced when their repressor, Stl, is sequestered away by an anti-repressor expressed early during the helper phage lytic cycle (e.g. helper phage 80α) (Tormo-Mas et al., 2010) (Figure 5). Derepression induces the expression of SaPI genes and couples the SaPI’s propagation cycle with that of the helper phage. Specialized SaPI structural proteins modulate the assembly of the helper phage’s capsid to produce a capsid that can only be packaged with the smaller SaPI genome. As a result, the host bacterium lyses, but primarily releases SaPI virions that infect neighbouring cells to disseminate the SaPI genomes and the virulence factors they encode. Recently, PICIs were found to also be widespread in gram-negative bacteria (Fillol-Salom et al., 2018). Instead of being regulated by a repressor, Gram-negative PICIs are induced by a PICI-encoded activator whose expression requires the helper phage.

To fight phage, *V. cholerae* encodes PICI-like elements (PLEs) (Seed et al., 2013), which are similar to other gram-negative PICIs though with somewhat different gene content. Upon infection of the host bacterium by the phage ICP1, the PLE-encoded recombinase Int excises PLE from the *V. cholerae* chromosome (McKitterick and Seed, 2018). PLE then replicates to high levels and inhibits ICP1 phage replication by unknown mechanisms. Interestingly, several ICP1 phage isolates encoded their own CRISPR system capable of neutralising PLEs (Seed et al., 2013), allowing ICP1 propagation in PLE^{+} *V. cholerae* strains.

Recently discovered anti-phage mechanisms

Given the astronomical number and diversity of bacteriophages in our planet, it is likely that we only know a minority of the anti-phage mechanisms present in prokaryotes. For example, a recent extensive screen of mycobacterial phages revealed varied mechanisms of phage-encoded Sie, which included a (p)ppGpp synthetase and a single-subunit RM system, as well as classical Sie modes like promoter repression and inhibition of entry (Dedrick et al., 2017). Sie, being as much a phage-phage interaction as a bacterium-phage interaction, is likely a more important and diverse resistance mechanism than previously appreciated.

Interestingly, bacterial defence systems often cluster in defence islands (Makarova et al., 2011). This has allowed a “guilt-by-association” approach to uncover new anti-phage mechanisms. The BREX (BacteRiophage EXclusion) system, which was discovered in this way, mediates methylation of a non-palindromic, six-nucleotide motif, most likely to achieve self/non-self discrimination (Goldfarb et al., 2015; Gordeeva et al., 2018). Phage DNA is inactivated after injection prior to DNA replication by an unknown mechanism that is not thought to involve cleavage. The BREX systems characterised recently share two genes

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(*pglX* and *pglZ*) with the Pgl system of *S. coelicolor* (see above) (Goldfarb et al., 2015), with the Pgl system being denoted type 2 BREX (of six types in total). Another recently discovered system is DISARM (Ofir et al., 2018), which provides broad anti-phage immunity through a novel RM-like mechanism that includes a methyltransferase modifying a five-nucleotide motif and a multi-component restriction element that probably cleaves unmodified phage DNA early in the phage life cycle.

Also using an approach based on identifying defence gene neighbourhoods, a recent study identified 26 candidate systems that were heterologously expressed in *E. coli* or *Bacillus subtilis* and assayed for anti-phage activity (Doron et al., 2018). Of these, nine provided robust protection against at least one type of phage (and one against plasmids), with one, named “Zorya”, most likely being an Abi system that may cause membrane depolarization of the host using a proton channel. Finally, the guilt-by-association approach was used to identify genes enriched near CRISPR loci (Shah et al., 2018; Shmakov et al., 2018). A diverse range of CRISPR-associated accessory candidate genes were identified, which likely complement or expand the functions of the core *cas* gene machinery.

Putting it all together

The phage resistance mechanisms discussed so far have mostly been studied in the lab individually, though this is rarely how a bacterium’s arsenal is applied against phage in nature. Bacteria employ several complementary lines of defence, none of which are mutually exclusive, and a phage has to overcome each system to allow successful infection. On the other hand, contrary to experimental settings, the environment typically contains a heterogeneous mix of phages.

Synergistic effects between anti-phage mechanisms are starting to be recognised. RM and CRISPR systems often co-exist, and spacer acquisition by the CRISPR system is enhanced in the presence of an RM system (Hynes et al., 2014). Cooperation between different CRISPR types has also been detected: *M. mediterranea* contains two CRISPR systems, and spacers incorporated from phage into the type I-B array can be used by the III-B machinery against phage (Silas et al., 2017). The cross-talk between the type I and III systems makes protection more robust since it is harder for phages to escape type III targeting through protospacer mutations (Pyenson et al., 2017). Although not experimentally demonstrated, it is tempting to speculate that there can also be synergy between dormancy-inducing components (TA systems) and effector components (i.e. CRISPR and RM systems), which are often clustered in genomic defence islands (Makarova et al., 2013). The rationale is that TA or Abi systems could “buy time” for the cell, inducing short-term dormancy while CRISPR or RM mechanisms eliminate the phage. This concept is illustrated in the type III-A CRISPR-Cas response, where the non-specific RNase Csm6 causes a transient growth arrest until the DNase activity of the Cas10-Csm complex has eliminated the plasmid invader (Rostøl and Marraffini, 2019). Induction of dormancy could also afford time for the acquisition of new spacers during CRISPR adaptation. Alternatively, if the invader is not cleared and the toxin remains active, the infected cell would die and prevent further spread of the phage.

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Outlook and future directions

Recently, novel technologies and experimental approaches, as well as renewed interest in bacteriophages, have dramatically boosted our knowledge of how bacteria resist their parasites. Scientists have probed both broader (new systems) and deeper (expanding repertoire of known systems). Future studies will surely continue this trend, which will most likely translate into clinical outcomes and technological innovations. Still, there are hurdles to overcome. The study of more integrative models where it is possible to appreciate how different immune mechanisms interact and complement each other, as well as more ecological approaches, where the fluxes of multiple and different bacteria and phages can be observed, will undoubtedly expand our understanding of prokaryotic immunity. The extent to which phages interfere with the life cycle of other phages is also underappreciated. Last but not least, given the importance of phages as mediators of horizontal gene transfer, the study of defence mechanisms will help us understand prokaryotic evolution. Considering the millions of years bacteria and phages have coevolved, there surely remains a cornucopia of unknown unknowns for us to discover.

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Bacteria are preyed upon by parasitic bacteriophages, which undergo continuous cycles of infection. In order to survive, bacteria have developed a complex arsenal of anti-phage mechanisms. Rostøl and Marraffini review the mechanisms employed by bacteria to resist their phages, with an emphasis on recent developments in the field.

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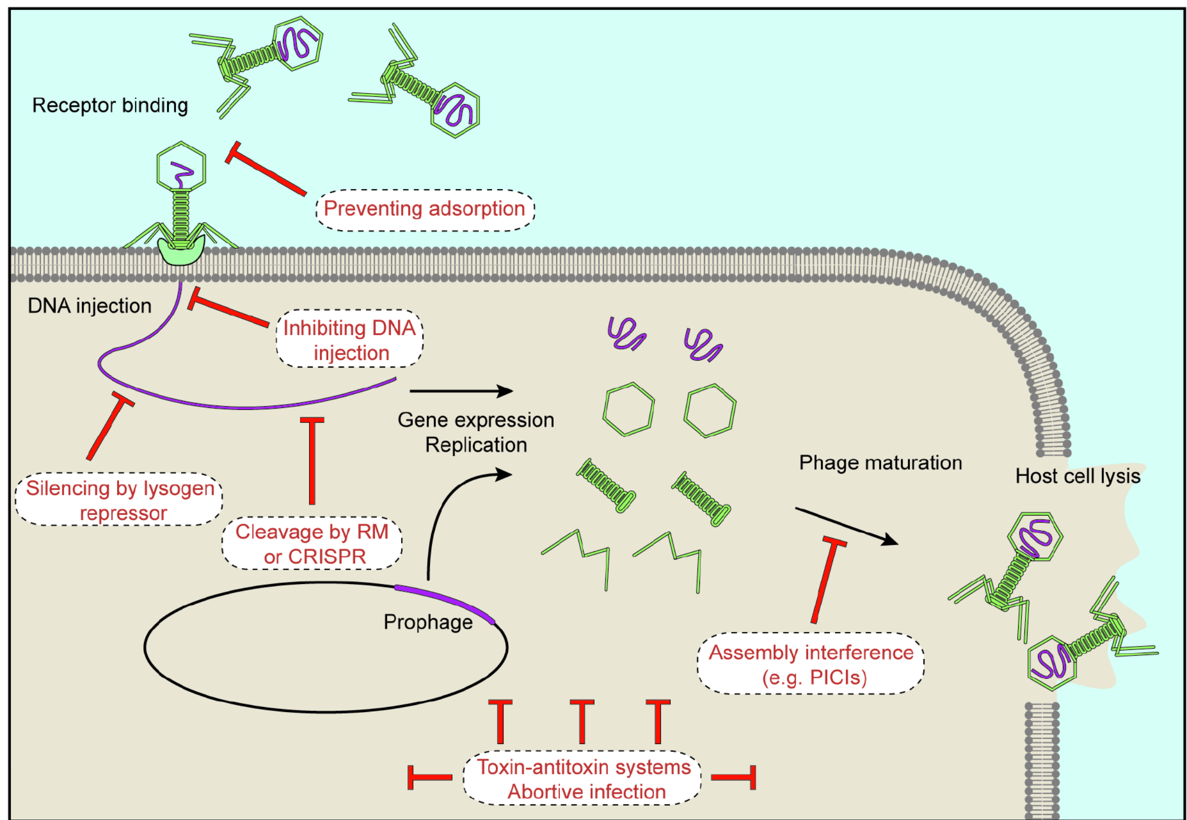

Figure 1. Stages of a phage's life cycle that can be targeted by different anti-phage mechanisms.
Upon recognising a surface receptor, a phage injects its DNA into the host cell. Either after injection or after prophage induction, the viral genome is subject to several rounds of replication and gene expression that leads to the assembly and accumulation of new viral particles, which are released upon lysis of the host cell. As indicated, anti-phage mechanisms can interfere with any part of this process.

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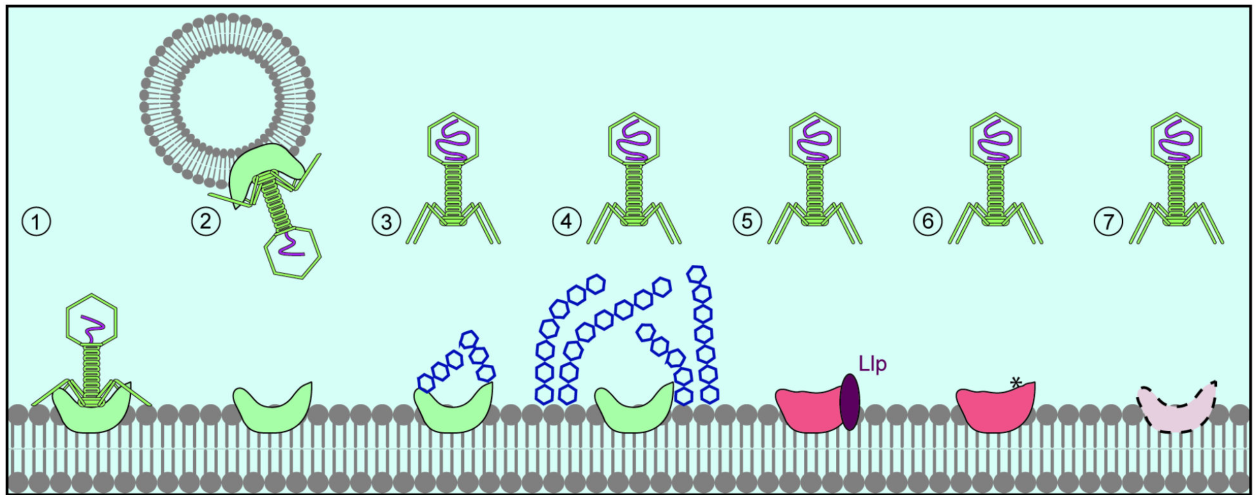

Figure 2. Prevention of phage adsorption.

1. Successful binding of the phage to its receptor (green). 2. Sequestration of phage particles by OMVs containing the phage receptor. 3. Prevention of phage adsorption due to receptor post-translational modifications (glycosylation). 4. Prevention of phage adsorption due to receptor occlusion by surface structures (glycan capsule). 5. Receptor modification through interaction with another protein. 6. Receptor mutations that abolish phage binding. 7. Regulation of receptor expression.

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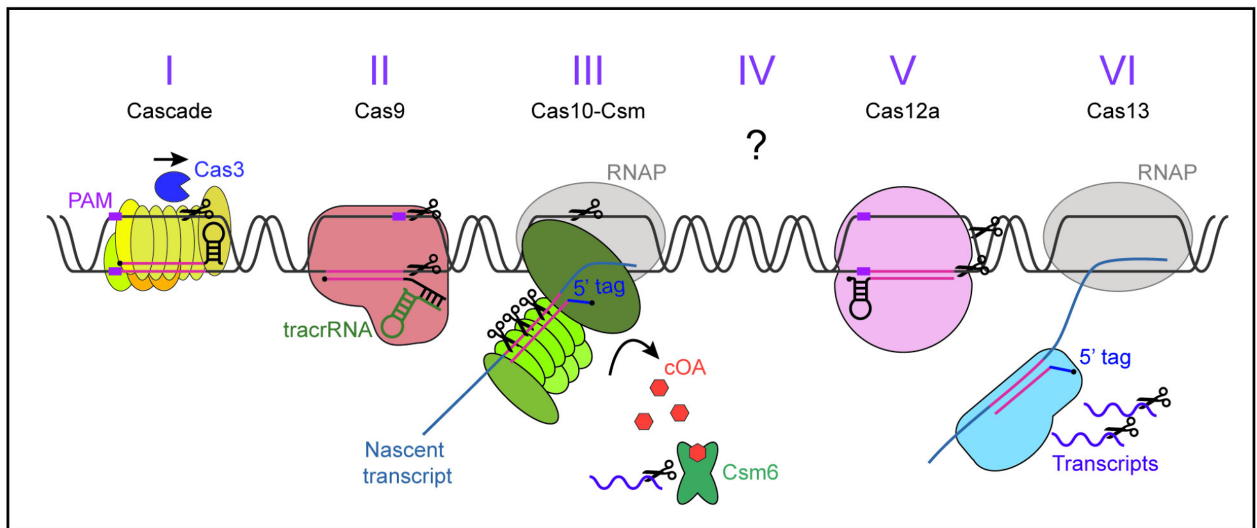

Figure 3. Different CRISPR targeting mechanisms.

See text for details. Purple box: PAM, black circles: crRNAs 5' end, pink: spacer/protospacer sequences, blue: 5' crRNA tags inhibiting type III/VI autoimmunity. For types I, II, and V, the DNA double helix is unwound by the main effector complex in a PAM-dependent manner, and DNA is cut by Cas3 (type I) or Cas9/Cas12a (types II/V). Type III and VI recognise the protospacer within a nascent transcript in a PAM-independent manner, this is followed by the cleavage of DNA and/or RNA from the invader. Type III also produces cyclic oligoadenylates (orange hexagons) which allosterically activate the accessory RNase Csm6 (Csx1 for III-B).

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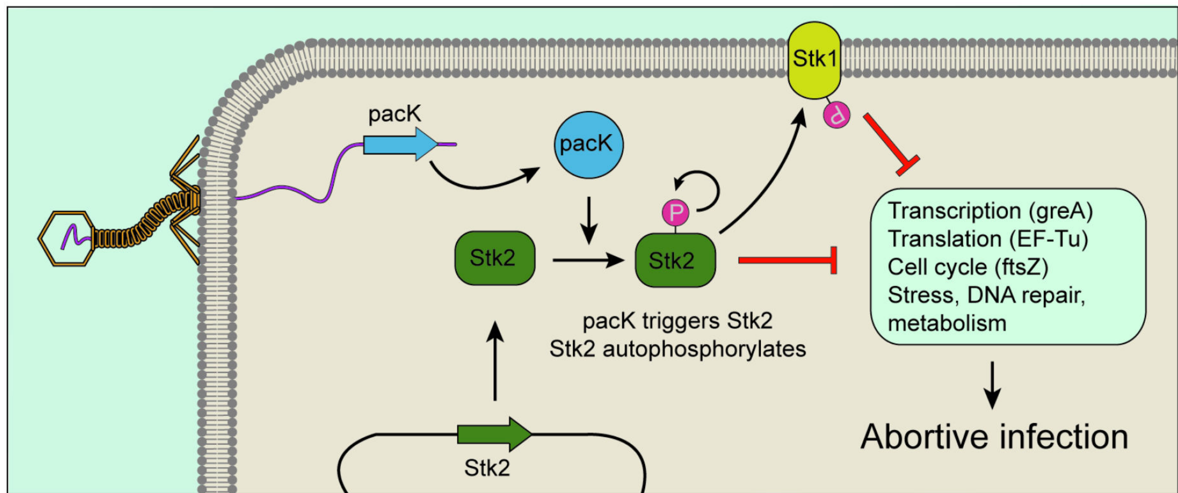

Figure 4. The staphylococcal Stk2 Abi system.

The phage protein pacK triggers autophosphorylation (and activation) of the *S. epidermidis* Stk2 kinase. Activated Stk2 phosphorylates Stk1 and miscellaneous cellular factors, eventually leading to the abortion of the viral infectious cycle and cell death.

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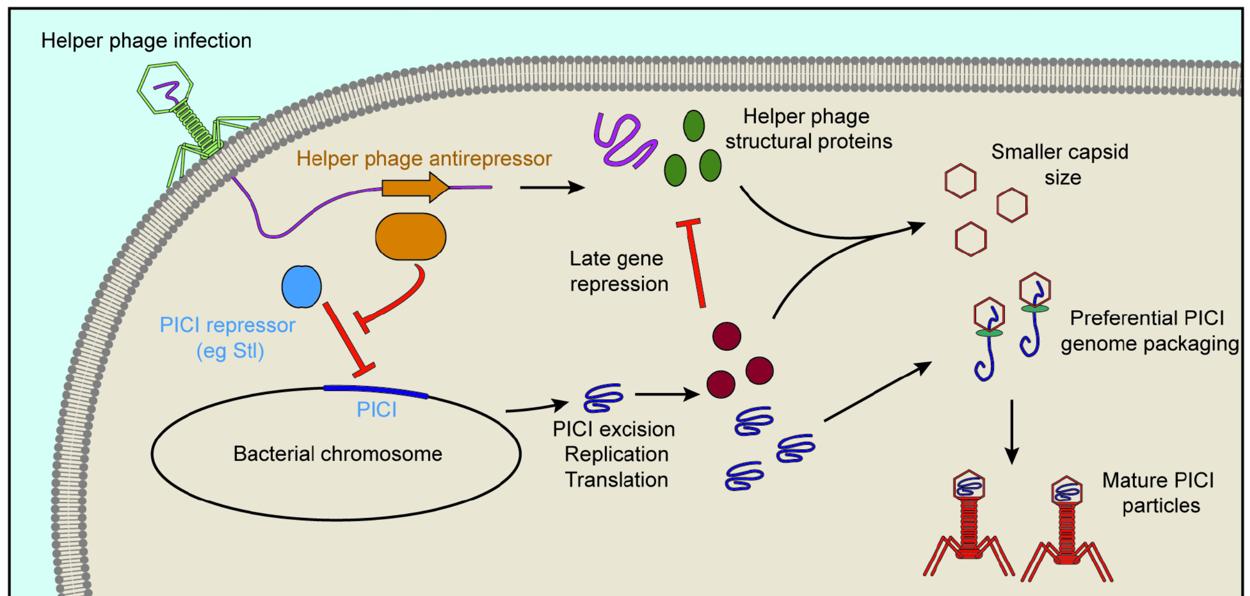

Figure 5. PICI-mediated interference of phage assembly.

In Gram-positive organisms, PICI expression is inhibited by a transcription repressor. Helper phages produce an antirepressor, leading to the excision of the PICI from the host chromosome. The PICI genome replicates, and expresses proteins that repress late helper phage genes and alter the phage capsid size to be more appropriate for the PICI genome size. This in turn leads to both the preferential packaging of PICI genomes and the prevention of the formation of helper phage virions.