The arms race between bacteria and their phage foes

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Bacteria are under immense evolutionary pressure from their viral invaders—bacteriophages. Bacteria have evolved numerous immune mechanisms, both innate and adaptive, to cope with this pressure. The discovery and exploitation of CRISPR–Cas systems have stimulated a resurgence in the identification and characterization of anti-phage mechanisms. Bacteriophages use an extensive battery of counter-defence strategies to co-exist in the presence of these diverse phage defence mechanisms. Understanding the dynamics of the interactions between these microorganisms has implications for phage-based therapies, microbial ecology and evolution, and the development of new biotechnological tools. Here we review the spectrum of anti-phage systems and highlight their evasion by bacteriophages.

Bacteriophages (phages) are viruses that infect bacteria and it has been estimated that there are 10^{31} phages present in the biosphere[1]. Their abundance accounts for 20–40% of bacterial mortality daily[2], and has a considerable impact on biogeochemical cycles[3]. The pressure of phage infection on bacteria has resulted in the evolution of multiple bacterial immune systems, each of which hampers different stages of the phage life cycle[4,5] (Fig. 1). Unsurprisingly, phages have evolved a myriad of ways to overcome these defences[5,6], which in combination with phage diversity, has contributed to the diversity of bacterial immune mechanisms.

Research interests in bacterial–phage interactions, and in particular bacterial defences, are manifold. First, although the importance of these interactions for global ecology is accepted, large sequencing efforts, such as the *Tara* Oceans project, are furthering our understanding by showing that phages drive rapid evolution through the daily transfer of approximately 10^{29} genes between bacteria[7]. Second, phage-based therapies are becoming feasible antibacterial treatments as alternatives to antibiotics, owing to the rise of antibiotic resistance[8]. For successful therapy, it is critical to understand how bacterial pathogens might become resistant to phages and, therefore, recalcitrant to treatment. Finally, phage-resistant strains are required in different industries[9] and fundamental research into phage-defence mechanisms has underpinned the development of these, and other, applications, such as gene editing and diagnostics[10]. The importance of bacterial immune systems to these areas has led to a resurgence in the discovery and characterization of phage-resistance mechanisms. Here we focus on the diverse systems that bacteria use to resist phages and how their phage invaders can evade these immune mechanisms.

Preventing adsorption

Phages exploit at least three different lifestyles to reproduce. Virulent phages replicate exclusively through the lytic cycle, exploiting bacteria to make new phages before their release by cell lysis[11] (Fig. 1). Alternatively, in addition to the lytic cycle, temperate phages can enter the lysogenic cycle and form prophages that are integrated into the bacterial chromosome or maintained extrachromosomally[1] (Fig. 1). By contrast, filamentous phages cause chronic infections and are continuously secreted from the bacterium without lysis[12]. For infection to occur, phages must adsorb to the cell surface by binding to phage receptors, and inject their genome (Fig. 2a). To prevent adsorption, bacteria can alter or disguise receptors through surface modification (Fig. 2a). For example, receptor mutations in *ompU* in *Vibrio cholerae* confer resistance to the vibriophage ICP2[13]. Bacteria can also use receptors as phage decoys. In this case, outer membrane vesicles (OMVs) that contain receptors bud off from *Escherichia coli* and *Vibrio*, and can bind to phages, reducing productive infections[14,15] (Fig. 2a). Nonetheless, OMVs have complex effects on phage dynamics because they can also extend the host range of phages. Indeed, phage receptors were transferred by OMVs to *Bacillus subtilis* cells that previously lacked the receptor, rendering *B. subtilis* and other phage-resistant species susceptible to phages[16] (Fig. 2b). Although this provides only transient susceptibility, the receptors may subsequently facilitate the transfer of receptor genes through generalized transduction, which could lead to a permanent heritable change in phage susceptibility. Inhibiting DNA entry into the bacterial cell is another defence strategy. For example, the Imm and Sp proteins of phage T4 prevent the DNA of other T-even phages from being translocated across the membrane[4]. However, systems that prevent DNA entry are typically encoded on prophages and inhibit infection by subsequent phages[4].

The fitness costs of receptor mutations have led to other strategies that impede attachment[5]. Phase variation enables the reversible expression of phage receptors, resulting in phage-resistant bacterial subpopulations[5,17]. Furthermore, receptors can be masked, preventing recognition while retaining function. For example, capsules or exopolysaccharides provide phage resistance in *Staphylococcus*, *Pseudomonas* and other species[18,19] (Fig. 2a). Subtle modifications can also disguise receptors from phages, such as in *Pseudomonas aeruginosa*, in which pilus and O-antigen modifications and type IV pili glycosylation occludes phages[20,21].

Receptor modification can select for phages that recognize the mutated, or alternative, receptors. In coevolution studies, it was shown

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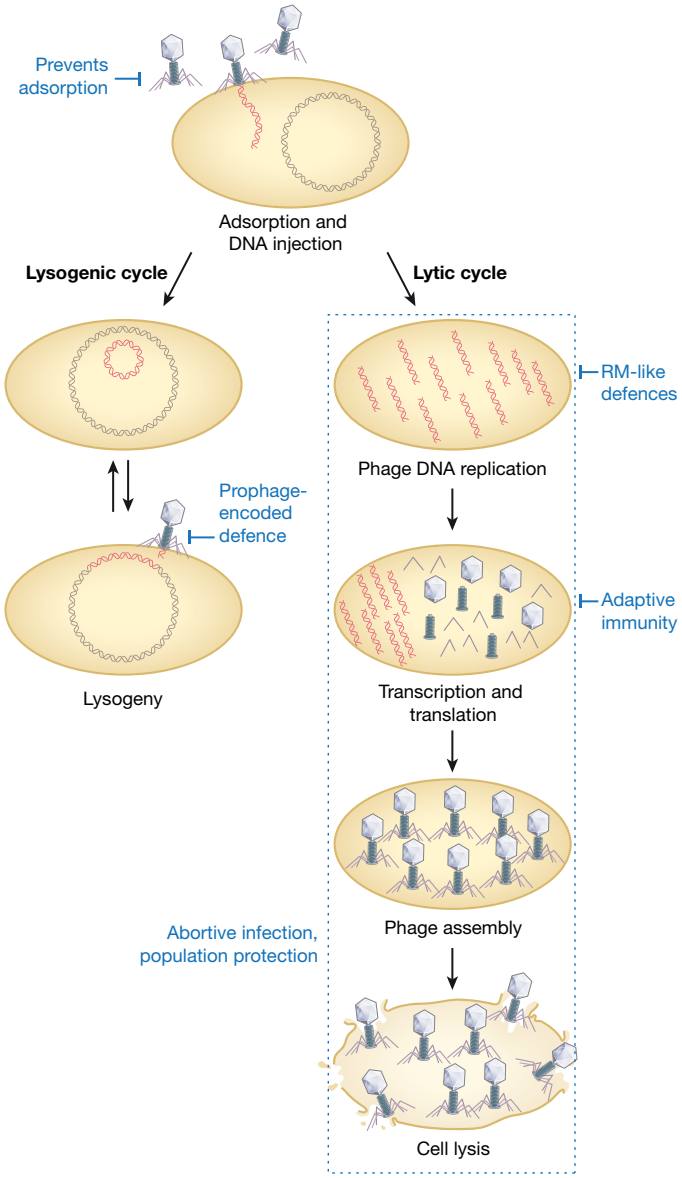

Fig. 1 | Anti-phage mechanisms act at different stages of the phage life cycle. Virulent phages replicate exclusively through the lytic cycle, whereas temperate phages may replicate through either the lytic or the lysogenic cycle. Bacteria have numerous anti-phage systems that function at different stages of the phage life cycle to prevent productive phage replication. Abortive infection mechanisms provide population protection and function at different stages of the phage life cycle, indicated by the blue dashed line.

that the receptor-binding proteins (RBPs) of phages—which bind to the bacterial receptors—are often mutated (Fig. 2b). For example, phage λ overcame a LamB receptor mutant of *E. coli* through tail fibre mutations, some of which caused stochastic protein folding into different forms that enabled the recognition of a new receptor (OmpF) or mutated LamB[22,23]. Access to a new receptor occurred through baseplate and tail fibre mutations in *B. subtilis* phage SPO1, which also resulted in an extended range of *Bacillus* hosts[24]. Instead of mutating, some phages—such as the *E. coli* phage phi92—have more than one RBP, enabling the recognition of multiple receptors[25]. RBP variability can be further expanded by diversity-generating retroelements that result in hypervariable RBPs. For example, the diversity-generating retroelement of the *Bordetella* phage BPP-1 creates variable tail fibres, changing the receptor tropism of the phage[4]. Finally, when receptors are masked by exopolysaccharide capsules, some phages produce depolymerases that enable them to gain access[26] (Fig. 2b). Recent research on receptor interactions is driven by the goal to understand and manipulate phage–receptor interactions to extend the host range for biotechnological and medical applications[27].

Restriction–modification and related defences

The biotechnological use of restriction–modification (RM) systems has led to these systems being the most well-characterized phage-resistance mechanism—they are highly diverse and ubiquitous, and are present in around 90% of bacterial genomes[28]. These systems distinguish self from non-self DNA to recognize and destroy phage DNA after its injection. Discrimination is due to DNA modifications at specific sequences and is characteristic of a number of anti-phage systems. Two components that are typically present in RM systems are a methyltransferase and a restriction endonuclease (Fig. 3a, b). Both recognize restriction-site sequences; the methyltransferase methylates DNA and the restriction endonuclease cleaves the unmethylated sequence. A comprehensive review of RM systems has been published previously[29]. A range of other phage-resistance systems have similarities to RM systems, but their functions appear to be more complex owing to the presence of additional genes.

One RM-like phage-defence mechanism is the phage growth limitation (Pgl) system in *Streptomyces coelicolor*, which modifies and cleaves phage DNA[30,31]. Pgl has three phases and requires four genes, *pglW*, *pglX*, *pglY* and *pglZ* (Fig. 3a, c). Phages become methylated after infecting Pgl$^+$ bacteria and, following release, these phages can infect other cells. During subsequent infections, the modified phage DNA is cleaved. Hence, although the initial infected cell does not survive phage infection, it is able to mark the phage to 'warn' neighbouring cells[31]. Genes similar to *pglZ* from the Pgl system were identified in six-gene clusters, including *brxABCL* and *pglX*[32] (Fig. 3a). These were termed bacteriophage exclusion (BREX) genes and have been characterized in Gram-positive (*B. subtilis*[32]) and Gram-negative (*E. coli*[33]) bacteria. Akin to RM, BREX acts after DNA injection to prevent phage replication and lysogen formation, but differs as DNA cleavage was undetectable[32]. BREX further differs from Pgl in that it restricts phages upon first exposure[32,33]; however, the precise mechanism by which BREX prevents infection remains unresolved.

Another RM-like system was recently identified—termed defence island associated with restriction–modification (DISARM)[34] (Fig. 3a). Class 1 and 2 DISARM share three core genes, with each class having two distinct additional genes. Class 2 DISARM includes a five-cytosine DNA methyltransferase and a system from *Bacillus paralicheniformis* prevented phage DNA accumulation by distinct families of double-stranded (ds)DNA phages. Notably, phages modified at a specific sequence, and therefore presumably masked from the RM-like system, were inhibited. Furthermore, DISARM offered protection against phages that lacked the sequence recognized by the RM-like system. These results indicate that the mechanism differs from classic RM systems. To add further mystery to the DISARM mechanism, the candidate nuclease was dispensable for resistance[34].

Phages have amassed strategies to counteract RM, and potentially RM-like, systems[6,35,36]. Phage DNA can become methylated by the host methyltransferase on entry, disguising the DNA from the host restriction endonuclease. The resulting phages become phenotypically RM-insensitive; however, this epigenetic avoidance is transient and is lost following infection of methyltransferase-deficient bacteria. In addition, RM sites are mutated, underrepresented or absent in phage genomes to prevent restriction[35,37,38] (Fig. 3d). Phages also exploit modified or unusual bases, such as hydroxymethylation, glycosylation, glucosylation and acetamidation to make these sites unrecognizable to the restriction endonuclease[36]. Specifically, coliphage 9g utilizes a deoxyarchaeosine modification to avoid restriction[39]. Some phage proteins (for example, Ral from λ and P22 phages) activate host methyltransferases

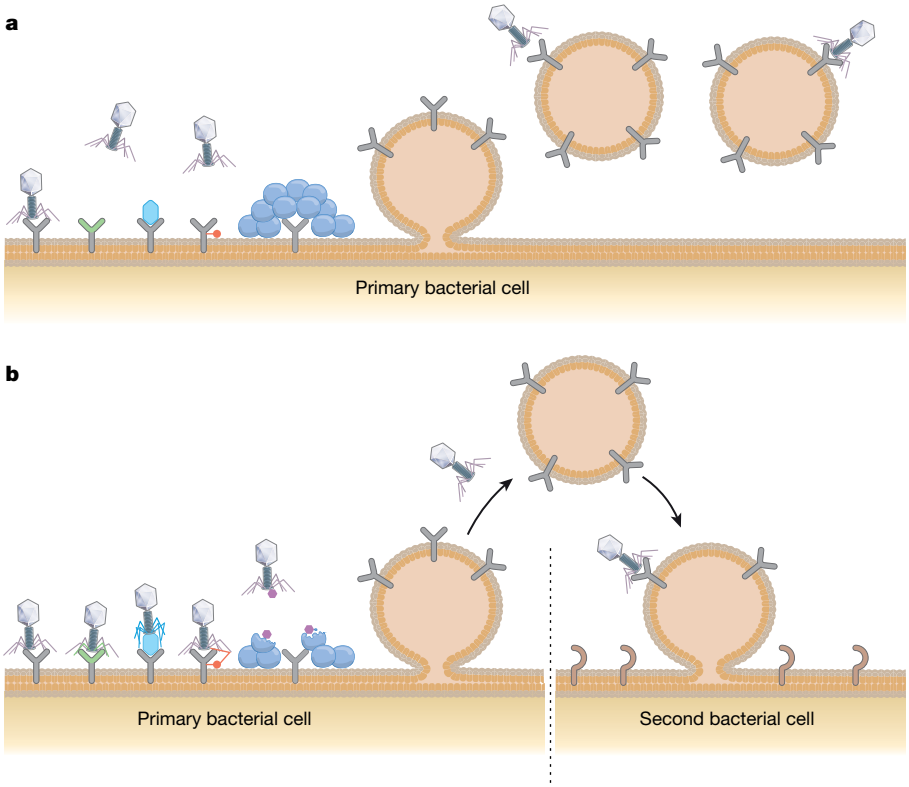

Fig. 2 | Preventing phage adsorption. a, Bacteria have developed a number of methods to prevent phage adsorption. These include altering (green), disguising (blue), modifying (red) or masking (blue circles) receptors and the use of decoy OMVs. **b**, Phages can co-evolve to recognize the modified receptor, through mutations, and produce extracellular-matrix-degrading enzymes. OMVs can also extend the host range of phages, by transferring receptors used by the phage to cells that previously lacked those specific phage receptors.

and promote DNA modification to protect against restriction endonucleases[40,41]. Phages can also encode methyltransferases, which protect their DNA from restriction[42,43] (Fig. 3d), such as the methyltransferase of the *Bacillus* phage SPR, which can modify three sites to protect against multiple nucleases[42,44]. Phages can also prevent degradation of their genomes using the defence against restriction (Dar) system. The Dar system of coliphage P1 limits DNA degradation by type I restriction endonucleases[45,46]. Dar proteins are injected along with the phage DNA and function in *cis*. Another successful anti-RM strategy is the direct inactivation of restriction endonucleases. The overcome classical restriction (Ocr) protein of coliphage T7 is expressed immediately after DNA injection, mimics DNA, and tightly binds and sequesters the EcoKI restriction endonuclease[47,48]. Routes of phage escape from the recently discovered, RM-like systems have yet to be thoroughly investigated. However, phages are likely to use similar anti-restriction mechanisms for DISARM and BREX. No phages that have escaped Pgl systems have been isolated[31,49], suggesting that bacterial protection by this system may be more robust than other RM-like systems.

CRISPR–Cas adaptive immunity

The ability to cleave phage DNA in a sequence-specific manner is shared by both RM and clustered regularly interspaced short palindromic repeats (CRISPR)–CRISPR-associated protein (Cas) systems. However, CRISPR–Cas provides 'adaptive' immunity through the generation of memories of past phage encounters that guide sequence-specific immunity[50]. CRISPR–Cas immunity is present in about half of sequenced bacteria and is mediated by three stages[51–53]: adaptation, expression and interference (Fig. 4a). The mechanistic diversity of CRISPR–Cas systems is considerable—currently there are two classes, six types and more than 30 subtypes[54,55].

Class 1 systems include types I, III and IV, which have multi-subunit Cas complexes. Various type I CRISPR–Cas subtypes have been shown to provide phage resistance[56–62], whereas type IV systems—which are most-closely related to type I—are poorly characterized and their role in phage resistance is unknown[63,64]. Type III systems differ from other class 1 systems, because they target both RNA and DNA[65,66]. Resistance to lytic infection has been demonstrated by the type III systems of *Staphylococcus epidermidis*[66–68], *Lactococcus lactis*[69] and *Streptococcus thermophilus*[70]; however, the RNA-dependent targeting provides tolerance to prophages[71]. An interesting feature of type III systems is that Cas10 synthesizes intracellular signals (cyclic oligoadenylates) that bind an accessory RNase and unleash its promiscuous activity[67,72,73]. The RNase may have an abortive infection effect (see 'Protecting the bacterial population' section), adding a further layer of defence by inducing dormancy through unspecific cleavage of both host and phage transcripts[74,75].

Class 2 CRISPR–Cas includes type II, V and VI systems, which are characterized by single-subunit effectors. The first direct evidence that CRISPR–Cas provides immunity against phages was provided by the type II-A system of *S. thermophilus*[50] and was later shown in *Streptococcus pyogenes*[76]. Type II systems use Cas9 to generate dsDNA breaks, whereas type V systems use Cas12[77]. Although there are few studies that have investigated phage resistance by type V systems, it has been shown that the *Francisella novicida* system protects against phage infection in *E. coli*[78]. The dsDNA breaks induced by class 2 systems have been exploited in biotechnology, but may be less effective for clearing phages. In support of this idea, class 2 systems are less common than type I, which have a potentially more destructive DNA-shredding mechanism[55]. Finally, class 2 systems can recognize and cleave phage RNA. Indeed, Cas13 from the type VI system of *Leptotrichia shahii* cleaved phage MS2 RNA in *E. coli*[79]. Upon target recognition, Cas13 not only

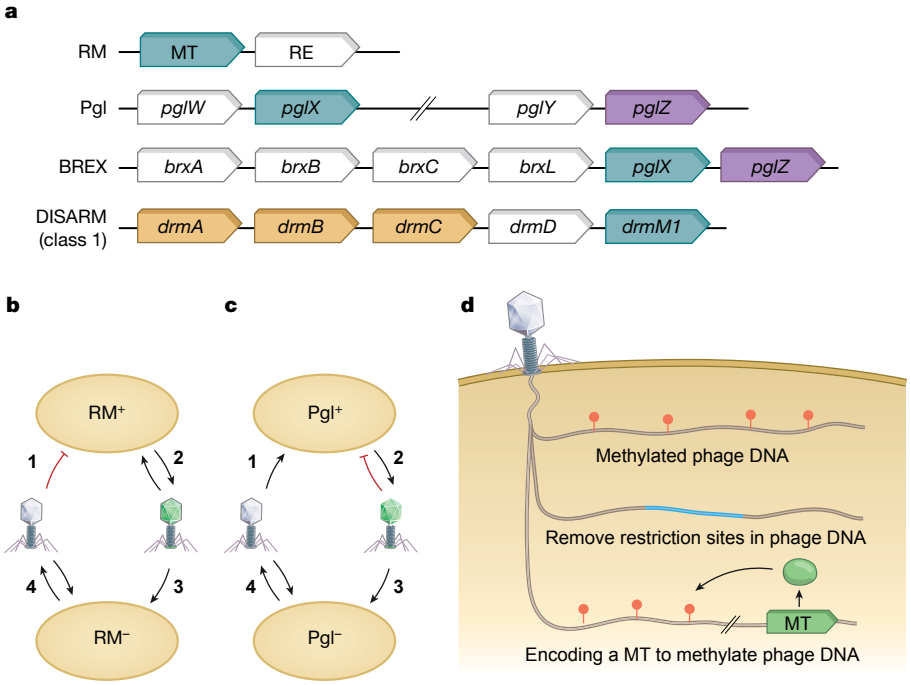

Fig. 3 | RM-like systems. a, Many proteins and protein domains are shared between the RM, Pgl, BREX and DISARM systems. The blue genes indicate enzymes that are responsible for DNA modification (methyltransferases (MT)), the purple gene (*pglZ*) encodes a conserved protein (an alkaline phosphatase) and orange genes in the DISARM system indicate core genes. RE, restriction endonuclease. *pglW* encodes a serine/threonine kinase; *pglX* encodes an adenine-specific methyltransferase; *pglY* encodes an ATP-binding protein; *brxA* encodes an RNA-binding anti-terminase; *brxB* encodes a protein with an unknown function; *brxC* encodes an ATP-binding protein; *brxL* encodes a protease; *drmA* encodes a putative helicase; *drmB* encodes a helicase-associated protein; *drmC* encodes phospholipase D/nuclease; *drmD* encodes an SFN2 helicase; and *drmM1* encodes an N^6-adenine DNA methyltransferase. **b**, (1) RM restricts any phage DNA that is not modified by methylation; (2) however, modified phages (green phage; see **d**) can replicate on RM^+ strains. (3) Modified or (4) unmodified phages can replicate on an RM^- strain but will lose any modifications. **c**, Pgl systems only restrict phages that have been previously exposed to the system. (1) A naive phage can replicate on Pgl^+, (2) but upon secondary infection of a Pgl^+ strain, the phage (shown in green) is restricted. (3) Modified (yellow) or (4) unmodified (grey) phages can replicate on Pgl^- strains. **d**, Mechanisms of phages for avoiding RM and RM-like systems include methylation of DNA, removing recognition sequences from their genome and encoding a methyltransferase to methylate the phage DNA.

cuts complementary transcripts, but also becomes a promiscuous RNase[79,80]. This promiscuous RNase activity can cleave phage mRNAs and host RNAs, inducing dormancy and providing Cas13-mediated resistance against dsDNA phages[80].

The sequence specificity of CRISPR–Cas selects for phages with mutations in targeted regions (Fig. 4b). Indeed, mutations in protospacer-adjacent motifs and spacer targets (i.e. protospacers) enable phages to overcome type I systems[57,59–61,81] and type II systems[82–85]. Insertions, deletions and recombination events can also mediate phage escape[50,59,81,84,85]. However, type I systems have a positive feedback mechanism to restore or enhance immunity by acquiring multiple new spacers that target escape phages—a process called priming[51]. There is now bioinformatic and experimental evidence that priming occurs in type II systems[86,87]. Nevertheless, phages can evade primed strains with multiple spacers by deleting the target region[81].

As type V and VI systems can also degrade non-specific single-stranded (ss)DNA (type V) or RNA (type VI), they might provide an additional layer of resistance, which may explain why escape phages are yet to be identified for these systems[75]. In agreement with this notion, dormancy induced by type VI systems suppressed the emergence of escape mutants and protected the bacterial population against phages[80]. Similar to RM system evasion, phages can modify DNA to reduce Cas complex binding and cleavage—as seen for T4 evasion of type I-E and II-A systems[59,78].

Escape mutations can lead to phage fitness defects, and if essential genes are targeted, escape might be impossible. As an alternative, some phages have anti-CRISPR (Acr) proteins that inactivate CRISPR–Cas systems[88,89] (Fig. 4b). Acrs have been identified for type I, II, III, V and VI systems and most interact with the Cas proteins to block activity[89–91]. Recently, an Acr has been shown to acetylate a type V system to prevent DNA binding[92], and another inactivates Cas12 by triggering cleavage of CRISPR RNA bound to Cas12[93]. Notably, some phages must cooperate to exploit their Acrs. Acrs produced by the first phage that infects can immunosuppress the host, but may fail to fully protect the phage from CRISPR–Cas, while enabling a productive infection by successive phages[94,95]. It is possible that Acrs have provided a selection for CRISPR–Cas diversity, but the ecological importance of their mechanistic diversity is unclear (see Box 1).

Phage defences such as CRISPR–Cas are sometimes encoded by phages[96,97]. For example, CRISPR arrays occur in prophages of *Clostridium difficile*, which target other *C. difficile* phages, and CRISPR–Cas systems are also present in 'huge' phages[98–100]. In many phages, these systems are incomplete—lacking genes for adaptation or interference[97,101]. Phages that contain these incomplete systems have been proposed to co-opt the required proteins from the host, or repress transcription without cleavage, akin to RNA interference[101]. These phage-encoded CRISPR–Cas components may also eliminate competing phages and manipulate the hosts[102]. Indeed, a complete system expressed by a *Vibrio* phage can protect against a host defence island[96]. Phages can also transduce CRISPR–Cas systems between bacteria, which can provide immunity against other phages[62,75]. These examples highlight how some phages have manipulated CRISPR–Cas systems as a way to avoid defence systems in the host and endow them with an advantage over competing phages.

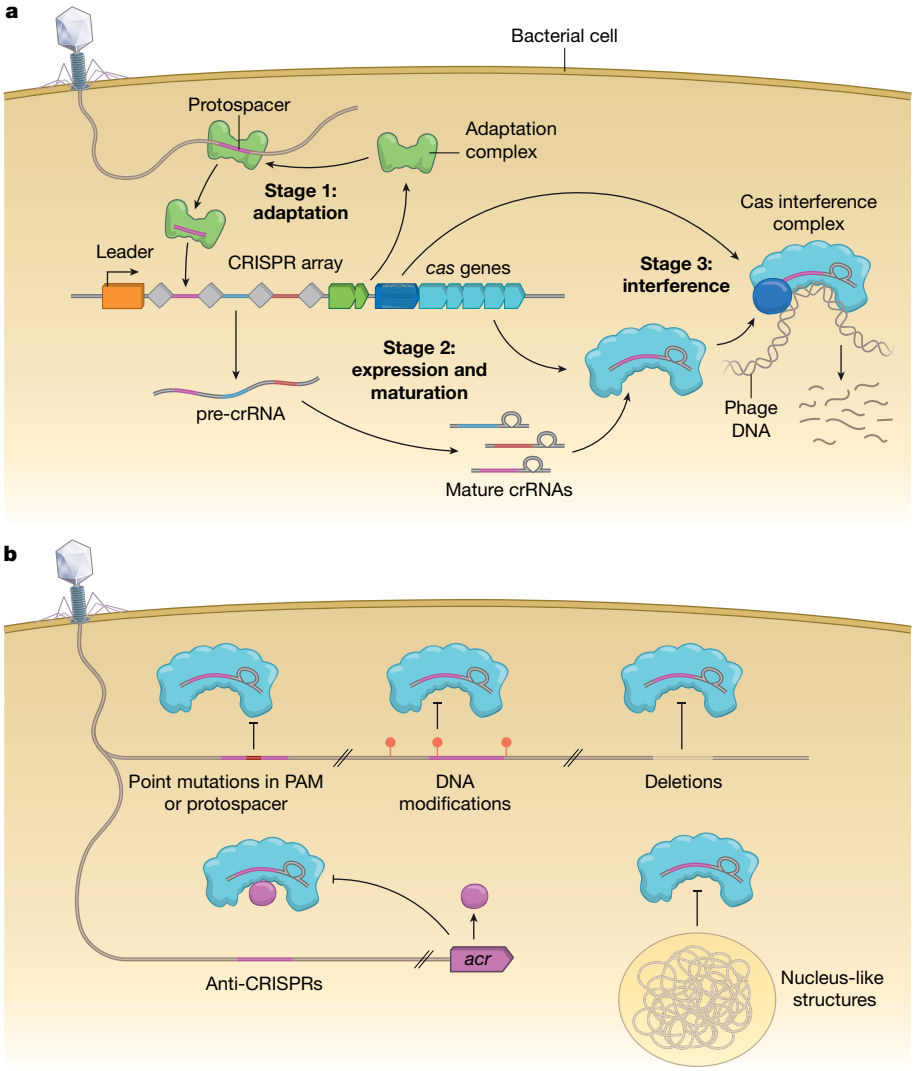

Fig. 4 | CRISPR–Cas adaptive immunity and how phages overcome the CRISPR–Cas adaptive immune system. a, Schematic of the three stages of CRISPR–Cas immunity, including adaptation (stage 1), expression and maturation (stage 2), and interference (stage 3). crRNA, CRISPR RNA. **b**, Phages have the ability to overcome CRISPR–Cas defences through point mutations in the protospacer-adjacent motif (PAM) or protospacer, deletions or modifications of the DNA so that the DNA cannot be bound by Cas complexes. Phages can also encode protein anti-CRISPRs that can interfere with CRISPR immunity, and jumbo phages produce a nucleus-like structure that excludes Cas complexes, thus preventing DNA targeting.

Protecting the bacterial population

In contrast to RM and adsorption inhibition, which confer individual benefits, abortive infection (Abi) anti-phage systems protect the bacterial population[5]. Abi is characterized by successful phage entry; however, development is interrupted, resulting in the release of few, if any, phages and the host cell dies, which prevents a phage epidemic and protects the bacterial population[103]. 'Altruistic' Abi systems are widespread in Gram-positive and Gram-negative bacteria[103]; however, as Abi systems are defined by phenotype, rather than genotype, their discovery has been sporadic[103]. Nevertheless, the presence of many Abi systems on plasmids has been used successfully to identify these systems, particularly in lactococci[103]. The mechanistic details of phage abortion are unknown for many systems, although disruption of essential processes, such as replication, transcription, translation and DNA packaging is common[4,104].

An Abi mechanism in *S. epidermidis* was recently shown to involve a serine/threonine kinase (Stk)[105]. Activated Stk phosphorylates proteins involved in translation, transcription, cell cycle control, the stress response, central metabolism, and DNA topology and repair[105]. Death of infected bacteria occurs through this phosphorylation pathway, decreasing phage release and protecting the population[105]. The presence of serine/threonine kinases in eukaryotic viral defences suggests there are shared immune strategies between these kingdoms. Kinases also play wider roles in viral defence in bacteria, with examples in the BREX and Pgl systems[30–32].

The phenotypic definition of Abi systems is also reflected in their mechanistic diversity. For example, *E. coli* lambda lysogens encode RexAB, which is activated by a poorly-characterized T4 phage protein-DNA complex[104,106]. When triggered, RexA activates RexB, which forms a membrane channel that leads to ATP leakage, lost membrane potential and phage exclusion[106]. RexAB-like systems are widespread, with their recent identification in actinobacteriophages. For example, *rexAB*-like genes in *Mycobacterium smegmatis* and *Gordonia terrae* prophages abort multiple phages[98,107]. In each host, phage escape mutants were identified and all contained mutations in the proteins that triggered RexAB activity[98].

A subset of Abi systems function through a toxin–antitoxin mechanism. Toxin–antitoxin systems are composed of a toxin and an antitoxin

Box 1

Ecology, evolution and phage defence systems

Interactions between bacteria and phages can have important consequences for microbial communities and it is essential to study these in more natural contexts[157–159]. Cocultures of bacteria and phages, which enable the assessment of changes in phage resistance and susceptibility, can provide insights into coevolutionary dynamics. For example, in early experiments with *E. coli* and T-even phages, bacteria became resistant through surface modification[160,161], whereas experiments with *Pseudomonas fluorescens* SBW25 and phage Φ2 showed coevolution of these microorganisms over time[162–164]. When monitored in soil, the coevolution of *P. fluorescens* and Φ2 still occurred, but in a different manner[165]. The differences that were observed were due to the reduced nutrients, which increased the growth-rate costs of phage resistance. This example highlights the importance of considering the ecological context and communities when studying phage resistance.

Evolutionary studies also provide insights into conditions in which bacteria might favour different resistance mechanisms. For example, *P. aeruginosa* used CRISPR–Cas when grown under nutrient-limiting conditions, whereas excess nutrients typically led to surface mutations[61]. Both resistance outcomes were costly to bacteria, yet each mechanism was favoured under different ecological conditions[61].

Although laboratory experiments that mimic natural ecological and evolutionary scenarios benefit from being easy to manipulate, they cannot replicate environmental complexities. Metagenomics provides one way to complement the laboratory approach and has enabled the high-resolution examination of bacterial and phage communities from complex environments[159]. This technique has been useful for following the evolution of CRISPR–Cas resistance and subsequent phage escape in diverse environments, including acid mine drainage, the human gut, hyper saline lakes and a fish farm[166–169]. Phages and bacteria can be monitored over time, providing valuable insights into the role of CRISPR–Cas immunity in shaping microbial communities. Furthermore, metagenomics has been key for identifying new anti-phage systems[127] and CRISPR–Cas variants[170,171].

Finally, mathematical modelling of bacteria–phage interactions provides insights into coevolutionary dynamics, helps to explain experimental observations and predict the influence of other ecological variables that can be difficult to manipulate experimentally[161,172,173]. To gain a more complete understanding of phage–bacteria interactions and phage resistance, we must use a multidisciplinary approach by combining these complementary research areas with molecular studies.

that are usually co-transcribed. The toxins targets essential cellular processes, leading to bacterial dormancy or death. There are six types of toxin–antitoxin systems, based on the identity of the gene products (RNA or protein) and whether, and how, the toxin and antitoxin interact[99]. ToxIN, a type III system from *Pectobacterium atrosepticum* was the first example of an Abi system that was shown to function as a toxin–antitoxin mechanism[100] and this has now been observed for other Abi systems[108,109]. Different toxin–antitoxin types can elicit phage resistance, but have not been strictly classified as Abi systems, as the outcome for the infected bacterium was not defined. Examples in *E. coli* include *hok/sok* (type I) and *rnlA/rnlB* (type II), which exclude phage T4[110,111], and *mazEF* (type II), which excludes phage P1[112]. Many of these toxins are RNases, a characteristic shared by several Abi systems. For example, *E. coli* PrrC is an RNase that cleaves lysine transfer RNA ($tRNA^{Lys}$) during infection, and only T4 phages that are able to repair this cleavage can replicate[4]. Thus, mutant T4 phages that lack a polynucleotide kinase or RNA ligase are aborted due to $tRNA^{Lys}$ cleavage[113].

To bypass toxin–antitoxin systems, phages can encode antitoxins. For example, T4 produces Dmd, an antitoxin that inhibits *E. coli* RnlA and LsoA toxins[114]. Dmd differs from the RnlB or LsoB antitoxins, suggesting it evolved independently, which is highlighted by its different toxin neutralization mechanism[114]. Phages can generate diversity for escape by acquiring host genetic material through recombination. Indeed, recombination between lytic phages and resident lactococcal prophages led to Abi escape through gene loss or gain[115]. Recombination can also promote antitoxin acquisition by phages. For example, to escape ToxIN, phages containing a short *toxI*-like sequence recombined with *toxIN* and directly gained *toxI*[116]. Notably, in other escape phages, *toxI*-like sequence duplications produced pseudo-ToxI RNAs that inhibited ToxN[116]. Rather than encoding its own antitoxin, coliphage T7 evades a toxin–antitoxin system by producing a protein that has been proposed to prevent antitoxin degradation by the Lon protease. This ensures that the toxin remains inactive by increasing the stability of the host antitoxin[117]. Finally, the T4 protein Alt (an ADP-ribosyltransferase) is injected with phage DNA, which chemically modifies the MazF toxin[118]. ADP-ribosylated MazF has reduced cleavage activity, enabling the survival of the phage[118].

Many new Abi systems await discovery and, indeed, new systems in different strains are still being uncovered. For example, Abiα was recently identified in *Enterococcus faecalis* and leads to asynchronous lysis[119]. To understand Abi responses, the phage genes involved can be revealed by isolating escape mutants. For example, ToxIN can be overcome by specific mutations in ϕM1 and T4-like phage proteins[120,121]. However, the often toxic and poorly characterized nature of the phage Abi-triggering proteins is a frequent challenge for mechanistic studies.

Prophage-encoded defence systems

Prophages can have immune systems that prevent subsequent phage infection of lysogens (for example, *rexAB*). These non-essential transcribed regions or genes within prophage genomes have been called 'morons' and can encode factors that benefit the host, such as defence systems[122]. For example, morons (or 'immunity cassettes') within *M. smegmatis* prophages provide phage defence by encoding RM and toxin–antitoxin components, and other defence systems[123]. These systems can be remarkably specific; for example, prophage Charlie encodes a defence system that offered protection against only one phage of many tested. A different *M. smegmatis* prophage encoded a (p)ppGpp synthetase similar to RelA/SpoT that is proposed to be inactivated by a prophage 'regulator' protein. Lytic phage replication leads to rapid dissociation of the synthetase from the regulator, (p)ppGpp accumulation, growth cessation and stalled phage development[123]. Another phage, Fruitloop, encodes an immunity protein that interacts with Wag31, a cell-wall synthesis protein in *M. smegmatis*. Fruitloop inhibits superinfection by other phages that are thought to require Wag31 for DNA injection[124]. Prophage-mediated phage defences are widespread. Indeed, a systematic study revealed that *Pseudomonas* lysogens have diverse prophage-encoded defences[21]. Furthermore, filamentous phages of the *Inoviridae* family that cause chronic infections were recently shown to have multiple toxin–antitoxin systems and superinfection systems[125]. As these systems are encoded by the phage, phage escape represents phage–phage coevolution. Accordingly, many genes of unknown function in prophages, especially within morons, may protect from superinfection and this knowledge may accelerate the identification of candidate resistance systems.

A new world of diverse resistance systems

Defence systems are often clustered in defence islands in bacterial genomes and unknown genes within these regions have been proposed to encode anti-phage systems[54,126]. This was supported by the discovery of BREX, DISARM and the Stk2 kinase[31,32,34,105], and was the premise for a search that uncovered 26 broadly distributed candidate defence systems[127]. Nine systems have been validated as anti-phage systems, some of which protect against specific phages, whereas others provided broader defence. Although the mechanisms are undetermined, multiple protein domains have been identified that are typical for phage-defence systems (for example, helicases and nucleases), in addition to proteins that have been proposed to be repurposed for phage defence. For example, components of the Zorya system, which is proposed to be an Abi system, show homology to the MotAB proteins that form the stator of the flagella complex, and are hypothesized to form a membrane channel that results in depolarization and cell death upon phage infection[127].

Prokaryotic Argonaute (Ago) proteins are also found in defence islands nearby other newly discovered and validated systems (for example, Thoeris), suggesting that they may also elicit phage defence[127,128]. Moreover, the eukaryote Ago proteins are key proteins in RNA-interference systems, and prokaryotic Ago proteins function as nucleic-acid-guided nucleases[129]. Generally, prokaryotic Ago proteins generate and associate with short-interfering DNA or RNA guides. The single-stranded guides facilitate the identification of the complementary sequence by prokaryotic Ago, which cleaves the target strand or produces double-stranded breaks[130–133]. Following the discovery of prokaryotic Ago proteins, further parallels are being drawn with the eukaryotic immune systems—for example, with the eukaryotic cGAS–STING pathway that senses viral DNA and activates an innate immune response. Recently, prokaryotic cGAS homologues, which cluster near defence islands, have been identified[134]. These cGAS-encoding genes reside in operons that include a phospholipase and two other genes that contain eukaryotic-like domains that are required for defence against some phages, but are dispensable for the defence against others. This pathway was named CBASS (cyclic-oligonucleotide-based anti-phage signalling system) and is triggered by an unidentified signal that causes cGAS to produce cyclic GMP–AMP (cGAMP). cGAMP activates the phospholipase, which aborts a range of dsDNA phages by eliciting membrane damage and cell death[134]. A second example is the eukaryotic-like HORMA proteins that are present in various bacteria, including *E. coli*[135]. These proteins sense unknown phage product(s) and, once activated, the HORMA domain activates a cGAS/DncV-like nucleotidyltransferase that produces the second messenger cyclic tri-AMP. Cyclic tri-AMP causes dsDNA cleavage by activating an endonuclease, which in *E. coli* confers λ immunity[135]. It is currently unknown whether this results in abortive infection or targeted destruction of the phage[135,136]. The discovery of eukaryotic-like defences in prokaryotes suggest that systematic searches for homologues in bacteria may uncover many new anti-phage systems.

Recently, a new type of phage defence was discovered that relies on small molecules rather than proteins[137]. This chemical defence is widespread in *Streptomyces*, a genus known for the prolific production of bioactive secondary metabolites. The metabolites block genome propagation by intercalating dsDNA. Because many secondary metabolites can diffuse and thus function outside of the cell, this has been proposed as an innate defence that protects bacteria before phage infection[137]. However, various aspects of the chemical defence strategy remain unclear, such as how the phage DNA is recognized as non-self.

With such a diversity of defence systems, the arms race has escalated. Indeed, jumbo phages produce nucleus-like structures inside the infected bacterium, in which phage DNA replication and transcription occur[138,139]. In *P. aeruginosa*, this nucleus-like structure protects ϕKZ from type I-C, II-A and V-A CRISPR–Cas and a type I RM system[139]. Moreover, in *Serratia*, a distinct nucleus-forming jumbo phage evades the native DNA-targeting type I-F and I-E CRISPR–Cas systems[140]. However, phage mRNA translated in the cytoplasm is susceptible to RNA-targeting by Cas13[139] or type III-A defence[140] in *P. aeruginosa* and *Serratia*, respectively. Therefore, this physical occlusion of the phage genome appears to be a widespread method to overcome anti-phage systems and this is supported by a paucity of type I spacers that target jumbo phages in nature, whereas type III-A spacers are over-represented[140].

Finally, extracellular chemicals not only engage in direct resistance against phages (for example, chemical defence[137]), but also facilitate communication to pre-empt bacteria to increase their immunity. Indeed, quorum sensing—cell-density-dependent signalling—upregulates bacterially encoded CRISPR–Cas and downregulates surface receptors when populations would otherwise be at increased risk of a phage epidemic[141–143]. Perhaps unsurprisingly, phages also use communication to ensure productive infection[144,145]. These peptide communication systems (which are also known as arbitrium) are diverse and widespread, and inform phages about host availability. Arbitrium has been proposed to limit phage-induced host decimation by determining whether phages enter the lytic or lysogenic life cycle[144–148]. Phages also encode LuxR-type proteins, which respond to Gram-negative quorum-sensing signals[149,150] and quorum-sensing genes are also present in Gram-positive phages[151]. Although the function of phage quorum-sensing genes remains to be elucidated, they might allow phages to sense host density[149]. These examples of communication between phages and bacteria raise the question whether bacteria and phages engage in 'espionage', where either party listens in to, or interferes with, the communications of the other to manipulate the outcome for their own benefit. However, the roles and implications of phage–phage and phage–bacteria communications remain to be understood.

Perspectives

There is a clear diversity of phage-resistance mechanisms and ways that phages evade these systems. This knowledge is informing microbiology, the potential of phage-inspired therapeutics and new biotechnological tools. Despite considerable advances, we are far from understanding bacterial defences and phage counter-adaptation across scales—from molecules, single cells, communities, ecosystems and through to the global scale (Box 1). Furthermore, the recent discovery of completely new systems demonstrates that our view of the defence arsenal is incomplete, and that their identification requires more systematic approaches. Increased sequencing data will expand the success of bioinformatics strategies, but these need to be complemented by high-throughput experimental techniques. For example, phage-based positive selection of new anti-phage systems from metagenomic libraries could be exploited in a similar manner to those reported for anti-CRISPR discovery[152]. To advance the field, both sides of this arms race, the bacteria and the phages, must be considered.

In terms of bacterial defences, critical gaps exist in our understanding of molecular mechanisms—for both old and new systems—and new techniques should be applied to uncover their mode of action. Determining the molecular mechanisms of diverse defences will undoubtedly lead to both fundamental biological knowledge and new technologies—as exemplified by the exploitation of CRISPR–Cas and RM systems. Furthermore, most defence systems have been studied without considering other co-existing immune mechanisms. Indeed, bacteria often have multiple CRISPR–Cas systems, in addition to other innate defences. How these function together—whether redundantly or synergistically—is not well understood, but they may help bacteria to resist diverse phages and overcome escape phages[153]. In fact, RM and CRISPR–Cas act together to increase phage resistance, and crosstalk between CRISPR–Cas systems can provide protection against escape phages[154,155]. In addition, each defence system is likely to have different

costs and benefits that depend on the niche inhabited and these factors may be key drivers in the evolutionary selection of defences.

Our understanding of phages is improving, in part due to the increased availability of sequencing data, but given their global abundance, we only have a tiny snapshot of this ever-changing community. Poor functional gene annotations highlight the gaps in fundamental phage biology and hinder our ability to understand their interactions with bacterial immune systems. We can focus on genes that probably influence bacterial immunity. For example, prophage-encoded defences and anti-defences are commonly found in particular genomic locations and their discovery has been facilitated by comparative genomics of phage families. Moreover, early expressed genes often have important roles in anti-defence or bacterial takeover[156]; however, studying these genes has been hampered by the paucity of genetic tools for phages. Reassuringly, phages are becoming genetically tractable due to CRISPR–Cas methods. To realize the ecological importance, and the therapeutic and biotechnological implications of bacterial immune systems, mechanistic studies must be complemented with evolutionary and ecological experiments to illuminate how molecular events scale to global microbial processes.

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