

## Review article



# The highly diverse antiphage defence systems of bacteria

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## Abstract

Bacteria and their viruses have coevolved for billions of years. This ancient and still ongoing arms race has led bacteria to develop a vast antiphage arsenal. The development of high-throughput screening methods expanded our knowledge of defence systems from a handful to more than a hundred systems, unveiling many different molecular mechanisms. These findings reveal that bacterial immunity is much more complex than previously thought. In this Review, we explore recently discovered bacterial antiphage defence systems, with a particular focus on their molecular diversity, and discuss the ecological and evolutionary drivers and implications of the existing diversity of antiphage defence mechanisms.

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## Introduction

Phages are viruses that infect bacteria and hijack the cell machinery to replicate. Phages exhibit two main types of lifestyle. The lytic cycle involves the expression of phage nucleic acids, leading to the production of both the phage genome and proteins that will be assembled into new virions<sup>1</sup>. At the end of a **lytic cycle**, the host cell is lysed, and the progeny phages are released. By contrast, the **lysogenic cycle** involves the integration of the phage genome into the host genome as a prophage transmitted across bacterial generations. Phages are extremely abundant and are thought to outnumber bacteria by a ratio of 10:1 in the ocean<sup>2</sup>.

Estimates suggest that phage infection could account for 20–40% of bacterial daily mortality. As such, phage infection represents a major evolutionary driver for bacteria<sup>2</sup>. In response to this strong evolutionary pressure, bacteria have developed an arsenal of antiphage systems, which can be defined as single genes or groups of genes that provide to their host partial or full resistance against phages. The antiphage arsenal of bacteria has long been thought to be limited to **restriction-modification (RM)**, **CRISPR–Cas** and a few other systems with unknown mechanisms. However, the development of high-throughput bioinformatic and experimental approaches (Box 1) has extended our knowledge of the antiphage arsenal of bacteria to more than a hundred (Supplementary Table 1), revealing an unsuspected wealth of diverse systems and mechanisms. Although existing knowledge of antiphage mechanisms was before almost exclusively limited to two systems that detect and degrade invading nucleic acids, recent findings revealed unsuspectedly complex bacterial immune strategies, which include systems that produce **small antiphage molecules**<sup>3,4</sup>, systems that rely on intracellular signal transduction through production of signalling molecules<sup>5–7</sup> and systems that recognize conserved structural patterns of viral proteins to trigger immune responses<sup>8</sup>. These new discoveries have made a compelling case to revisit existing paradigms on antiviral immunity in bacteria, moving away from a simplistic perspective to a much more detailed one.

Early discovered defence systems were previously reviewed elsewhere<sup>9–11</sup>. In this Review, we first describe the diversity of antiphage mechanisms, focusing on the most recently discovered ones, and discuss the evolutionary drivers and the ecological consequences of this diversity.

## Genomic diversity of antiphage defence systems

The genomic features of antiphage systems reflect their diversity. First, the repertoire of genes associated with phage defence includes diverse functions and domains, comprising **nucleases**, **helicases**, **proteases**, **kinases**, **ATPases**, **reverse transcriptases** and so on. The variety of this repertoire seems relatively limited and comprises mostly nucleic acid **interacting domains** and a few other domains (for example, SIR2, TIR and transmembrane (TM) domains) (Supplementary Table 1). However, antiphage genes can be combined in unique ways to compose diverse defence systems. Certain defence systems comprise **single genes** (for example, AbiH, Lit, NixI and BstA systems), whereas other systems can encode **five genes or more** (for example, CRISPR–Cas, BREX, DISARM, Dnd and Ssp systems).

The abundances of different defence systems in bacteria vary greatly from one system to another. Analysis of the RefSeq database with DefenseFinder, a tool developed to systematically detect defence systems in prokaryotic genomes, reveals that RM and CRISPR–Cas systems are encoded in, respectively, 84% and 40% of bacterial genomes,

whereas more than half of the detected systems are found in <3% of bacterial genomes<sup>12</sup> (Fig. 1b, Supplementary information and Supplementary Tables 2 and 3). The distribution of defence systems appears to be **long-tailed**<sup>12,13</sup> and contains a wealth of rare systems that are the main source of diversity in antiviral arsenals.

Multiple defence systems can also be combined in unique ways into an antiviral arsenal that is specific to a given strain. Out of 21,364 fully sequenced bacterial genomes, 78% of them encode more than two defence systems<sup>12</sup> (Fig. 1a), with **important variations between strains**. For instance, certain intracellular parasitic bacteria, including members of the Chlamydiae phylum (Fig. 1b), harbour few or no defence systems, whereas other species encode more than 50 (refs. 12,13).

It is also notable that many of the defence systems encoded by bacteria can also be detected in archaea. Among the systems detected by DefenseFinder, more than a third can be detected in **archaea**, showing strong conservation of antiviral immunity between prokaryotic kingdoms.

## Mechanistic diversity of antiphage defence systems

To target phages without harming the host, most defence systems combine two elements: a **sensor** that detects the infection and an **effector** that either targets the phage or kills the infected host before the phage can complete its reproduction cycle, effectively protecting the rest of the population from newly released virions<sup>14</sup>. This specific mechanism is called ‘Abortive infection’ (Abi). The various mechanisms used by bacteria to sense and resolve phage infection, as well as the articulation between the two, are described in the following part (Fig. 2).

### Detection of phage infection: sensing modules and mechanisms of bacterial immunity

Following the specific binding of a phage to its host bacteria during **adsorption**, the phage genome is injected into the host. Viral genes are then expressed by the host cell machinery, and newly synthesized nucleic acids are packaged with the newly translated phage proteins to produce mature virions. All these steps can be sensed by the defence systems of the bacterial cell as cues of viral infection.

**Detection of invading nucleic acids.** Detection of invading nucleic acid is a widespread strategy to sense phage infection (Fig. 2). As nucleic acid injection and synthesis are among the first steps of phage infection, this sensing mechanism allows for an early immune response. These sensors are linked to two types of mechanisms. Certain systems introduce **DNA modifications** on specific motifs in the host genome and recognize invading nucleic acids that lack those modifications. As in the case of RM systems, the effector domain is triggered by the recognition of non-modified motifs in the invading DNA (except for type IV RM systems that target modified invading DNA, the host DNA being without alterations)<sup>15</sup>. Classically, the modification of the host DNA involves **methylation**<sup>15</sup>. Some newly discovered defence systems, generally described as **RM-like**, also rely on methylation of the host DNA to recognize invading nucleic acids. This is the case of **BREX** (bacteriophage exclusion)<sup>16,17</sup> and **DISARM** (defence islands system associated with RM)<sup>18</sup>, two systems for which the precise defence mechanisms remain to be elucidated. Other systems use different types of host DNA modifications. For example, **DndACDE**<sup>19,20</sup> and **SspABCD**<sup>21,22</sup> protein complexes perform, respectively, **double-stranded and single-stranded phosphorothioate modification of the host DNA**, by replacing a non-bridging oxygen with a sulfur in the DNA sugar-phosphate backbone.

Similarly, the **anti-plasmid Dpd system** inserts 7-deazaguanine derivatives in the host DNA to distinguish it from the phage invading and non-modified DNA<sup>23</sup>.

Defence systems can also use a **nucleic acid guide** to specifically recognize complementary sequences. An example of this are **CRISPR–Cas systems**, which rely on the Cas machinery to process

## Box 1

### High-throughput methods for prediction of antiphage systems

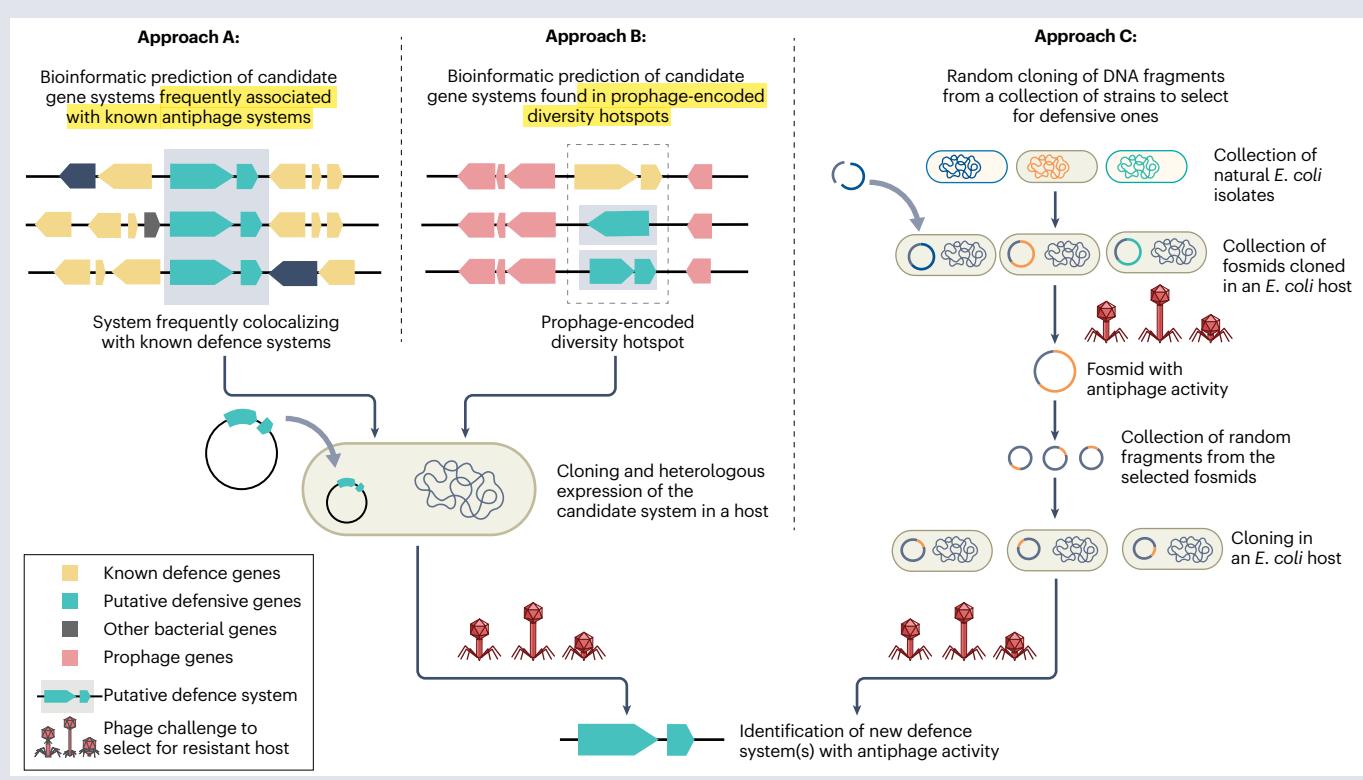
The development of novel methods to uncover antiphage systems in bacteria has drastically increased the rate of defence systems discovery. The initial approach that revealed dozens of novel systems (see the figure, approach A) relies on the fact that defence systems often colocalize with one another on the bacterial genome<sup>54,124</sup>. This observation is used to bioinformatically predict genes involved in antiphage defence on the basis of their **tendency to be encoded close to known defensive genes**. The genomic context of homologues of the genes predicted to be defensive is then used to determine whether they are **single-gene systems** or whether they **work in association with others**. The newly predicted system is then validated experimentally. This approach was fine-tuned to lead to the prediction of thousands of genes potentially involved in antiphage defence<sup>13,37</sup>.

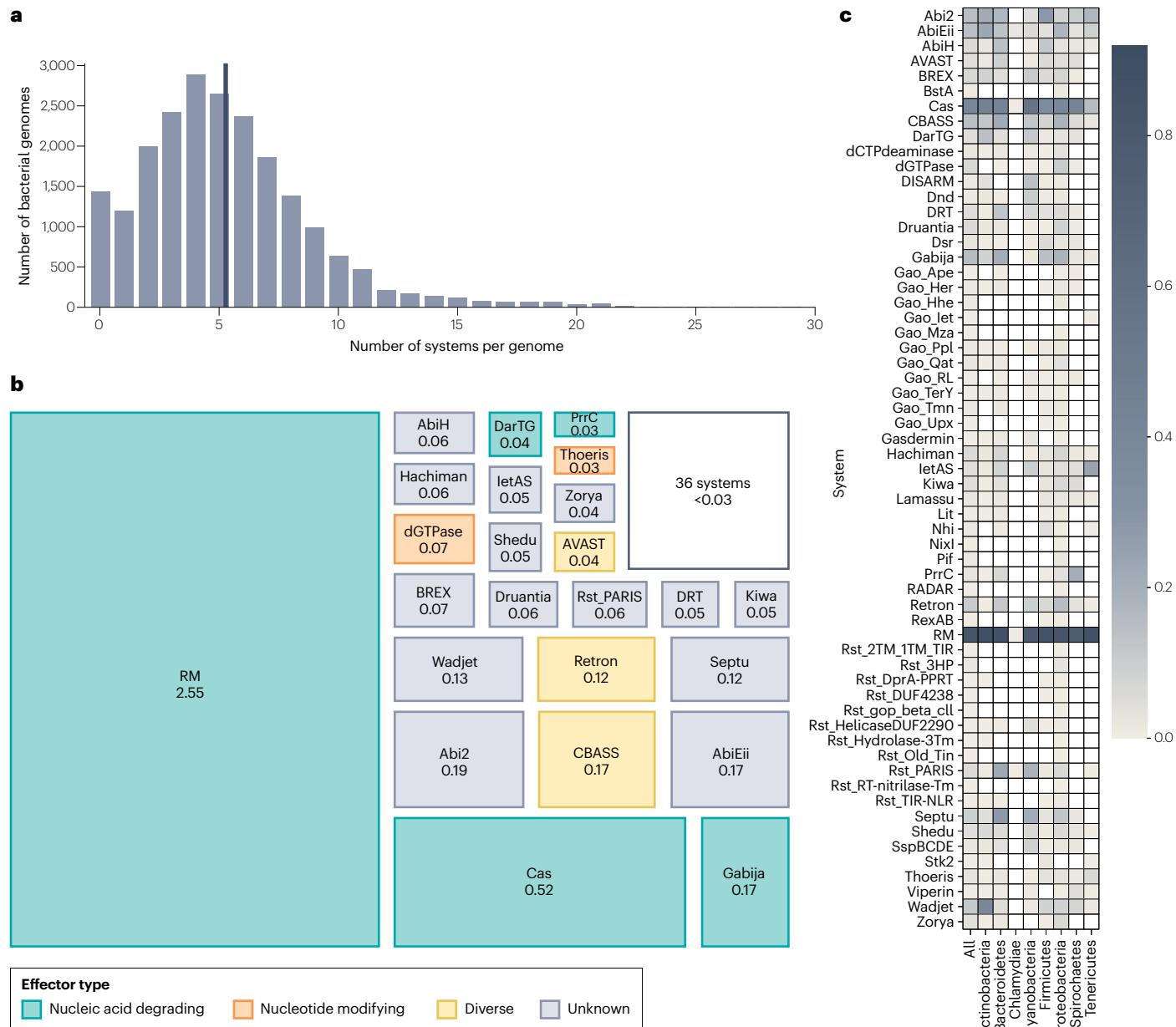
Although this guilt-by-association logic allowed the discovery of dozens of new systems<sup>16,18,25,35,37,54,64</sup>, only systems that are frequently encoded in defence islands can be uncovered, potentially overlooking many systems. For example, an important diversity of defence systems was unravelled in genomic hotspots of mobile genetic elements<sup>32,53,75,85,125</sup>. Screening combinations of genes of unknown function found in these **hotspots for antiphage activity** constitutes another method to uncover new systems (see the figure, approach B).

Whereas both approaches A and B are based on **bioinformatic prediction**, a third approach consists in an **experimental screening**<sup>76</sup>. Through random **cloning of genome fragments** from **natural Escherichia coli isolates**, expression in an *E. coli* K12 strain and **screening for antiphage properties**, 21 new systems were identified (see the figure, approach C). Upon studying the genomic context of these systems, the authors reported that only 3 out of 21 systems are part of defence islands, suggesting that this method could help to uncover a new repertoire of defence systems.

The **use of heterologous expression** is one of the **limitations** of these approaches. Mainly, two model organisms have been used up to now: *E. coli* and *Bacillus subtilis*. Phylogenetically distant clades may encode different types of systems, which might not be active in these two model organisms.

The number of new systems discovered in recent years is striking. Bioinformatic predictions provide thousands of candidate systems to test, but current experimental validation methods could be improved to keep up with the constant flow of new bioinformatic predictions. Efforts to increase the efficiency and yield of these methods would undoubtedly help to discover more systems.





**Fig. 1 | Diversity of bacterial antiphage defences.** Re-analysis of the results of Defense Finder v1.0.2 on the 21,364 fully sequenced bacterial genomes of RefSeq<sup>12</sup> (Supplementary information and Supplementary Tables 2 and 3). **a**, Distribution of the total number of antiphage systems per genome. The vertical dark grey bar indicates the mean of defence systems per bacteria (mean = 5.3). The x-axis was cut at 30 for data visualization purposes. Maximum number of systems per genome is 57 (*Desulfonema limicola*). **b**, Frequency of

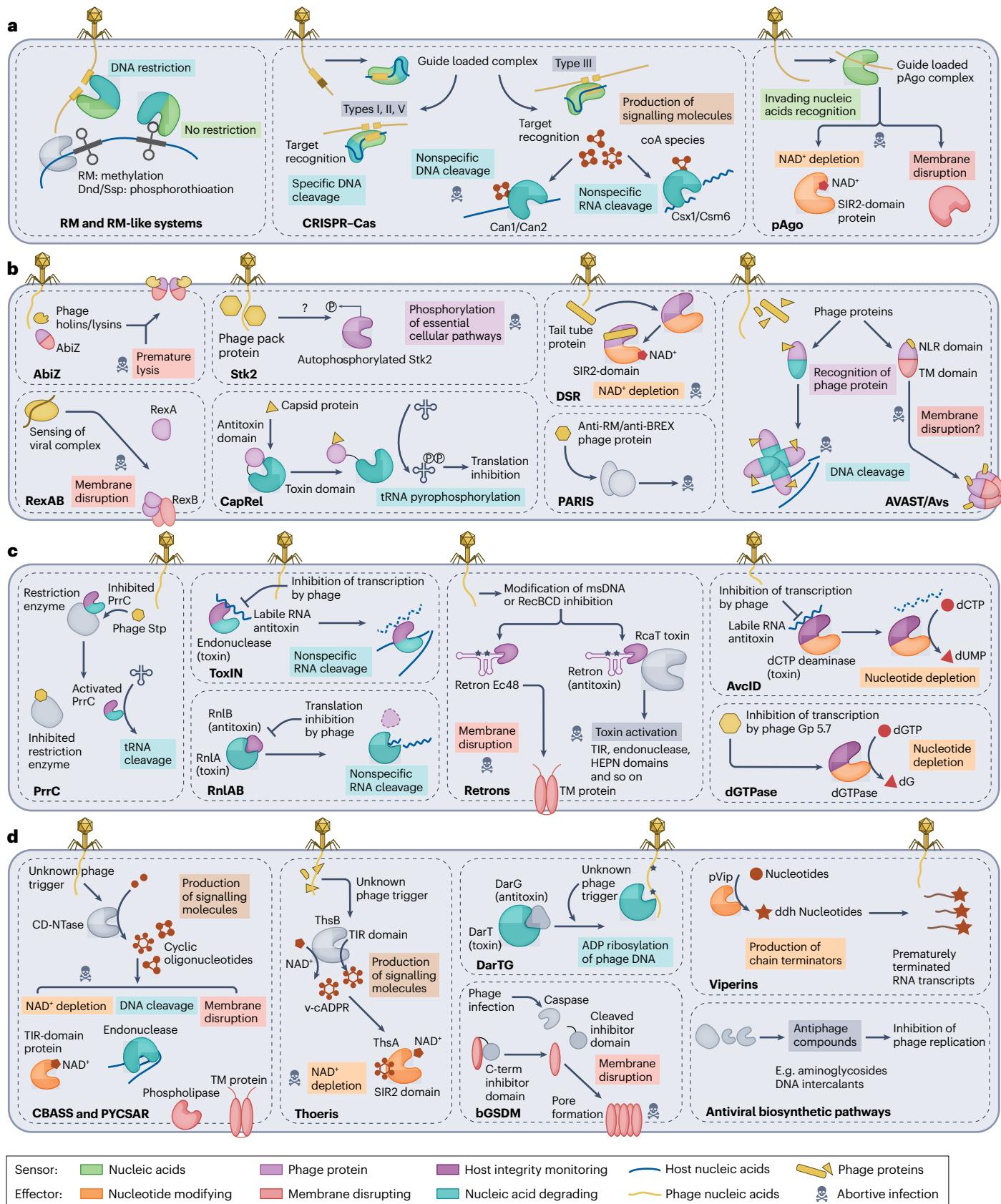
each of the known defence systems per prokaryotic phylum. The colours of the heatmap represent the frequency of a system (rows) in the genomes of a phylum (columns). **c**, Relative abundance of the different systems in bacterial genomes. In the treemap, each rectangle corresponds to a given system. The area of the rectangle corresponds to the mean number of copies of the system encoded in one bacterial genome (number also indicated in each square). The fill colour indicates the type of effector encoded by the system.

small fragments of the invading viral nucleic acids that are integrated in the CRISPR array<sup>24</sup>. Thus, the CRISPR array provides its host cell with a **memory of past infections**, making CRISPR–Cas the first reported bacterial adaptive immune system. The CRISPR array is transcribed and processed, providing mature guide RNAs that allow the Cas machinery to specifically recognize their nucleic acid target<sup>24</sup>. CRISPR–Cas

systems are generally classified into two classes and six types on the basis of the set of Cas genes they encode and the nature of their effector (single protein or protein complex)<sup>24</sup>.

**Prokaryotic Argonautes (pAgO)** seem to rely also on invading nucleic acid recognition to achieve phage defence. Argonaute proteins are found in all three domains of life. In eukaryotes, they are key players

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Sensor:	Nucleic acids	Phage protein	Host integrity monitoring	Host nucleic acids	Phage proteins
Effector:	Nucleotide modifying	Membrane disrupting	Nucleic acid degrading	Phage nucleic acids	Abortive infection

## Fig. 2 | Antiphage molecular mechanisms. a, Nucleic acid sensing systems.

Restriction-modification (RM) and RM-like systems modify specific motifs of the host DNA (via methylation for RM<sup>15</sup> and DnD<sup>19</sup>; phosphorothioate modifications for Ssp<sup>21,22</sup>). Non-modified invading DNA will be restricted by nucleic acid degrading effectors. During infectious events, fragments of foreign nucleic acids can be processed by the CRISPR–Cas machinery and integrated into the CRISPR array as part of the host genome. Expression and processing of the CRISPR array will produce mature guide RNAs, which can be used by the CRISPR machinery to recognize invading nucleic acids. Recognition of foreign nucleic acids triggers the immune response<sup>24</sup>. The activation of immune effectors is either direct (CRISPR–Cas system types I, II and V) or mediated by cyclic oligoadenylate (cOA) signalling molecules produced upon target recognition. Prokaryotic Argonautes (pAgo) proteins use nucleic acid fragments as guides to recognize and bind complementary nucleic acids. Upon target recognition, various toxic effector domains or proteins are activated and trigger cell death<sup>26,27</sup>. b, Phage protein sensing systems. The AbiZ protein can team with phage-encoded holins and lysins to cause premature lysis of the cell before the phage infection cycle is completed<sup>34</sup>. In the RexAB system, sensing of phage infection by RexA triggers the toxic effector of the system, the ion channel RexB, leading to cell death<sup>33</sup>. Phage-encoded PackK protein activates the serine–threonine kinase Stk2, which triggers cell death through a phosphorylation cascade<sup>30</sup>. In the fused toxin–antitoxin (TA) system CapRel, binding of phage capsid protein to the antitoxin domain releases the toxin domain from its inhibition. The toxin domain then phosphorylates transfer RNA (tRNA), inhibiting translation<sup>31</sup>. Binding of a phage tail tube protein triggers the nicotinamide adenine dinucleotide (NAD<sup>+</sup>) degrading activity of DSR2 proteins, leading to ‘Abortive infection’ (Abi) caused by NAD<sup>+</sup> depletion<sup>25</sup>. Phage anti-restriction-induced system (PARIS)-mediated Abi is triggered by the phage-encoded Ocr protein, known for inhibiting both RM and BREX defence systems<sup>32</sup>. Avs systems recognize conserved structural features of phage proteins through direct binding by the sensor domain, activating various types of effector domains<sup>8</sup>. c, Systems monitoring the integrity of the host. The PrrC protein is bound to and inhibited by one of the restriction enzymes of its host under normal conditions. Binding of the phage-encoded anti-restriction protein Stp to the restriction enzyme during phage infection releases PrrC, which then cleaves tRNA, interrupting protein synthesis and consequently the phage infection cycle<sup>41,42</sup>. In the type III TA system ToxIN, inhibition of transcription by phages leads to the depletion of the labile RNA antitoxin of the system. Once released from its inhibited state, the toxic effector of the system mediates Abi through

nonspecific RNA cleavage<sup>36</sup>. In the type II TA system RnlAB, degradation of the unstable RnlB antitoxin following phage infection leads to the activation of the toxin RnlA, a nuclease that in turn mediates nonspecific RNA cleavage<sup>44</sup>. Retron Ec48 is triggered by the inhibition of the bacterial RecBCD complex during phage infection<sup>38</sup>. Retron Sen2 is triggered by the modification of its multicopy single-stranded DNA (msDNA) synthesis during phage infection<sup>39</sup>. In both cases, the detection of phage infections triggers toxic effectors that mediate Abi. In the TA system AvlCD, the deoxyctidine triphosphate (dCTP) deaminase toxic effector of the system is inhibited by an RNA antitoxin under normal conditions. During phage infection, the synthesis of the labile RNA antitoxin is stopped and the dCTP deaminase toxin becomes activated, depleting its host nucleotide pool through dCTP to deoxyuridine monophosphate (dUMP) conversion<sup>43</sup>. Inhibition of bacterial transcription mediated by phages during infectious events triggers antiphage deoxyguanosine triphosphatase, depleting the host nucleotide pool through (deoxyguanosine triphosphate) dGTP to (deoxyguanosine) dG conversion<sup>40</sup>. d, Other mechanisms. During phage infection, oligonucleotide cyclases of the pyrimidine cyclase system for antiphage resistance (PYCSAR) and cyclic oligonucleotide-based antiphage signalling system (CBASS) produce signalling molecules, which in turn activate a diversity of immune effectors to mediate Abi responses<sup>748</sup>. When triggered by phage infection through an unknown mechanism, the TIR domain containing the ThsB sensor of the Thoeris defence systems produces signalling molecules, which activate the NAD<sup>+</sup> degrading activity of ThsA, leading to the depletion of the host NAD<sup>+</sup> pool and Abi<sup>0</sup>. Upon phage infection, the DarT toxin of the DarTG TA system becomes activated and performs adenosine diphosphate (ADP) ribosylation of phage DNA, inhibiting viral replication<sup>35</sup>. In bacterial gasdermin (bGSDM) systems, sensing of phage infection leads to the cleavage of the C-terminal inhibitor domain of bGSDM proteins by associated caspase-like proteases. Cleavage of their C-terminal domains relieves bGSDM proteins of their auto-inhibited state, leading to the formation of large pores in the bacterial membranes and subsequent cell death<sup>65</sup>. Prokaryotic Viperins catalyse the conversion of nucleotides triphosphate into their 3'-deoxy-3',4'-didehydro analogues. During phage infection, the modified nucleotides inhibit viral transcription, presumably by acting as chain terminators once integrated into the nascent viral RNA chains<sup>64</sup>. Certain secondary metabolites produced by members of the *Streptomyces* genus were shown to have antiphage activity. Notably, both some DNA intercalants (for example, doxorubicin and daunorubicin)<sup>4</sup> and members of the aminoglycoside antibiotic class<sup>3</sup> were reported to have antiphage activity.

in RNA interference, whereas in prokaryotes, shorter pAgos provide defence against phages in association with diverse known antiphage effectors<sup>25–27</sup>. In vitro, the pAgo subunit of pAgo-containing systems (either alone or in a complex with ancillary proteins) uses nucleic acid guides to recognize and bind nucleic acid targets<sup>26,27</sup>, suggesting that pAgos are the sensors of these systems.

Finally, other systems might also detect invading nucleic acids through a mechanism independent of sequence recognition. The anti-plasmid Wadjet system was recently reported to recognize invading DNA through topology sensing and to cleave closed-circular DNA<sup>28</sup>. Similarly, preliminary evidence suggests that certain CBASS (cyclic oligonucleotide-based antiphage signalling system) could be activated through binding of a folded fragment of RNA, the secondary and/or tertiary structure of the RNA being essential for CBASS activation<sup>29</sup>. Sensing of characteristic structural features of newly injected phage DNA might also be used for antiphage defence, although supporting evidence for such mechanism is currently lacking.

**Sensing of phage protein.** An alternative mechanism to the sensing of phage nucleic acids is that of sensing the phage proteins. If infection is not detected and resolved during the first stages of the phage

reproduction cycle, phage protein synthesis will start. These viral proteins can be detected by sensor modules, a common strategy in defence systems. Interestingly, all the systems reported to detect phage proteins (for example, Avs<sup>8</sup>, Stk2 (ref. 30), CapRel<sup>31</sup>, DSR<sup>25</sup>, phage anti-restriction-induced system<sup>32</sup>, RexAB<sup>33</sup> and AbiZ<sup>34</sup>) function through an Abi mechanism, meaning that recognition of the trigger phage protein ultimately activates a toxic effector (Fig. 2). In the case of detection of structural or functional proteins involved in progeny virion assembly, which tend to be synthesized late in the phage reproduction cycle, this could be explained by the difficulty of resolving phage infection at advanced stages of infection. Indeed, late stages of infection offer a reduced time window for resolving phage infection and are characterized by the presence of many copies of phage proteins and nucleic acids in the cell. Many phages cause irreparable damage to the host and its genome during infection, and several studies report that the host does not necessarily die as a result of the activity of the abortive systems, but rather because of the damage caused by the phage before the activation of the system<sup>35,36</sup>. It thus seems that fitness-costly Abi defence strategies could be kept as a last resort to protect the rest of the population and intervene when the infection is too advanced to be contained, or when the phage is resistant to the other defence systems of the host.

In general, defence systems can sense two types of proteins. On the one hand, **proteins with functions that are essential for phage replication**. This is the case of Avs (formerly AVAST) systems, which are found in up to 4% of fully sequenced bacterial genomes<sup>8,32,37</sup>. Two **Avs enzymes**, one from *Salmonella enterica* and one from *Escherichia coli*, were documented to recognize and bind viral terminases and portal proteins, respectively, which are both necessary for viral replication<sup>8</sup>. On the other hand, defence systems can **target structural proteins**. For instance, the defence-associated sirtuins (DSR) system in *Bacillus subtilis* recognizes phage SPR infection by binding to its **tail tube protein**<sup>25</sup>, whereas the CapRel system found in *E. coli* is activated by the binding of the **major capsid proteins** of diverse phages<sup>31</sup>.

Viral proteins with important biological roles are submitted to strong selective constraints, and therefore recognizing them to trigger phage defence presents major advantages. First, it **limits the possibility of escape through mutations of the phage protein**. For instance, the Stk2 system found in *Staphylococcus epidermidis* recognizes the **Pack protein** of phage phiNM1. Stk2 escapees have a reduced fitness and encode **mutated versions of Pack**. Sequencing of the Stk2 escapees shows incomplete DNA packaging in the virions, suggesting that Pack is essential to complete DNA replication or packaging and that its mutation is deleterious to the escapee phages<sup>30</sup>. Second, it allows for a potentially **wide defence range**, as such viral proteins are generally **conserved throughout evolution**. As an example, Avs sensing of viral proteins does not rely on sequence but rather on conserved structural features, allowing the enzymes to recognize triggers with little sequence homology in viruses as distant as human herpesvirus<sup>8</sup>.

Proteins that are not essential to viral replication but that have a role in **inhibiting defence systems** can also trigger antiphage systems. For example, the phage anti-restriction-induced system is triggered by the Ocr protein encoded by certain phages, an anti-restriction protein that inhibits both BREX and RM systems<sup>32</sup>.

**Monitoring the integrity of the bacterial cell machinery.** A third mechanism to detect viral invasion is monitoring the bacterial cell machinery. During infection, the host machinery is rapidly hijacked to stop the production of bacterial components while forcing it to produce viral ones. This process is detected by the sensor modules of several defence systems. In most cases, this type of sensing could be described as undirect sensing of a phage protein. Such a strategy has been described in two systems that use a retronelement as their sensor. Retrons are genetic elements composed of a specialized reverse transcriptase and a non-coding RNA, which is processed by the reverse transcriptase to produce an RNA–DNA hybrid. In *E. coli*, the retron Ec48 was shown to have antiphage activity. Although the exact mechanism remains elusive, the **retron Ec48** was shown to **monitor the activity of the RecBCD complex**, meaning that **inhibition of RecBCD triggers the activity of the system**<sup>38</sup>. In the RcaT system of *Salmonella enterica* subsp. *enterica* serovar Typhimurium, which also includes a retron sensor, two phage triggers were identified. Both the triggers interfere with the RNA–DNA complex of the retron element, one by methylating it (in the case of Dam) and the other by degrading it (in the case of RecE)<sup>39</sup>. Similarly, mutation of the phage protein gp5.7 allows T7 phages to escape two single-gene defence systems: the deoxycytidine triphosphate (dCTP) deaminase and the deoxyguanosine triphosphatase (dGTPase). As gp5.7 is responsible for shutting down oS-dependent host RNA polymerase transcription, this observation suggests that **both dCTP deaminase and dGTPase systems could sense transcription inhibition**<sup>40</sup>. The PrrC system, by contrast, is activated when the phage T4 tempers

with its *E. coli* host restriction endonuclease (*EcoprrI*). Indeed, the PrrC protein is bound to and inhibited by *EcoprrI* under normal conditions. However, **binding of the phage T4-encoded protein to *EcoprrI* during infection alleviates PrrC inhibition, triggering the immune response**<sup>41,42</sup>.

Antiphage **toxin–antitoxin (TA) systems** can also monitor the integrity of the host cell machinery. These systems often encode a stable toxic effector protein, which is inhibited by a labile RNA or protein antitoxin under normal conditions. During phage infection, the **degradation of the labile antitoxin releases the toxin, killing the host**. In type III TA systems, which encode RNA antitoxins, phage hijacking of the host cell machinery and subsequent cessation of bacterial transcription result in the depletion of the labile antitoxin and release of the toxin inhibition. This strategy is used by both ToxIN<sup>36</sup> and AvcD<sup>43</sup> systems. A similar mechanism can be found in type II TA RnlAB system, in which the RnlB antitoxin protein is a lot less stable than its cognate toxin, RnlA. Shutoff of the host transcription during phage infection and degradation of RnlB by the host proteases results in RnlA activation<sup>44</sup>.

An interesting evolutionary consequence of this type of sensor is that they force the phages to a trade-off between taking over the resources of their host and escaping detection by antiphage systems, potentially resulting in reduced fitness of the resistant phages.

## Activation of downstream bacterial immune response

Once viral infection is detected by the sensor module, the information must be transmitted to activate the effector module. This can occur either through direct activation or indirect activation by signalling molecules.

When activation of the effector is **direct**, the **sensor and effector modules physically interact**. This is the case of all the single-gene systems, in which the sensor and effector domains are found either on the same protein or fused, for example, Avs<sup>8</sup>, CapRel<sup>31</sup>, dGTPase and dCTP deaminase<sup>40</sup>, Lit and PrrC<sup>45</sup> and pAgo-Sir2<sup>25,26</sup> systems. It is also the case of RM and RM-like systems, and of most CRISPR–Cas systems (except for type III), in which the effector directly recognizes foreign nucleic acid.

In mechanisms involving **indirect activation through signalling molecules**, the sensor produces messenger molecules that activate the effector. This signal transduction strategy was first discovered in **CRISPR–Cas systems type III**. When recognizing its target RNA, the cyclase domain of the large Cas10 subunit polymerizes ATP into a cyclic oligoadenylate species (cOA)<sup>46</sup>. The signalling molecule is then recognized by an ancillary toxic effector that mediates an Abi response<sup>47</sup>. A **CBASS** constitutes another type of signalling antiphage systems. Upon infection, the cyclase (CD-Ntase) encoded by a CBASS produces **cyclic oligonucleotides signalling molecules** (for example, cGMP–AMP, cUMP–UMP, cCMP–UMP and so on). These signalling molecules activate various antiphage immune effectors, such as Cap4 endonucleases or Patatin-like phospholipases<sup>48,49</sup>. Pyrimidine cyclase system for antiphage resistance (PYCSR) is similar to CBASS but produces the **cyclic mononucleotides deoxycytidine monophosphate (cCMP)** or **uridine monophosphate (cUMP)**<sup>7</sup>.

Finally, in the **Thoeris system**, the sensor protein ThsB detects phage infection through an unknown mechanism, activating its TIR domain to produce a variant of cyclic ADP ribose (v-cADP). This **signal molecule binds to and activates the toxic effector of the system, ThsA**<sup>6,50</sup>.

The presence of intermediate **signalling molecules** offers interesting possibilities. As their concentration gradually builds up during infection, the **cell can tune its response** via degradation or dilution. For example, ring nucleases have been shown to be associated with CRISPR type III and to degrade the cyclic oligoadenylate molecules produced

during infection, allowing an interruption of the Abi pathway before cell death if the infection is otherwise resolved<sup>51</sup>. Conversely, signalling systems offer the possibility of signal amplification. For instance, the CRISPR type III encoded by the archaea *Sulfolobus solfataricus* produces 1,000 signalling molecules per RNA molecule detected, which could allow for an early immune response<sup>51</sup>.

Interestingly, the CD-Ntases of CBASSs share distant but clear homology with cyclic GMP-AMP synthase (cGAS), an eukaryotic enzyme that performs immune signal transduction in the cGAS-STING (stimulator of interferon genes) antiviral pathway<sup>48</sup>. Thoeris also resembles a eukaryotic immune pathway, as TIR-domain receptors in plants produce a similar signal molecule upon pathogen recognition, leading to cell death via a mechanism reminiscent of Abi. Therefore, the discovery of signal transduction through messenger molecules for antiphage defence opens fascinating parallels with eukaryotic immunity.

### Interruption of the phage reproduction cycle

Once infection is detected, antiphage systems exploit several strategies to interrupt the phage reproduction cycle. They mainly target three types of elements: nucleic acids, single nucleotides and the cell membrane.

**Nucleic-acid-degrading effectors.** Systems that degrade nucleic acids are very abundant in bacterial genomes<sup>12</sup> (Fig. 1c) and they do so by two types of mechanisms: specific cleavage of viral nucleic acids or untargeted cleavage of both viral and host nucleic acids.

Although type I, II and V CRISPR–Cas systems differ in their organization and mechanism, they all recognize and specifically cleave target phage DNA<sup>52</sup>. RM systems also specifically cleave phage DNA when recognizing unmodified motifs (or modified motifs in the case of type IV RM systems). In the RM-like systems Ssp and Dnd, effector modules DndFGH and SspE also cleave unmodified nucleic acids<sup>19,22</sup>. The NixL nuclease, which is encoded on phage parasites and inhibits the Vibrio-phage ICPI, also appears to act through specific cleavage of the ICPI genome, although the exact mechanism behind self-discrimination and non-self-discrimination is not yet fully understood<sup>53</sup>.

Two types of CRISPR–Cas systems, types III and VI, can activate untargeted nucleic acids (DNA and/or RNA) cleavage by the nucleases associated to the system upon detection of invading nucleic acids, leading to either cell death or dormancy following infection<sup>24</sup>. CBASS, Avs and Lamassu systems can also be found to be associated with endonuclease effectors<sup>8,13,48,54–56</sup>. Among the TA systems, both RnLAB and ToxIN have an RNase toxin that nonspecifically cleaves host and phage RNA upon phage infection<sup>36,44</sup>. The Gabija system effector GajA has also been proposed to function as a nonspecific DNA nicking endonuclease when activated<sup>57</sup>. Finally, both PrcC and RloC, respectively, nick and cleave tRNALys molecules when activated<sup>42,58</sup>.

Generally, targeted nucleic acid degradation allows the host to resolve and survive phage infection, whereas untargeted nucleic acid degradation leads to an Abi mechanism. However, although untargeted DNA degrading systems offer little chance of survival to the infected cell, cleavage of RNA transcripts only leads to cell dormancy. Therefore, if the trigger of the system is destroyed, the host can potentially recover from phage infection. For instance, cells can recover from the dormancy induced by Cas13 collateral RNase activity in type VI CRISPR systems if they also encode an RM system<sup>59</sup>.

Some systems do not cleave nucleic acids but modify viral nucleic acids to achieve defence. For example, in the DarTG system,

the DarT toxin performs ADP ribosylation of the phage DNA, which inhibits both viral DNA and RNA synthesis<sup>35</sup>. Similarly, the toxin domain of the CapRel system pyrophosphorylates tRNAs, blocking translation<sup>31</sup>. Systems such as NHI, BREX or DISARM seem to target phage nucleic acids without cleaving them through a mechanism not yet deciphered<sup>16,18,60</sup>.

**Nucleotide-modifying effectors.** As phage replication requires an abundance of intracellular nucleotides to use as building blocks for the fast-replicating phage genome, certain bacterial antiphage effectors directly target the single nucleotides pool of the host. This deprives the replicating phages of essential building blocks. Two classes of enzymes that degrade deoxynucleoside triphosphates have been described as effectors of antiphage systems: dCTP deaminases and dGTPases, which, respectively, degrade dCTP and dGTP<sup>40,43</sup>.

NAD is an essential co-enzyme of many essential metabolic reactions, and depletion of cellular NAD<sup>+</sup> can cause growth arrest or cell death. Thoeris, DSR and pAgo systems have effectors that use the NAD<sup>+</sup> degrading activity of their SIR2-domain effector to kill infected cells<sup>6,25,26</sup>. PYCSAR and CBASS, by contrast, have TIR domains that also kill the cell through NAD<sup>+</sup> depletion<sup>48,61</sup>. TIR domains are also associated with retrons and Avs, suggesting a similar mechanism<sup>8,38</sup>. Finally, SEFIR domains can also be antiphage effectors acting through NAD<sup>+</sup> depletion<sup>13</sup>. Although RADAR was initially reported to function through deamination of its host cellular RNAs during phage infection<sup>37,62</sup>, depletion of cellular ATP through adenosine deamination was recently suggested to be involved in the antiphage activity of the system<sup>63</sup>.

Prokaryotic viperins are enzymes that can modify the triphosphate substrates of nucleotides and remove the hydroxyl group at the 3' carbon of the ribose, producing 3'-deoxy-3',4'-didehydro (ddh) nucleotides. Viperins were shown to inhibit transcription by T7 RNA polymerase, probably because the viral polymerase incorporates ddh nucleotides in the nascent chain of the viral RNA. Ddh nucleotides would then act as chain terminators, causing premature termination of the RNA chain<sup>64</sup>.

**Membrane-disrupting effectors.** Many defence systems encode TM proteins or membrane-targeting proteins that kill the infected cell by disrupting its membrane. There are several types of membrane-disrupting effectors.

Bacterial gasdermins (bGSDMs) are generally found next to one or several proteases, which are activated during phage infection to cleave an inhibitory C-terminal peptide of the bGSDMs, releasing them of their autoinhibition. Activated bGSDMs assemble and form large pores in the membrane, eventually killing the host<sup>65</sup>. Interestingly, gasdermins are also known effectors of programmed cell death in mammals<sup>65</sup>. Although the primary mode of action of most CRISPR systems is to interfere with nucleic acids, the CsxB2 protein encoded by the type VI-B2 system forms pores in the membrane of the infected cell upon target phage RNA detection by Cas13, resulting in membrane depolarization. The presence of CsxB2 increases antiphage defence by several orders of magnitude compared with CRISPR–Cas alone<sup>66</sup>. Other systems relying on pore-forming effectors include RexAB<sup>33</sup>, Pif<sup>67,68</sup> and probably Zorya, which encodes homologues of proton-channel-forming proteins<sup>54</sup>.

Phospholipases are common CBASS effectors and when activated, they degrade the phospholipids in cell membrane<sup>5,48</sup>. The archaeal pAgo found in *Sulfolobus islandicus* also encodes a toxic effector

(Aga2), which binds anionic phospholipids and triggers membrane depolarization<sup>27</sup>.

Proteins containing TM domains have been shown to impair membrane integrity and lead to cell death in CBASS, PYCSR and Avs systems<sup>78,48,69</sup>. The retron system Ec48 from *E. coli* also encodes a TM domain-containing effector, which causes premature loss of membrane integrity during phage infection<sup>38</sup>. Intriguingly, the single-protein system AbiZ, which encompasses two TM domains, teams up with phage-encoded holins or lysins to cause premature lysis<sup>34</sup>.

Overall, effectors that disrupt the membrane integrity appear to be common in antiphage defence systems and respond to phage infection by killing the infected host.

From systems that monitor the host integrity to signalling systems and membrane-degrading ones, the diversity of antiphage sensors, signal transductors and effectors is striking. Yet, the mechanisms of more than half (58%) of the reported antiphage systems are not yet deciphered<sup>12</sup> (Fig. 1c and Supplementary Table 1), and most of the 7,000 gene families predicted to be antiphage on the basis of their propensity to be encoded next to known defence genes (Box 1) remain to be studied<sup>37</sup>, suggesting the existence of many more systems and mechanisms. Importantly, the articulation of sensor–transductor–effector does not seem to apply to all defence systems. For example, chemical defence against phages relies on the constitutive production of small antiphage molecules, such as aminoglycosides<sup>3</sup> and DNA intercalants<sup>4</sup>.

The diversity of strategies to resist phage infection goes beyond antiphage systems. Other layers of antiphage mechanisms exist, although they are not always considered to be strictly defence systems (Box 2). These mechanisms can participate in determining the set of strains a given phage can successfully infect, that is, its host range. For instance, mutating or masking the bacterial receptors that allow

phages to adsorb to the bacterial membrane prevents the injection of viral nucleic acids. In this Review, we discuss only defence systems that act intracellularly (after phage infection) and that do not have housekeeping functions.

## Drivers of antiphage defence diversity

The outstanding diversity of the defence strategies implemented by bacteria to resist phages has both ecological and evolutionary origins. The coevolution between bacteria and phages, as well as between bacteria and other mobile genetic elements (MGEs), has driven the emergence and diversification of antiphage systems for billions of years. This phenomenon is facilitated by the genetic organization of antiphage systems: the reservoir of antiphage genetic elements (domains, sensors and effectors) is limited, but such elements can be combined in a multiplicity of novel associations to create defence systems.

## Coevolution between bacteria and phages drives the diversification of antiphage systems

Ecological studies of bacteria–phages interactions in natural populations hint at a fast-paced coevolution. For instance, a matrix of interactions of 195 *Vibrio crassostreae* strains against 243 vibriophages isolated over a 5-month time period shows that only 2.2% of the host–phage interactions tested are positive and that a given phage is more likely to infect a host isolated in a closed space and time area<sup>70</sup>. Behind the coevolution of phage and bacteria, there is a continuous arm-race process between the two. The diversity of antiphage defence systems seems to be mirrored by a similar diversity of antidefence systems encoded by phages<sup>10,71,72</sup> (Box 3).

The selective pressure maintained by the continuous emergence of escaper phages probably drives the fast acquisition and diversification of phage defence systems in bacteria (Fig. 3 and Box 3).

## Box 2

### Definition and limits of defence systems

Although the concept of defence systems has become increasingly popular, a unified definition of what constitutes a defence system is still elusive. Various bacterial elements that were not considered to be defensive in this Review can prevent phage replication. For instance, mutation, loss or masking of bacterial receptors can prevent phage adsorption to its host. Notably, bacterial capsules were proposed to defend against certain phages in *Escherichia coli*, *Pseudomonas fluorescens* and *Staphylococcus simulans*<sup>126–128</sup>. Mutations<sup>129</sup> or modifications such as glycosylation<sup>130</sup> of bacterial receptors were also shown to provide bacterial resistance. However, these modifications once acquired become constitutive and are present even in the absence of phage infection. Therefore, these systems can be considered ‘passive’ resistance mechanisms, but probably not defence systems. By contrast, the expression of the stress-response RNA polymerase sigma factor σX (SigX) was reported to be enhanced in non-infected *Bacillus subtilis* cells when neighbouring bacteria were infected by phages<sup>131</sup>. The authors report that sigX expression confers resistance to the cell by inhibiting phage adsorption to its secondary receptor. Similarly, transient loss of the

cell wall was reported to be an antiphage response in *B. subtilis*, *Listeria monocytogenes*, *Enterococcus faecalis* as well as in several *Streptomyces* species<sup>132,133</sup>.

Besides receptor-based resistance, certain housekeeping genes also have antiphage properties. For instance, the RecBCD complex is an essential element of various metabolic processes in *E. coli*. Notably, its ability to process double-stranded DNA ends is used to either repair double-stranded DNA breaks or degrade unwanted DNA<sup>134</sup>. This second function of RecBCD allows it to degrade phage DNA, giving antiphage properties to the complex. Another example is the DynA protein of *B. subtilis*, which is normally involved in membrane remodelling processes but was also shown to delay host lysis during phage infection, possibly by stabilizing the membrane<sup>135</sup>. As the antiphage activity appears to be almost a side effect of the normal activity of these proteins and not their primary function, they do not necessarily qualify as defence systems. A defence system could thus be defined as protein (proteins) and/or nucleic acid molecules primarily dedicated to antiphage defence and that actively inhibits phage replication.

**Box 3**

## Diverse immune escape strategies in phages

Phages often carry antidefence systems, which can target and escape virtually any step of bacterial defences. First, phages can escape immune sensing through loss or modification of the trigger of a defence system. In the case of nucleic sensing systems, epigenetic modification or mutation of the sequence recognized is a frequent antidefence strategy<sup>10</sup>. In the case of systems detecting phage proteins, either by direct recognition or through monitoring of the host integrity, mutations of the protein detected can allow phages to escape detection<sup>38,136</sup>. Targeted proteins can also be fully deleted or exchanged. For instance, phage SPR is normally sensitive to the DSR defence system, but can become resistant by replacing through recombination its tail tube protein with an homologue taken from resistant phages Spbeta or phi3T<sup>25</sup>. Not only are the ways to escape bacterial immunity numerous but also escape can be relatively easy. In a recent study, escaper phages could be successfully isolated for 15 out of 54 defence systems tested (in *Escherichia coli* or *Bacillus subtilis* hosts), resulting in 177 resistant phages<sup>136</sup>. For 79% of them, escape occurred through a single mutation, probably in the element detected by the defence systems. Given this result, the main obstacles to the emergence of phage resistance seem to be the fitness cost of mutating these triggers and the existence of a diversity of defence system sensors.

A second mechanism relies on phages interfering with signal transduction. For instance, CRISPR-Cas type III, cyclic oligonucleotide-based antiphage signalling system (CBASS) and pyrimidine cyclase system for antiphage resistance systems can be escaped by phages encoding proteins that degrade the signalling molecules of the systems<sup>120,137</sup>. A different way of resisting signalling

systems is found in a small protein named Tad1, which acts as a 'molecular sponge' by binding and sequestering the signalling molecule of Thoeris or CBASSs<sup>50,138</sup>. In all cases, reducing the cellular concentration of signal molecules prevents the activation of the effector of systems, protecting the phage. Resistance mechanisms in phages could explain the diversity of signalling molecules found in bacteria. As an example, none of the phage proteins shown to degrade CBASS and pyrimidine cyclase system for antiphage resistance signalling molecules could degrade all of the diverse signals produced by these systems<sup>120</sup>.

Effector inhibition is also common in phage resistance. Proteins of several antidefence systems directly bind and inhibit the systems effector. For instance, anti-CRISPR proteins can bind and inhibit Cas effectors<sup>139</sup>, whereas mimic of antitoxins can be used by phages to inhibit toxin-antitoxin systems<sup>123</sup>.

Finally, certain phage resistance systems do not inhibit bacteria defence per se, but rather allow the phages to accommodate the defence system activity. For example, DarTG-mediated ADP ribosylation of phage DNA still occurs during infection by resistant SECφ18 phages. The presence of mutations in their DNA polymerase suggests that they acquired the ability to accommodate the modification of their DNA substrates<sup>35</sup>.

Importantly, antidefence systems do not only benefit individual phages but can also benefit viral communities. For instance, it was demonstrated that certain Acr proteins have an activity too weak to inhibit CRISPR on their own, but the accumulation of Acr proteins in the cell with each round of unsuccessful infection will eventually allow a phage to escape bacterial resistance<sup>121,122</sup>.

### Mobile genetic elements and horizontal gene transfer promote diverse antiphage arsenals in bacteria

Defence systems have a higher turnover rate than any other class of genes<sup>73</sup>. The patchy distributions of most defence systems suggest that horizontal gene transfer (HGT) is key in explaining this dynamic<sup>12,13,74</sup>. Consequently, the defensive content of very closely related strains can be highly variable.

MGEs are major contributors to the horizontal transfer of defence systems. Prophages, integrative and conjugative elements, plasmids and transposons often carry defence systems<sup>32,75–80</sup>. Studies suggest that the majority of *E. coli* defence arsenal could be carried by MGEs<sup>76,78,81</sup>. However, MGEs and the defence elements they encode can take a toll on their host, both owing to the resources their expression consumes and to the risk of autoimmunity<sup>82</sup>. MGEs can be lost by bacteria. Therefore, the strong association between MGE and defence systems is probably responsible for their high gain-and-loss rate through HGT. As MGEs often seem to integrate at specific hot-spots, this could explain the existence of defence islands<sup>32,78</sup> (Box 1). The defensive content of closely related bacterial strains can vary, reflecting the rapid rate at which defence systems are gained and lost. For instance, in a set of 23 nearly clonal *Vibrio lentus* strains, defence elements were shown to account for 95% of the flexible region<sup>83</sup>.

The importance of HGT in the spread of bacterial defence systems has led to the hypothesis of a pan-immune system, in which various systems are distributed and shared among bacterial communities, increasing the diversity of accessible systems while lowering their fitness cost<sup>84</sup>.

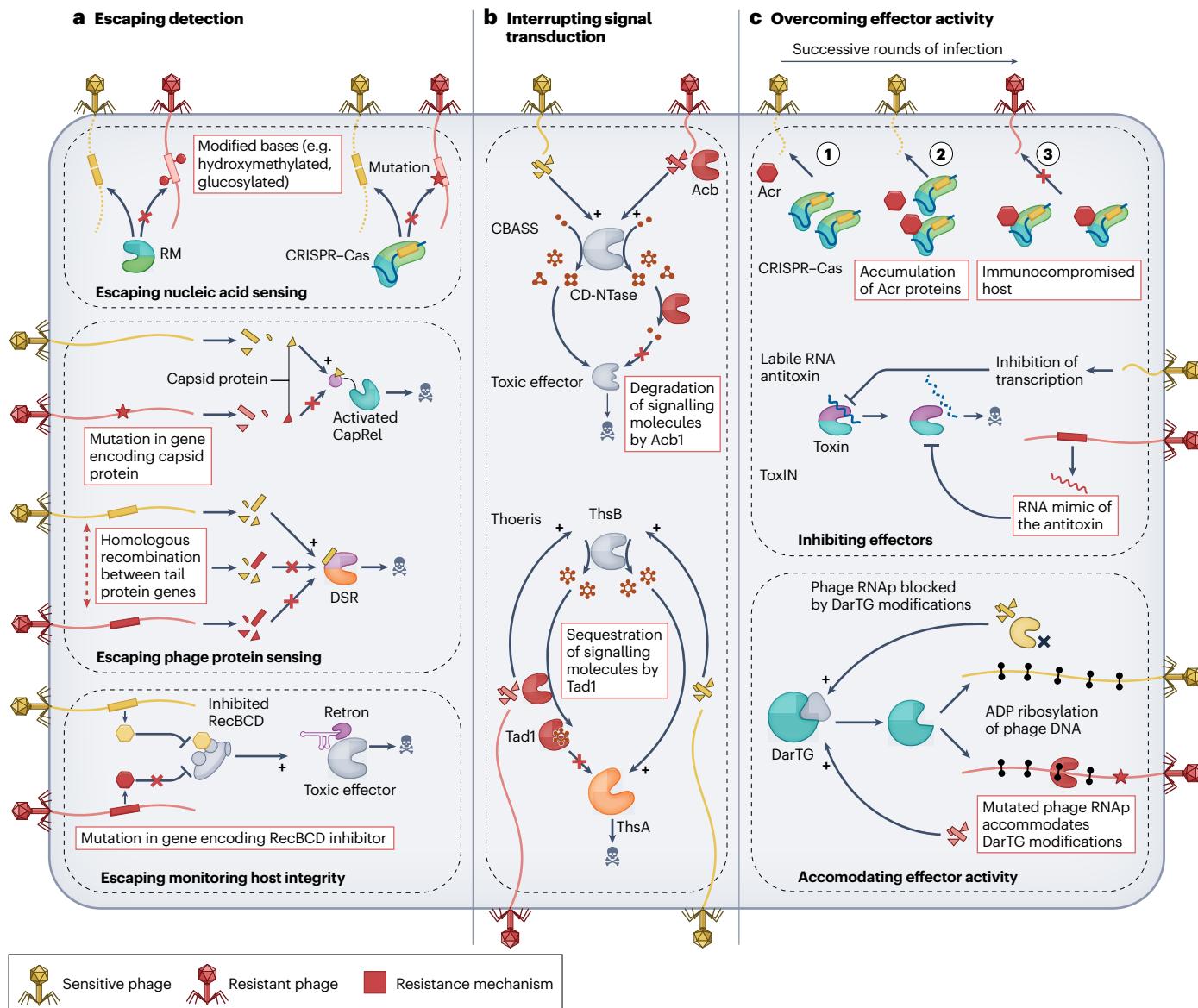
Certain systems appear to be specifically associated with certain types of MGEs. For instance, prophages often encode defence systems that protect the host they infect from other phages<sup>85–87</sup>. Certain defence systems are almost always found on prophages and rarely on the chromosome or other types of MGEs<sup>12,78</sup>. For example, BstA<sup>88</sup>, NixI<sup>53</sup>, RexAB<sup>33</sup>, Ltp<sup>89</sup> and Lit<sup>90</sup> are associated with phages and prophages. By contrast, some systems appear to be frequently associated with plasmids, as in the case of TA systems<sup>9</sup>. The extent to which systems exclusively encoded on MGEs and not by bacterial chromosomes themselves can be considered antiphage systems remains uncertain and raises questions about the limits of the definition of a defence system (Box 2).

Although MGE-encoded defence systems can be beneficial to their hosts, they are also selfish genetic elements. For instance, prophages encoding BtsA also encode an anti-BstA (*aba*) element that prevents autoimmunity and allows them to undergo their lytic cycle. Because of the selfish nature of MGEs, it has been proposed that the defence

systems they encode could be mere weapons in the inter-MGE war<sup>82,91</sup>. This hypothesis highlights how ecological conflicts between bacteria and MGEs, and between MGEs themselves, drive the diversification of defence systems under changing evolutionary pressures.

## Modularity as a source of diversity

The evolution and diversification of defence systems are probably supported by their modularity. Both in terms of domains, sensors and/or effectors, the diversification of defence systems seems to



**Fig. 3 | Phage resistance mechanisms target all steps of antiphage defence.** **a**, Escaping detection. Mutations or incorporation of modified bases in the recognition sequence or motif allows phages to escape restriction-modification (RM) or CRISPR systems<sup>118,119</sup>. Modifications in its capsid protein allow phage SECphi27 to escape detection by the CapRel system<sup>31</sup>. SPR phages are normally susceptible to defence by defence-associated sirtuins (DSR), but exchanging their tail protein gene with its distant counterpart from coinfecting resistant phages allows them to escape detection by the bacterial immune system<sup>25</sup>. Mutations in genes encoding RecBCD inhibitor allow phages Lambda and T7 to escape defence by the Ec48 retron system<sup>38</sup>. **b**, Interrupting signal transduction. Protein Acb1 from phage T4 degrades cyclic nucleotide signals of the cyclic oligonucleotide-based antiphage signalling system (CBASS) and prevents

activation of the effector<sup>120</sup>. Tad1 proteins encoded by multiple phage families bind and sequester signal molecules of the Thoeris system, which allows phages to resist the system<sup>50</sup>. **c**, Overcoming antiphage effectors, through the inhibition of effectors or accommodation of their activity. Weak anti-CRISPR proteins (Acr) fail to inhibit CRISPR-mediated DNA cleavage but accumulate in the cell over successive failed rounds of infection by phages encoding them; the host immunity is eventually compromised when a threshold concentration is reached, allowing phages to reproduce<sup>121,122</sup>. Phages can encode RNA mimics of the antitoxin of ToxIN, which binds and inhibits the toxin activity<sup>123</sup>. Mutations in genes encoding their RNA polymerase allow phages to accommodate DarTG-mediated modifications of their DNA and reproduce themselves<sup>35</sup>.

be driven by an evolutionary mix-and-match game played at different and imbricated scales.

First, at the gene level, certain domains appear to be co-opted by many defence systems. For instance, HEPN domains are common RNase effectors in nucleic acid degrading systems such as CRISPR–Cas Type III, Prrc, RloC, RnlAB and ApeA<sup>92</sup> and were also found in defensive hotspots<sup>32</sup>. Similarly, SMC domains are encoded by several systems, including Lamassu<sup>13,54</sup> and Wadjet<sup>28</sup>, and are also found in defensive hotspots<sup>32</sup>.

At the system level, several studies suggest that a given sensor module can activate multiple effector modules. For instance, the DNA-modifying module of the Dnd system (DndACDE) is not only found with the DNA-cleaving effector (DndFGH) but can also be associated in archaea with an effector module (PbeABCD) that does not degrade or cleave viral DNA but halts phage replication<sup>19,20</sup>. Similarly in Ssp, DNA-modifying SspBCD can be associated with either SspE (DNA-nicking) or SspFGH (unknown mechanism)<sup>21,22</sup>, suggesting additional mechanisms. In the Avs defence systems, the NLR sensor domain can be fused with diverse effector domains. Gao et al.<sup>8</sup> created a chimeric system in which the natural Mrr-like nuclease effector domain of Avs4 of *E. coli* was swapped with the TM domain effector domain from an Avs4 homologue encoded by *Sulfurospirillum* sp. The chimeric system retained its antiviral activity, further demonstrating the modularity of the Avs systems.

Conversely, an effector module can also be activated by multiple sensor units. For instance, in genomes encoding a Thoeris system, the *thsA* effector gene is often found to be accompanied by several *thsB* sensors. It appears that different ThsB proteins recognize different phages and all activate the same effector, widening the activity spectrum of the system<sup>6</sup>. Other examples are the endonucleases NucC, which is an effector of both CRISPR–Cas and CBASS systems<sup>46</sup>, and Cap4, which can be an effector of both Lamassu and CBASS<sup>93</sup>. Preliminary evidence also suggests that ATP nucleosidases can be effectors of CBASS, pAgO and Avs systems<sup>94</sup>.

It has also been proposed that domains and modules associated with defence systems can either evolve from or be domesticated into non-defensive cellular processes, as it has reported for certain RM enzymes<sup>91,95,96</sup>. Highlighting even more the modular nature of defence systems are the many newly discovered multi-effector or multisensor families of systems, such as CBASS, Lamassu, retrons and pAgos<sup>26,27,38,39,48</sup>.

The co-optation of domains, sensors and/or effectors, from other defence systems or other cellular pathways, could allow the evolution of novel antiphage mechanisms not de novo but as a combination of existing modules.

## Impact of antiphage systems diversity

Defence systems have strong ecological repercussions, both on phage–bacteria interactions and on phage and bacteria separately.

### Impact on host range

The effect of the diversity of antiphage defence systems on host range is not fully understood yet. However, several tendencies have already emerged. First, the combination of different defence systems is often synergistic. On the one hand, encoding several defence systems active against the same phage can increase the host resistance, in comparison to expressing a single system<sup>70,81,97</sup>. On the other hand, most defence systems only defend against specific phages. For instance, preliminary data revealed that when 15 systems of *Pseudomonas aeruginosa* were cloned, 11 of them were specific to one or two phage families when expressed in a *P. aeruginosa* host<sup>98</sup>. The expression of several defence systems

active against different phages can result in an increase of the resistance range, which can be equal or superior to the sum of the resistance profiles conferred by individual systems<sup>80,98</sup>. Second, encoding diverse defence systems could hinder the emergence of resistant phages, as reported, for instance, for the combination of ToxIN and RexAB<sup>99</sup>, BREX and RM systems<sup>80</sup>, CRISPR and RM systems<sup>100</sup>. Overall, the diversity of antiphage sensors and effectors could allow defence systems to target a wider range of phages while preventing the emergence of resistance.

### Defence systems shape microbial genomics

Defence systems can affect HGT and shape the spread and fixation of mobile genetic elements within microbial populations in various ways. First, the diversity of defence systems affects MGEs. Antiphage defence systems can restrict not only phages but also other types of MGEs. This could prove detrimental to the host, given that MGEs can bring beneficial elements to the host (for example, antibiotic resistance genes). In *V. cholerae*, the presence of genetic elements encoding defence systems was shown to limit the acquisition of two beneficial MGEs<sup>79</sup>, and the introduction of a CRISPR–Cas system in a *Bacillus* strain naturally lacking one resulted in reduced growth, sporulation, biofilms and adaptability to environmental stresses<sup>101</sup>. Depending on their molecular mechanisms, defence systems are more or less permissive towards invading nucleic DNA and therefore towards MGEs. For instance, systems that detect RNA can coexist with unexpressed foreign DNA. Second, the diversity of antiphage mechanisms influences HGT in bacteria. For instance, ‘addictive’ systems such as TA systems can participate in stabilizing MGEs in the bacterial population by killing any progeny cells that did not inherit the MGE encoding them. DNA degrading systems such as RM could facilitate homologous recombination by introducing breaks in double-stranded DNA<sup>102</sup>. CRISPR–Cas systems have also been proposed to promote HGT, possibly by favouring homologous recombination between CRISPR spacers and phage protospacers<sup>103,104</sup>.

Although the impact of viral predation on microbial communities is probably still underestimated, several studies highlight the importance of phage–bacteria interactions on microbiomes and ecosystems<sup>2,105,106</sup>. Phages not only affect the species they target but also indirectly affect competing species of the ecological niche. As an example, a study showed that introduction of lytic phages in the gut microbiome of gnotobiotic mice led to stable coexistence of the phage and the targeted host, and had cascading indirect effects on non-targeted species, and ultimately affected the gut metabolome<sup>105</sup>. As defence systems shape phage–bacteria interactions and influence microbial genomics, they probably have major ecological implications that remain to be fully comprehended.

### Conclusion

The diversity of antiphage mechanisms in bacteria is striking and comprises unexpectedly complex immune strategies. Despite recent mechanistic advances in the field, many questions remain to be explored. Very few systems have been characterized outside classic model organisms, and future research on distant bacteria or archaea could provide fascinating insights and allow the discovery of novel mechanisms. In the past, the mechanistic richness of bacterial antiphage defence has been the source of major biotechnological breakthroughs. Systems such as RM, CRISPR–Cas and retrons have provided invaluable tools in fields as diverse as molecular biology, genome editing and clinical diagnosis, and many more applications are probably yet to be uncovered<sup>107–111</sup>.

The giant leaps made in our understanding of antiphage defence systems have major ecological and evolutionary repercussions. These

advancements compel us to shift our perspective towards an integrated understanding of the role of defence systems within a complex network of interactions, moving away from a simplistic view of how a single system affects the interaction between a phage and its host. The overwhelming presence of abortive infection systems<sup>8,26,43,62,69</sup>, despite their important fitness cost for the bacteria expressing them, demonstrates that defence systems should not be considered exclusively at the single-cell level, but rather at the population level. Reports on the antiphage activity of certain molecules excreted by certain Actinobacteria also support the importance of considering antiphage defence at the level of microbial communities. Studies suggest that real-life factors such as interspecies competition<sup>112</sup> and spatial heterogeneity could affect the dynamics of antiphage defence in bacteria<sup>113</sup>. Yet, the role of antiphage systems has rarely been explored outside simple and controlled laboratory settings. Notably, very few antiphage systems have been studied in their original host, and studying antiphage mechanisms in their native conditions will probably be key in a better understanding of antiphage defence in nature.

The interplay between defence systems and the genetic elements that encode them appears far more complex than initially envisioned. The diversity of defence systems not only stems from an ongoing war between bacteria and MGEs but also shapes the fate of all protagonists involved. Bacteria, plasmids, phages, prophages and phage satellites can all develop and/or exploit pre-existing defence systems, antidefence systems and even anti-antidefence systems. These findings challenge our perceptions of defence systems as mere weapons in a war between bacteria and phages and suggest that antiphage defence systems can be envisioned as a toolbox used by both MGEs and bacteria to interfere with gene fluxes, alongside notably antiplasmids and viral antidefence systems<sup>82</sup>. Investigating a potential continuum among antiphage defence, HGT and/or MGE regulation will undoubtedly help refine current knowledge of microbial genomics.

Finally, from an evolutionary perspective, defence systems appear to be an excellent example of the concept of evolutionary tinkering developed by François Jacob<sup>114</sup> in 1977, which argues that natural selection is akin to a tinkerer assembling existing objects, in this case defensive modules, until they combine into something useful. Interestingly, some defensive modules appear to be conserved across domains of life. As mentioned earlier, many antiviral systems can be conserved between Bacteria and Archaea. Even more surprising, both protein and domain conservation between prokaryotic and eukaryotic immunity have been reported, as for Viperins<sup>64</sup>, Gasdermins<sup>65</sup>, but also TIR and NLR domains<sup>6,115</sup>. Several hypotheses could explain the conservation of antiviral immune systems between prokaryotes and eukaryotes, one of them suggesting that these systems were inherited from the last common ancestor (ancestors) of prokaryotes and eukaryotes<sup>116</sup>. It is also possible that some defence systems were acquired during later events of HGT between prokaryotes and early eukaryotes. Combining ideas of evolutionary tinkering and immune building blocks opens fascinating research avenues, where we could delimit what can be considered antiphage building blocks, map them in chromosome genomes and study their possible combinations to systematically explore the diversity of antiphage systems in bacteria<sup>95</sup>. Additionally, the conservation of these building blocks across domain of life presents an opportunity to discover and comprehend eukaryotic immune mechanisms that share similarity with known antiphage systems<sup>50,117</sup>.

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## Author contributions

The authors contributed equally to all aspects of the article.

## Competing interests

H.G. is employed by Generare Biosciences. A.B. declares no competing interests.

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