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Remarkable Mechanisms in Microbes to Resist Phage Infections

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Abstract

Bacteriophages (phages) specifically infect bacteria and are the most abundant biological entities on Earth. The constant exposure to phage infection imposes a strong selective pressure on bacteria to develop viral resistance strategies that promote prokaryotic survival. Thus, this parasite-host relationship results in an evolutionary arms race of adaptation and counter-adaptation between the interacting partners. The evolutionary outcome is a spectrum of remarkable strategies used by the bacteria and phages as they attempt to coexist. These approaches include adsorption inhibition, injection blocking, abortive infection, toxin-antitoxin, and CRISPR-Cas systems. In this review, we highlight the diverse and complementary antiphage systems in bacteria, as well as the evasion mechanisms used by phages to escape these resistance strategies.

INTRODUCTION

Bacteriophages: Early History

Phage therapy: the use of phage in an antibacterial therapeutic, such as in agriculture and medicine

Bacteriophages (a term derived from the Greek words *baktērion*, meaning "rod," and *phagein*, meaning "to devour"), or phages, are ubiquitous parasitic viral entities that specifically infect bacteria. Discovered by Frederick Twort and Félix d'Hérelle in 1915 and 1917, respectively, phages are considered to be the most abundant biological entities, and the oldest viruses, on Earth (1). d'Hérelle recognized the therapeutic potential of phages, and this recognition led to the development of a phage preparation to treat dysentery in soldiers during World War I (2). The use of phages as bactericidal agents, later termed phage therapy, was practiced for several decades before the discovery of antibiotics (2). Due to the shift to antibiotics, phage therapy was discontinued in the West; however, its usage continued in Eastern Europe. Soon thereafter, research shifted to the use of phages as model genetic systems. Phage research led to significant discoveries that collectively contributed to our fundamental understanding of the central dogma of molecular biology and resulted in countless reagents that underpin modern biotechnology (3). Today, phage research is undergoing another renaissance. Firstly, there is a renewed interest in phage therapy, due to the inexorable rise of multidrug resistance in bacterial pathogens. Secondly, phage and bacterial genomics have provided new insights and stimulated research aimed at addressing the important ecological roles of phages and their impact on the evolution and pathogenesis of bacteria. Finally, the study of phage resistance systems has continued to provide tools for molecular biology, from restriction enzymes, phage display, and resistance systems used to protect cultures in the dairy industry to the rapid recent emergence of the use of adaptive immune systems for genome editing and related applications (4).

Phage-Bacterium Interactions

Microbes, such as bacteria and viruses, do not exist in isolation but shape intricate ecological interaction webs (5). Such biological contacts can yield beneficial (win), detrimental (loss), or negligible impacts on the species involved. Classic nonmutual interactions are parasitism and predator-prey relationships, where one species benefits at the expense of the other (5). For host-parasite systems, continuing adaptation is essential for a species to maintain its relative fitness. In the Red Queen hypothesis, Leigh Van Valen (6) posited that every positive adaptation in an organism causes a decline in fitness in those species with which it interacts. Such coevolutionary interactions create the natural cycle of adaptation and counteradaptation of ecologically interacting species, thereby driving rapid molecular evolution (6, 7). Nowhere is this dynamic arms race as prevalent as in microbe-phage interactions. Phages in any given environment can be 10-fold more abundant than bacteria (8). In addition, $\sim 10^{25}$ phage infections per second are thought to occur on Earth, thereby imposing a strong selection pressure on bacteria and a resultant bacterial lysis and turnover that impacts global nutrient cycling (9, 10). Furthermore, phage-mediated horizontal gene transfer influences bacterial evolution, including the acquisition of virulence determinants in human pathogens (11). As hosts of predatory viruses, bacteria have evolved numerous antiphage mechanisms to ensure their survival (4, 12), and through coevolution, phages counteradapt and develop strategies that bypass these defenses (13). In this review, we highlight the remarkable spectrum of antiphage systems in bacteria that function at every stage of the phage infectious cycle (**Figure 1**).

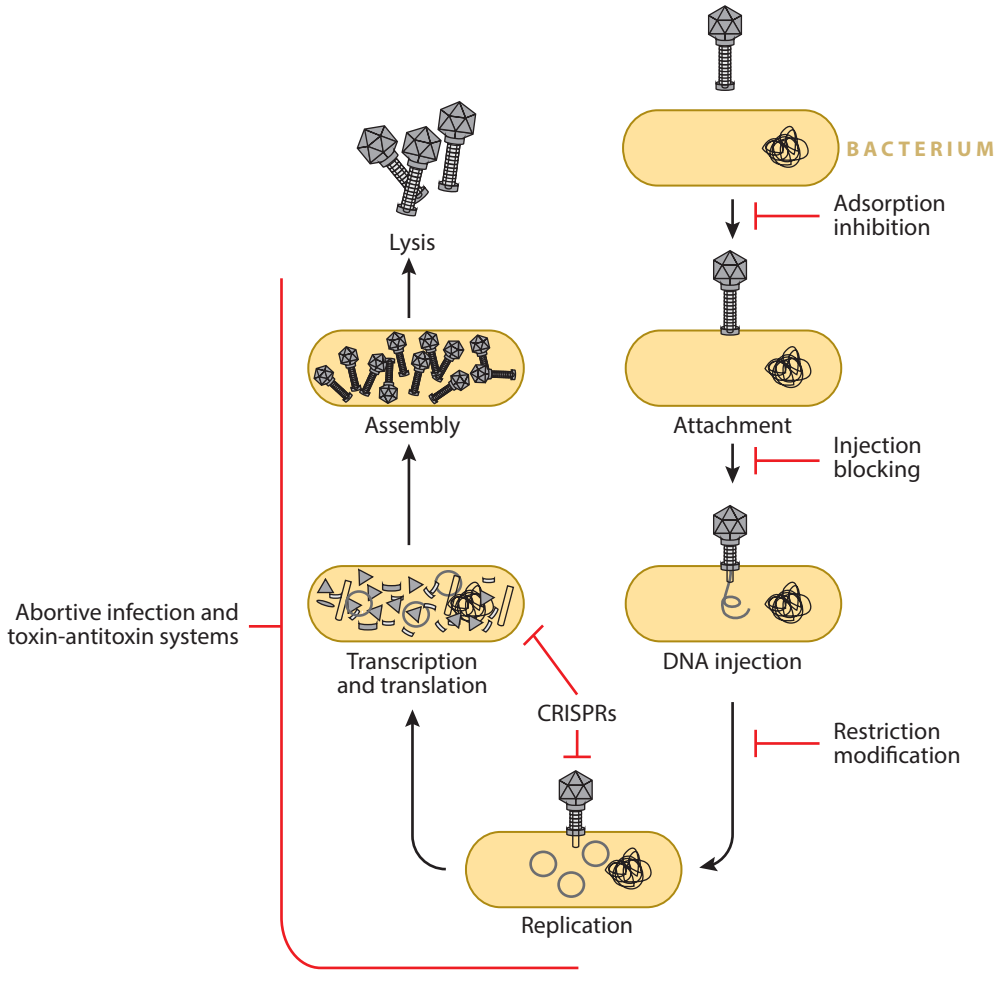

Figure 1

The phage lytic life cycle and the bacterial antiphage systems. Bacteria possess a range of defense strategies that target various phases of the phage life cycle.

MICROBIAL ANTIPHAGE SYSTEMS

Adsorption and DNA Injection Inhibition

There are two antiphage systems known to target the start of the phage infection life cycle. In the following section, we provide examples of known adsorption- and DNA injection–blocking strategies in bacteria.

Adsorption inhibition. To initiate an infection, phage tails recognize specific cell-surface receptors, such as lipopolysaccharide (LPS), membrane proteins (such as porins), pili, or flagella. In *Escherichia coli* phages like T4, irreversible attachment to the cell receptor induces a conformational change in the phage baseplate leading to tail contraction and DNA injection (14). Adsorption inhibition is a mechanism in which bacteria alter or block these receptors to avoid phage attachment.

LPS: lipopolysaccharide

Phase variation: a heritable and reversible genetic switch that typically results in all-or-none expression of specific genes

There are several known adsorption-blocking strategies in bacteria, such as mutating or masking phage receptors, production of extracellular matrix to occlude receptors, and exploitation of competitive receptor inhibitors (12).

Some bacteria produce proteins that mask phage receptors. For example, outer-membrane protein A (OmpA) serves as the entry receptor for many T-even-like *E. coli* phages (15). An outer-membrane lipoprotein, TraT, encoded by the F plasmid interacts with OmpA and inhibits phage attachment (16). As many receptors are nonessential, mutants arising in the population can survive by avoiding phage binding. For example, point mutations, rearrangement, and insertions altering the OmpA surface lead to phage resistance (17). Bacteria also produce extracellular polymers, which act as physical barriers against harsh environments while also impeding phage attachment (18, 19). Exopolysaccharides (EPSs) such as alginate and hyaluronan constitute bacterial capsules, and plasmids encoding EPSs can be horizontally acquired and impart an adsorption-blocking phenotype (20). Finally, bacteria can produce small molecules that occupy the active sites of particular receptors and interfere with phage attachment. In *E. coli*, FhuA is an iron transporter protein and the receptor of phages T1 and T5. Microcin J25 (MccJ25) is a 21-amino-acid antimicrobial peptide produced under nutrient limitation that binds to FhuA for transport into the cell. MccJ25 binding competitively blocks FhuA, preventing the initiation of T5 adsorption (21).

Phages can overcome adsorption-inhibiting defenses by modifying their tail fibers to recognize new or altered receptors. For example, phage λ evolved to target a new receptor when expression of its preferred receptor was suppressed. Phage λ binds to the LamB outer-membrane receptor of *E. coli* using tail protein J, and mutations that decrease LamB expression reduce phage adsorption (22). Decreased LamB expression led to mutations within the *j* gene, which enabled phage λ to infect by binding another receptor, OmpF (22). Four nucleotide changes were required for the altered receptor binding, and all mapped to the distal end of *j*, which is the domain involved in host-receptor binding (22). Similarly, phages can adapt to recognize altered target receptors. T7 infects *E. coli* K12 by binding LPS, which is a lipid embedded in the bacterial membrane with several sugar moieties attached and presented at the cell surface (23). For attachment, T7 can recognize the first glucose, the penultimate glucose, or the terminal heptose of LPS (24). The process of T7 adsorption was recently visualized using cryo–electron tomography, which revealed a phage "walk" in search of receptors (25). Mutations in genes involved in LPS biosynthesis resulted in differing LPS structures and abrogated T7 adsorption. Following successive rounds of infection of *E. coli* LPS mutants, a T7 variant was isolated that had evolved the ability to infect using the wild-type or the mutant LPS structures. The T7 variant had mutant tail proteins that now enabled infection of *E. coli* independently of LPS (24).

Phages can acquire EPS-hydrolyzing enzymes that enable penetration of the extracellular matrix that can mask cellular receptors. These EPS-hydrolyzing enzymes can either reside within the tail fibers for direct penetration of the polymeric barrier or be dispersed after phage burst to aid infection of neighboring bacteria by new viral progeny (26). Phage H4489A has a hyaluronan lyase attached to its tail fibers that degrades the capsule of *Streptococcus pyogenes* to assist phage adsorption (27). Similarly, *Klebsiella* phages have tail-associated glycanases that depolymerize the bacterial capsule layer to ease phage adsorption (28). Furthermore, phage PT-6 encodes a soluble alginate lyase, which is liberated after burst and improves phage infection of *Pseudomonas aeruginosa* (29).

Phase variation. Genes encoding surface components that act as phage receptors can be mutated or deleted, leading to decreased susceptibility to phage attack. However, these mutations can be detrimental, because many phage receptors play important cellular roles. In some cases, surface proteins are subject to reversible or temporal expression known as phase variation (30). Phase

variation is a heritable, but reversible, form of regulation that typically results in all-or-none expression of specific genes. The result is reversible bacterial heterogeneity within the population. Thus, some cells express a surface protein rendering them phage sensitive, whereas other bacteria repress the protein and have reduced phage sensitivity. This can be viewed as a form of bet-hedging, in which the generation of different phenotypes can be used to increase overall population fitness by mitigating risk imposed through variable selective conditions (31).

Slipped-strand mispairing: a mutagenic process that occurs during replication and is often caused by homopolymeric nucleotide tracts

One example of phase variation is seen in the control of the infectious cycle of pathogenic *Bordetella* species, which utilize the two-component regulatory system BvgAS. Through a phosphorelay, BvgS, a transmembrane sensor kinase, together with its transcriptional regulator, BvgA, couples environmental cues to the expression of surface proteins and other virulence factors. In the virulent Bvg^+ phase, characterized by high BvgAS activity, several adhesins, toxins, and Type III secretion systems are expressed, which are important in pathogenesis (**Figure 2*a***). In the Bvg^- phase, BvgAS is inactive, virulence genes are temporally suppressed, and motility genes are induced (**Figure 2*a***). Aside from temporal control of expression by BvgAS, phase variation of *bvgS* can dictate the transition between Bvg^- and Bvg^+ phases (32). The *bvgS* gene contains a 6-bp polycytosine tract that expands through slipped-strand mispairing, resulting in a BvgS frameshift, which negatively affects expression of pertactin (Prn) (33). Phage BPP-1 recognizes Prn, which is produced only during the Bvg^+ phase. Thus, Bvg^+ *Bordetella* cells are 10^6 times more susceptible to BPP-1 infection than are Bvg^- cells (**Figure 2*a***) (34). However, phase variation of Prn does not offer absolute BPP-1 protection because the phage still infects cultures of Bvg^- cells, albeit at a lower rate. These BPP-1 escape phages undergo a tropism switch, enabling either specific

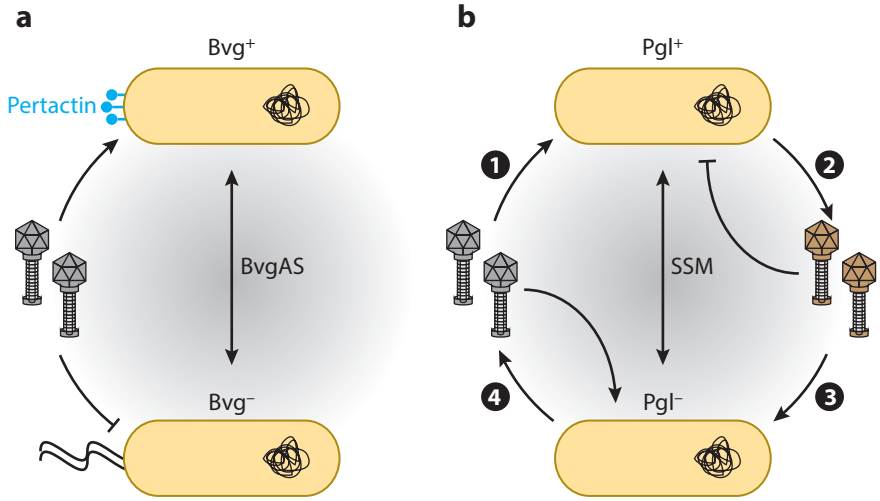

Figure 2

Dodging phage infection through phase variation. (*a*) *Bordetella* species have two phase variants determined by the two-component regulatory system BvgAS. Pertactin is an outer-membrane protein expressed through BvgAS, present only in Bvg^+ phase variants and recognized by phage BPP-1. *Bordetella* Bvg^- variants with low BvgAS activity do not express pertactin and therefore are protected from phage BPP-1 infection. (*b*) The Pgl system of *Streptomyces coelicolor* enables phage protection by supporting a normal lytic life cycle upon the first infection. However, the progeny phage produced are modified such that they are ineffective in infecting Pgl^+ hosts and can only propagate in Pgl^- variants. The Pgl methyltransferase (PglX) of *S. coelicolor* is predicted to modify the phage DNA, marking it for degradation during subsequent infection. PglX is phase-variably expressed through slipped-strand mispairing (SSM) of an 8-bp polyguanine tract within the *pglX* gene.

infectivity of Bvg$^-$ cells or broad infectivity in both Bvg$^+$ and Bvg$^-$ cells. Comparison of BPP-1 with the escape phages revealed a mutational hotspot within a variable repeat (VR1) at the distal end of *mtd* (major tropism determinant) that encodes the tail fiber. Nucleotide substitutions are introduced to VR1 by a family of diversity-generating retroelements, which are genetic cassettes that use site-specific and error-prone reverse transcription to diversify sequences and the proteins they encode (35, 36). A template (TR1) homologous to VR1 acts as the intermediate transcript for reverse transcription, during which adenine residues are substituted for other nucleotides (36). Mutagenesis of VR1 in *mtd* results in tropism switching of BPP-1 that enables recognition of new or altered receptors (35, 36). Bacteria can also use diversity-generating retroelements. In *Legionella pneumophila* a repertoire of 10^{19} distinct LtdA lipoproteins can be generated through diversity-generating retroelements (37), indicating another mechanism in bacteria to generate receptor variants that might prevent phage adsorption.

Sie: superinfection exclusion

Temperate phages: phages that can undergo lytic or lysogenic life cycles; during lysogeny, the prophage replicates stably with the host bacterium

A similar situation occurs in phage ICP1, which infects *Vibrio cholerae* by specifically binding the LPS O1 antigen. The phase variation of two genes (*manA* and *wbeL*), both important for O1 antigen biosynthesis, enables phage ICP1 escape (38). Both biosynthetic genes contain homopolymeric adenosine [poly(A)] tracts within their reading frames that are mutated through slipped-strand mispairing (38). Phase variants defective in producing LPS (e.g., *wbeL**) are highly resistant to ICP1 phage infection, but as a consequence, they are severely attenuated in virulence (38). Similarly, phase variation of the glucosylation state of the O12 antigen in *Salmonella* Typhimurium is mediated by the *gtrABC1* cluster and enables transient resistance to phage SPC35. The host receptor for SPC35 is BtuB, but the O12 antigen assists efficient binding (39).

DNA injection blocking. After phage attachment, in phages like T4, a needle within the phage baseplate initiates penetration of the bacterial envelope (40). Phage hydrolytic proteins are released and locally digest the cell wall murein/peptidoglycan layer, easing DNA injection (41, 42). Injection-blocking superinfection exclusion (Sie) systems are commonly phage or prophage encoded and block phage DNA entry, but their mechanisms are not well understood. The Sie proteins (Imm and Sp) from T4 are membrane associated and stop phage DNA translocation of other T-even phage infections (i.e., T2, T4, and T6) (43). Imm directly blocks DNA injection, whereas Sp inhibits phage-encoded lysozymes, blocking DNA translocation (43). Similarly, in gram-positive bacteria, Sie_{2009} is a membrane protein of the P335-type temperate phages of *Lactococcus lactis* that exclude members of 936-type phages. Sie_{2009} is predicted to interfere with the release of phage DNA from the capsid by masking a membrane component that triggers DNA translocation (44). In the *Streptococcus thermophilus* temperate phage TP-J34, the lipoprotein $Ltp_{TP\text{-}J34}$ excludes 936-type phages (45), and escape phages display shorter tails due to mutations within the tape measure protein gene, which is important for tail assembly, ion-channel formation, and DNA passage into the cell (46). $Ltp_{TP\text{-}J34}$ is predicted to inhibit DNA injection by directly interacting with, and blocking, tape measure protein entry into the bacterial membrane from the phage tail tube. Thus, these exclusion systems protect bacteria from secondary infections by the same, or closely related, phages.

Degrading Phage Nucleic Acids

Bacteria possess numerous nucleic acid degrading systems that enable protection from invading DNA. In the following sections, the restriction-modification and CRISPR-Cas systems are described.

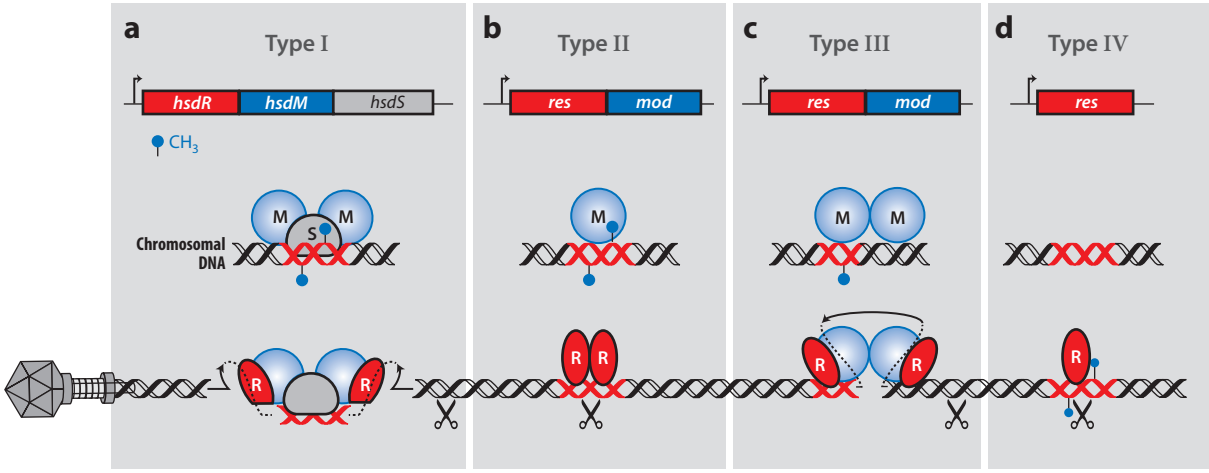

Figure 3

The mechanisms for methylation and restriction for the four types of restriction-modification (RM) systems. (*a*) Type I RM systems are composed of *hsdRMS*. HsdS is the specificity subunit that binds to an asymmetrical DNA sequence. Two HsdM subunits and one HsdS subunit ($HsdM_2$:HsdS) are required for methylation. For restriction, two complexes of $HsdR_2$:$HsdM_2$:HsdS bound to unmethylated recognition sequences pull the DNA in a bidirectional manner, forming two loops for each complex (one complex shown). The DNA is cleaved kilobases away from the recognition site following collision of the two complexes. (*b*) Type II RM methyltransferase (MTase) and restriction endonuclease (REase) function separately and independently. A monomeric MTase recognizes a palindromic sequence and methylates both strands of the DNA. A monomeric or homodimeric REase cuts precisely within or outside the unmethylated palindromic sequence. (*c*) Type III RM systems are also composed of two components. The homodimeric MTase binds DNA and methylates one strand. Two $MTase_2$:$REase_2$ complexes bound inversely at two adjacent unmethylated recognition sites pull DNA in a unidirectional manner, forming one loop for each complex. The DNA is cleaved at a fixed location >20 bp away from the recognition sites following collision of the two complexes (one complex shown). (*d*) In Type IV RM systems, the REase recognizes methylated DNA or other modifications (i.e., hydroxymethylcytosine and phosphorothioation) and cleaves DNA within or away from specific sequences. Abbreviations: M, MTase; *mod*, MTase-encoding gene; R, REase; *res*, REase-encoding gene; S, specificity subunit.

Restriction-modification systems. Following passage of phage DNA into the cell, host functions may be redirected, and several phage genes are temporally regulated. Restriction-modification (RM) systems are considered as the innate bacterial immune system against foreign DNA. RM systems display two contrasting activities: (*a*) a methyltransferase (MTase) that modifies DNA bases of a specific sequence through the transfer of a methyl group to both strands and (*b*) a restriction endonuclease (REase) that recognizes and cleaves the same DNA sequence if nonmethylated (47). The methylated sequences are thus discriminated as self and are protected, whereas nonmethylated foreign sequences are considered nonself and are degraded. Bacterial genomes can contain multiple RM systems, and there are four families of RM systems classified by their subunit composition, DNA sequence recognition, cleavage position, cofactor requirements, and substrate specificity (**Figure 3**) (for a detailed review, see 47).

Phages have several antirestriction strategies to escape RM systems. Recognition sites within the phage DNA can be substituted through point mutations to prevent or reduce the likelihood of restriction by RM systems (47). However, bacteria can regain RM-mediated protection through altering the sequence specificities of RM systems. For example, in *L. lactis*, recombination of two *hsdS* genes facilitated the formation of chimeric HsdS subunits with altered RM specificities (48). Phages can also acquire MTases that modify their own DNA to mimic that of the host (49). In a remarkable example of molecular mimicry, phages can evolve proteins that are structural analogs

RM: restriction modification

MTase: methyltransferase

REase: restriction endonuclease

of specific phage DNA sequences that then sequester restriction enzymes and thereby prevent digestion of the viral genome. The Ocr protein is produced from the first gene expressed following DNA injection of phage T7. Dimeric Ocr mimics DNA, and *Eco*KI MTase and REase enzymes have a 50-fold greater binding affinity for Ocr than for the target DNA; thus, Ocr efficiently blocks phage genome restriction (50, 51). In addition, phages can utilize other modified bases such as uracil, hydroxymethyluracil, and hydroxymethylcytosine (hmC) to prevent recognition by RM systems. For example, instead of cytosine, T4 incorporates 5-hmC into its DNA, which can be further modified by glucosylation. Type IV REases are unlike the other RM Types in that they cleave only modified DNA. The MrcBC (previously named Rgl system) of *E. coli* K12 limited the population of T4 phages that contained hmC-substituted DNA (52). MrcBC recognizes two hemimethylated dinucleotides separated by 40–3,000 bp, and cleavage occurs 30 bp from this site (**Figure 3**) (53). Similarly, *Pvu*Rts1I restricts glucosylated 5-hmC DNA (54). Thus, these Type IV RM systems play an important and additive role, enabling bacteria to be protected from heavily modified phages that have escaped other RM systems.

RM systems can be regulated by phase variation such as the phage growth limiting (Pgl) system of *Streptomyces coelicolor* (55). The Pgl phenotype is characterized by the ability of Pgl^+ hosts to support a lytic cycle of phage ϕC31. However, after the burst, progeny phage cannot infect a new Pgl^+ host but can propagate in Pgl^- strains (**Figure 2*b***) (56). Pgl is composed of two closely located operons, *pglWX* and *pglYZ*, encoding proteins with poorly understood roles (57). It is proposed that the Pgl proteins modify the phages released from the first lytic cycle and target them for restriction during the second round of infection. Mutations that result in Pgl^- phenotypes arise with frequencies of 10^{-3} to 10^{-4} per spore, and reversion occurs at similar frequencies (56, 58). Most of the mutations can be mapped to the two *pgl* operons. PglX, a predicted adenine-specific DNA MTase, is predicted to modify the phage DNA during replication in Pgl^+ hosts, allowing ϕC31 to be detected by REase for cleavage and degradation. The expression of *pglX* is phase variable through slipped-strand mispairing (56). An 8-bp polyguanine tract within *pglX* expands and contracts during chromosomal replication and results in frameshifts and mistranslation of PglX. The resulting cells cannot modify ϕC31, and the phage can therefore infect those bacteria effectively.

siDNA: small interfering DNA

Clustered, regularly interspaced short palindromic repeats (CRISPRs): loci containing repeat-spacer units that form the heritable genetic memory of the CRISPR-Cas adaptive immune systems

CRISPR-associated (Cas) proteins: the proteins involved in CRISPR-Cas resistance; they are diverse and are typically encoded near the CRISPR arrays

Bacterial Argonaute. Eukaryotic Argonaute proteins are known to bind and guide small RNAs to complementary transcripts for gene silencing—a process known as RNA interference. Argonaute homologs are abundant in archaeal and bacterial genomes (59). Recently, bacterial Argonaute proteins were shown to act as a barrier for the uptake and propagation of foreign DNA. Despite structural similarity to eukaryotic homologs, bacterial Argonaute drives the DNA-directed interference of foreign genetic elements (60–62). Argonaute of *Thermus thermophilus* (*Tt*Ago) binds to 5′-phosphorylated small DNA fragments (termed small interfering DNAs, or siDNAs), which guide *Tt*Ago to complementary DNA sequences and cause strand cleavage (60). Each *Tt*Ago:siDNA complex causes single-stranded DNA (ssDNA) cleavage. In contrast, Argonaute from *Rhodobacter sphaeroides* (*Rs*Ago) acquires RNA guides from mRNA, represses expression of plasmid-encoded genes, and causes some plasmid degradation (62). The guides of both Argonautes are overrepresented for plasmid sequences (60, 62). Because many phage resistance systems, like RM and CRISPR-Cas systems (see below), also target plasmids, it is tempting to speculate that Argonaute may also provide phage resistance in bacteria.

CRISPR-Cas systems. Clustered, regularly interspaced short palindromic repeats (CRISPRs) and CRISPR-associated (Cas) proteins are widespread in bacteria and archaea and provide adaptive immunity against phages and other mobile genetic elements. The CRISPR arrays form the genetic memory of past infections by incorporating short sequences derived from the invader

genomes (**Figure 4*a***). Resistance is conferred when small CRISPR RNAs (crRNAs), derived from CRISPR array transcripts [precursor crRNAs (pre-crRNAs)], bind to complementary sequences (protospacers) in the invading genome, resulting in their subsequent degradation. For recent in-depth reviews of CRISPR-Cas systems, see References 63–66.

Three steps lead to phage resistance, and the Cas proteins are important for all stages (**Figure 4*a*–*c***): (*a*) adaptation, during which new spacers are incorporated into the CRISPR array; (*b*) crRNA biogenesis, whereby the CRISPR transcript is processed into short interfering crRNA fragments; and (*c*) interference, during which the crRNAs aid the recognition and degradation of the phage target. Currently, CRISPR-Cas systems are classified into three Types (Types I, II, and III) (67). The Cas1 and Cas2 proteins are present in all systems and are required for acquisition of immunity (67–69). Each CRISPR-Cas Type is further divided into subtypes (>11 total) possessing unique combinations of proteins (67). Despite the apparent diversity of the systems, the overall mechanisms are similar and most systems target DNA (although the Type III-B system targets RNA) (70).

In a key study, Barrangou et al. (71) demonstrated that upon phage challenge, *S. thermophilus* developed resistance, which was accompanied by acquisition of new virus-derived spacers. Subsequently, CRISPR-Cas adaptation was detected in other bacteria (reviewed in 72). The most convincing evidence of the evolutionary and ecological importance of CRISPR-Cas systems was provided by metagenomic studies of a variety of niches, which revealed rapid CRISPR evolution during phage exposure (73, 74). The mechanisms by which phage sequences are integrated into the CRISPRs are now becoming clear. In the *E. coli* Type I-E system, spacer uptake requires the Cas1 and Cas2 nucleases, one repeat, and a short AT-rich region preceding the CRISPR array (the leader) (69, 75). The presence of Cas1 and Cas2 in all types suggests mechanistic conservation for CRISPR-Cas adaptation. New spacers are derived from coding and noncoding regions and from either strand of the phage genome and are typically incorporated at the leader end of the CRISPR array (**Figure 4*a***) (76). In Type I and II systems, short sequences of 2–8 nt are adjacent to protospacer targets in the phage genomes; these are termed protospacer-adjacent motifs (PAMs) (77). PAMs are important for spacer selection and for interference, but additional factors can influence the efficiency of spacer acquisition (68, 69, 78, 79). CRISPR-Cas systems appear to preferentially acquire DNA from phages and other mobile genetic elements instead of bacterial chromosomal DNA (69), but the mechanism underlying this discrimination is unclear. When host DNA is occasionally acquired, it can lead to cell suicide due to DNA damage from self-targeting or can result in large-scale bacterial genomic deletions (80).

The guide crRNAs produced from the CRISPR transcript include both repeat- and spacer-derived sequences (**Figure 4*b***) (81, 82). The generation of crRNAs in Type I and III systems typically requires the Cas6-family endoribonucleases (81–84), and most crRNAs have a 5′ repeat handle of 8 nt (summarized in 85). In Type III-A systems, these 5′ handles engage in a base-pairing self/nonself discrimination to avoid "autoimmune" targeting of the CRISPR array that produces the crRNA (86). In contrast, in Type I systems, nontarget avoidance is provided by PAM detection (87). Following crRNA generation in Type I systems, the endoribonucleases stay associated with the 3′ end of the crRNA and form targeting complexes with additional Cas proteins (see below) (**Figure 4*c***) (81, 88–91). In Type III systems, Cas6 is not a part of the interference complex (70, 92–94). Moreover, the crRNA is further processed, resulting in removal of the 3′ repeat remnants (70, 92–95).

Biogenesis of crRNAs in Type II systems is entirely different. First, *trans*-activating crRNAs (tracrRNAs), encoded near the CRISPR, base-pair with the pre-crRNA repeats, and crRNAs are generated by Cas9 and host RNase III (**Figure 4*b***) (96). Cas9, guided by the crRNA-tracrRNA hybrid, is then sufficient for interference by introducing double-strand breaks in the targeted

CRISPR RNAs (crRNAs): small guide RNAs produced following cleavage of the CRISPR array transcript

Precursor crRNA (pre-crRNA): the RNA transcript produced from the CRISPR array prior to cleavage into guide crRNAs

Protospacer: the invader sequence that is targeted by a specific crRNA during CRISPR-Cas interference

Spacers: sequences derived from foreign DNA, such as phages, that are located between repeats in CRISPR arrays

Protospacer-adjacent motif (PAM): a short nucleotide motif adjacent to invader target DNA that is required for CRISPR-Cas adaptation and interference

tracrRNA: *trans*-activating crRNA

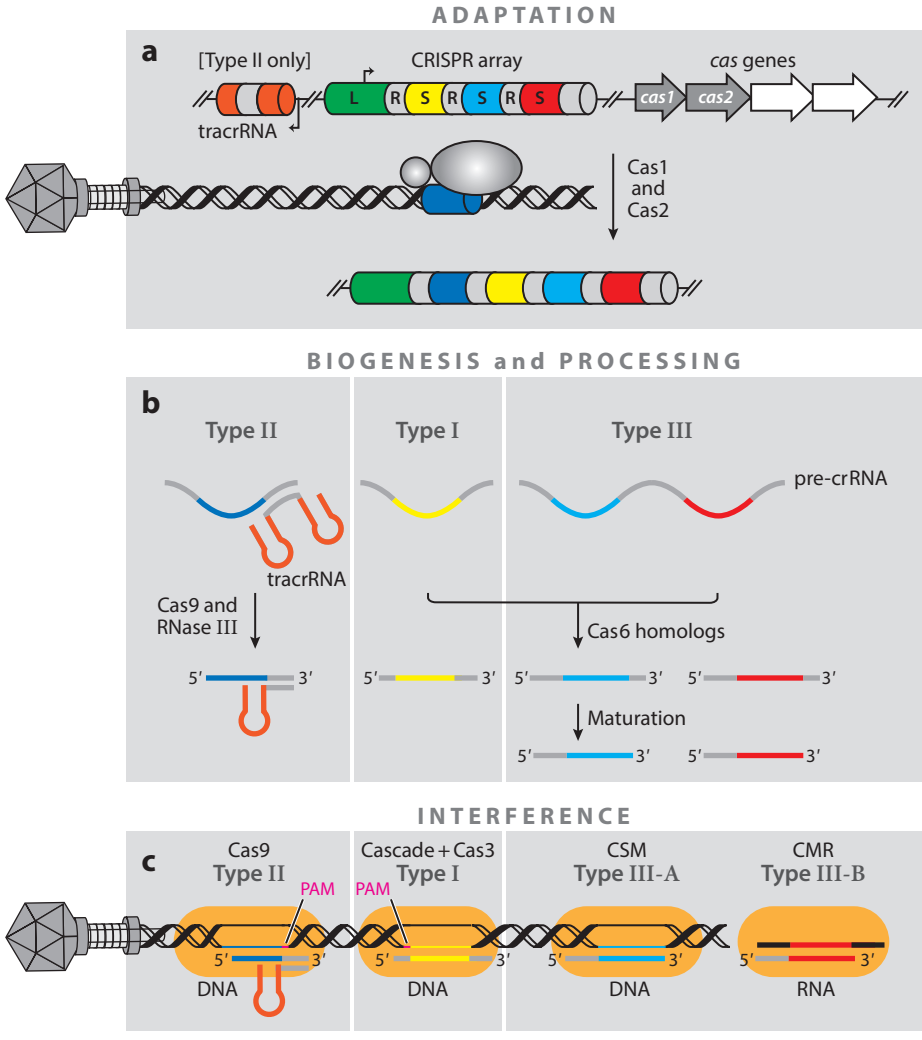

Figure 4

The three different types of CRISPR-Cas systems and their mechanisms for phage interference. CRISPR-based interference is divided into three sequential steps: (*a*) adaptation or the acquisition of phage-derived spacers; (*b*) CRISPR and *cas* expression, CRISPR RNA (crRNA) biogenesis, and processing; and (*c*) crRNA-directed interference. (*a*) The CRISPR array is composed of repeats (R) interspaced by spacers (S) and a leader (L) sequence. Typically, near the CRISPR array is the *cas* operon. Part of the phage DNA is incorporated as a spacer at the leader-proximal end of the CRISPR through the action of Cas proteins. Type II CRISPR-Cas systems also possess a *trans*-activating crRNA (tracrRNA) that aids crRNA processing and interference. (*b*) Once transcribed, the long precursor crRNA (pre-crRNA) is processed into crRNA by Cas proteins and host RNase III (in the Type II systems). (*c*) The crRNA guides the Cas complex to complementary sequences (protospacers) in the phage genome and elicits their degradation. In Type I systems, Cas3 is required for degradation. Next to each protospacer is a protospacer-adjacent motif (PAM; *pink*) important for spacer acquisition and interference.

phage DNA (**Figure 4*c***) (97, 98). A short seed sequence of ~13 nt in the 3′ region of the crRNA spacer is required for efficient target recognition and cleavage (98).

Seed: a short sequence of the spacer that is the most important region for complementary base pairing and CRISPR-Cas interference

In contrast to Type II systems, interference by Type I and III systems requires the formation of a multiprotein complex of the subtype-specific proteins (81, 88–94, 99). Strikingly, all Type I and III complexes share a degree of structural homology, which includes a helical backbone that binds the crRNA spacer portion as well as a large subunit putatively involved in target recognition (recently reviewed in 100). In Type I-E systems, the large subunit protein Cse1 binds phage targets via PAM recognition (101). Subsequently, the target invader DNA is incorporated into the targeting complex via formation of an R-loop (99). In Type I systems, a short seed sequence (8–10 nt) at the 5′ end of the spacer, complementary to the protospacer, is essential for target binding (89, 102). Once the phage DNA is bound, the displaced ssDNA strand leads to recruitment of Cas3, a protein with nuclease and helicase activity that participates in subsequent DNA degradation (103).

One apparent Achilles' heel of CRISPR-Cas immunity is that deletions and point mutations in the protospacer or the PAM region of the targeted phage genome result in evasion of CRISPR-Cas interference (76). However, some Type I CRISPR-Cas systems have a positive feedback loop, termed priming, that allows the rapid uptake of additional spacers upon encounter with an invader that has escaped interference via point mutation (68, 78, 104, 104a). The additional spacers restore phage resistance, and acquisition of multiple spacers further reduces the probability of evasion, because mutation of each target sequence is required (68, 78, 104, 104a). In contrast to naive adaptation (described above), which utilizes Cas1 and Cas2, priming requires the presence of crRNA and the complete set of Cas proteins (68, 104). Surprisingly, even with multiple mismatches (up to 13) between the invader protospacer-PAM region and the priming spacer, rapid primed spacer acquisition occurs, indicating that CRISPR-Cas immunity is more robust than previously thought (105). Whether priming occurs in other CRISPR-Cas types and subtypes is currently unknown (72).

Phages have evolved other strategies to avoid CRISPR-Cas immunity. Anti-CRISPR genes were recently identified in temperate phages that infect *P. aeruginosa* (106). The infectivity of CRISPR-sensitive phages was significantly increased in *P. aeruginosa* lysogens encoding anti-CRISPR genes or in strains expressing any of the anti-CRISPR genes from a plasmid (106). Some phages have acquired other potential anti-CRISPR genes during their evolution, including some encoding regulatory proteins. Metagenomics revealed an H-NS homolog encoded by a virus predicted to infect *Candidatus* Accumulibacter phosphatis. Based on a putative binding site, H-NS was proposed to repress the CRISPR system(s) of the predicted host (107). In the *E. coli* Type I-E system, expression of the CRISPR arrays and *cas* genes is repressed by the H-NS DNA-binding protein (108, 109). Phages also appear to have hijacked CRISPR-Cas systems, potentially to compete with other mobile elements. *Clostridium difficile* prophages contain CRISPR arrays (110), and ICP1-related phages of *V. cholerae* O1 have recently been shown to contain Type I-F CRISPR-Cas systems (111). Spacers in the CRISPR arrays matched a phage-inducible chromosomal island–like element, which is excised upon phage infection and provides viral resistance through an unknown mechanism. Indeed, for productive infection, the phage required the CRISPR-Cas system and spacers targeting the island-like element host (111). In summary, the widespread CRISPR-Cas systems provide a sophisticated resistance strategy against unwanted phage infection. However, phages have also evolved ways to subvert these defenses (13).

Abortive Infection and Toxin-Antitoxin Systems

Bacteria can utilize cell suicide systems to abort phage propagation. The two known phage exclusion systems in bacteria, namely abortive infection and toxin-antitoxin systems, are discussed in the following sections.

Abi: abortive infection

Abortive infection systems in *Escherichia coli*. Abortive infection (Abi) systems block phage propagation at the expense of the infected bacterium. This phage exclusion strategy is broadly categorized as a postinfection defense mechanism, interrupting various stages after DNA injection (**Figure 1**). The infected bacterium does not survive, and few, if any, new phage progeny are released, protecting the clonal bacterial population from viral infection. Therefore, Abis may be considered an altruistic trait for the preservation of bacterial populations. Recent work has shown that these suicide strategies are successful only in the presence of a spatial structure (112). Due to the phenotypic definition of Abi systems, their mechanisms are varied and diverse, but some common themes emerge. In general, Abis are dormant proteins that are activated by phages and elicit cellular inhibition by interfering with essential metabolic processes.

The first Abi system discovered was *rexAB*, which is present in phage λ. Prophage-encoded RexAB in the *E. coli* lysogen excludes T4*rII*-mutant phage (113, 114). The *rII* locus encodes RIIA and RIIB, which allow circumvention of RexAB-mediated phage abortion. However, *rII* does not confer complete protection because overexpression of *rexAB* leads to wild-type T4 exclusion (115). RexA is an intracellular sensor that activates RexB, a membrane-anchored protein containing four transmembrane helices (**Figure 5*a***) (116). A protein-DNA complex, produced as an intermediate

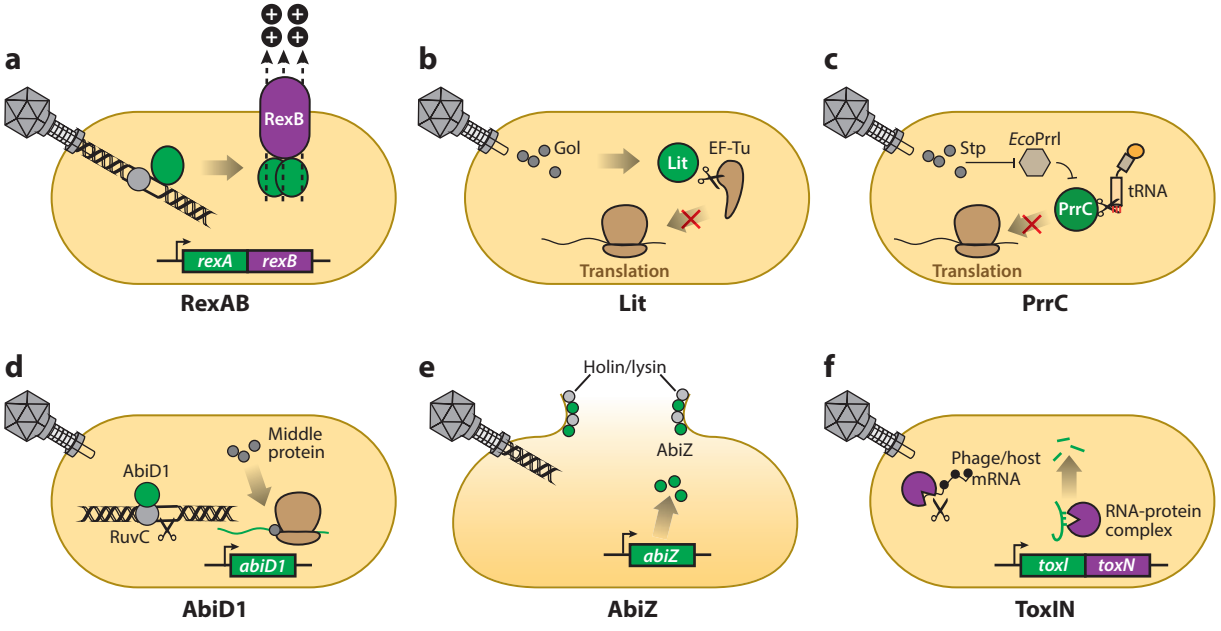

Figure 5

The mechanisms of phage abortive infection (Abi) systems. (*a*) RexAB is activated by a phage protein–DNA complex formed during replication. RexB, once activated by RexA, forms an ion channel that depolarizes the bacterial membrane, causing cell death. (*b*) Lit targets the translational machinery by cleaving elongation factor Tu (EF-Tu), stopping production of phage and host components. A major capsid protein, Gol, activates Lit. (*c*) PrrC also targets the cell's translational machinery. The phage-encoded Stp protein inactivates the Type I restriction-modification system *Eco*PrrI, which inhibits PrrC activity. (*d*) The *abiD1* mRNA is unstable, and AbiD1 is not translated during normal growth. A phage middle protein stabilizes *abiD1* mRNA, therefore activating protein translation. AbiD1 inhibits a phage-encoded RuvC-like nuclease that resolves branched DNA structures formed during phage DNA replication, consequently impairing phage DNA maturation and packaging. (*e*) AbiZ acts with the phage-encoded holin and lysin to fast-track cellular lysis before phage assembly. (*f*) ToxIN is a Type III toxin-antitoxin (TA)-Abi system, whereby an endoribonuclease toxin ToxN is neutralized by the noncoding *toxI* RNA. ToxI is labile and might be preferentially degraded upon phage infection. Free ToxN causes cytotoxicity and inhibits phage propagation by degrading phage and host RNA.

of phage recombination and replication, activates RexA (**Figure 5*a***) (116). Two RexA proteins activate RexB, which forms an ion channel that depolarizes the bacterial membrane (116). The decrease in intracellular ATP leads to bacterial death and consequent abortion of T4*rII* infection. On the *E. coli* lambdoid prophage 933W, the *stk* gene is positioned similarly to *rexAB*. Stk is a tyrosine kinase that inhibits phage HK97 infection. However, whether HK97 resistance is mediated via an Abi mechanism is unclear. Phosphorylation of the Stk protein is activated by Orf41, a protein analogous to the *erf* gene product of phage P22; the Erf recombinase is essential for phage P22 growth on a *rec*$^-$ host (117). It is unknown how phosphorylation leads to phage protection. Another *E. coli* Abi system is PifA, which excludes T7 by using an activity similar to that conferred by RexB. The *pifA* gene is part of the F plasmid *pifABC* operon. PifA is the single gene responsible for T7 resistance, because an F plasmid without *pifBC* still aborted T7 (118). PifA binds the cytoplasmic membrane and contains a predicted Walker-A/P-loop motif involved in ATP/GTP binding (118). Activation of PifA requires the phage proteins Gp1.2 and Gp10 (119), and mutations in *gp1.2* or *gp10* allow phage growth in cells containing the F plasmid (120). Activation of PifA leads to leakage of ATP, through the loss of membrane integrity (121).

Lit and PrrC both achieve phage abortion by inhibiting translation. Lit is encoded by a defective *e14* prophage of *E. coli* K12 (122), and it possesses a zinc metalloprotease domain and cleaves elongation factor Tu (EF-Tu) (**Figure 5*b***) (123, 124). Lit is activated by the major capsid protein Gp23 (or Gol peptide) (**Figure 5*b***) (125). Likewise, *prrC* is carried on a cryptic element in the *E. coli* CT196 chromosome, and it excludes T4 mutants deficient in *rli* (RNA ligase 1) or *pnk* (polynucleotide kinase) (126, 127). PrrC is an endoribonuclease that cleaves $tRNA^{Lys}$ at its anticodon loop (128) (**Figure 5*c***). PrrC is directly inhibited by a Type I RM system *Eco*prrI (129). A 26-amino-acid peptide called Stp (suppressor of the three-prime phosphate) is encoded by T4; it inactivates *Eco*prrI and hence activates PrrC (**Figure 5*c***) (130, 131). Cleavage of EF-Tu and $tRNA^{Lys}$ stops translation of phage and host proteins, causing phage abortion.

Abortive infection systems in *Lactococcus lactis*. *L. lactis*, an important bacterium in the dairy and fermentation industries, contains many Abis, which play a vital role for phage protection in this genus (132). Currently, there are 23 classified lactococcal Abis, consisting of one to three genes, and most of these are plasmid encoded and readily transferred by conjugation.

AbiD1, one of the most well characterized lactococcal Abi systems, excludes lactococcal phage 936 and c2 families (133). The *abiD1* mRNA is unstable and does not increase during phage infection (134). Furthermore, translation of the *abiD1* mRNA is inefficient owing to a stem-loop structure within the translation initiation region, suggesting a *trans*-acting factor might be required for activation (134). A middle protein of phage bIL66 (936 group), ORF1, binds to the translation initiation region of *abiD1* mRNA to stabilize and activate protein translation (**Figure 5*d***) (134, 135). AbiD1 subsequently blocks the phage protein ORF3, a RuvC-like endonuclease, that resolves branched DNA structures during phage DNA replication (**Figure 5*d***) (136). The inhibition of ORF3 stops phage DNA maturation and packaging, resulting in abortion of phage multiplication. Phages escape AbiD1 through spontaneous mutations in their *orf1* gene, which affect Orf1 in its ability to activate *abiD1* mRNA translation (137).

Abi systems such as AbiA and AbiK provide immunity against the three broad families of lactococcal phages (936, c2, and P335). AbiA and AbiK are 23% identical, which indicates that they may be related (138, 139). AbiK contains a reverse-transcriptase motif that catalyzes long, random, nontemplated nucleotide polymerization, analogous to terminal transferases (140); however, how this leads to phage abortion is unclear. Phages escape AbiK through mutations in *sak* (sensitivity to AbiK). Sak belongs to the RAD and Erf family of recombinases, which have ssDNA binding properties (141, 142). These ssDNA-annealing proteins are involved in dsDNA break repair,

which is important during DNA replication and for phage genome circularization following entry to the cytoplasm (140). How Sak activates AbiK-mediated phage abortion is unclear.

A distinct Abi mechanism is conferred by AbiZ, which inhibits phage ϕ31 (P335 group) by inducing premature lysis in response to phage infection. The accelerated killing is mediated by the interaction of AbiZ with the phage-encoded holin and lysin proteins, leading to membrane permeabilization (**Figure 5*e***) (143). By fast-tracking bacterial lysis, the phages have insufficient time to be completely assembled and therefore are unable to initiate further infection.

There is evidence that some Abis are toxic proteins that, upon activation or expression, cause growth inhibition. AbiK, AbiN, and AbiO, when cloned into high-copy-number plasmids or under foreign promoters, could not be maintained in *Lactococcus* (138, 144, 145). In addition, overexpression of AbiD1 and AbiB is toxic to both *E. coli* and *L. lactis* (132, 133). However, not all Abis encode toxic proteins. For those Abis that are toxic, tight control is required to prevent premature cell suicide in the absence of phage infection. Neutralization of toxic Abis can be achieved at transcriptional, translational, and posttranslational levels. Although the transcription of most *abi* genes examined appears constitutive, some are repressed by regulatory proteins. For example, a helix-turn-helix protein Orf4 represses AbiK transcription, whereas AbiU2 downregulates AbiU1 (139, 146). Abi levels might be translationally limited because most contain codons for rare tRNAs (132). The discovery of the ToxIN Abi system in *Pectobacterium atrosepticum* revealed that some Abis are controlled at the posttranslational level by antidotes that specifically neutralize their toxic Abis, forming functional TA systems (147).

Toxin-antitoxin systems. TA systems typically encode a toxin gene, which is preceded by an antitoxin gene, and both are transcribed from a common promoter (148, 149). Toxins interfere with diverse essential cellular processes such as translation (e.g., ToxN, MazF, RelE, HipA), replication (e.g., CcdB, ParE) and cytoskeletal/cell wall formation (e.g., CbtA, PezT). Antitoxins neutralize their cognate toxins in various ways and often regulate the cognate TA operon. TA systems were first discovered on plasmids and enable plasmid maintenance by killing daughter cells that lose the plasmid—a process termed postsegregational killing (PSK) (150, 151). "Addiction" to these systems occurs because antitoxins generally have shorter half-lives than their toxin partners, resulting in rapid turnover of the antitoxin unless it is continuously produced. The unequal stability can exist due to molecular differences (e.g., RNA versus protein) (147), disordered protein folds (152), and vulnerability to host proteases (153). The negative transcriptional autoregulation of TA operons by antitoxins ensures a steady toxin:antitoxin equilibrium whenever antitoxin levels decrease (154, 155). In response to particular stimuli, antitoxins are degraded, and the toxins are liberated to act on their targets, causing either reversible bacteriostasis or cell death. However, reversible bacteriostasis, if not neutralized within a window of time, can also lead to eventual cell death; this phenomenon has been referred to as the point of no return (156).

To date, there are five known types of TA systems, classified by the mode of toxin repression (for a recent review, see 157). Briefly, Type I encodes an antitoxic RNA that is antisense to, and hybridizes with, the TA mRNA. Formation of the duplex dsRNA inhibits toxin translation and promotes TA transcript degradation (158). Types II and III encode proteic (148) and untranslated RNA antitoxins (147), respectively, that directly interact with their cognate toxins for inhibition. Type IV antitoxins interact with toxin targets and catalyze opposing reactions to neutralize toxicity (155, 159). The single Type V antitoxin characterized has a nuclease activity that selectively degrades toxin transcripts (160).

Some TA systems can be activated in response to invading phages and provide phage resistance through abortive infection (147, 155). Many phages hijack host transcription and translation for phage replication. Thus, it was hypothesized that either the degradation of host

DNA or the shutdown of host transcription limits TA synthesis, resulting in free toxin due to faster turnover of the antitoxin (147, 161). The toxin could then induce cell death, with phage multiplication disrupted as collateral damage. The best characterized system for TA-mediated phage abortion is the plasmid-encoded Type III ToxIN system of *P. atrosepticum* (147, 162, 163). The bicistronic *toxIN* locus encodes a cytotoxic endoribonuclease (ToxN), which is neutralized by the repetitive noncoding ToxI RNA through the formation of a heterohexameric RNA:protein complex (**Figure 5*f***) (147, 162). Once activated, ToxN cleaves cellular and phage RNA, stopping phage production and inducing bacteriostasis. ToxIN provides high-level resistance against various phages and is functional in different bacterial genera (147, 163). ToxIN homologs were also identified bioinformatically in many bacteria, including *Bacillus thuringiensis*, *Photorhabdus luminescens*, *Ruminococcus torques*, *Coprococcus catus*, and *Eubacterium rectale*, and some have been shown to possess TA properties (147, 164, 165). Likewise, the ToxN homolog, AbiQ, also acts as a TA system in *Lactococcus* for phage exclusion (166, 167). TA-acting Abi systems also exhibit TA-related phenotypes such as plasmid maintenance (147, 155, 165). Therefore, these modules likely play additional roles in bacteria aside from phage exclusion.

Intriguingly, *P. atrosepticum* ϕTE phage mutants can be isolated that evade ToxIN. Genome analyses of wild-type and mutant phages revealed phage-encoded *toxI* pseudorepeats that were expanded to mimic the host ToxI antitoxin to suppress ToxN activity and inhibit abortive infection (168). In one instance, a ϕTE mutant phage incorporated the full-length *toxI* into its genome through recombination, providing immunity to ToxN (168). Lactococcal phages can also escape the related AbiQ system by acquiring mutations in genes involved in nucleic acid metabolism (169). Thus, phages can utilize several mechanisms to counteract or prevent activation of phage-induced abortive infection.

Other TA systems, such as RnlAB/LsoAB (Type II) and *hok/sok* (Type I), exclude T4*dmd*- mutant and wild-type T4 phage infection, respectively (161, 170). RnlA and its homolog LsoA are endoribonuclease toxins that are inhibited by direct interaction with their cognate antitoxins, RnlB and LsoB (170). The RnlAB and LsoAB systems appear to act as an Abi system against T4*dmd* phage mutants (170). RnlB and LsoB are rapidly degraded following T4 infection (170, 171). Wild-type T4 counteracts RnlA and LsoA to facilitate normal phage infection, because Dmd acts as an antitoxin to directly inhibit RnlA or LsoA toxins (170). Dmd binds and blocks the C-terminal domain of RnlA proposed to be responsible for RNA cleavage activity (172). Deletion of the chromosomal *mazEF* locus, which encodes another Type II TA system, caused increased P1 infection (173). For the *hok/sok* and MazEF systems, it is not clear whether resistance is mediated via an Abi cell-suicide mechanism. Nonetheless, inducing cell death for population-level phage protection is a phenomenon reminiscent of Abi systems.

Given the interest in TA and Abi systems, novel approaches have assisted in identifying new phage exclusion systems (155, 174, 175). Recently, the functional link between Abi and TA systems was further investigated, using lactococcal Abi systems to discover new TA systems (155). This approach led to the identification of the AbiE system as a widely disseminated TA-acting Abi system in bacteria (155). AbiE of *L. lactis* aborted infection of the phage 936 family, preventing DNA packaging through an unknown mechanism (176). The toxin, AbiEii, is a nucleotidyltransferase that catalyzes the addition of GTP to an unknown target to elicit bacteriostasis (155). The antidote protein, AbiEi, represses transcription of the *abiE* operon by binding to a conserved inverted repeat within the promoter and antagonizes preexisting toxin without direct protein-protein interaction via a separable novel domain (155). Antitoxicity is likely mediated at the substrate level (Type IV). The AbiE-related SanaTA system of *Shewanella* was identified through shotgun sequencing, and this also acts as a TA system and protects *E. coli* from T7 phage infection (175). Phage resistance required mutation of the T7 gene *gp4.5*, which

encodes a protein that interacts with the Lon protease (175). Gp4.5 presumably blocks Lon, preventing degradation of the antitoxin and suppressing activation of the TA system.

CONCLUSIONS

In their so-called arms race, bacteria and phages have developed diverse strategies to defend themselves from each other. Bacteria possess antiphage systems that enable resistance to phage infection or actively limit phage growth. The characterization of these defenses and their underlying mechanisms has contributed significantly to basic knowledge of molecular biology and to our understanding of bacterial evolution.

Phage resistance systems in bacteria are normally studied individually, under controlled laboratory conditions and using simple phage-host models. Because phages vastly outnumber bacteria in any ecosystem, multiple antiphage systems likely act in conjunction to restrict a variety of phages. A recent study showed that infection with multiple phages can reveal complex interaction responses for the CRISPR-Cas system that were not observable when examining single phage-host systems (177). Another recent study has shown CRISPR-Cas and RM systems are compatible and together act to increase phage resistance in *Streptococcus* (178). In agreement, Abi systems in *Lactococcus* often cluster with multiple phage resistance systems [e.g., pNP40 (176, 179)] and provide multilayered protection to hosts. Future studies with more heterogeneous models that test the interactions between bacterial defense systems and phages are required to shed light on the complexity of these immune systems and their likely action in nature.

Lastly, phages can rapidly adapt systems to subvert host defenses and can even use horizontal gene transfer to reprogram a population of cells to resist other phages (168). In our quest to exploit phages as antimicrobial agents for biocontrol or treatment of bacterial diseases, it is imperative to elucidate the complex and dynamic evolutionary interplay between phages and their hosts. The molecular mechanisms of many antiphage systems and phage escape strategies still remain elusive, highlighting the need for further study of the interactive subtleties of phage-host relationships.

SUMMARY POINTS

1. The antagonistic relationship between bacteria and phages has led to the evolution of incredibly diverse antiphage systems in bacteria and subsequent evasion strategies in phages to overcome these defenses.
2. Bacteria possess resistance systems that target every stage of the phage infectious life cycle.
3. Surface proteins of bacteria can be mutated, blocked, masked, or phase-variably expressed to prevent phage attachment.
4. A diverse range of restriction-modification systems direct the degradation the nucleic acids of invading phage genomes.
5. Bacterial Argonaute is a recently identified DNA-targeting system that might interfere with phages.
6. CRISPR-Cas systems are highly adaptable immune systems in bacteria and archaea that utilize small memory RNAs to guide protein complexes to phage genomes to elicit their degradation.

7. Abortive infection systems are postinfection modules activated in a virally infected bacterium that result in cell death, which limits viral propagation and spread through the clonal bacterial population.
8. The study of phage-bacterium interactions has contributed significantly to our understanding of biology and has provided major advances in molecular biotechnology.

FUTURE ISSUES

1. Are phage resistance systems regulated (or triggered) in response to phage infection, and if so, how?
2. Can Argonaute systems protect bacteria from phage infection?
3. How do CRISPR-Cas systems discriminate between host and phage genomes during spacer acquisition? Also, how do CRISPR-Cas systems adapt to, and interfere with, RNA phages?
4. What are the molecular mechanisms underlying the various abortive infection systems, and are there commonalities?
5. Do any antiphage systems interact synergistically to reinforce a more robust immunity against phage infection, or are they completely separate lines of defense?
6. How do the various phage resistance systems contribute to the phage-bacterium interplay that occurs in complex ecosystems in natural environments?

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