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Evolutionary Ecology of Prokaryotic Immune Mechanisms

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SUMMARY

Bacteria have a range of distinct immune strategies that provide protection against bacteriophage (phage) infections. While much has been learned about the mechanism of action of these defense strategies, it is less clear why such diversity in defense strategies has evolved. In this review, we discuss the short- and long-term costs and benefits of the different resistance strategies and, hence, the ecological conditions that are likely to favor the different strategies alone and in combination. Finally, we discuss some of the broader consequences, beyond resistance to phage and other genetic elements, resulting from the operation of different immune strategies.

INTRODUCTION

Bacteria are under constant threat by viruses (bacteriophages [phages]) and have evolved multiple immune strategies to combat phage infections (Fig. 1). Phage immune mechanisms can act at different stages of the phage life cycle, including receptor binding, genome injection into the host cell, and intracellular genome replication. First, immunity by surface modification results from masking, mutation, or loss of host receptor proteins, which serve as entry points for the phage. Second, the host can block phage DNA injection or phage replication, known as superinfection exclusion (Sie). Third, injected phage genomes can be cleaved by at least three distinct intracellular immune mechanisms: restriction-modification (RM), the CRISPR-Cas (clustered regularly interspaced short palindromic repeat–CRISPR-associated gene) system, and prokaryotic Argonaute (pAgo). Finally, bacteria

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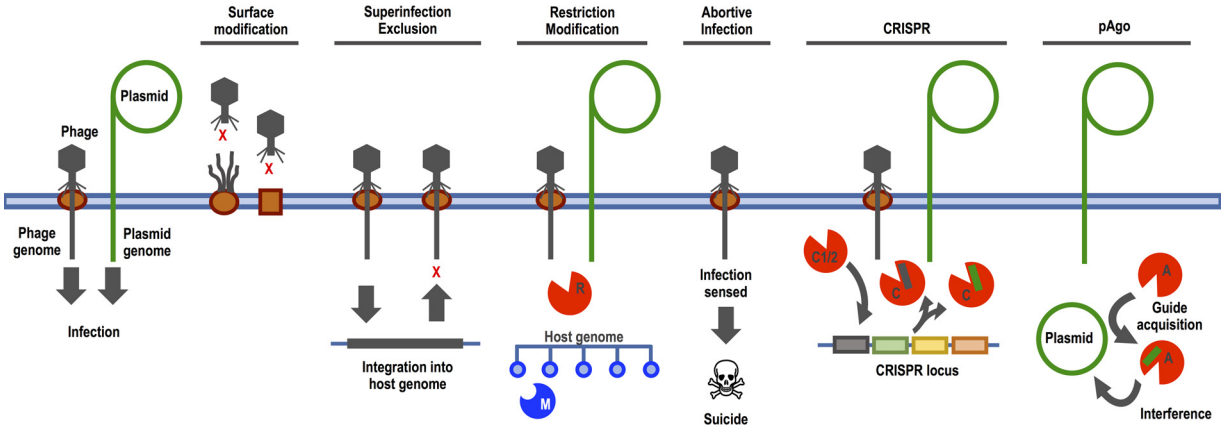

FIG 1 Bacterial immune mechanisms. Letters indicate protein components involved in the immune mechanism (M, methylase; R, restriction enzyme; C1/2, Cas1 and Cas2; C, Cas effector-nuclease complex; A, prokaryotic Argonaute enzyme).

can trigger a suicide reaction upon phage infection, known as abortive infection (Abi), resulting in infection failure. The molecular mechanism of these immune strategies has been the subject of several excellent reviews (see references 1–3) and are reiterated below. Below, we discuss how ecological selection pressures have driven the evolution of diverse bacterial immune mechanisms and the broader ecological and evolutionary consequences of their existence beyond resistance to viruses and other mobile genetic elements. In this review, we use the terms resistance, defense, and immunity interchangeably.

DIFFERENT RESISTANCE MECHANISMS OF BACTERIA

Surface Modification

The initial step of phage infection is the adsorption of a phage ligand (usually tail protein) to specific host surface receptors. To prevent phage adsorption, bacteria can either (i) lose the receptor or downregulate its expression, (ii) mutate the receptor, or (iii) block or mask the receptor.

Receptor loss is commonly seen under laboratory conditions, especially when phages use motility organelles, such as the flagellum or the pilus, to enter the host cell (4, 5) or receptors that are not essential under laboratory conditions, such as the LamB receptor, which is necessary for maltose uptake and hence is dispensable when bacteria are grown in LB medium (6). The loss of these organelles leads to complete resistance but may be associated with a large fitness cost in a natural environment (see below).

Unlike the other defense strategies described below, many examples of resistance mediated by surface modification are the result of mutation and selection rather than an inherent system that confers or predisposes bacteria toward resistance. However, there are examples where bacteria can temporarily downregulate the expression of phage receptors, which may have evolved in response to phage-imposed selection. Some bacteria alter surface receptors through "phase variation," a process in which bacteria vary protein expression depending on environmental conditions (2, 7). By doing so, surface receptors are produced only under specific conditions or upon a specific stimulus so as to limit the risk of phage infection. In addition, some bacteria use quorum sensing to regulate phage receptor expression (8, 9). For example, *Escherichia coli* can reduce the expression of LamB, the receptor for phage λ, in response to quorum sensing signals (8). A recent study on the fish pathogen *Vibrio anguillarum* and its phage, KVP40, showed that quorum sensing is used by bacteria to alternate between two different phage protection mechanisms. At low population densities, *Vibrio* shows high-level expression of OmpK, the main receptor for phage KVP40, but is protected against phage infection by increased biofilm formation. As population densities increase, however, OmpK expression is downregulated through quorum sensing regulation, which renders *Vibrio* almost fully resistant to the phage (9).

Where receptor loss is too costly, receptor mutation, blocking, or masking may be favored by selection. Evidence for frequent receptor mutation follows from increases in the numbers of nonsynonymous mutations in phage receptors (10) and adaptive receptor mutations in response to phage in natural populations (11); a study on the human pathogen *Vibrio cholerae* showed that in response to phage, bacteria acquired point mutations in the outer membrane porin OmpU, the receptor for the lytic phage ICP2 (11). Many phages of Gram-negative bacteria use outer membrane lipopolysaccharides (LPSs) to enter the host cell, which are macromolecules consisting of a lipid and a polysaccharide group. Modification of LPS as a response to phage infection has been observed for a range of bacterial species, including *E. coli* (12) and *Pseudomonas fluorescens* (13).

Receptor blocking or masking occurs when bacterial molecules interfere with phage adsorption. For example, *Staphylococcus aureus* produces a molecule (protein A) that likely prevents phage adsorption by masking of the phage receptor (14). *E. coli* uses the lipoprotein TraT, encoded by the F plasmid, to modify the conformation of the OmpA protein, which is a common receptor for *E. coli* phages (15). The production of extracellular matrices can provide protection against phages when they form a physical barrier between the phage and the receptor. A well-known example is the production of alginate by pseudomonads. This polysaccharide causes a mucoid colony morphology that is associated with phage resistance (16).

Superinfection Exclusion

Superinfection exclusion (Sie) prevents the entry or replication of phage DNA, but as its name suggests, it is a resistance mechanism encoded by the infecting phage. Sie is commonly achieved by interfering with the injection or replication of a

superinfecting phage through alterations to the cell surface or repression of replication, both of which are discussed below (reviewed in reference 1).

Relatively well-studied Sie systems include the *cor* gene, expressed by *E. coli* prophages F80 and N15 (17). This gene blocks DNA injection of related superinfecting phages by inactivating the ferrichrome uptake protein FhuA, which serves as a phage receptor (18). Another Sie system is the *gp15* gene carried by the temperate *E. coli* phage HK97, which blocks the DNA entry of the lytic phage HK97 and the closely related phage HK95, presumably by the insertion of the Gp15 protein in the *E. coli* inner membrane, where it interacts with phage tail proteins (19). Further Sie systems include the *ltp* gene of the *Streptococcus thermophilus* temperate phage TP-J34. This gene encodes a lipoprotein that inhibits DNA release into the host cell during infection with a lytic phage, presumably by targeting the phage-encoded tape measure protein (TMP), which is necessary for channel formation to allow DNA passage into the cell (20, 21).

Pseudomonas aeruginosa prophage D3 mediates Sie through modification of the O-antigen of LPS on the host surface (so-called "seroconversion," as this modification changes the host serotype). Many phages require the O-antigen to attach to the host cell and establish a successful infection, which is inhibited by this Sie mechanism (22). This is analogous to the mechanism of another phage-encoded protein (twitching-inhibitory protein [Tip]) that modifies the type IV pilus on the surface of *P. aeruginosa*. As many *Pseudomonas* phages require type IV pili for successful infection, this protein may represent a general Sie strategy (23, 24).

Although the majority of Sie systems appear prophage encoded, they are also carried by some lytic phages, such as the *E. coli* phage T4, which encodes one of the best-characterized Sie systems to date. This Sie system consists of the Immunity (Imm) and Spackle (Sp) proteins, which act independently and through distinct modes of action. The Imm protein blocks the translocation of phage DNA into the cytoplasm by altering the conformation of the DNA injection site on the host membrane. Sp is a membrane protein that inhibits the activity of T4 lysozyme that is contained in the phage tail and functions in creating holes in the peptidoglycan layer to facilitate phage DNA injection (reviewed in reference 25).

Apart from mechanisms that interfere with phage DNA injection, prophages can also confer repressor-mediated immunity. Repressor proteins silence phage genes in order to maintain cell viability during the lysogenic life cycle (26). These repressors bind specific DNA sequences located in intergenic regions on the phage genome, and phages vary with respect to the specificity of repressor-DNA interactions (27). A prophage can provide immunity to a phage that carries a repressor with the same specificity, as this will result in the blocking of the lytic cycle of the superinfecting phage. The phage may also capture repressor genes from an unrelated phage, presumably in order to confer immunity to superinfection (27).

Restriction-Modification

Restriction-modification (RM) systems are diverse and widespread immune mechanisms that function by cleaving nonself, unmodified DNA, while modified self DNA is left untouched. The majority of RM systems consist of two components: an enzyme that methylates DNA (methyltransferase [MT]) and an enzyme that cleaves unmethylated DNA (restriction endonuclease [RE]) by recognizing specific DNA sequences (restriction sites). Self-cleavage of the host genome is prevented by MT-catalyzed methylation of chromosomal restriction sites. Based on subunit composition and biochemical characteristics such as protein structure, restriction site recognition, cofactor requirements, and substrate specificity, RM systems are classified into four different types (types I to IV) (28). Type I systems encode a protein complex that contains restriction (HsdR), modification (HsdM), and specificity (HsdS) subunits. Unmodified target sequences trigger the DNA translocation activity of HsdR, while HsdS remains bound to the recognition sequence, leading to loop formation in the DNA. Cleavage occurs when two complexes collide, and the cleavage site can therefore be tens of thousands of base pairs away from the actual recognition site. The majority of the type II RM systems contain separate REs and MTs. Type II REs are typically homodimers or homotetramers that cleave DNA at or very close to their recognition site. These type II REs represent the restriction enzymes that became a crucial tool for DNA analysis and cloning (29) and are therefore by far the best-studied REs. The type III RM systems encode a protein complex that is usually a heterotrimer of the RE and MT (Res_1Mod_2) (30), and cleavage requires two recognition sites that are inversely oriented with respect to each other. Type IV systems are very different from the others, as they cleave only DNA sequences that have been modified (methylated, hydroxymethylated, or glucosyl-hydroxymethylated) and appear to have evolved independently. Based on the sequence homology, codon usage, and GC content of RM systems, it has been hypothesized that high levels of horizontal gene transfer (HGT) have contributed to the evolution and spread of RM systems (31–33). The effectiveness of RM systems in host protection against phage infection has been demonstrated in various studies reporting 10- to 10^8-fold protection against phage infection (reviewed in reference 34). Interestingly, the expression of many RM systems is phase variable (35–38), which has important evolutionary implications, which are discussed below.

CRISPR-Cas Systems

CRISPR-Cas systems are the adaptive immune systems of bacteria and archaea. Their molecular mechanism has been extensively reviewed elsewhere (39–43). Here we briefly explain the mechanism of these different variants of the system.

CRISPR-Cas systems consist of CRISPR-associated (*cas*) genes and CRISPR loci. *cas* genes encode the protein machinery that carries out the immune response. CRISPR loci consist of invader-derived sequences separated by direct repeats and provide a genetic memory of previous infections. CRISPR-Cas systems are extremely diverse and are currently classified into 2 distinct classes, 6 types, and 16 subtypes based on phylogeny, *cas* gene composition, and CRISPR sequences (44, 45). Despite differences, all systems rely on the same basic principle of spacer acquisition from foreign DNA followed by integration of these sequences into CRISPR loci on the host genome (adaptation). Next, CRISPR loci are transcribed, and the resulting RNA molecule is processed to generate mature CRISPR RNA (crRNA), which forms a complex with one or more Cas proteins (expression). Finally, crRNA-Cas complexes bind and cleave complementary nucleic acids (in some cases, this step involves additional Cas nucleases), resulting in host immunity (interference).

Class 1 CRISPR-Cas systems. Class 1 systems encode multisub-

unit crRNA-Cas complexes and can be further subdivided into type I, III, and IV CRISPR-Cas systems. The latter awaits molecular characterization. Type I systems are characterized by the nuclease/helicase Cas3 and encode a Cascade (CRISPR-associated complex for antiviral defense)-like complex (46). Type III systems are typified by the presence of Cas10. In agreement with the phylogenetic relationships between type I and type III systems (44, 47, 48), their associated multisubunit crRNA-Cas complexes share key structural features (39, 49–51). Adaptation in type I systems requires only Cas1 and Cas2 (52), which form a heterotetrameric complex that binds to the leader end of the CRISPR array to catalyze the integration of spacers (53–57). During "expression," the CRISPR is transcribed, followed by Cas6-mediated cleavage, to yield mature crRNA molecules (46, 58). Cas6 is a subunit of Cascade, which consists of multiple Cas proteins and a single crRNA molecule (59). During the "interference" stage, Cascade binds the genome of an infecting parasite and marks it for destruction by the Cas3 effector nuclease (60–65). The affinity of target DNA binding is strongly increased if the target sequence (protospacer) is flanked by a protospacer-adjacent motif (PAM) (60, 66, 67). As the PAM is absent from the CRISPR loci on the host genome, it serves to avoid autoimmunity problems (68). Some type III systems target both single-stranded RNA and transcriptionally active DNA (69–77). This RNA cleavage is important when DNA cleavage is delayed, for example, due to mismatches or because the target gene is expressed late during the phage life cycle (75). Type III-A systems also have a PAM-independent self/nonself discrimination mechanism, which relies on the inhibition of CRISPR interference when target sequences are flanked by CRISPRs (78), which avoids self-targeting of CRISPR loci on the host genome.

Class 2 CRISPR-Cas systems. Although Class 2 systems are less common than class 1 systems (44, 79), they have received much more attention recently due to their application in genome editing. Class 2 systems are uniquely suited for this application since a single protein carries out all functions of the multisubunit crRNA-Cas complexes of class 1 systems. Class 2 systems can be subdivided into type II systems (44), which encode the Cas9 enzyme that is presently widely used for genome editing (80, 81); type V systems, which encode the Cpf1, C2c1, or C2c3 effector enzyme (44); and type VI systems, which encode the C2c2 effector enzyme (45). Type II systems require Cas1 and Cas2 for spacer acquisition as well as Cas9 for PAM specificity (82). The expression stage requires a *trans*-encoded crRNA (tracrRNA) molecule that pairs with pre-CRISPR RNA repeat sequences, which, in the presence of Cas9, triggers RNase III-mediated cleavage in the resulting stretch of double-stranded RNA (83). The tracrRNA remains bound to the processed crRNA and forms an essential component of the tracrRNA-crRNA-Cas9 effector complex (84). During interference, the effector complex binds the double-stranded target molecule (85), followed by PAM-dependent Cas9-mediated cleavage (85). Type V and VI systems do not encode Cas9, and their molecular details of expression and interference are distinct from those of type II systems. For example, Cpf1 and C2c1 lack a requirement for tracrRNA during the expression and interference stages (45, 86). Cpf1 also has different PAM requirements and yields different cleavage products (87).

pAgo

Argonaute (Ago) proteins are present in all domains of life and are key enzymes of the RNA interference (RNAi) pathway in eukaryotes (88). In eukaryotes, Ago plays a key role in a range of cellular functions, including gene regulation and host defense (88). Ago proteins are part of the PIWI (P-element-induced wimpy testis) protein superfamily. In addition to the PIWI domain, Ago proteins contain the N-terminal domain (N domain), the PAZ (PIWI-Argonaute-Zwille) domain, and the MID (middle) domain, and these domains are linked together by two linkers, L1 and L2. Eukaryotic Ago proteins interact with RNA molecules of well-defined lengths (ranging from 20 to 30 nucleotides [nt], varying between different paralogs), and these RNA molecules function as guides for Ago to bind (and sometimes cleave) cRNA sequences (RNA-guided RNA interference).

The discovery that Ago is also found in prokaryotic genomes (89, 90), together with the finding that prokaryotic Ago (pAgo) often colocalizes with other defense genes, led Makarova and coworkers to hypothesize that pAgo comprises a prokaryotic defense system (91). Prokaryotes have both long pAgo proteins, which carry the same domains as their eukaryotic counterparts, and short pAgo proteins, which consist of only the MID and PIWI domains (92). Of the long pAgo enzymes, only 28% are predicted to be catalytically active, and inactive enzymes often colocalize with other nucleases that may carry out target cleavage (92). Interestingly, many pAgo proteins preferentially bind DNA guides rather than RNA guides (93–96), although pAgo from *Rhodobacter sphaeroides* was found to associate with RNA guides (97). These guides are typically 15 to 19 nt long and have a well-conserved 5′ nucleotide (96, 97). Both the DNA-guided pAgo proteins from *Thermus thermophilus* and *Pyrococcus furiosus* and the RNA-guided pAgo protein from *R. sphaeroides* were reported to interfere with plasmids (96–98), and Ago deletion mutants resulted in increased plasmid gene expression (97) and plasmid transformation efficiencies (96, 98). *In vitro* analyses revealed that both the *P. furiosus* and *T. thermophilus* pAgo proteins carry out DNA-guided DNA cleavage (96, 98). DNA-guided *T. thermophilus* pAgo, but not *P. furiosus* pAgo (98), could also cleave cRNA *in vitro* (96), but the enzyme does not appear to interfere with mRNA *in vivo* (99). Like *P. furiosus* pAgo, *Methanocaldococcus jannaschii* pAgo is also unable to cleave cRNA (100). Hence, a picture emerges in which pAgo enzymes are generally (but not always) guided by short DNA molecules and typically interfere with cDNA by either pAgo-mediated cleavage or, possibly, the recruitment of additional nucleases if pAgo lacks catalytic residues. The enzymatic activity seems to be directed primarily against plasmids. Many key questions remain concerning the mechanism of pAgo, most prominently how the enzymes discriminate self from nonself and how guide DNA molecules are acquired from plasmid targets.

Abortive Infection

Abortive infection (Abi) systems cause programmed cell death of an infected bacterium, thereby preventing phage replication and, thus, phage spread to neighboring uninfected cells. As such, abortive infection is essentially an altruistic response to phage infection; the infected cell will die, but the rest of the bacterial population is likely to survive as phage spread is aborted (101). Several Gram-negative strains carry Abi systems, of which the rapid II (rII) exclusion (Rex) system found in phage λ-lysogenic *E. coli* strains is probably the most well-characterized one (1). Rex systems consist of two proteins, RexA and RexB, both of which are needed for phage protection. Upon infection, phage protein-DNA complexes are produced as replication intermediates that activate

the intracellular sensor molecule RexA. Subsequently, RexA activates the membrane-anchored ion channel protein RexB. RexB activation causes a sudden drop in the cellular ATP level, thereby aborting ATP-dependent processes, including virus replication (102). Another Abi system is the late inhibitor of T4 (Lit) system, which is found in a defective prophage integrated into the genome of the *E. coli* K-12 strain. Upon activation by phage protein, Lit cleaves a translation elongation factor, thereby inhibiting protein synthesis (102, 103). This finally leads to abortion of virus infection and bacterial cell death. Abi systems are highly abundant in Gram-positive bacteria, in particular in the lactococci, where they are commonly encoded on plasmids (101, 104). To date, over 23 lactococcal Abi systems have been identified, all of which are thought to interfere with different steps of the phage replication cycle (1, 101). Recent studies have shown that certain toxin-antitoxin (TA) systems can also act as Abi systems upon activation by phage infection (104–106). For example, the plant-pathogenic bacterium *Pectobacterium atrosepticum* encodes the TA system ToxIN, which aborts phage infection. This mechanism functions through the action of the RNA antitoxin ToxI and the endoribonuclease toxin ToxN, whereby ToxI neutralizes ToxN under normal conditions (101, 107). However, upon phage infection, host gene expression is arrested, and as the antitoxin is less stable than the toxin, ToxI levels drop more rapidly than do ToxN levels. This causes ToxN activation, leading to the induction of cell death. Abi-inducing TA systems have also been identified in *E. coli* to prevent infection with phage T4 (108) and phage P1 (109). As TA systems are extremely widespread in bacteria, and only a few have been studied in detail, it is anticipated that many more TA systems that function through an Abi mechanism will be identified in the future.

SELECTIVE FORCES DRIVING THE EVOLUTION OF IMMUNE MECHANISMS

Why did bacteria evolve these different immune mechanisms? Despite the progress in unraveling the mechanism of bacterial defenses, we know relatively little of the selection pressures that drive their evolution. Studying the evolutionary ecology of immune mechanisms is important if we are to understand, predict, and manipulate bacterial adaptation to phage infection.

Is More Immunity Best?

Is it simply the case that having multiple defense mechanisms is best, analogous to vertebrate immune systems that are composed of both innate and adaptive mechanisms? Consistent with this idea, RM and CRISPR-Cas systems frequently cooccur (33), and their combination results in increased levels of immunity (110) and more rapid spacer acquisition (111). Moreover, immune mechanisms may even interact synergistically. For example, transcriptome analyses show that pAgo deletion impacts the expression levels of CRISPR-Cas components (99), suggesting that these systems may show genetic interactions, and synergistic interactions between Abi and CRISPR systems have been suggested based on data from *in silico* analyses (112).

However, if it is better to have multiple mechanisms, why is it that not all bacteria have all mechanisms? There are two possibilities: either not all mechanisms have evolved in all organisms yet or immune mechanisms are associated with a fitness cost that can select against the maintenance of the system. We can rule out the first explanation, since there are many examples of the presence and absence of mechanisms in closely related bacteria where immune mechanisms have been gained and lost or inactivated (see, for example, references 113–116). Fitness costs associated with immune mechanisms are therefore a more probable explanation as to why not all bacteria have all mechanisms.

Fitness Costs of Immunity

For obvious reasons, host immunity confers a clear selective advantage in the presence of parasites. However, investment in immunity is also typically associated with fitness costs and can therefore be selected against in the absence of parasites (117). Such costs of resistance appear to be very general among both prokaryotes and eukaryotes (reviewed in references 118 and 119). Fitness costs associated with immune systems can arise due to immunopathology (e.g., autoimmunity); due to an allocation of resources to defense, which would otherwise be used to increase reproductive success; or due to pleiotropic effects of resistance that decrease host fitness, as is often the case for surface modification (120).

Surface modification. Surface modifications are typically associated with a constitutive fitness cost (i.e., a fixed cost that is independent of the presence or absence of phage). The cost of losing surface receptors, such as the flagellum or pilus, is likely to be lower in laboratory settings, where nutrients are readily available and broth is continuously mixed, than in natural environments. For example, pili and flagella are associated with key bacterial functions such as movement (swimming, swarming, and twitching) and biofilm formation (121), and the loss of these receptors can have a high competitive cost in spatially structured and resource-limited environments (122, 123). Moreover, modification of surface receptors such as LPS can also result in a reduction of fitness in some contexts (120).

Superinfection exclusion. Fitness costs associated with Sie mechanisms have not been studied in great detail. One *P. aeruginosa* prophage was found to encode a Sie system that had a subtle effect on host motility and was cost-free during growth in soil and during infection of a *Caenorhabditis elegans* host (124). Prophage integration into the host genome may be associated with a constitutive fitness cost if host genes are disrupted (e.g., tRNA genes), but this cost may be compensated for by phage-borne tRNA genes and other accessory genes that confer a benefit to the host (125, 126).

Restriction-modification. A recent study investigated fitness costs associated with RM and found that increased levels of SOS responses were elicited, suggestive of autoimmunity effects (127). Furthermore, the fitness cost of carrying RM systems was manifested under conditions of low but not high resource levels (127), which is often observed for parasite resistance (123, 128). In agreement with autoimmunity, bacterial genomes harboring RM systems generally have a decreased frequency of the cognate restriction site (129, 130). This phenomenon, named restriction site avoidance or palindrome avoidance, is even more pronounced in host than in phage genomes (129). Palindrome avoidance itself may also be associated with a fitness cost to the bacterium, as it could affect gene functioning through mutations. In addition, some (but not all) RM systems are extremely energy-consuming, with RE translocation over the DNA consuming 1 ATP per base pair (131). Whether ATP consumption provides a benefit (e.g., through more rapid detection or destruction of phage genomes) is unknown. Phase-variable expression of many RM systems may help to reduce the associated fitness costs of carrying these systems.

CRISPR-Cas. The class 2 CRISPR-Cas system of *S. thermophi-*

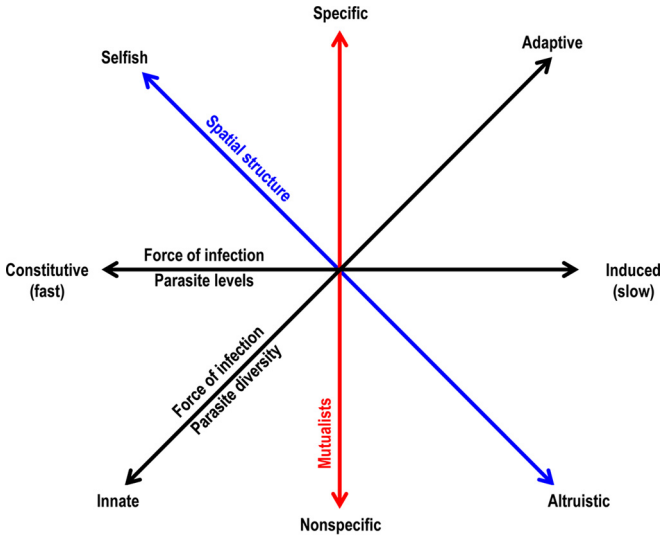

FIG 2 Four-dimensional space defined by the four axes that capture different features of immune mechanisms is sufficient to explain the existing diversity of immune mechanisms in nature. The ecological factors indicated drive the evolution of the feature indicated on the corresponding axis. Details are provided in the text.

lus was found to be associated with both a constitutive cost of carrying the system as well as an inducible cost of using the system (132). An inducible cost of mounting a CRISPR immune response was also detected in *P. aeruginosa* (133). The inducible cost is consistent with the induced expression of CRISPR-Cas adaptive immune systems upon infection (134–136). A constitutive fitness cost of CRISPR-Cas systems could select for a loss of the systems in the absence of parasites (137). At present, the mechanistic basis for the observed fitness cost associated with the CRISPR-Cas system is unclear but may be related to autoimmunity (138–144) or allocation of resources to defense that would otherwise be invested in growth.

Abortive infection. Abi was found to be associated with a constitutive cost of carrying the system (145), but the mechanistic basis of this cost is unknown. Moreover, there are inevitable individual costs associated with suicidal behavior following the induction of the Abi system.

Costs of Resistance Can Help To Explain Diversity in Immune Mechanisms

One important feature of immune mechanisms is whether they are constitutive (always active) or inducible (triggered upon infection) (Fig. 2). Inducible defenses are typically associated with an induced cost of resistance, whereas constitutive defenses are associated with a fixed cost. As a consequence, the force of infection is predicted to be a key ecological factor driving the evolution of these different immune strategies, since the overall cost of an inducible resistance strategy will depend on the frequency of infection (146). Recently, it was demonstrated that the force of infection can tip the balance from CRISPR immunity to surface modification immunity (133), and this was explained by the CRISPR-Cas system and surface modification being associated with inducible and fixed costs of resistance, respectively. Hence, depending on the risk of infection, either the CRISPR system or surface modification is favored (Fig. 3). Consistent with the idea that the CRISPR system is better when the risk of infection is low, thermophiles tend to have more and longer CRISPRs (147, 148) and have lower host and parasite population densities (149). Similar effects are expected in the context of other immune mechanisms that have inducible costs.

How Does Symbiont Diversity Contribute to Immune Diversity?

A second factor that is likely to be important in the evolution of immune strategies is the level of diversity in mobile genetic ele-

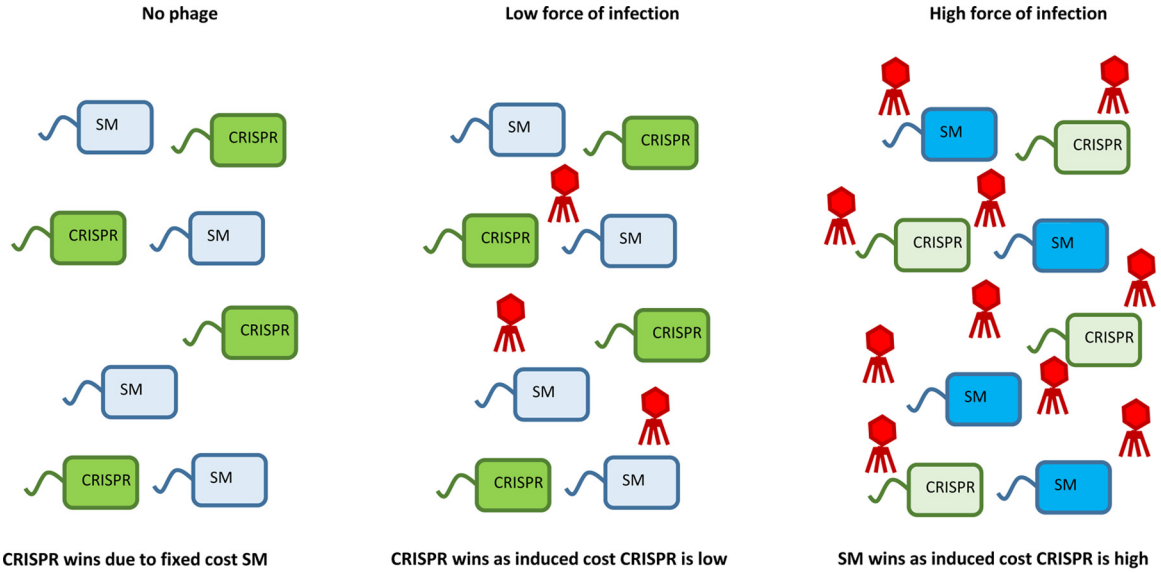

FIG 3 The force of infection is an important determinant of the relative fitness associated with CRISPRs and surface modification (SM). In the absence of phage or at a low force of infection, CRISPRs are favored over SM, since the latter is associated with a fixed cost of resistance. At high phage exposure, SM is favored over the CRISPR, because the latter is associated with an inducible cost of resistance that increases with an increasing force of infection. Empirical support for this was reported previously (133).

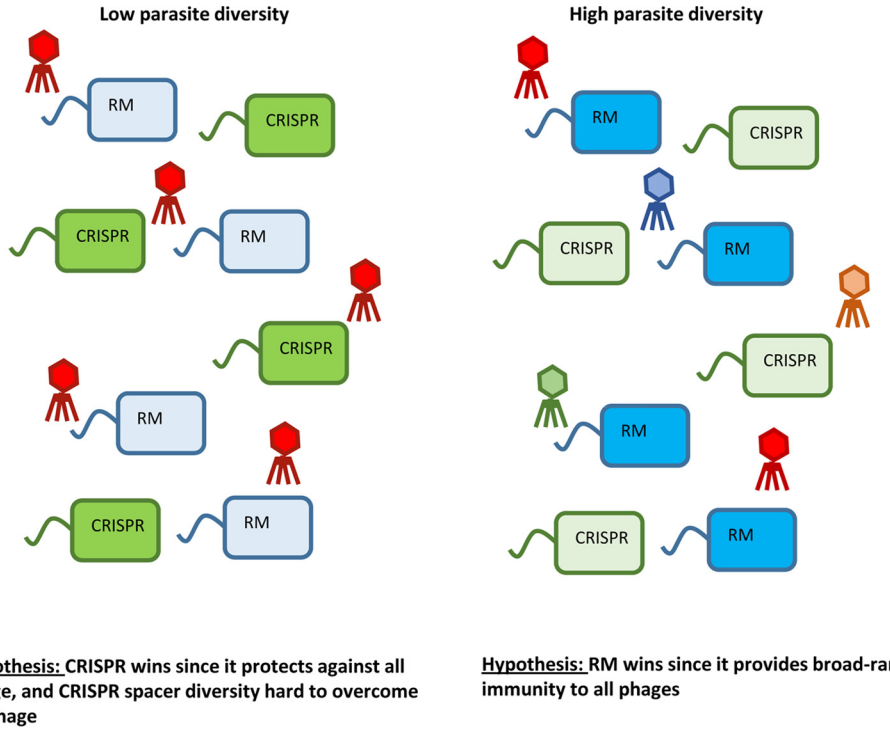

FIG 4 Although empirical support is currently lacking, it may be the case that increasing phage diversity can select for broad-range innate immune mechanisms, such as RM, over specific adaptive immune systems, such as the CRISPR-Cas system. While the CRISPR system is extremely effective if there is low genetic variation in the phage population, theory predicts that the system becomes less effective if the host is exposed to a phage population with high levels of genetic diversity (149).

ments (here broadly referred to as symbiont diversity). Diversity of symbionts may drive diversity of host immune mechanisms in at least two ways. First, parasite diversity can drive the evolution of a division of labor, whereby different immune mechanisms coexist in the same bacterium but each one is effective against different parasites. Anecdotal evidence suggests that this may be the case. For example, surface modification is generally more effective against phages than plasmids, although mucoid phenotypes were found to confer protection against a virulent plasmid (150). The RM and CRISPR-Cas systems can target both phages and plasmids (76, 151–154), and pAgo appears to predominantly target plasmids (96), while Abi is typically induced by phages. Second, symbiont diversity is likely to also impact the evolution of stand-alone immune strategies, in particular along the innate-versus-adaptive-immunity axis and the specific-versus-nonspecific-immunity axis (Fig. 2). Common wisdom suggests that an adaptive immune system is particularly beneficial if a host is exposed to an unpredictable range of different parasites, since it allows the acquisition of immunity to all these parasites. Exposure to many parasites could also select for nonspecific immune mechanisms (Fig. 4); however, such indiscriminate, generalized immune strategies are likely to interfere with host-mutualist interactions (e.g., acquisition of plasmids that encode antibiotic resistance). Hence, the presence of mutualists may impose selection on the evolution of specific versus nonspecific defenses (Fig. 5) (see the section on mutualists, below).

Diverse pathogens: intraspecific diversity. Genetic diversity in phage populations is predicted to drive the evolution of generalized defense, since phage mutants readily evolve to overcome specific defenses (137, 149). For example, it has been shown that phage can rapidly evolve to overcome CRISPR-Cas-mediated immunity by point mutation, resulting in invasion by surface mutants (155, 156). However, phage was unable to evolve infectivity in bacterial populations with high levels of CRISPR allele diversity (156). Yet, the outcome may depend on the relative levels of phage and host genetic diversities; the diversity-generating benefit of CRISPR-Cas may be more limited at increased levels of phage diversity (149), which may therefore favor more broad-range (nonspecific) defenses.

RM systems provide nonspecific immunity but are also prone to phage evolving to overcome resistance, which typically occurs through an "accidental" modification of the phage genome before cleavage by the restriction enzyme. To deal with rapid phage evolution, some RM systems also generate diversity in the specificity subunits through recombination (157, 158). As with CRISPRs, this diversity-generating property of these RM systems may limit the evolution of phage to overcome host resistance. In addition, phase-variable expression of RM systems (35–37) may also help to limit the evolution of escape phage, since infection of a bacterial clone that has the RM system switched off will result in phage progeny that are not modified and therefore subject to RM of related bacteria in the population.

Some forms of broad-range (nonspecific) defense, such as surface modification by receptor loss, seem harder to overcome by phage, but even then, phage can evolve infectivity against initially resistant hosts through the recognition of novel receptors (6). Some phages even carry mechanisms that specifically generate diversity in genes involved in host receptor recognition (159, 160).

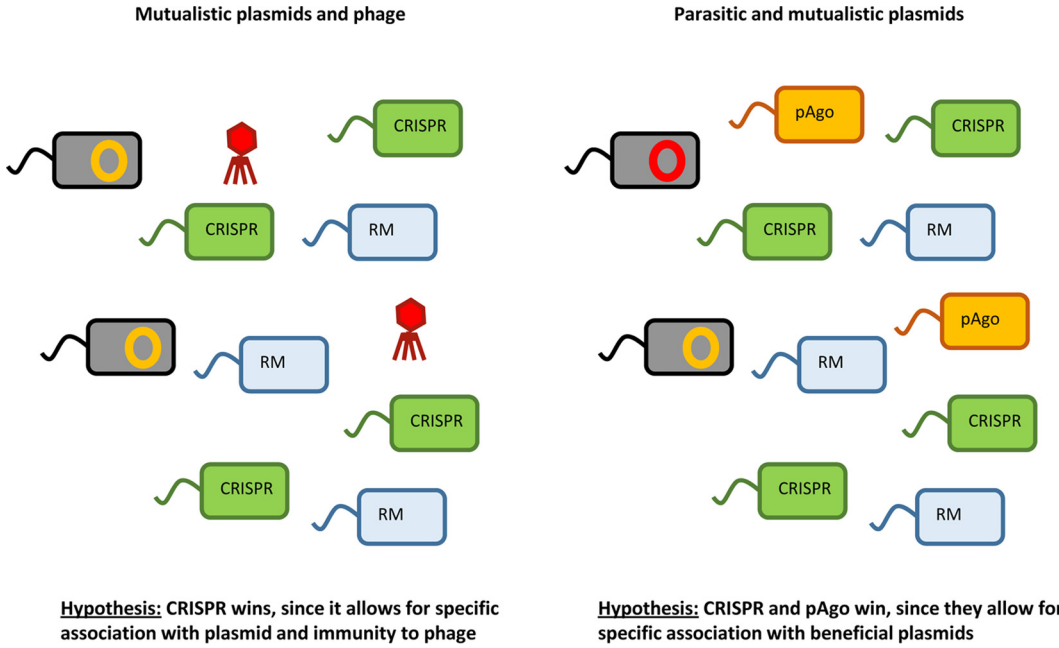

FIG 5 Mutualists (e.g., plasmids that confer a fitness benefit to the bacterial host) can select against immune mechanisms (177). If both mutualists and parasites are present (e.g., plasmid and phage [left] or beneficial and harmful plasmids [right]), CRISPRs and pAgo may be beneficial since their specificity allows bacterial hosts to specifically acquire resistance against the parasite. Empirical data to support this idea are currently lacking.

These diversity-generating retroelements (DGRs) function through a reverse transcriptase-mediated process that introduces adenine-specific substitutions in a gene that encodes a distal tail fiber protein. The DGR allows the phage to rapidly adapt to the rapidly changing host surface structures associated with phase variation (159, 160). Similar DGRs have since been discovered in a range of phages (161) and in archaeal viruses (162).

Diverse pathogens: interspecific diversity. While intraspecific diversity may be neutralized by a sufficiently high diversity of host resistance alleles, interspecific diversity (i.e., many different viruses) is likely to select for broad-range innate immune mechanisms, such as RM, Abi, and Sie, which typically provide protection against a range of phages (124, 163) (Fig. 6). Surface modification can also provide broad-range immunity against multiple phages, but the range of immunity depends on the type of modification and the phage receptors involved; for example, a point mutation in a receptor is likely to be associated with a lower fitness cost than the complete loss of the same receptor but is also more likely to provide relatively narrow-range resistance against a single or few phage species compared to receptor loss. In agreement with this, evolution of broad-range immunity has been shown to be more costly than narrow-range immunity (164), but the mechanistic basis of immunity in these experiments was not assessed. In some cases, surface modification-based resistance against one phage can increase susceptibility to other phages, as is the case for the cyanobacterium *Prochlorococcus* (165).

The benefit of the CRISPR-Cas system in the face of diverse viruses is unclear. On the one hand, an adaptive immune system allows a host to adapt to many different threats. On the other hand, the specificity of adaptive immune systems means that there is little cross-resistance. Theory and metagenomics data suggest that in the context of two different viruses, CRISPR-Cas systems may be associated with selective sweeps in host populations if a single host genotype acquires resistance against two phages that are present in the environment (166, 167). Consistent with theory (167, 168), experiments suggest that multiple phages prolong CRISPR-virus coevolution (144). Furthermore, it was found that viruses can escape CRISPRs through recombination (144, 169).

Mutualists. Apart from inter- and intraspecific parasite diversity, the relative importance of mutualistic DNA elements is also likely to impact the relative benefits of different immune strategies. For example, accessory genes carried by plasmids can confer a fitness benefit to the host (170–173), and specific immune mechanisms, such as the CRISPR-Cas system or pAgo, which can selectively evolve immunity against parasites but not mutualists, are expected to be favored over broad-range defenses such as RM (174), which can form an important barrier for gene transfer (175) (Fig. 6). However, phase variation of RM systems may allow the uptake of beneficial DNA by bacteria in which the RM system is switched off (35). That said, there is some evidence that even the CRISPR-Cas system may restrain horizontal gene transfer. First, both in the laboratory and in nature, bacteria evolve CRISPR immunity against plasmids (76, 154), but this may be due to the fact that plasmids can act as parasites. Second, correlational studies demonstrate that the CRISPR-Cas system limits horizontal gene transfer of antibiotic resistance genes (176). It has been suggested that species can lose CRISPR-Cas systems under conditions where horizontal gene transfer is important (reviewed in reference 177). However, a recent bioinformatics analysis of >1,300 genomes failed to detect any clear effect of CRISPRs on rates of horizontal gene transfer (178).

Impact of Spatial Structure on Evolution of Immune Mechanisms

Apart from phage abundance (force of infection), phage diversity, and the relative importance of plasmids, one further ecological

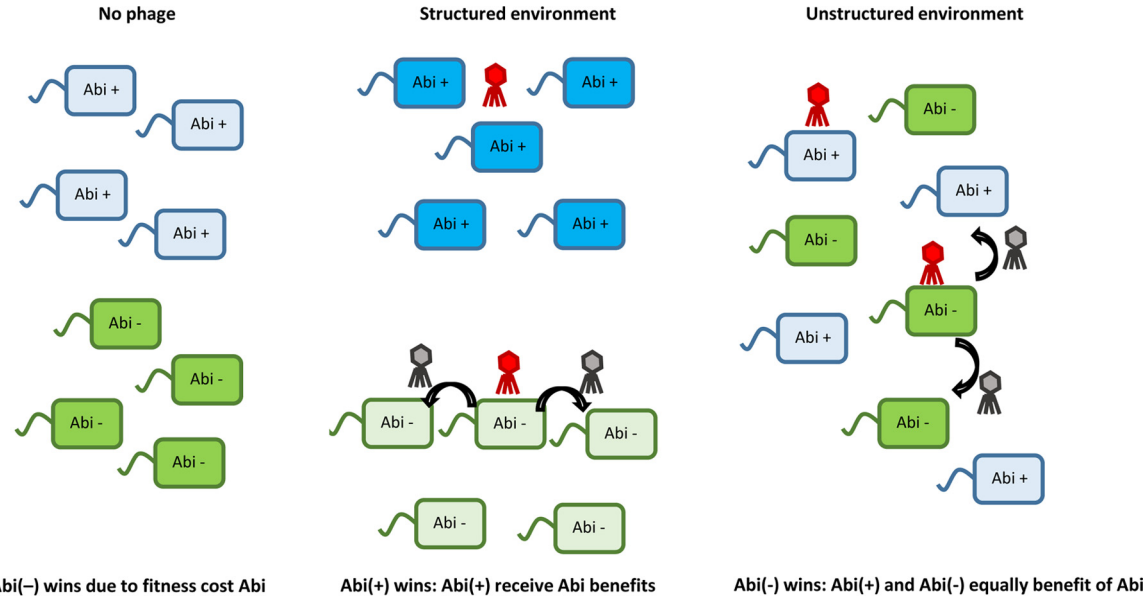

FIG 6 Spatial structure is an important fitness determinant for Abi, since it impacts relatedness. In the absence of phage, there will be selection against Abi because of the cost of carrying the system. In a structured environment, the benefits of Abi are directed toward related individuals that also carry the Abi gene. The phage will die out rapidly in the presence of Abi, while the phage will cause an epidemic in the absence of Abi (progeny phage is indicated in gray). In a well-mixed environment, bacteria lacking the Abi system benefit from the altruistic defense of bacteria that encode the Abi system, but they do not pay the cost. Both strains equally suffer from the epidemic that results from infection of bacteria that lack the Abi system. Empirical support for these findings was reported previously (181–183).

factor that is key for host-parasite interactions and the evolution of immune mechanisms is spatial structure (Fig. 2). Spatial structure increases the likelihood that neighboring individuals are clone mates, which in turn affects selection for different immune strategies. Most immune strategies confer an individual benefit, but Abi is a clear exception, since it protects neighboring bacteria at the expense of the individual expressing that trait. Theory suggests that altruistic behaviors (i.e., individually costly but group beneficial) such as Abi are likely to evolve when altruism preferentially benefits individuals who share the same altruism genes (179, 180). Studies using an artificially engineered suicide system in *E. coli* (181) and the naturally occurring *E. coli* Abi system Lit (145) show that spatial structuring is indeed needed for abortive infection systems to evolve, with the latter also showing that levels of mixing that are too low may prevent the evolution of abortive infection due to the absence of parasite spread (and thus epidemics) under these conditions (145). These findings were largely confirmed for the well-known *E. coli* Rex system (182), and this study also showed that abortive infection is likely to evolve even when genetic similarity between neighboring strains is relatively low, as long as the cost for abortive infection is also low (182). Apart from Abi, the evolution of other immune mechanisms may also be subject to spatial structure. Since spatial structure is an important factor for the evolution of lysogeny (183), it will also, indirectly, impact the evolution of superinfection exclusion, which is encoded by phages.

CONSEQUENCES OF OPERATION OF DIFFERENT MECHANISMS

While it is clear from these studies that ecology affects the evolution of immune mechanisms, it is less clear what the broader consequences of the operation of different immune mechanisms might be. As a result of the differences in the underlying genetics, different mechanisms—and their combinations—are also likely to be associated with distinct coevolutionary dynamics (184, 185). As well as affecting the extent to which bacteria are resistant to their cooccurring phage populations, different coevolutionary dynamics can have correlated effects on the evolution of important bacterial phenotypes. We first discuss what little we know about coevolution resulting from the different immune mechanisms and then discuss some of these broader consequences.

Coevolution with Different Immune Mechanisms

Surface modification. Many laboratory studies on bacterium-phage coevolution report receptor modification-based resistance, often using B strains and T phages of the model bacterium *E. coli*. These studies show that coevolution in the laboratory takes place in an asymmetrical fashion; i.e., the evolutionary potentials for host and phage are different. This leads to resistance-conferring host adaptations (e.g., receptor loss or modification), which cannot be overcome by the phage on a short time scale. Loss of surface receptors is generally not associated with coevolution (186, 187), although phages could evolve to adapt to a novel receptor (1, 6). In these cases, only one or a few cycles of resistance-infectivity coevolution are typically seen, after which bacterial surface mutants emerge, which the phage is unable to infect in the short term (reviewed in reference 188). This is also seen in studies of coevolution between the cyanobacterium *Plectonema boryanum* and its cyanophage, LPP-1, which, after several rounds of coevolution, typically resulted in full host resistance that could not be overcome by the phage (189–191). One of the exceptions to this is the coevolution between *P. fluorescens* and phage ϕ2, where coevolution persists over long time spans, with bacteria modifying their receptors in response to phage infection and phage evolving to

overcome bacterial resistance (192–194). By using this experimental system, it was found that bacterium-phage coevolution under high-nutrient conditions is associated with selective sweeps that lead to the fixation of bacterial resistance phenotypes (192, 195) and novel phage phenotypes that carry mutations to overcome bacterial resistance (196). This type of coevolution leads to the evolution of genotypes with increasing ranges of resistance (i.e., genotypes able to resist a wide range of phages) and infectivity (i.e., able to infect a wide range of bacterial hosts) and can be described as an arms race (197). Later studies assessing the genetics of bacterium-phage coevolution found that coevolving bacteria often have numerous mutations in the genes encoding LPS, the presumed ϕ2 receptor (13). Phages that evolved toward generalism were found to carry a number of mutations in the phage tail fiber genes (encoding proteins necessary for host receptor adsorption) (193, 194). While coevolution in nutrient-rich broth showed arms race dynamics (at least initially [196]), ecological conditions can change the dynamics. Specifically, lower nutrient levels result in fluctuations in resistance and infectivity ranges through time (198), while spatial structure results in temporal fluctuations in the frequency of different specialist resistance and infectivity genotypes (199, 200). These altered dynamics were associated with costs of increasing resistance and infectivity ranges, which started to outweigh the benefits when host-parasite encounter rates were reduced by low nutrient levels and spatial structure.

Superinfection exclusion. Evidence that phages are able to overcome superinfection exclusion comes from a study by Bailone and Devoret, who examined the effect of the expression of the superinfection repressor protein cI from phage λ on the emergence of virulent (i.e., lytic) phages. By increasing cI expression levels in *E. coli* cells, phages insensitive to superinfection could be readily isolated (201). These phages had accumulated mutations in the phage λ operator (*oLoR*), which, when bound by cI, keeps the host cell in a lysogenic state and prevents the replication of a superinfecting phage. However, compensatory mutations in the *cI* gene can restore superinfection suppression (202). Based on these studies, Berngruber et al. (203) generated a theoretical model that suggests that virulence and superinfection exclusion can coevolve. Virulent phages insensitive to superinfection can infect lysogens, but their insensitivity to superinfection repression keeps them in a virulent state, which can be costly for phages. As such, selection can subsequently favor superinfection suppression through compensatory mutations in the repressor (203).

Other Sie mechanisms rely on blocking phage DNA injection (reviewed in reference 1). Mechanistic insight into how injection blocking could evolve was provided by a study by Meyer et al. (6). By using experimental evolution, this study followed the fate of phage λ that had evolved to recognize a novel receptor on the surface of its *E. coli* bacterial host. After shifting receptor usage (from LamB to OmpF), bacteria acquired resistance through mutations in either the *manY* or the *manZ* gene. Both of these genes encode the transmembrane channel of the ManXYZ mannose permease, which is required for λ DNA to cross the inner membrane (204–206). Thus, these mutations conferred resistance by blocking DNA transport of phage DNA, akin to some Sie mechanisms.

Restriction-modification. In the short term (i.e., on ecological time scales), coevolution associated with RM is usually short-lived. Numerous studies have demonstrated the ability of RM systems to confer immunity against phages under laboratory conditions (reviewed in reference 34). However, even though many RM systems readily cleave unmodified phage DNA upon entry, there is a 10^{-2} to 10^{-6} probability that phage will be "accidently" modified by the host MT, which renders all host cells carrying the same RM system susceptible to that phage (207). This is likely to be a coevolutionary dead end, as a host cannot overcome this immune evasion in the short term. Hence, RM systems appear to be generally relatively unimportant for coevolution on ecological time scales compared to rapidly evolving immune responses such as surface modification or CRISPR-Cas. However, a recent theoretical study suggested that higher RM diversity may allow the stable coexistence of multiple bacterial strains due to the generation of different epigenetic variants of a phage species (due to RM-mediated modification of phage DNA) that restrict bacterial strains from dominating the population (208). Rapid evolution of the restriction site specificity of a type I RM system through DNA inversions within the HsdS-encoding gene was first described for *Mycoplasma pulmonis* (158), and similar mechanisms to rapidly alter type I RM specificity by DNA inversion appear to exist in *Streptococcus pneumoniae* (209) and *Bacteroides fragilis* (157, 210). This could potentially lead to RM-based bacterium-phage coevolution on ecological time scales.

Furthermore, over longer evolutionary time scales, coevolution between phages and RM systems takes place, as is evident from the extensive array of strategies that phages have evolved to avoid restriction (reviewed in references 211 and 212). Active evasion of RM systems is seen, for example, for phages that encode their own MTs (34, 213, 214). This ensures proper modification and thereby protection of the phage genome against cleavage by host REs that are compatible with that modification. Some phages have evolved the ability to modify their own DNA by adding bulky groups to it (213, 214). For example, coliphage Mu encodes a protein (Mom) that modifies adenine residues by adding an acetamide group to it, thus protecting it against cleavage by a wide range of REs (215). An intriguing example of a fierce coevolutionary arms race is seen for phage T4 and its host, *E. coli* K-12. Phage T4 incorporates the unusual base hydroxymethylcytosine (HMC) instead of a cytosine in its genome. As a response, hosts evolved the ability to specifically recognize and cleave this modified DNA (216), after which the phage evolved the ability to glucosylate its already modified DNA. This resulted in the evolution of RM systems that specifically recognize glucosylated DNA. In turn, phages evolved a strategy to inhibit these specialized RM systems, and recently, bacterial proteins that block these phage inhibitors were found (217). Phages can also encode proteins that mask recognition sites on the phage genome from host RE cleavage, for example, the "defense against restriction" (Dar) proteins encoded by coliphage M1 (218). Furthermore, several phages produce proteins that mimic stretches of DNA, which then bind to REs with high affinity, thereby blocking RE cleavage activity. A well-known example of such a protein is the "overcome classical restriction" (OCR) protein encoded by T7 phages (219), which binds to type I RM systems by mimicking B-form DNA to prevent cleavage activity. Similar types of mimicking proteins, named "alleviation of restriction of DNA" (Ard) proteins, are encoded by a range of plasmids, and these proteins also inhibit type I enzymes (220). Selection on phage genomes to evade restriction has led to restriction site avoidance in the phage genome (221). Furthermore, a strand bias in T7 phages ensures that all type III recognition sites are in the same orientation instead of the inverse orientation re-

quired for cleavage, which leads to immunity evasion from type III RM systems.

CRISPR-Cas. CRISPR-Cas-mediated immunity relies on a perfect sequence match between a spacer and a virus. As a consequence, virus can overcome CRISPR-Cas-mediated resistance by a simple point mutation in the target sequence. The high specificity of the interaction between CRISPRs and escape viruses led to predictions of persistent coevolution (149, 222, 223). However, both theory and data show that CRISPR-virus coevolution can be short-lived (149, 156).

Under some conditions, phage escape mutants can be readily picked up under laboratory conditions (68, 224, 225), and metagenomics data show that escape phage can increase in abundance *in vivo* (226–228). These associated mutations are typically located in a confined sequence area: either in the PAM sequence or in the adjacent seed sequence (68, 225, 229). The seed sequence is a 7- to 12-nt part of the protospacer immediately adjacent to the PAM (225) and is thought to be the area where R-loop formation starts (225, 230). Single or multiple point mutations in other parts of the protospacer typically do not lead to phage escape from the immune response (225). Type III CRISPR-Cas systems appear to be generally more tolerant to mismatches, making it more difficult for phage to overcome immunity by point mutation (231, 232). A bioinformatics study showed that PAM sequences are underrepresented on phage genomes, probably as a result of CRISPR-mediated selection (233).

Under other conditions, phage cannot evolve to escape by point mutation (156). What, then, determines the ability of phage to escape CRISPR-Cas systems? Theory predicts that virus mutation rates and host spacer acquisition rates are key factors during CRISPR-virus coevolution (149). If bacterial host populations generate high levels of spacer diversity, as is the case with *P. aeruginosa*, the virus is driven extinct (156, 234). This is because the virus can no longer evolve infectivity against the mix of host genotypes, even though the same virus can rapidly evolve infectivity against the individual clones in monoculture (156). This shows that spacer diversity increases overall population resistance (herd immunity). The propensity to generate spacer diversity is therefore an important fitness determinant of the CRISPR-Cas system. The selective pressure imposed by diversity-generating CRISPR-Cas systems may have driven the evolution of phage-borne anti-CRISPR genes in many *Pseudomonas* phages (235, 236). Anti-CRISPR proteins bind CRISPR-Cas components to interfere with either target DNA recognition or DNA destruction (237). Anti-CRISPRs are encoded by an extremely diverse set of genes that are often located in a conserved locus on phage genomes (235, 236) and other mobile genetic elements (153, 236).

Interestingly, *in vitro* long-term bacterium-phage evolution experiments with *Streptococcus thermophilus* DGCC7710 and phage D2972 revealed persistent coevolution, with phage acquiring mutations in the PAM and the seed sequence and hosts acquiring novel spacers (143, 144). How are these different dynamics explained? It seems that *S. thermophilus* generates much less diversity (CRISPR populations are dominated by a single spacer [143, 144; our unpublished data]), which is therefore predicted to lead to ongoing coevolution (149). The lower levels of diversity may be explained the requirement for a longer PAM by *S. thermophilus* CRISPR-Cas systems, which reduces the putative number of protospacers by a factor of ~10 compared to the *P. aeruginosa* type I-F system. An increase in the mutation fixation rate by the virus would also be expected to result in more persistent coevolution (137, 149).

Abortive infection. Similar to restriction-modification systems, coevolution between Abi systems and phage is less important on short time scales. However, on a longer time scale, coevolution between Abi systems and phages is evident, as phages have several distinct strategies to evade Abi mechanisms (238, 239). Cell death through the action of the above-mentioned ToxIN system from *P. atrosepticum* can be circumvented by mutants of its phage, ΦTE, that express molecules that mimic the antitoxin (238, 239). The Rex system (described above) can be circumvented by phages that carry mutations in the *motA* gene (240). This gene encodes a transcription factor that directs RNA polymerase from the early to the middle promoters during the infection cycle, and mutations in this gene likely allow phages to complete their replication cycle (211). Likewise, some phages are able to escape Lit abortive infection mechanisms (see above). These phages carry mutations in the grow on Lit (*Gol*) gene, which normally encodes a peptide that activates the Lit system (241). For several of the numerous Abi systems present in the lactococci (*Lactococcus* species), phages that can overcome a particular Abi system have been identified, although the exact mechanism by which they do so is in most cases unknown (211).

Broader Consequences of Different Immune Mechanisms

The way in which bacteria evolve immunity against their viral predators can have important implications. First, resistance mechanisms can lead to different coevolutionary dynamics, which can impact microbial community composition (and therefore functioning). Phage can both increase host diversity by selecting for host defense polymorphisms (e.g., see references 242 243) and reduce diversity through population bottlenecking and selective sweeps. Generally, coevolution characterized by fluctuating selection dynamics allows the maintenance of diversity, as different genotypes coexist through negative-frequency-dependent selection (197). Arms race dynamics, on the other hand, results in low diversity levels since it is associated with selective sweeps. Furthermore, as the continuous escalation of bacterial resistance and phage virulence becomes increasingly costly, arms race dynamics and fluctuating selection dynamics may also have different impacts on microbial performance and virulence. As explained above, the fitness cost associated with resistance depends on the type of immune mechanism that evolves. For example, the acquisition of CRISPR-Cas-mediated phage resistance is cost-free in the absence of phage (133), but *sm* generally reduces bacterial fitness (164, 244) and may affect host colonization or immune evasion (245). Indeed, phage-mediated control of *Flavobacterium psychrophilum*, a fish pathogen, resulted in surface mutants with attenuated virulence (246). The human pathogen *Vibrio cholerae* lost the ability to spread between patients after it acquired a surface mutation that conferred resistance against phage (11), and an *E. coli* strain infecting calves had greatly reduced virulence following resistance evolution (247). Hence, understanding the conditions under which different types of resistance evolve may therefore be important for predicting pathogen virulence, particularly in light of the resurgent interest in the therapeutic use of phages.

Apart from effects resulting from differences in coevolutionary dynamics, resistance mechanisms can have a general impact on microbial adaptation, including the evolution of virulence, through their impact on microbial mutation rates and horizontal

gene transfer. Viruses can select for bacteria with increased mutation rates (248), which increases bacterial adaptability. However, this effect may be reduced if the host carries a diversity-generating immune mechanism. In addition, immune systems can also have a long-term impact on microbial adaptation through their impact on horizontal gene transfer. Several correlational studies indicate that the CRISPR-Cas system limits gene transfer. For example, genome sizes of strains of the opportunistic pathogen *P. aeruginosa*, which often causes lung infections in cystic fibrosis patients, are significantly smaller if they encode CRISPR-Cas systems than if they lack CRISPR-Cas systems (153). In accordance, CRISPR-Cas systems are absent from genomes of species that rely on gene transfer, such as *Streptococcus pneumoniae*, which causes pneumonia and requires natural transformation for capsule switching during infection (177). Strains of *Streptococcus pyogenes*, which causes pharyngitis, sepsis, and necrotizing fasciitis, are more likely to carry prophage-encoded virulence factors (249, 250) if they lack CRISPR-Cas systems (177). Furthermore, the presence of CRISPR-Cas immune mechanisms is inversely correlated with antibiotic resistance in *Enterococcus faecium* and *Enterococcus faecalis* (176). Immune mechanisms can thus impact adaptation and evolution of virulence in bacteria by blocking HGT.

Alternative Function of Immune Mechanisms

Apart from their role in immunity, defense systems are also involved in other cellular processes. For example, the CRISPR-Cas system has been reported to be involved in DNA repair as well as the regulation of genes involved in virulence and group behaviors (251–253). Usually, either *cas* genes or CRISPR arrays are involved in these noncanonical functions, rather than the combined action of both elements (252). Alternative roles for RM systems have also been proposed (reviewed in reference 212). These roles include stabilization of genomic islands, stimulating recombination and genome rearrangements, and modulating the rate of genome evolution. In addition, it has been hypothesized that type II RM systems may behave as selfish genetic elements (254, 255), in a fashion similar to that of toxin-antitoxin addiction systems. To increase their own frequency within the population, these systems maintain themselves in the host through postsegregational killing (32), a strategy that is used by a wide variety of selfish genetic elements to render the cells dependent on (addicted to) the residing RM systems for survival. Like toxin-antitoxin systems, selfish type II systems are generally associated with mobile elements, which may allow them to easily invade new genomes (33). Theoretical modeling showed that spatial structuring is an important factor in the spread of addiction systems such as type II RM and TA systems (256). In a spatially structured environment, the frequencies of these genetic elements are likely to increase but not in an unstructured environment.

Although Sie protects hosts against phage infection, the question of whether Sie systems are selected for their role in immunity is a subject of ongoing debate. Sie systems encoded by temperate phages prevent subsequent infection by similar phages and perhaps primarily comprise a phage strategy to successfully compete with other phages, known as "phage warfare." That said, prophage-encoded Sie systems can clearly benefit the host by either providing immunity to other (potentially virulent) phages or enhancing the competitive abilities of the lysogen over prophage-free hosts; in this context, prophages can be used as a "biological weapon." For example, several *Salmonella enterica* strains carry prophages, and these populations produce low virus titers that are sufficient to eliminate competing bacterial populations devoid of such prophages (126, 257). However, this benefit disappears when the competing bacteria also become lysogenized (258). The level of lysogenization of sensitive competitors was shown to be much reduced when the lysogens carry multiple prophages, and this was associated with greatly increased host fitness when competing with phage-sensitive bacteria compared to a lysogen carrying a single prophage (259). The protective effect of lysogens may be key to explaining the observed selection for temperate phages at high bacterial densities (260).

APPLICATIONS, CONCLUSIONS, AND OUTLOOK

The ability to understand, predict, and manipulate the evolution of bacterial immune mechanisms will be extremely powerful to generate virus-resistant bacterial strains for use in industrial fermentations, agriculture, and probiotics. The type of evolved resistance could be tailored to minimize tradeoffs and unwanted coevolutionary consequences.

Apart from applications in industry, understanding bacterial resistance evolution and coevolutionary consequences is also important for phage therapy. Specifically, the ability to expose bacteria to phages in a way that results in resistance evolution associated with large tradeoffs can reduce pathogen virulence or potentially limit the acquisition of novel genes through horizontal gene transfer. Understanding the conditions under which different immune mechanisms evolve can therefore be important for manipulating the evolution of pathogen virulence.

Taken together, it is becoming increasingly clear that ecological factors are key for the evolution of distinct bacterial immune mechanisms and bacterium-phage coevolution (188). Bioinformatics analyses predict the existence of many more immune mechanisms, often clustered together in defense islands (112). One such mechanism, coined bacteriophage exclusion (BREX), is widely distributed across bacteria and appears to share some mechanistic features with RM systems (261). We are starting to understand how all these different immune mechanisms work, but the next challenge is to examine when these mechanisms are favored over one another and how they are integrated during antiviral responses.

Finally, as noted above, phages can encode a range of immune strategies themselves and are likely important vectors for moving these systems around between bacterial strains and species. In addition to Sie mechanisms, phages have been found to carry RM (262) and CRISPR-Cas (263) systems. Phage-encoded CRISPRs can play key roles in phage-phage interactions (263) and in interactions between phage and other genetic elements (264). Understanding how selection acts on diverse immune strategies will require their movement by phage to be explicitly taken into account.

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