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Review

Contemporary Phage Biology: From Classic Models to New Insights

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Bacteriophages, discovered about a century ago, have been pivotal as models for understanding the fundamental principles of molecular biology. While interest in phage biology declined after the phage "golden era," key recent developments, including advances in phage genomics, microscopy, and the discovery of the CRISPR-Cas anti-phage defense system, have sparked a renaissance in phage research in the past decade. This review highlights recently discovered unexpected complexities in phage biology, describes a new arsenal of phage genes that help them overcome bacterial defenses, and discusses advances toward documentation of the phage biodiversity on a global scale.

Bacteriophages (or phages for short) are viruses that infect bacteria. Like any virus, they are obligatory parasites, requiring the host cellular machinery to reproduce. Infection begins by attachment of the phage particle to its host cell through specific recognition of a receptor on the host surface, followed by delivery of the phage nucleic acids into the infected cell. Once inside the bacterium, the phage takes over the bacterial cell, hijacks its cellular components and shuts down its defense mechanisms. Phage genes are expressed, and the phage genome is replicated and eventually packed into self-assembled phage particles. At the end of the lytic infection cycle, progeny phage particles emerge from the cell in a process that usually involves cell lysis by phage proteins (Calendar and Abedon, 2006) (Figure 1A). Most isolated phages (>95%) discovered to date have linear, double-stranded DNA (dsDNA) genomes packed into a tailed proteinaceous capsid (Ackermann, 2007). Other groups of phages can have non-tailed capsids with dsDNA genome or non-tailed capsids with single-stranded DNA (ssDNA) or RNA genomes (Ackermann, 2006) (Figure 1B).

Phages were independently discovered twice: by Twort in 1915 (Twort, 1915) and d'Hérelle in 1917 (D'Herelle, 1917). They were initially studied as anti-bacterials and were later widely used in the clinic, mainly in the former Soviet Union (Abedon et al., 2011). Starting the 1940s, phages became model organisms that facilitated the birth of molecular biology and were utilized to derive the most basic discoveries on the nature of life at the molecular level, including the random nature of mutation (Luria and Delbrück, 1943), the discovery that DNA is the genetic material (Hershey and Chase, 1952), and the understanding of gene-expression control (Jacob and Monod, 1961). The history of phage research and its contributions to molecular biology has been recently reviewed (Salmond and Fineran, 2015).

The deep understanding of phage biology that stemmed from these early studies led to the development of fundamental molecular tools. Such tools are very widely used to date and include gene-expression systems based on the phage T7 RNA polymerase (Studier and Moffatt, 1986), the phi29 DNA polymerase that allows single-cell genomics and is an essential component of the PacBio SMRT sequencing technology (Eid et al., 2009), and the phage P1-derived Cre-Lox system that is used for site-specific recombination in numerous applications (Sauer, 1987). The phage display technology, where filamentous phages are used as carriers of peptide libraries displayed on the virion surface, is commonly applied for antibody development and protein interaction studies (Pande et al., 2010). In addition, phage-centered research led to the discovery of restriction-modification systems, which opened the door to genetic engineering, and more recently the CRISPR-Cas system that became the basis for the genome-editing revolution. Both of these systems are primarily naturally used by bacteria as anti-phage defense systems (Roberts, 2005; Sorek et al., 2013). Together, these molecular tools are estimated to sell at many millions of dollars per year, exemplifying the huge impact that phage-derived biology had and still has on modern biotechnology.

The early decades of phage research led to very deep understanding of phage biology, but the overwhelming majority of knowledge was derived from a very small set of model phages, primarily those infecting *Escherichia coli*. Between the 1980s and the early 2000s, phages received much less attention as a research subject, presumably because the topic was perceived as largely understood (Young, 2006). However, advances in genomics and molecular ecology, as well as the discovery that bacteria have an adaptive immune system against phages—CRISPR-Cas—have revived interest in phage research and sparked a renaissance in the field.

The purpose of this review is to describe major new knowledge from the past decade or so in phage biology. We note that, as the phage literature is vast, no single review can cover all aspects of phage biology; rather, we focus on novel emerging concepts that stem from studying new, non-model phages or from applying new techniques to study established phage models. Specifically, we highlight discoveries in three areas.

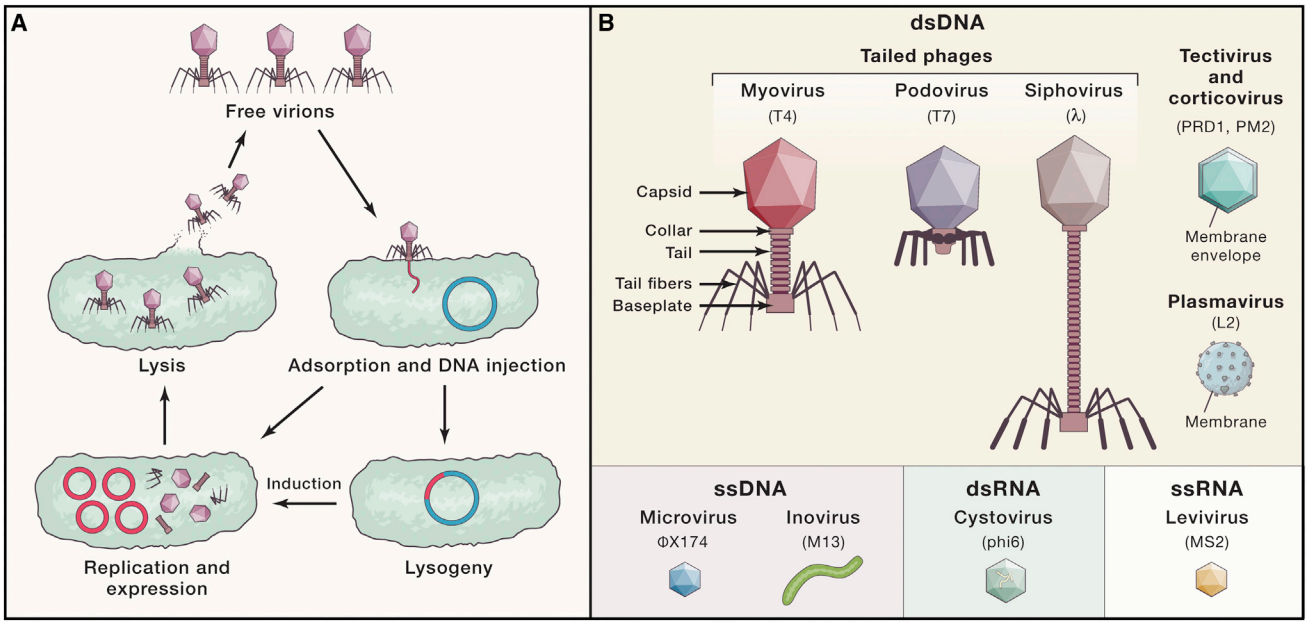

Figure 1. Phage Life Cycle and Morphologies
(A) Phage life cycle.
(B) Phage taxonomy based on morphology and genome composition. A representative type phage for each taxonomical group is in parenthesis.

First, we discuss the massive expansion of known phage sequence space that occurred in recent years. Second, we describe the complexity of molecular circuits in phages' lysis-lysogeny decisions that was recently revealed to deviate from the known paradigm of the well-studied lambda phage and also discuss an expanding set of cases where lysogenized phages became themselves decision-making switches in bacteria. Third, we review new discoveries regarding the molecular mechanisms employed by phages in their arms race against bacterial defenses and specifically the ways they interact with CRISPR-Cas systems. We conclude with new insights into phage biology revealed by advances in high-resolution microscopy. Through the advances described below, our aim is to highlight the major impact that the recent revitalization of phage research had on our understating of their biology.

Expansion of the Known Phage World

Phages are known to be highly abundant in multiple environments, and in most environments they outnumber their bacterial hosts (Parikka et al., 2017). In seawater, where environmental phages were intensively studied, they are consistently documented to exist in 10^6–10^7 particles per milliliter (Parikka et al., 2017) and are known to have major ecological roles (Weinbauer, 2004). For example, apart from directly shaping the bacterial communities through killing of bacteria, phages rewire bacterial metabolism through phage-encoded metabolic genes (Hurwitz and U'Ren, 2016) and can terminate bacterial blooms through the induction of lysogens (Brum et al., 2016).

A major development in phage ecology research, which was derived from the genomic and metagenomic revolution, is our ability to begin appreciating the diversity and abundance of phage species in global scales. Prior to this revolution, there was no good way to really assess the extent of this diversity, although hints for the existence of a huge "dark matter" of unexplored phages was available from early studies on phage isolates (Rohwer, 2003) and from analyses of CRISPR spacers, revealing that only 2% of spacers had hits to known phage sequences and suggesting that 98% of the world "phagome" was unknown (Mojica et al., 2005). Recent applications of the metagenomic approach, in which DNA extracted from environmental samples is directly sequenced and studied, indeed revealed that the majority of phage genomes discovered through this approach are new to science, exposing an overwhelming diversity of phage genomes that were not encountered before (Andersson and Banfield, 2008; Brum and Sullivan, 2015; Brum et al., 2015; Hurwitz and Sullivan, 2013; Paez-Espino et al., 2016; Roux et al., 2015, 2016). For example, a 2015 study that examined 43 phage-enriched metagenomic samples from 26 surface ocean sites revealed 5,476 populations of phage genomes, of which only 39 were previously known (Brum et al., 2015). A later 2016 study, in which 104 ocean samples were analyzed, yielded over 15,000 epipelagic and mesopelagic populations, roughly tripling the number of known ocean phage populations (Roux et al., 2016). Despite the huge diversity of phage genomes revealed via metagenomics, deep sampling of certain habitats brings us closer to documenting a significant portion of their biodiversity. For example, in the surface photic ocean, the most extensively sampled habitat for phage genomes, the discovery rate of new phage gene clusters in metagenomic samples is approaching saturation (Roux et al., 2016). In more sparsely sampled habitats, such as the deeper ocean, discovery rates of new genes and new phages still seem far from saturation (Mizuno et al., 2016; Roux et al., 2014, 2016).

Although the most deeply studied habitat for phage diversity is the marine environment, a new frontier is the study of phages in the human microbiome (Mirzaei and Maurice, 2017), where saturation in new phage gene discovery also seems to be within reach. Phages are highly abundant in the human gut microbiome and were suggested to be involved in shaping the healthy gut microbiome as well as having a role in pathogenic conditions (Mirzaei and Maurice, 2017). Although gut phages are very diverse, high-intensity, deep sampling of gut microbiomes revealed a core set of phages that are common among healthy individuals (Manrique et al., 2016; Stern et al., 2012). One of the most abundant phages in the human gut microbiome is called crAssPhage, probably infecting a *Bacteroides* host (Dutilh et al., 2014). Although this phage is responsible for up to 90% of viral reads in some gut metagenomic samples (Manrique et al., 2016), it was only recently discovered (Dutilh et al., 2014). With the intensive sampling of gut phageomes, discovery of new phage genes in gut microbiome samples now seems to approach saturation (Paez-Espino et al., 2016).

Apart from sampling efforts targeted at viral communities, the repository of phage genomes has recently been significantly expanded by applying new analysis tools to existing sequence databases. Specifically, new tools that detect prophages in bacterial genomes identified close to 13,000 phage genomes integrated within their host genomes, identifying for the first time phages infecting important phyla of bacteria with no previously known phage (Roux et al., 2015). In another study, a massive search for virus-associated genes in metagenomic data from over 3,000 geographically diverse samples documented more than 125,000 new viral contigs (large pieces of viral genomes) (Paez-Espino et al., 2016). Such analyses of metagenomic data revealed novel strategies in phage biology, including extensive utilization of non-canonical codons and stop codons in phage genomes (Ivanova et al., 2014).

While metagenomics is the most rapid approach for massive discovery of new phage genomes, deep functional phage research usually necessitates isolation of cultivable phages and their hosts. A recent endeavor, called SEA-PHAGES, attempted to characterize the entire repertoire of phages infecting a single host using a parallel effort of phage isolation, performed by high-school and undergraduate students as part of their early scientific training (Jordan et al., 2014). This resulted in the largest collection of phages infecting a single host—currently containing ~8,500 isolated phages infecting *Mycobacterium smegmatis*, over 1,300 of them fully sequenced and annotated (Pope et al., 2015). Comparison of phage genomes within this large dataset revealed extensive genome mosaicism among these phages, suggesting extremely frequent genetic exchange between phages infecting the same host, even to a point of a "continuum of diversity" (Pope et al., 2015). Studying phages from this set revealed new modes of lysogeny regulation (Broussard et al., 2013) and exposed an abundant phenomenon of prophage-mediated protection against attack by other phages (Dedrick et al., 2017).

The expanding set of known phage genome sequences has implications on our understanding of their evolution—for example, a recent study, which analyzed 1,440 genomes of dsDNA viruses, showed evolutionary relatedness between phages and metazoan *Herpesvirales* based on shared capsid proteins and packaging components (Iranzo et al., 2016). Analysis of over 2,000 complete dsDNA phage genomes also revealed two distinct evolutionary strategies of high versus low horizontal gene transfer flux among phage genomes, which are influenced by both the phage ecology and genetic modules (Mavrich and Hatfull, 2017). A more complete record of the phage sequence space should enable a more detailed and fine-grained understanding of their evolutionary paths.

While we are still very far from documenting the entire space of phage groups in the world, the studies described above demonstrate that very deep sampling can yield near-saturated documentation of the phages that exist in specific habitats or infecting specific hosts, a task considered largely unachievable until recently. While this holds true for the abundant tailed phages, recent discoveries suggest that non-tailed dsDNA, ssDNA and RNA phages are underrepresented in conventional metagenomic studies (Brum et al., 2013; Kauffman et al., 2018)—a challenge that needs to be addressed. With the exponential growth of available genomic and metagenomic data, we can expect extensive phage documentation in more and more habitats in the foreseeable future, which should ultimately enable true ecology-scale characterization of phage global abundance and diversity.

New Insights into Phage Lysogeny

Another field in which key insights have been recently obtained from application of new techniques is phage lysogeny. Lysogeny is a very common alternative life cycle that temperate phages can employ, where instead of replicating and lysing their host, they become latent, either by integrating into their host genome or by forming an episome within the host cell. Once lysogenized, these phage genomes are replicated together with the host genome and can eventually initiate their lytic cycle upon specific cues that usually involve host stress (Howard-Varona et al., 2017). Temperate phages affect bacterial communities on multiple levels—they transfer new genes to their hosts, alter the expression of host genes, provide protection against infection by other phages, and kill host populations upon induction. These phenomena were recently reviewed (Howard-Varona et al., 2017).

New Discoveries on the Lysis-Lysogeny Decision

A temperate phage needs to make a decision every time it infects a bacterial cell—either to execute the lytic cycle or to become a prophage. The lysis-lysogeny decision (or "molecular switch") has been a paradigm for molecular decision-making processes since the very early days of molecular biology (Golding, 2011; Herskowitz and Hagen, 1980). This topic was thoroughly investigated in the *E. coli* phage lambda, where it was found to be a complex process involving an intricate network that includes transcriptional repressors and transcriptional activators, as well as RNA degradation, transcription antitermination, and proteolysis. The network integrates information on the metabolic state of the cell and the phage multiplicity of infection to make the eventual decision (Oppenheim et al., 2005).

The basic understanding of the lambda lysis-lysogeny decision began by studying phage mutants in the 50s (Kaiser, 1957) and was considered largely solved in the 1980s (Herskowitz and Hagen, 1980). However, this decision was thought to be strongly

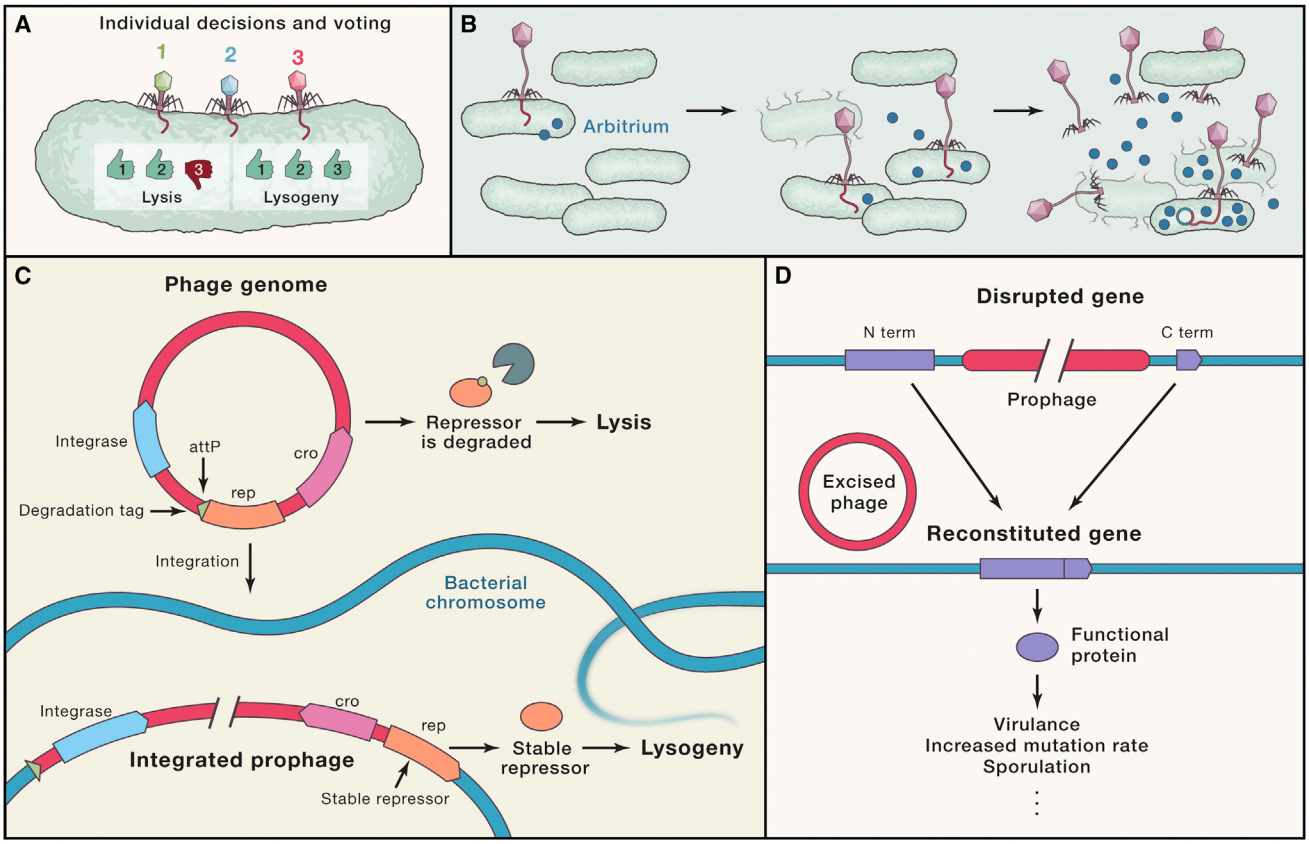

Figure 2. Mechanisms of Phage Lysogeny Decisions

(A) When multiple lambda phages infect a single *E. coli* host, a unanimous "vote" for lysogeny by all infecting phages is necessary for eventual establishment of lysogeny (Zeng et al., 2010).

(B) Communication-based lysogeny decisions. When phage phi3T infects its *Bacillus* host, it releases a measured amount of a 6 aa peptide called arbitrium (blue dots). After several cycles of infection, the rising concentrations of the arbitrium peptide will lead the phage to lysogeny (Erez et al., 2017).

(C) In several mycobacteriophages, the site of lysogenic integration (attP) is found within the repressor (rep) gene. Prior to genome integration, the repressor contains a C-terminal tag (green) leading it to degradation, thus promoting lysis. Following integration, the tag is separated, and the repressor becomes stable, allowing lysogeny maintenance (Broussard et al., 2013).

(D) Prophages as genetic switches in bacteria. Phage integration into the bacterial genome disrupts a gene, turning it inactive. When the gene is needed, the phage genome is excised, reconstituting the functional gene.

influenced by stochasticity (or "noise") (Oppenheim et al., 2005). New studies that examined this process using single-cell techniques now showed that much of the stochasticity can be explained by deterministic decisions at the cellular or subcellular levels (Golding, 2016; St-Pierre and Endy, 2008; Trinh et al., 2017; Zeng et al., 2010). In these studies, fluorescent markers allow visualization of single phages infecting single cells and report on the individual lysis/lysogeny decision that each phage makes. It was shown that lambda phages infecting cells of larger volumes have lower chances of choosing lysogeny, presumably due to dilution of phage lysogeny-promoting proteins in the larger cell (St-Pierre and Endy, 2008). Moreover, when multiple phages infect a single cell, the decision of all phages is integrated within the cell, such that only a unanimous vote for lysogeny by all phages eventually leads to a lysogenized bacterium (Zeng et al., 2010) (Figure 2A).

While the lambda lysis-lysogeny switch was subject to hundreds of studies, the decision-making process in other phages remained almost completely unknown. A recent study that examined the lysogeny dynamics of the *Bacillus* phage phi3T found that small-molecule communication between phages is used to coordinate the lysogeny decision (Erez et al., 2017) (Figure 2B). It was found that during infection, the phage releases a 6-amino-acid peptide called arbitrium (Latin for "decision") into the medium. In successive infections, offspring phages measure the concentration of this peptide and lysogenize if the concentration is sufficiently high. This form of communication allows descendant phages to estimate the number of predecessor phages that completed successful infections in recent previous cycles. As the chances of progeny phages to find a viable host diminish after multiple cycles of infection, it makes sense that in that case the phage will prefer to lysogenize and reproduce through the replication of its host genome (Erez et al., 2017). In a sense, this is parallel to the observation that infection of the same cell by multiple lambda phages increases chances of lysogeny (Zeng et al., 2010) as the number of co-infecting phages is also a proxy for reduced chances of finding a viable host in the next cycle. The arbitrium system was

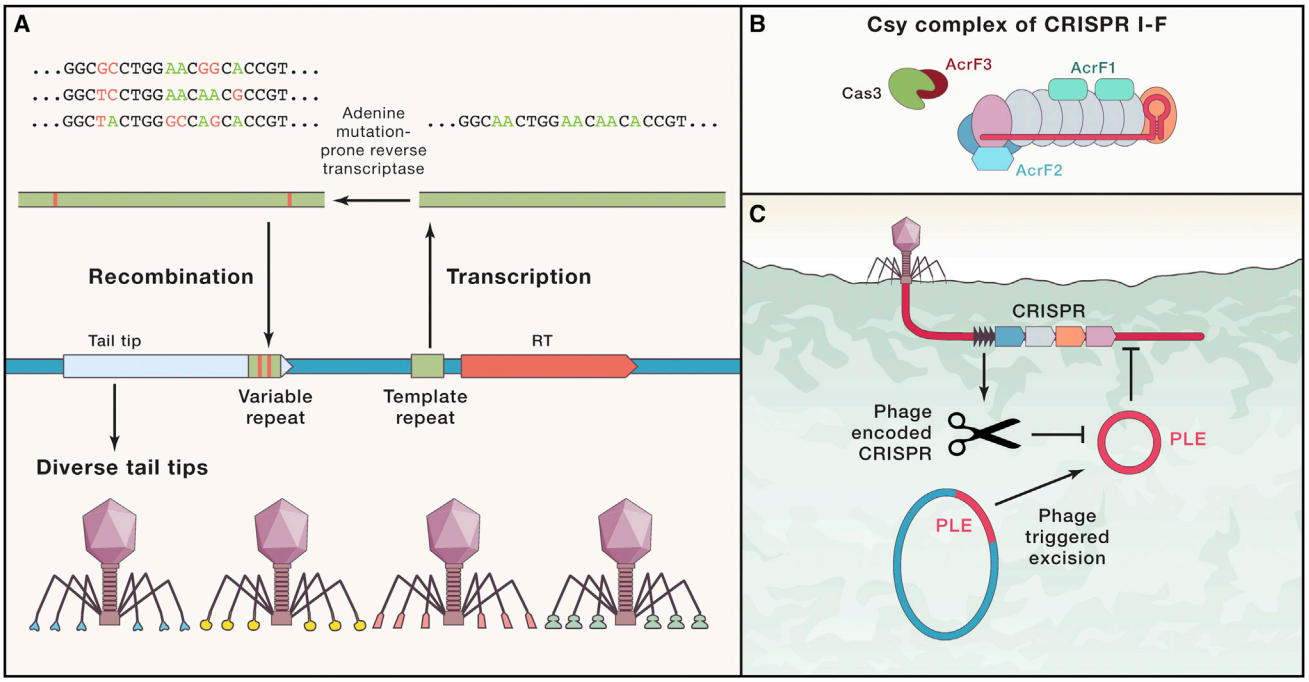

Figure 3. Mechanisms of Phage Escape from Bacterial Defenses

(A) Diversity-generating retroelements (DGRs) diversify phage tail tips. The template repeat is transcribed and then reverse-transcribed by a phage-encoded error-prone reverse transcriptase (RT) that converts adenines into random nucleotides. The cDNA is then recombined with the C terminus of the gene encoding the tail-tip, generating a population of phages that can encode an extremely diverse repertoire of tail tips.

(B) Anti-CRISPR proteins can target various components of the CRISPR machinery.

(C) Phage-encoded CRISPR targets a chromosomal island in *Vibrio cholerae* that excises upon phage infection and interferes with the phage-replication process.

documented in a large group of phages infecting *Bacillus* species and is the first small-molecule communication system discovered in viruses. It is not unlikely that other phages, and maybe even non-phage viruses, will be found in the future to use such communication systems to coordinate group decisions.

Another demonstration that new insights can be derived when non-model phages are studied comes from the compact lysogeny switch that was discovered in several mycobacteriophages (Broussard et al., 2013). In contrast to the complex decision-making network observed in phage lambda, the mycobacteriophage system appears to involve only 3 genes: repressor, Cro, and integrase. The integration site on the phage genome (attP) resides within the ORF (open reading frame) of the repressor gene itself, and successful integration into the bacterial genome truncates the repressor gene and removes a proteolytic degradation signal from its C terminus, thus stabilizing the repressor protein and maintaining lysogeny (Figure 2C). In the lytic cycle, the repressor and the integrase are degraded by cellular proteases, preventing lysogeny (Broussard et al., 2013; Villanueva et al., 2015).

Prophages as Bacterial Regulatory Switches

Apart from the decisions made by the phage itself, temperate phages can also become adapted to serve as decision-making switches within bacteria. This occurs when a prophage is integrated within a bacterial gene and disrupts it. Under specific conditions, when the bacterial gene product is required, the prophage is excised from the genome, and the bacterial gene is reconstituted and regains its function (Feiner et al., 2015) (Figure 3D). Such switches were found to control the hypermutation phenotype in both *Streptococcus* (Scott et al., 2008, 2012) and *Vibrio* (Chu et al., 2017) by excision of phages naturally integrated into the *mutS-mutL* operon and the adaptation to cold temperatures in *Shewanella oneidensis* by excision of a cryptic phage from a tmRNA gene (Zeng et al., 2016). Phage excision was also found as required for successful intracellular infection of mammalian cells by *Listeria monocytogenes* serovar 1/2, by activating the *comK* gene, which is naturally interrupted by a prophage (Rabinovich et al., 2012). Interestingly, in the cases described above, the phage excision is reversible, and the phage episome reintegrates into the gene once its expression is no longer required. Non-reversible phage excision as a regulatory switch has also been documented (Abe et al., 2014; Takemaru et al., 1995).

The recent discoveries that shed new light on such a deeply characterized topic as lysogeny demonstrate, again, how the field of phage research has benefited from the recent shift of interest into non-model phages as well as from application of modern technologies to previously studied phenomena. Both of these trends also converge in the study of the phage-bacteria arms race, a field that provided molecular biology with models for co-evolution on one hand and molecular tools such as restriction enzymes on the other. New transformative discoveries regarding the mechanisms that allow phages to overcome bacterial resistance are described in the following section.

New Phage Strategies to Counteract Bacterial Defense

Phages are thought to have been co-evolving with their bacterial hosts for billions of years. The strong selection pressure imposed on bacteria by phages resulted in the evolution of multiple bacterial defense strategies against phage infection. It is now understood that bacteria carry multiple lines of innate and adaptive defense (Dy et al., 2014), while phages came up with complex strategies to overcome these defenses (Samson et al., 2013). Bacterial defenses target multiple stages in the phage infection cycle: surface modifications and receptor mutations prevent phage adsorption, restriction enzymes and CRISPR-Cas systems identify and cleave phage nucleic acids, and abortive infection (Abi) systems lead to cell death or stasis when phage replication takes place. Additional defense mechanisms identified more recently include bacterial Argonautes (Swarts et al., 2014) and multiple additional defense systems with yet unknown mechanism of action (Goldfarb et al., 2015; Ofir et al., 2018; Doron et al., 2018). It is thought that many unknown defense mechanisms are still awaiting discovery (Makarova et al., 2011; Stern and Sorek, 2011). While the arms race between bacteria and phages has been studied extensively in the past, new discoveries that stem from studying non-model phages unveiled a new arsenal of tools used by both phage and bacteria in their endless conflict.

Hyper-Diversification of Phage Receptor-Binding Proteins

An essential prerequisite for phage infection is the recognition and adsorption of the phage to its cognate receptor on the bacterial surface. Both lab experimental evolution and observations in natural environments point to receptor mutations as a major avenue to acquire resistance against infecting phages (Rodriguez-Valera et al., 2009). Phages, on the other hand, must change their own receptor-binding proteins (typically located on their tail fibers) to match the mutated receptor or to adsorb to a new target (Rodriguez-Valera et al., 2009). Cycles of receptor mutations and counter mutations were long appreciated as major facilitators of bacteria-phage coevolution (Lenski, 1984), but recent discoveries show that both phages and bacteria do not rely solely on passive mutations to survive in this arms race.

An intriguing mechanism for phage tail fiber hyper-diversification is encoded by diversity-generating retroelements (DGRs), which were first discovered in the temperate phage BPP-1 infecting *Bordetella* (Doulatov et al., 2004). The DGR system is comprised of two repeats (variable repeat [VR] and template repeat [TR]) and a reverse transcriptase gene. The VR lies in the gene encoding the tip of the phage tail fiber, which is responsible for binding the bacterial receptor (Figure 3A). The TR is found in a non-coding RNA that is transcribed and then reverse-transcribed by the reverse transcriptase in an error-prone manner, generating random substitutions specifically at adenine residues. This mutated copy of the second repeat undergoes recombination with the variable repeat to generate a new polypeptide sequence in the binding region of the tail tip protein, generating a huge diversity of potential tail fibers that can reach 10^{14} different variations (Doulatov et al., 2004). Thus, the phage uses the DGR system in order to rapidly diversify its tail tips and potentially overcome bacterial receptor mutations.

The absolute dependence of phages on receptors present on their host surface represents a weak point that can be utilized by bacterial defenses. It was shown that bacteria can release "decoy" extracellular vesicles that carry phage receptors; these vesicles lure phages to attach and inject their genome into a compartment that cannot support phage replication, thus preventing infection of a viable cell and titering the phage out (Biller et al., 2014; MacDonald and Kuehn, 2012). On the other hand, vesicles were recently demonstrated to enable transfer of phage receptors from susceptible cells to resistant cells lacking the receptor, thus enabling phages to infect receptor-less hosts (Tzipilevich et al., 2017).

CRISPR-Cas and Anti-CRISPRs

CRISPR-Cas is the adaptive immune system of bacteria, enabling them to generate and memorize resistance against foreign invading DNA. This system acquires fragments of phage DNA, incorporates them into CRISPR memory arrays (spacers) in the bacterial genome, and uses RNA probes transcribed from these arrays to identify invading DNA that is then cleaved and destroyed (Amitai and Sorek, 2016; Sorek et al., 2013).

The adaptive nature of CRISPR-Cas came as a surprise, as adaptive immune systems were believed to be limited to "higher" organisms. The fact that, in contrast to vertebrate immunity, the acquired CRISPR-Cas immunity is inherited by the progeny of the acquiring bacteria has implications on the nature of the co-evolution of phages and their hosts. Bacteria no longer need to "wait" for the appearance of mutations to escape phage infection. It was therefore suggested that CRISPR adaptation can be perceived as Lamarckian evolution, where the "environment" (phage pressure) directly causes heritable genetic mutations that are beneficial against the challenges faced by the organism (Koonin and Wolf, 2016). Intriguingly, the study of bacterial resistance to phage infection resulted in both the direct demonstration of the Darwinian random nature of mutations and selection by Luria and Delbrück (1943) and the best example of Lamarckian inheritance through CRISPR adaptation (Koonin and Wolf, 2016).

Early on, it was found that phages can escape CRISPR-Cas interference by mutations in the DNA region (protospacer) targeted by the CRISPR RNA. However, in a diverse bacterial culture, individual bacteria can acquire different spacers, generating a group immunity that cannot be avoided by a single-point mutation in the phage (van Houte et al., 2016). Additionally, a mechanism called CRISPR priming (or primed adaptation) enables bacteria to use partial matches between the CRISPR RNA probe and the target to promote acquisition of new CRISPR spacers (Fineran et al., 2014), reducing the efficiency of point mutations as an escape strategy for phages.

Recently, phages were found to encode specific proteins, called anti-CRISPRs, which inhibit the activity of CRISPR-Cas systems (Borges et al., 2017). Anti-CRISPR genes were first discovered in prophages of *Pseudomonas aeruginosa* through the observation that bacteria harboring these prophages were phage-sensitive even though they possessed CRISPR spacers targeting these phages (Bondy-Denomy et al., 2013). Structural and biochemical studies showed that different anti-CRISPRs target different elements of the CRISPR-Cas system. For example, one anti-CRISPR (AcrF2) inhibits the *P. aeruginosa* type IF CRISPR surveillance complex (Csy) by directly binding the complex and occupying the site of interaction with the DNA target. A second anti-CRISPR (AcrF1) binds a different

position on the Csy complex backbone and distorts it to prevent the CRISPR RNA from interacting with the DNA target (Bondy-Denomy et al., 2015; Chowdhury et al., 2017). A third anti-CRISPR directly binds and inhibits Cas3, the nuclease that cleaves the target DNA after recognition (Bondy-Denomy et al., 2015). Anti-CRISPRs were generally found to be very specific, such that a certain anti-CRISPR will only target one CRISPR subtype (Pawluk et al., 2014), although one anti-CRISPR was found to inhibit both type IE and type IF CRISPR-Cas systems in *P. aeruginosa* (Pawluk et al., 2016a).

Anti-CRISPR proteins were initially found in *Pseudomonas aeruginosa* phages, but recent genomic analyses found such proteins in phages infecting other bacteria (Pawluk et al., 2014, 2016a). Most recently, multiple anti-CRISPR genes that inactivate Cas9, the workhorse of genome editing, were also discovered (Pawluk et al., 2016b; Rauch et al., 2017). These proteins, originating from multiple prophages and mobile genetic elements residing in CRISPR-Cas9-containing bacteria were shown to inhibit the activity of the Cas9 itself. It was then shown that these anti-Cas9 proteins can modulate the activity of Cas9 and inhibit genome editing, marking them as promising biotechnological tools for "off-switching" genome editing (Pawluk et al., 2016b; Rauch et al., 2017).

Phages Can Also Carry Anti-phage Systems: Phage versus Phage

The intricacies in the evolutionary processes of phage defense and counter-defense reveal ever-growing complexities in this perpetual arms race. Apparently, phages are not only the targets of anti-phage defense; sometimes their own genomes carry systems targeting other phages. Two recent studies analyzing a large collection of temperate phages infecting either *Pseudomonas* or *Mycobacterium* revealed that many prophages provide their host with defense against other types of phages (Bondy-Denomy et al., 2016; Dedrick et al., 2017). Temperate phages of *P. aeruginosa* were found to frequently manipulate the host receptors, thus providing immunity against other phages that rely on these receptors (Bondy-Denomy et al., 2016). *Mycobacterium* phages were found to provide their host with defense by carrying restriction-modification systems that protect against other phages as well as genes that mediate intracellular defenses via mechanisms yet unclear (Dedrick et al., 2017).

Recently, phages were documented as carrying functional CRISPR-Cas systems with spacers targeting other phages, so that the lysogen of the former phages becomes resistant to the latter (Bellas et al., 2015; Chénard et al., 2016; Seed et al., 2013). Perhaps the most intriguing case of phage utilization of CRISPR-Cas is that of the *Vibrio cholerae* phage ICP1 that is abundant in stool samples of cholera patients. Some *V. cholerae* strains are protected against infection by this phage via phage inducible chromosomal-like element (PLE), which are elements that excise from the bacterial genome upon phage infection and prevent phage progeny release (Penadés and Christie, 2015). The ICP1 phage that overcomes this defense contains a fully functional CRISPR-Cas system with spacers against the PLE, thus degrading the PLE DNA and avoiding its effect (Seed et al., 2013) (Figure 3C).

These recent discoveries add a great deal of information on the molecular drivers of the bacteria/phage co-evolution. As such, they form a model for the evolutionary arms race between hosts and parasites. The widespread utilization of CRISPR-Cas as a biotechnological tool adds another dimension of importance to the understanding of the ways by which phages can evade and modify these systems. Anti-CRISPR proteins are now marked as potential CRISPR-Cas modifiers in applied contexts, and CRISPR-carrying phages are developed as tools in the fight against antibiotic-resistant bacteria (Bikard et al., 2014; Yosef et al., 2015).

New Insights into Phage Biology Revealed by Modern Microscopy

Although the major steps of phage replication within its host cells were studied in high detail for decades, recent examination of non-model phages have revealed surprising new features in phage intracellular replication, as in the case of phage 201ϕ2-1 that infects *Pseudomonas chlororaphis*. This phage was found to encode a structural homolog of tubulin, and super-resolution microscopy revealed that this tubulin generates cytoskeletal filaments that position the replication center of the phage DNA, during lytic infection, at the center of the bacterial cell (Kraemer et al., 2012; Erb et al., 2014). In a subsequent study, the tubulin-positioned phage-replication center was surprisingly found to be encapsulated in a nucleus-like shell made of the phage-encoded protein gp105 (Chaikeeratisak et al., 2017). Cryoelectron microscopy (cryo-EM) then revealed that the phage capsid is assembled outside of this shell and is then docked onto the shell to receive and package the phage genome (Figure 4A). While phages were previously known to replicate in spatially distinct positions in the cell (Jakutytė et al., 2012), the involvement of such nucleus-like structures is a completely new concept. A possible role for such compartmentalization is to protect the phage replicative center from bacterial defense systems.

The above-mentioned discovery was facilitated, to a large extent, by advances in cryo-EM and super-resolution fluorescent microscopy. These recent advances, often referred to as the "resolution revolution," now allow examination of well-studied processes in model phages in unprecedented, often atomic resolution. For example, cryo-EM was used to examine the adsorption and DNA injection process of the model phage T7. This phage belongs to the *Podoviridae* phage family and has a tail that is too short to span the cell envelope. Recent cryo-EM studies revealed how a structure within the T7 virion, denoted the "internal core," is ejected from the virion following adsorption to form an extended tail that spans the double membrane and penetrates the cell cytoplasm to allow DNA delivery (Hu et al., 2013) (Figure 4B). Cryo-EM studies also documented the stepwise conformational changes that the baseplate in the T4 tail undergoes during adsorption to its host (Taylor et al., 2016) (Figure 4C). It is likely that additional high-resolution understanding of various processes in phage biology will be derived from applying these modern microscopy techniques on both model and non-model phages.

Conclusions

In this review, we highlighted recent discoveries in phage research in multiple scales: the massive expansion of known phage sequence space has implications in the global biosphere scale, phage genetic diversity is in turn the basis for discovery of novel molecular decision making circuits, and the study of

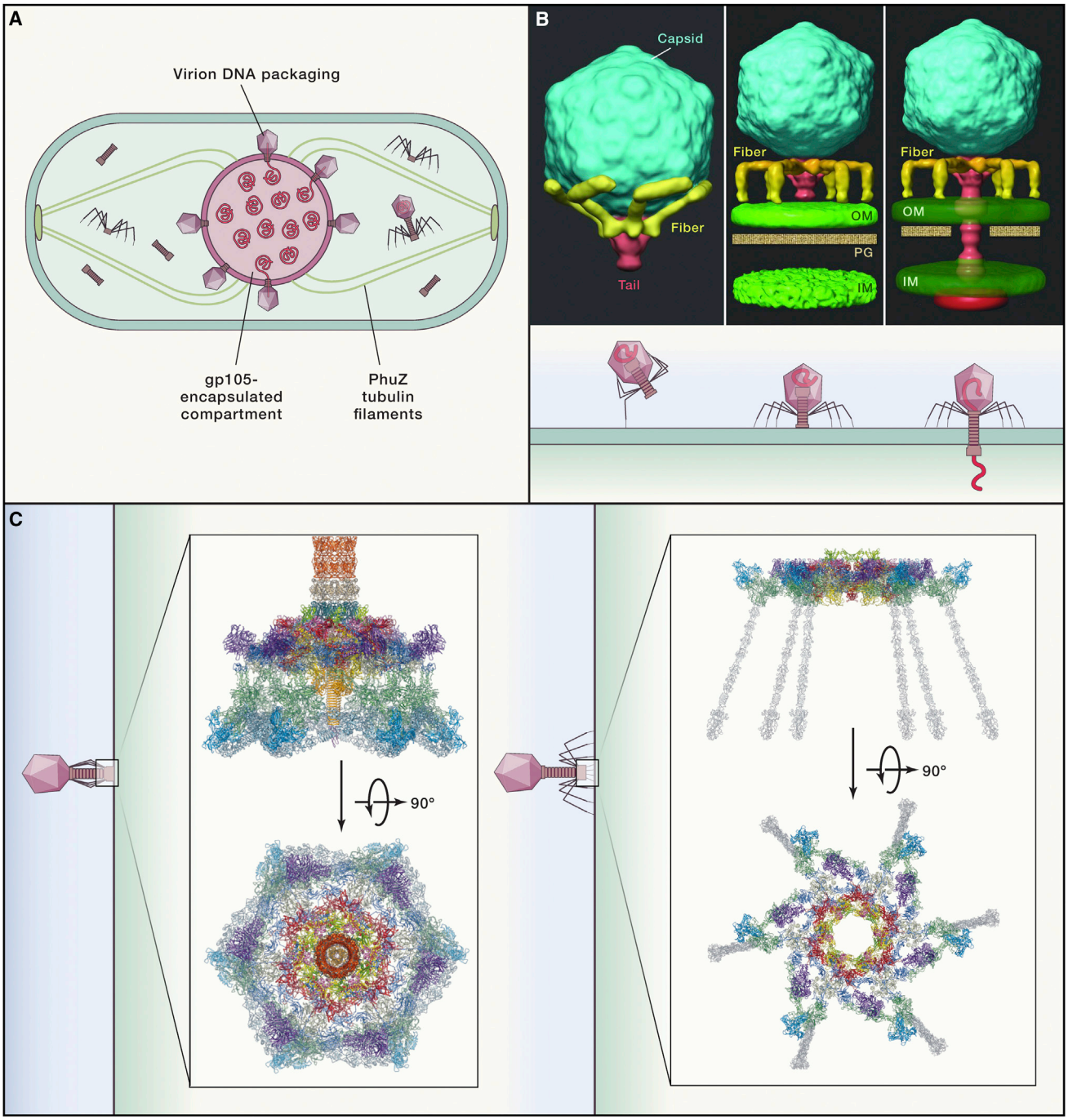

Figure 4. High-Resolution Microscopy Reveals New Dynamic Structures in Phages

(A) A nucleus-like shell encompassing the replication center of phage 201ϕ2-1 during infection of its host *Pseudomonas chlororaphis* (Chaikeeratisak et al., 2017). Tubulin-like filaments form a spindle that positions the shell inside the bacterial cell (Erb et al., 2014). The phage replication machinery and the replicating DNA are contained within the shell. Phage capsids are assembled outside of the shell and migrate to its surface to be packed with DNA. The shell was shown to constantly rotate, but the function of this rotation is unknown (Chaikeeratisak et al., 2017).

(B) Cryo-EM structural reconstruction of T7 virions during adsorption and DNA infection. Before adsorption, most tail fibers are folded onto the virion and extend only upon attachment to the cell. The internal core is then ejected and spans the double membrane. From Hu et al. (2013). Reprinted with permission from AAAS.

(C) Cryo-EM structure of the T4 baseplate before and after adsorption. The baseplate undergoes extensive structural changes, and the short tail fibers are extended to allow irreversible adsorption. Reprinted by permission from Macmillan Publishers: Nature (Taylor et al., 2016), copyright 2016.

phage-bacteria interactions provides insights on the nature of evolution in large scales and on the mechanisms that arise through the arms race in molecular scales.

The history of phage research suggests that discoveries regarding basic phage biology can be translated into valuable tools. The massive expansion of phage genome space revealed an abundance of phage metabolic genes that take part in core bacterial biological processes or modify them. These genes might be utilized in the future as parts of synthetic biology circuits. For example, synthetic carbon fixation cycles are of great biotechnological interest (Antonovsky et al., 2016), and phages are known to dramatically influence these processes in marine photosynthetic bacteria (Hurwitz and U'Ren, 2016). The new molecular decision-making circuits that are being revealed in lysogenic phages can be valuable tools in synthetic biology of biological computation circuits, as phage-recombinases were already used to construct such circuits (Siuti et al., 2013). The study of the arms race between phage and bacteria provided us with restriction enzymes and CRISPR-Cas, and the discovery of novel defense systems is a promising field of research for novel tools for DNA manipulation and genome editing. Phage anti-CRISPR mechanisms are a promising tool for genome-editing regulation (Rauch et al., 2017).

Another field that attracts renewed interest is phage therapy. Phages were used for therapeutic purposes shortly after their discovery, but the development of antibiotics in the 1940s caused phage therapy to be largely abandoned in Western countries (Abedon et al., 2011). The looming global antibiotic-resistance crisis now calls for new antimicrobials to be developed, and phages are once again being considered as potential therapeutic agents. In addition, we now understand that microbiome bacteria have beneficial roles in human health, marking phages, which can selectively eliminate pathogens while avoiding harm to the healthy microbiome, as ideal narrow-range antimicrobials. The much deeper understanding we now have of phage biology and evolution, as well as understanding of defense and counter-defense mechanisms, is expected to accelerate the development of next-generation phage therapy (Abedon et al., 2017; Roach and Debarbieux, 2017).

The past decade has shown remarkable progress in both deepening our understanding of model phages and broadening the discovery realm beyond the well-studied models. The reinvigorated interest in phage research promises further progress in uncovering their ecological roles, host-takeover mechanisms, and potential utilizations for human health. 60 years ago, phage research laid the foundations to molecular biology; contemporary phage research shows that the most abundant biological entities on Earth have many more secrets to reveal.

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DECLARATION OF INTERESTS

R.S. is a scientific cofounder and advisor of BiomX. G.O. and R.S. are inventors on U.S. provisional patent applications 62/512.219 and 62/512.216 as well as on Israel patent application 250479.

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