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Defining the expanding mechanisms of phage-mediated activation of bacterial immunity

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Due to recent discovery efforts, over 100 immune systems encoded by bacteria that antagonize bacteriophage (phage) replication have been uncovered. These systems employ direct and indirect mechanisms to detect phage infection and activate bacterial immunity. The most well-studied mechanisms are direct detection and activation by phage-associated molecular patterns (PhAMPs), such as phage DNA and RNA sequences, and expressed phage proteins that directly activate abortive infection systems. Phage effectors may also inhibit host processes and, therefore, indirectly activate immunity. Here, we discuss our current understanding of these protein PhAMPs and effectors expressed during various stages of the phage life cycle that activate immunity. Immune activators are predominantly identified from genetic approaches that isolate phage mutants that escape a bacterial immune system, coupled with biochemical validation. Although the mechanism of phage-mediated activation remains uncertain for most systems, it has become clear that each stage of the phage life cycle has the potential to induce a bacterial immune response.

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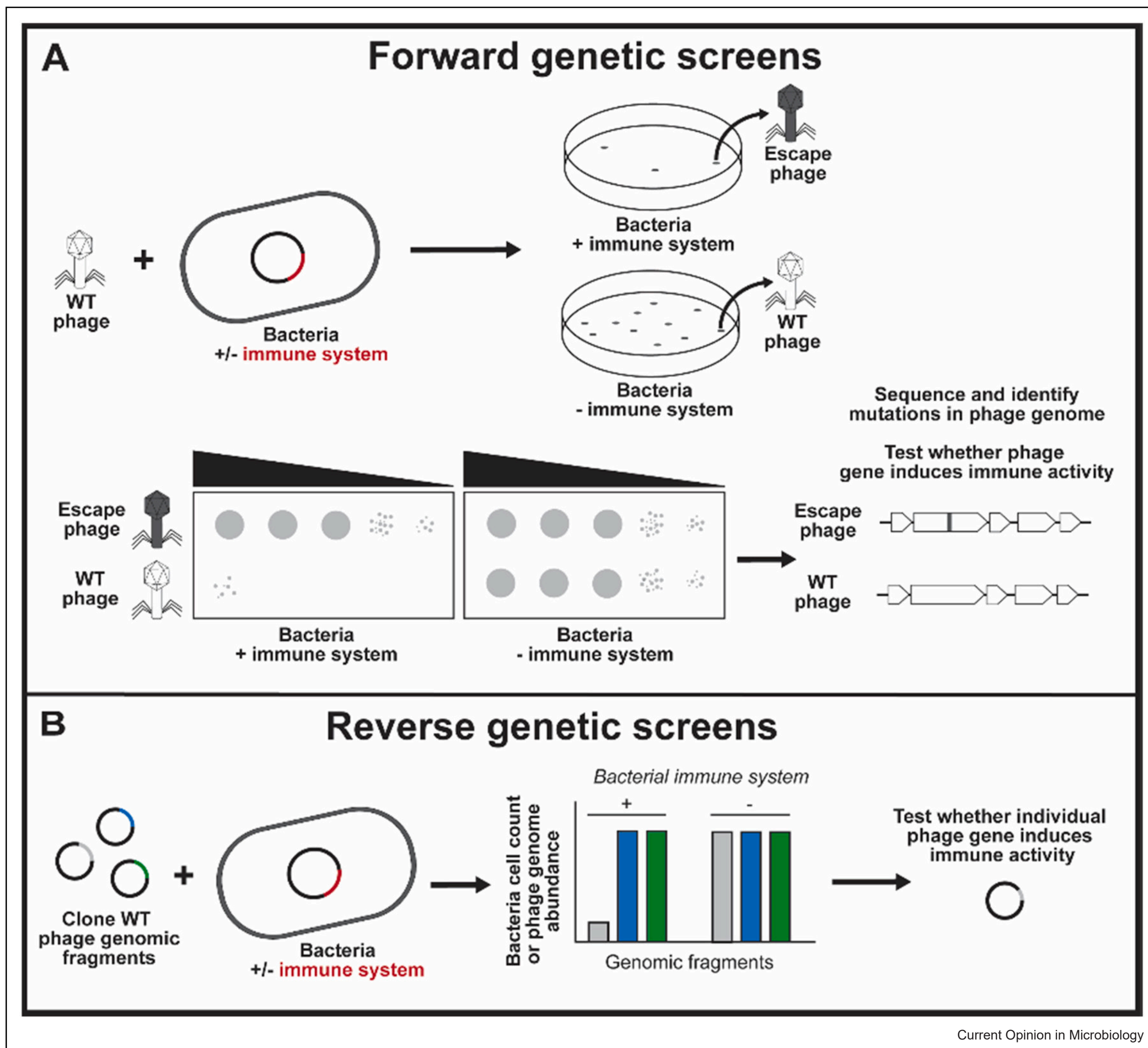
Introduction

Anti-phage bacterial immune system discovery efforts have accelerated in the past five years alone [1–6], yet the mechanisms of immune activation are largely unaddressed. Notably, several anti-phage systems are evolutionarily conserved in eukaryotic anti-viral innate immune pathways through related structures and functions of core immune proteins [7–15], suggesting that mechanisms of immune activation may also be conserved. In eukaryotic cells, viruses directly or indirectly activate innate immunity. Viruses harbor conserved features, referred to as pathogen-associated molecular patterns (PAMPs), that directly bind to the host's cognate pattern recognition receptor and then activate an immune response [16]. By contrast, viruses may produce an effector (typically a protein) that manipulates host cell structures or processes, which activates immunity [17]. Although viral effectors are diverse and rapidly evolving — making them 'bad' PAMPs — the manipulated host structures or processes are widely conserved. These two strategies of immune activation are not mutually exclusive and ensure that host cells can respond to numerous, variable 'patterns of pathogenesis' [17,18].

In bacteria, there is evidence of both direct and indirect activation mechanisms in response to phage infection akin to those observed in eukaryotic anti-viral innate immunity. We define these mechanisms of phage-mediated activation as (i) detection of phage-associated molecular patterns (PhAMPs) that directly activate bacterial immunity, and (ii) detection of phage-associated effector activities that indirectly activate bacterial immunity. Well-studied examples of PhAMPs include phage DNA and RNA sequences, which directly activate Clustered regularly interspaced short palindromic repeats (CRISPR)–Cas [19] or restriction-modification (RM) systems [20]. Numerous phage proteins or protein complexes have also been discovered to directly activate bacterial immune systems, which tend to be abortive infection (Abi) or cell death systems [7,12,14,21–23]. In parallel, phage proteins that inhibit conserved host processes, such as RM and RecBCD, have been shown to activate bacterial immunity [3,24–28]. The mechanisms of indirect immune activation are generally not well understood.

To gain mechanistic insight into how phages interface with host immunity, phage mutants can be isolated that escape immune function. These mutations may be in the gene(s) or sequence(s) activating immunity (i.e. the 'trigger'), mutate a component that is the target of bacterial immune effectors, or activate expression of an anti-immune inhibitor. Isolating escape mutants has the potential to be an efficient and successful genetic approach

Figure 1

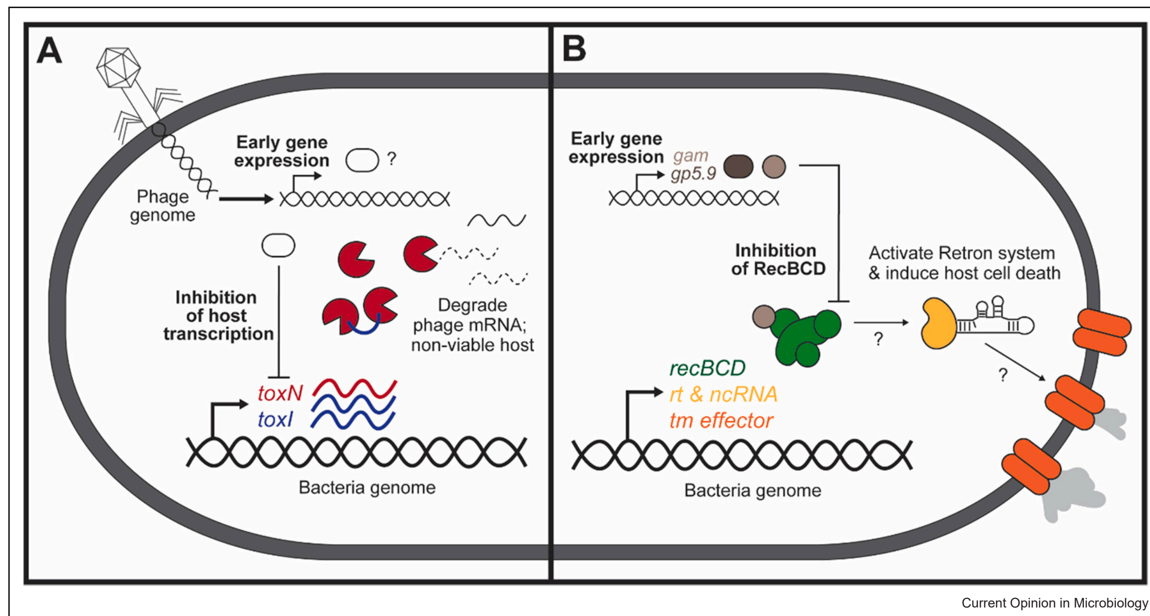


Phage genetic approaches used to identify putative activators of bacterial immune systems. **(a)** Forward genetic screen via evolved phage genetic mutants or **(b)** reverse genetic screen via unbiased phage genome fragments and then observing bacteria cell death and phage gene abundance.

because of the ability to generate large populations of phage in the lab, coupled with the strong selective pressure imparted by bacterial immune systems. Mutations can be present naturally in the population, introduced by mutagenesis, or result from recombination with related phages. A mutation in a PhAMP or effector that activates immunity will suppress its activator function and result in phage resistance to immunity, which may be referred to as an 'escape' phage (Figure 1a). Another successful approach is a reverse genetic screen, where phage genomic fragments are co-expressed with an immune system and then immunity-dependent cell death is identified (Figure 1b). This approach has been used to identify PhAMPs that activate Abi or toxin–antitoxin (TA) systems.

Combining these genetic approaches with immune function assays has validated several new protein PhAMPs and effectors as *bona fide* activators [3,7,12,29], where the immune activity is dependent on the addition of the PhAMP or effector, which is standard for validating eukaryotic antiviral immune activity [16,17,30]. Several new bacterial immunity studies have also applied biochemical and structural approaches to further define the mechanism of detection and activation [14,21–25,28]. However, in many studies that yield phage escape mutants, it is unknown whether the identified gene(s) pinpoint the activator, target, or another stage of bacterial immunity that has yet to be characterized [11,26,31–33]. Moreover, these collective studies focus on lytic phage infection, but it is also likely that unique PhAMPs or effectors activate immune

Figure 2



Phage-mediated effector activities that indirectly activate bacterial immune systems at the early stage of the phage life cycle. Examples of inhibition of host transcription and DNA repair are shown. **(a)** ToxIN system becomes activated following phage-mediated inhibition of host transcription of the *toxIN* locus [24]. **(b)** Retron–Ec48 system becomes activated following phage-mediated inhibition of the host RecBCD complex [3]. Schematics are adapted from their respective studies.

systems targeting lysogen establishment and is a potential avenue of future discovery. Here, we review evidence of the fundamental mechanisms of phage-mediated activation of bacterial immunity, focusing on protein PhAMPs and effector activities within the context of the phage life cycle, and the genetic approaches and escape phages that led to these insights.

Early-stage inhibition of host proteins or processes

Following adsorption, phages inject their nucleic acid into the bacterial cell and immediately express early genes that often inhibit or manipulate host processes, make lysis or lysogeny decisions, and initiate DNA replication. Numerous studies have shown that early phage genes encode protein inhibitors of conserved host functions, which we refer to here as phage-mediated effector activities, and serve as activators of bacterial immunity (Figure 2).

Host transcription

Inhibition of host transcription has been implicated in the ToxIN and dCTP/dGTP depletion systems. ToxIN is a TA system composed of a *toxI* RNA antitoxin and a ToxN RNase toxin that disrupts phage and host transcription [24] (Figure 2a). RNA-seq and northern blotting demonstrated that T4 phage infection inhibits host transcription, including the *toxIN* locus, so that *toxI* expression is rapidly stopped and residual *toxI* is degraded by host RNases. Following *toxI* depletion, the ToxN RNase is released and cleaves a sequence-specific motif in phage mRNA. Many T4-encoded proteins inhibit host transcription, but no single-phage protein was identified as being essential for *toxIN* inhibition. Similarly, inhibition or disruption of the host RNA polymerase (RNAP) is hypothesized to activate dCTP/dGTP depletion systems [11], but how inhibition is detected and induces activation of these systems is unknown. During phage infection, *E. coli* dCTP deaminase or *S. putrefaciens* dGTPase activity resulted in a reduction in dCTP or dGTP levels, respectively. As a result of reducing the pool of available dNTPs, phage replication stopped. T7 escape phages of each system were isolated, and all acquired mutations in *gp5.7* and/or *gp5.5*. Gp5.7 shuts down σ^S-dependent bacterial RNAP transcription, and the upstream position of *gp5.5* suggests that it regulates *gp5.7*. dCTP deaminase-expressing cells infected with Gp5.7 mutant phage incurred a smaller decrease in dCTP levels compared with infection with Gp5.7 WT phage. Future studies will need to test the dGTPase system, but current evidence suggests that phage-mediated inhibition of host RNAP activates both systems.

Host DNA production

Manipulation of host DNA synthesis activates a Retron system expressed in *Salmonella enterica* (Sen2) [25]. This Retron system consists of a reverse transcriptase (RT)

and multicopy single-stranded DNA (msDNA) complex, serving as the antitoxin and inhibiting the RcaT toxin. RcaT is proposed to target nucleic acids or nucleotides to induce cell death. To identify the activator, a reverse genetic screen was performed and *dam* and *recE* genes were identified to induce Retron-dependent cell death. Dam is a bacterial DNA methyltransferase with homologs encoded on phage genomes, and both versions can induce Retron–Sen2 toxicity. *In vitro* experiments showed that the bacterial Dam protein recognizes and methylates a dsDNA motif in the msDNA, and Dam expression levels during phage infection are sufficient to do so. RecE is a prophage-encoded nuclease that degrades msDNA and disrupts the RT–msDNA complex. Dam or RecE-mediated disruption of the RT–msDNA complex activates the RcaT toxin. Future studies are necessary to address the RcaT mechanism of toxicity and Retron–Sen2 activation in the context of phage infection.

Host DNA repair

Inhibition of the host DNA repair complex, RecBCD, activates *E. coli* Retron (Ec48) and Old nuclease systems. This Retron system's RT–msDNA complex is proposed to activate a toxic effector protein in response to phage infection (Figure 2b). λ-vir and T7 escape phages acquired mutations in *gam* and *gp5.9*, respectively, which are RecBCD inhibitors [3]. Another study also identified λ-vir *gam* mutants that evade a *Salmonella* Retron system (Se72) [26]. Co-expression of WT *gam* and Retron–Ec48 or Se72 reduced bacterial growth, whereas co-expression of the mutant *gam* did not. In parallel, expression of Retron–Ec48 or Se72 in cells with a disrupted RecBCD complex (deletion of *recB*) was toxic, providing additional evidence that inhibition of RecBCD activates specific Retron systems [3,26]. By contrast, other Retron systems do not appear to detect RecBCD inhibition (Eco8 [3,26]), and in other cases, there is not sufficient evidence to support an Abi/cell death strategy (Eco1 [25]), suggesting a diversity of Retron mechanisms. However, it remains unknown how Retrons detect RecBCD inhibition and how activation proceeds. Inhibition of the RecBCD complex (or deletion of *recB* or *recC*) also activates the Old nuclease encoded by the P2 prophage in other *E. coli* strains, which degrades phage and host DNA and causes cell death [27,34]. Additionally, λ mutants that escape Old harbor deletions encompassing *gam*, similarly to Retrons, suggest that Gam-mediated inhibition of RecBCD is a common phage effector activity. Follow-up *in vivo* and *in vitro* experiments are required to determine how the Gam–RecBCD complex is detected by each respective anti-phage immune system.

Host restriction enzymes

Inhibition of the host restriction enzymes (RE) has been studied in the context of PrrC and phage anti-restriction-induced system (PARIS). PrrC is a tRNA^{lys}-specific anticodon nuclease that is turned on by a phage anti-RM protein (Stp) [35]. *In vitro* experiments showed that the Stp protein inhibits Ecoprrl restriction activity and PrrC directly binds to and monitors Ecoprrl [28]. PrrC therefore detects Stp-mediated inhibition of Eco*prrI* and then activates its ribonuclease domain to cleave tRNA^{lys}, inhibits translation, and abrogates phage replication [36,37]. A recent study also hypothesized that inhibition of host RE activates PARIS, which is an ATPase and TOPRIM-based system that causes cell death [4]. Heterologous expression of PARIS in an *E. coli* strain that naturally harbors EcoKI was initially used for experiments. T7 escape mutants acquired mutations in *ocr*, which is a DNA mimic protein that inhibits EcoKI. However, it was also shown that T7 escape phages can acquire *ocr* mutations in the presence of PARIS alone, suggesting that PARIS may respond to the Ocr protein itself, or interactions between Ocr and another unidentified host protein. In turn, it is possible that PARIS has evolved to detect both protein PhAMPs and effector activities to induce immune activation.

Middle-stage phage DNA replication, recombination, or repair

As phage infection progresses, so does DNA replication and the expression of middle-stage genes that are involved in DNA recombination, repair, and nucleotide metabolism. These genes have been implicated in several studies of bacterial immune systems as putative PhAMPs, which by themselves or in complex with other phage components, activate immunity. However, in nearly all studies, the molecular mechanisms appear to be complex and specific PhAMPs have yet to be validated as *bona fide* activators.

DNA replication proteins

Phage DNA replication or recombination intermediates, and the phage proteins mediating DNA replication, are connected to several different bacterial immune systems. Intermediates of DNA replication or recombination are hypothesized to activate the Rex system, which is encoded in *E. coli* λ prophages and inhibits T4 rII mutant phage replication [38,39]. The main evidence lies in a heterologous expression system of RexA/B, where RexB activation is observed at the point of T4 DNA replication and recombination. In turn, RexA is proposed to detect a phage DNA-protein complex, likely a by-product of DNA replication or recombination, and then directly activates the RexB transmembrane protein [40]. RexB then disrupts cell membrane potential and ATP levels, abrogating phage replication. Phage proteins involved in replication have also been implicated in the recently identified Borvo and AbpA/B systems. Phage DNA polymerase (DNAP) interactions with DNA and/ or proteins are hypothesized to activate the Borvo

system [26]. SECΦ4 mutant phages that escape *E. coli* Borvo acquired multiple mutations across its genome, but each phage harbored mutations in its predicted DNAP. Co-expression of WT phage DNAP and Borvo reduced bacterial growth, whereas co-expression of the mutant phage DNAP only partially inhibited growth. Additional DNAP mutants were isolated in T5 and SECΦ18 phages, yet there is no detectable sequence identity between the DNAP of T5 and SECΦ4 or SECΦ18 phages, so it is proposed that the DNAP structure, its complex with other proteins or DNA, or the downstream product activates Borvo. Characterizing Borvo protein function and its binding partners is required to test this hypothesis. Lastly, phage DNA helicase mutants were shown to evade the AbpA/B system, which is encoded in the *E. coli* CP4–57 prophage and inhibits T4 phage replication [31]. T4 escape phages were isolated and most, but not all, acquired mutations in the T4 DNA helicase gene. The study has yet to determine the function of AbpA/B proteins or the mechanistic connection to the phage DNA helicase.

DNA modification proteins

Phage DNA-modifying proteins, as well as modified phage DNA itself, have been implicated in bacterial immunity. Specifically, DNA methylation has been connected to the Dazbog system [26]. *Bacillus mycoides* Dazbog was heterologously expressed and used to isolate escape phages, which acquired mutations in its DNA cytosine methylase gene. Co-expression of WT phage methylase and Dazbog reduced bacterial growth, while co-expression of the mutant phage methylase did not. *In vitro* experiments showed that WT protein methylates phage DNA and the mutant protein abolishes methylation, suggesting that methylated phage DNA or the active phage methylase enzyme induces Dazbog-dependent cell death. However, like many new systems, the molecular function of Dazbog and its putative sensing mechanism remain uncharacterized. Lastly, phage DNA modifying, packaging, and binding proteins induce restriction by an adenosine deaminase acting on RNA (RADAR) [2], yet the mechanisms of detection and activation are unknown. RADAR was proposed to edit host and phage RNA and induce in cell death, but recent studies presented evidence that RADAR functions in ATP mononucleotide deamination [41,42]. To identify the activator, a reverse genetic screen with T2 phage genomic fragments was performed in RADAR-expressing cells and then ATP deamination was quantified. Several phage genomic fragments induced RADAR-dependent deamination, and then individual genes were identified as DNA-interacting proteins. Notably, co-expression of WT phage genes *dam* (DNA adenine methyltransferase), *a-gt* (DNA alpha-glucosyltransferase), and *rnh* (Rnase H) induced RADAR-dependent deamination, while active-site mutations in those enzymes reduced it. This suggests the outcome of the phage protein activity may activate RADAR, or multiple, diverse PhAMPs are directly detected.

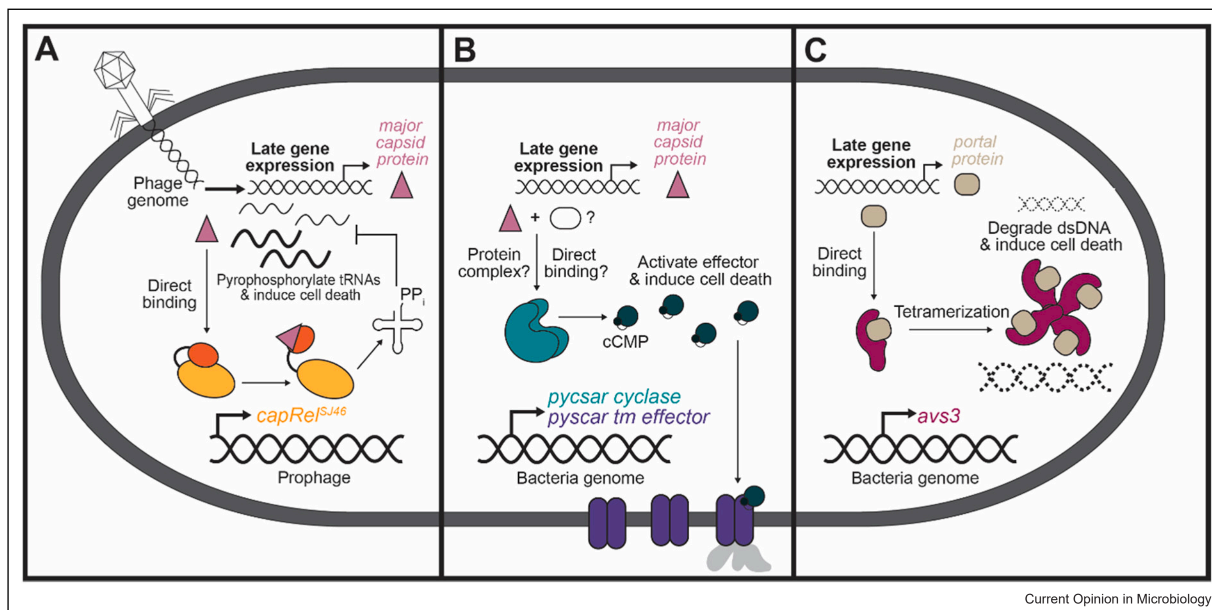
Single-stranded DNA-binding proteins

Phage single-stranded DNA-binding (SSB) proteins have roles in replication, recombination, and repair, and this conserved phage protein may activate many different bacterial immune systems. SSB interactions with DNA during replication or recombination are implicated in T-even inhibition (Tin) immunity [43]. T4 escape phage acquired mutations in its SSB gene, which encodes a protein that is involved in the formation of multiprotein–nucleic acid helical filaments. In turn, Tin is proposed to target and disrupt the phage SSB–DNA filament complex, preventing downstream DNA replication and recombination. *In vitro* experiments demonstrate that Tin and the phage SSB protein directly interact [44]. Since phage targets and activators are not mutually exclusive, SSB/DNA complex may serve both functions for Tin Phage SSB protein interactions with replicating or recombining DNA are also hypothesized to activate the Hachiman system [26]. In the same study, phage SSB mutants were shown to evade Retron systems. Escape phage (from parental phages SPR, rho14, and SBSϕJ) that evade *B. cereus* Hachiman harbored mutations in their SSB genes. SPR escape phage also contained large (~4 kb) deletions that encompassed the DNA ligase and uncharacterized or hypothetical proteins. In parallel, heterologous expression of *E. coli* Retron (Eco8) led to T7, SECϕ4, SECϕ6, and SECϕ18 escape mutations in, or upstream, of the phage SSB genes. Co-expression of WT SSB proteins with either Hachiman or Retron–Eco8 reduced bacterial growth, while the SSB mutations partially restored growth of Hachiman-expressing cells and fully restored growth of Retron–Eco8-expressing cells. Follow-up work with the Retron-Eco8 system demonstrated that the WT SSB protein pulled down with the Retron msDNA while the mutant SSB did not, suggesting that Retron-Eco8 directly detects and is activated by SSB-msDNA binding interactions. By contrast, the mechanisms of phage detection for the Hachiman system still remains unknown. Lastly, SSB proteins are hypothesized to directly activate nuclease–helicase immunity (Nhi) [32]. Nhi is a nuclease–helicase enzyme that likely degrades phage DNA, inhibiting phage replication while leaving host cells viable. The Nhi-sensitive phage JBug18 acquired resistance to Nhi through exchange of its truncated SSB with another phage's full-length SSB. *In vitro* experiments with Nhi, phage DNA, and SSB variants are necessary to determine whether full-length SSB protein is an activator, target, or protecting against Nhi activity.

Late-stage phage structural or lysis proteins

The final stage of the phage life cycle involves assembly and organization of the mature virion, such that capsid

Figure 3



PhAMPs that directly activate bacterial immune systems at the late stage of the phage life cycle. Examples of phage structural proteins are shown. **(a)** The CapRel^{SJ46} system becomes activated following direct binding to the phage major capsid monomer to the CapRel^{SJ46} protein [23]. **(b)** Pycsar becomes activated by the phage major capsid protein in an indirect manner [29]. **(c)** Avs system becomes activated following tetramerization and binding of phage portal (or terminase) protein [14]. Schematics are adapted from their respective studies.

proteins come together, packaging proteins shuttle nucleic acid into the capsid, and then the tail is attached. Several studies have demonstrated the phage capsid, packaging, and tail proteins serve as PhAMPs that directly bind to and activate their respective bacterial immune systems (Figure 3). We anticipate that phage proteins involved in cell lysis are also PhAMPs, but they have yet to be identified. Therefore, PhAMPs that directly activate bacterial immune systems are another essential and widely observed strategy of immune activation.

Capsid proteins

Phage major capsid proteins directly activate the CapRel^{SJ46} protein, whereby its C-terminal antitoxin domain autoinhibits its N-terminal toxin domain. Following phage infection, the major capsid protein directly binds to and stabilizes CapRel^{SJ46} into its open, active state so that it can pyrophosphorylate tRNAs, inhibit translation, and cause cell death [23] (Figure 3a). To identify the activator, CapRel^{SJ46} was heterologously expressed and genetically diverse phages were used to identify escape mutants. SECΦ27 and Bas8 escape phages acquired mutations in their major capsid protein. Co-expression of the SECΦ27 WT major capsid proteins, but not the mutant version, induced a CapRel^{SJ46}-dependent reduction in translation and bacterial growth. In parallel, co-expression of the Bas5 or Bas8, but not Bas4 major capsid protein, resulted in CapRel^{SJ46}-dependent reduction in bacterial growth and led to the identification of an amino acid residue (F113) that likely binds to the CapRel^{SJ46} protein. Co-IP and ITC experiments validated direct binding of the SECΦ27 major capsid protein to CapRel^{SJ46}, and AlphaFold software predicted a heterodimer structure. These genetic and biochemical results demonstrate that the major capsid protein is a *bona fide* PhAMP, and suggest that CapRel^{SJ46} may detect diverse phage capsids. Similarly, the phage major capsid protein is hypothesized to directly activate phage inhibition by F factor A (PifA), which is a part of the *pif* operon encoded on the F plasmid that is typically found in *E. coli* [45–47]. T3 and T7 escape phages acquired mutations in a GTPase inhibitor and its major capsid protein. PifA-dependent cell death and inhibition of phage replication occurs in cells expressing T3 or T7 WT major capsid genes; however, follow-up studies are required to determine the connection between PifA activity and the phage GTPase inhibitor and major capsid proteins.

Phage major capsid protein complexes directly activate Lit, and may also be implicated in pyrimidine cyclase system for antiphage resistance (Pycsar) and cyclic-oligonucleotide-based antiphage signaling system (CBASS) immunity. A complex of the phage major capsid protein with the host elongation factor EF-Tu activates Lit (late inhibition of T4) [22]. Lit is a protease that directly binds to and cleaves EF-Tu, inhibiting protein synthesis

and causing cell death. T4 escape phages acquired mutations within a 29-amino acid sequence of the major capsid protein N-terminal domain, which was later dubbed as the Gol (grow on Lit-producing bacteria) peptide [48]. Biochemical and structural experiments determined that Lit directly detects the Gol–EF-Tu complex, and in doing so, serves as the PhAMP that directly activates its protease function.

Phage major capsid mutants were demonstrated to limit Pycsar-mediated production of cyclic mononucleotide signaling molecules [29], but the mechanism of detection and activation remains unclear. LC–MS revealed that *E. coli* or *X. perforans* Pycsar expression coupled with phage infection leads to an increase of cCMP or cUMP molecules, respectively. T5 escape phage acquired mutations in the major capsid gene, which abolished cCMP production. However, the WT major capsid protein alone was insufficient to induce Pycsar-dependent cell death nor did it pull down with the Pycsar cyclase. These data suggest a higher-ordered complex with the phage major capsid protein and an additional phage or host component may activate Pycsar (Figure 3b). Lastly, phage major capsid mutants evade CBASS [33], which utilizes a variety of cyclic nucleotides to activate a downstream effector that typically results in cell death [8,49]. A native *P. aeruginosa* Type-II-A CBASS host was identified that generates cGAMP molecules following PaMx41 phage infection. Following the removal of an identified anti-CBASS gene, genetically distinct phages escaped CBASS with acquired mutations in their major capsid gene. However, like Pycsar, co-expression of the WT major capsid gene did not induce CBASS-dependent cell death. Future studies will need to focus on understanding how the WT and mutant major capsid proteins impact the initial step of CBASS — cGAMP production — and therefore determine whether the phage capsid is involved in CBASS activation. Similarly, a recent study on Type-I-B CBASS identified escape phage with mutations in its scaffold gene, which is an essential componente for mature capsid assembly. However, the authors suspect that phage capsids are not involved in CBASS activation, but rather direct binding of a structured, double-stranded phage RNA to the systems' cyclase activates cGAMP production [50]. However, it is important to note that this CBASS system's cyclase specifically accomodates viral RNA binding, while others may not, suggesting that there are multiple different mechanisms of phage-mediated activation of CBASS immunity.

Virion assembly proteins

Phage proteins involved with efficient transport of DNA into the mature capsid directly activate antiviral STAND (Avs) NTPases and an serine/threonine kinase (STK) system. Phage portal or terminase proteins directly bind to and activate Avs, inducing dsDNA degradation and cell death [14] (Figure 3c). Portal proteins are critical for virion assembly, serving as a channel for genome transport into the capsid and a site for tail attachment, while terminase proteins use ATP hydrolysis to cut and package the phage genome into the capsid. To identify these activators, a reverse genetic screen was performed with *E. coli* phage ΦV-1 fragments co-expressed in cells harboring Avs4 or Avs3 and then the cells were deep-sequenced. Gene fragments eliminated from cells in an Avs-dependent manner were further analyzed and identified the portal and terminase proteins as putative PhAMPs that directly activate Avs4 and Avs3, respectively. A follow-up genetic screen was performed that cloned portal and terminase proteins from 24 different phages and co-expressed them in cells harboring one of 15 different Avs systems. Diverse portal and terminase proteins activated the Avs, highlighting the breadth of detectable PhAMPs. Structures of the portal-Avs4 or terminase-Avs3 complex revealed tetramerization of the Avs protein, and identified key contact residues in the phage proteins. *In vitro* experiments validated that the PhAMPs directly induce Avs-mediated dsDNA degradation. This combination of genetic, biochemical, and structural approaches thoroughly validates phage portal and terminase proteins as PhAMPs.

A new phage DNA packaging protein (PacK) is proposed to directly activate the STK2 system [7], yet the mechanism of detection and activation is unclear. Following phage infection, STK2 is activated through autophosphorylation and then phosphorylates downstream host proteins, disrupting normal cell function and inducing cell death. STK2 was initially identified using *S. epidermidis* RP62a deletion strains. In the native host, STK2-dependent phage targeting was observed and then STK2 was heterologously expressed for downstream experiments. ϕNM1 escape phages acquired mutations in a gene of unknown function. DNA sequencing of the escape phages showed high DNA coverage up until the phage pac site and then gradually dropped for the remainder of the genome, suggesting that capsids contain only part of the phage genome that is packaged first. Therefore, the authors concluded that the gene is involved in DNA packaging and renamed it to *pacK*. Co-expression of PacK and STK2 resulted in STK2-dependent cell death and phosphorylation of PacK, STK2, and multiple cellular homeostasis proteins. However, it is still unclear how PacK proteins are detected and whether other host kinases are involved in this immune system.

Tail proteins

Phage tail proteins directly activate the defense-associated sirtuin 2 (DSR2) system [12]. DSR2 is an NADase that depletes NAD^+ and causes cell death following phage infection. A unique system was established to isolate escape phages, where two distinct bacterial

Table 1

PhAMPs that directly activate bacterial immunity.

	Immune system (subtype)	PhAMP	Experimental model		Mechanism of immunity	Mechanism of activation	Genetic approach and mutants	Ref.
			Bacterial host (strain)	Phage				
Middle-stage phage DNA replication, recombination, or repair	Rex	Hypothesized phage replication or recombination intermediates	*Escherichia coli* (B and K) native and heterologous expression	T4	RexA actives the TM protein RexB, which disrupts ATP levels and inhibits phage replication; likely results in cell death	Hypothesized that RexA detects phage DNA complexed with proteins that initiate replication or recombination	-	[38–40]
	Borvo	Hypothesized DNAP protein complex	*Escherichia coli* (403116) heterologous expression in *E. coli* host (MG1655)	T5, SECϕ4, SECϕ18	Unknown; contains CHAT protease domain protein [6] and results in cell death	Hypothesized that phage DNAP structure or interactions with other proteins and/or DNA activate the system	Naturally evolved mutations in phage DNAP (T5 *pol,* SECϕ4 *dnap I,* SECϕ18 genome not annotated, and predicted *dnap*)	[26]
	AbpA/B	-	*Escherichia coli* (K12) native strains	T4	Unknown AbpA/B protein functions, but they inhibit phage DNA replication; viable host cells	-	Naturally evolved mutations in DNA helicase (T4 *gp41*)	[31]
	Dazbog	Hypothesized methylated DNA or methylase protein	*Bacillus mycoides* (GOE9) and *Bacillus cereus* (FORC_013) heterologous expression in *B. subtilis* (BEST7003)	SPR	Unknown; contains DUF262 domain protein [6] and results in cell death	Hypothesized that phage methylation of DNA or the phage DNA methylase protein activates the system	Naturally evolved mutations in DNA cytosine methylase (SPR *gp161*)	[26]
	RADAR	Hypothesized DNA modification or interacting proteins	*Citrobacter rodentium* (DBS100) heterologous expression in *E. coli* host (K12)	T2	RNA editing of host and phage and/or deanimation of host ATP; results in cell death	Hypothesized that phage DNA-protein interactions activate the system	Genetic expression screen that induces RADAR-dependent edits (T2 DNA-interacting proteins *dam, a-gt*, *gp50*, *gp2*, *rnh*, and *dsbA*)	[2,41,42]
	Tin	Hypothesized SSB–DNA complex	*Escherichia coli* (B) native and heterologous expression	T4	Unknown Tin protein function, but it inhibits phage DNA replication and results in cell death	Hypothesized that Tin detects the phage SSB–DNA complex and disrupts its role in replication or recombination	Naturally evolved mutations in SSB protein (T4 *gp32*)	[43,44]
	Hachiman	Hypothesized SSB–DNA complex	*Bacillus cereus* (B4087; phylogroup III-12 9) heterologous expression in *B. subtilis* (BEST7003)	SPR, rho14, SBSϕJ	Unknown; contains helicase domain protein [1] and results in cell death	Hypothesized that phage SSB–DNA complex activates the system	Naturally evolved mutations in DNA-binding proteins (SPR SSB *gp147;* ~4-kb deletion including DNA ligase *gp136,* rho14 SSB *gp40*, SBSϕJ genome not annotated, and predicted SSB)	[26]

Table 1 (*continued*)

	Immune system (subtype)	PhAMP	Experimental model		Mechanism of immunity	Mechanism of activation	Genetic approach and mutants	Ref.
			Bacterial host (strain)	Phage				
	Retron (Eco8)	Hypothesized SSB–msDNA complex	*Escherichia coli* (200499) heterologous expression in *E. coli* host (MG1655)	T7, SECϕ4, SECϕ6, SECϕ18	RT–msDNA complex proposed to activate nuclease effector, target host, and result in cell death	Phage SSB directly binds to–msDNA and this complex is hypothesized to activate the system	Naturally evolved mutations in SSB proteins (T7 *gp*2.5, SECϕ4 *gp41,* SECϕ6, and 18 genomes not annotated; predicted SSB)	[26]
	Nhi	Hypothesized SSB protein	*Staphylococcus epidermidis* (RP62a) native and heterologous expression in *S. epidermidis* (LM1680) or *S. aureus* (RN4220)	Andhra, JBug18, ISP	Nhi is a nuclease–helicase that degrades phage DNA replication intermediates; viable host cells	Hypothesized that the N-terminal domain of the phage SSB recruits Nhi to bind phage DNA and degrade its genome	HDR-mediated genetic exchange with related phages (JBug18 exchanges its SSB protein with Andhra, both *gp03*); naturally evolved mutations in SSB protein (ISP *gp067*)	[32]
Late-stage phage structural or lysis proteins	CapRel (CapRel^{SJ46})	Major capsid protein	*Salmonella* temperate phage (SJ46) heterologous expression in E. coli host (MG1655)	SECϕ27, Bas4, Bas5, Bas8	CapRel^{SJ46} C-terminal domain (antitoxin) and autoinhibits the N-terminal domain (toxin) that pyrophosphorylates tRNAs and inhibits phage translation; results in no viable phage production and host cells	Phage major capsid protein directly binds and disrupts the CapRel^{SJ46} C-terminal antitoxin domain	Directed evolution of phage with mutations observed in phage major capsid gene (SECϕ27 *gp57*); naturally evolved mutations in major capsid gene (Bas8, *gp8*)	[23]
	PifA	Hypothesized major capsid protein	*Escherichia coli* (BL21) native and heterologous expression	T3, T7	Unknown PifA protein function, but inhibits phage DNA replication and results in cell death	Hypothesized that PifA directly binds to the capsid protein; changes in quantity or structure may activate PifA function	Naturally evolved mutations in major capsid gene (T3 and T7 *gp10*)	[45–47]
	Lit	Major capsid protein–EF-Tu complex	*Escherichia coli* (K12) native host	T4	Lit is a protease that cleaves EF-Tu and inhibits translation; results in cell death	Phage gol peptide (of the major capsid protein) directly binds to EF-Tu; Lit protease detects this complex and becomes activated	Naturally evolved mutations in major capsid gene (T4 *gp23*)	[22,48]
	Pycsar	Hypothesized major capsid protein complex	*Escherichia coli* (E831) or *Xanthomonas perforans* (GEV1011) heterologous expression in *E. coli* host (MG1655)	*Ec*: T5; *Xp:* T7	cCMP signals to TM or cUMP signals to TIR effector, both proposed to target host and result in cell death	Hypothesized that Pycsar detects the major capsid protein in complex with another phage or host protein	Naturally evolved mutations in major capsid (T5 *pb8*)	[29]

Table 1 (*continued*)

Immune system (subtype)	PhAMP	Experimental model		Mechanism of immunity	Mechanism of activation	Genetic approach and mutants	Ref.
		Bacterial host (strain)	Phage				
CBASS (Type II-A)	-	*Pseudomonas aeruginosa* (BWHPSA011) native host and heterologous expression in *P. aeruginosa* host (PAO1)	PaMx41, JBD67, JBD18	cGAMP signals to phospholipase effector, proposed to target host and likely results in cell death	Unknown	Naturally evolved mutations in major capsid gene (PaMx41Δacb2 *orf11*, JBD67Δacb2 *orf32*, and JBD18 *orf35*)	[33]
Avs (Avs4)	Portal protein structure	*Escherichia coli* (NCTC1132; EcAvs4) heterologous expression in *E. coli* host (NovaBlue; DE3)	ϕV-1	Avs4 N-terminal domain is a nuclease that degrades DNA and results in cell death	Avs4C-terminal domain detects the portal protein and activates nuclease activity	Genetic expression screen that induces Avs4-dependent cell death (PhiV-1 portal protein *gp8*)	[14]
Avs (Avs3)	Terminase protein	*Salmonella enterica* (NCTC13175; SeAvs3) heterologous expression in *E. coli* host (NovaBlue; DE3)	ϕV-1	Avs3 N-terminal domain is a nuclease that degrades DNA and results in cell death	Avs3C-terminal domain detects the terminase protein and activates nuclease activity	Genetic expression screen that induces Avs3-dependent cell death (PhiV-1 terminase *gp19*)	[14]
STK (STK2)	DNA PacK protein	*Staphylococcus epidermidis* (RP62a) native and heterologous expression in *S. epidermidis* (LM1680) or *S. aureus* (RN4220)	CNPx, ϕNM1, ϕNM2	Stk2 phosphorylates intracellular proteins and results in cell death	Phage PacK protein activates Sk2 autophosphorylation and phosphorylation of downstream proteins; unknown how PacK is detected	Naturally evolved mutations in predicted DNA PacK protein (PacK; ϕNM1 and ϕNM2 *gp14)*	[7]
DSR (DSR2)	Tail protein	*Bacillus subtilis* (29R) heterologous expression in *B. subtilis* host (BEST7003)	SPR, ϕ3T, SPβ	DSR2 is an NADase that depletes NAD+ and results in cell death	Phage tail tube protein directly binds and activates DSR2; unknown what DSR2 domain interacts with activator	HDR-mediated genetic exchange between related phages (SPR tail tube gene exchanged with ϕ3T or SPβ)	[12]

Table 2

Phage-associated effector activity that indirectly activates bacterial immunity.

	Immune system (subtype)	Effector activity	Experimental model		Mechanism of immunity	Mechanism of activation	Genetic approach and mutants	Ref.
			Bacterial host (strain)	Phage				
Early-stage inhibition of host proteins or processes	ToxIN	Inhibition of host transcription	*Escherichia coli* (GCA_001012275) heterologous expression in *E. coli* host (MG1655)	T4, T7	*toxI* RNA (antitoxin) releases ToxN nuclease (toxin) that degrades phage mRNA; results in no viable phage and host cells	Phage inhibits *toxIN* transcription and turnover of *toxI;* does not identify specific T4 inhibitor(s)	-	[24]
	dCTP deaminase	Hypothesized inhibition of host transcription	*Escherichia coli* (AW1.7) heterologous expression in *E. coli* host (MG1655)	T7	dCTP depleted and phage DNA replication inhibited; viable host cells	Hypothesized that dCTP deaminase monitors host RNAP activity or integrity	Naturally evolved mutations in inhibitor of host RNAP (T7 gp5.7)	[11]
	dGTPase	Hypothesized inhibition of host transcription	*Shewanella putrefaciens* (CN-32) heterologous expression in *E. coli* host (MG1655)	T7	dGTP depleted and phage DNA replication inhibited; viable host cells	Hypothesized that dGTPase monitors host RNAP activity or integrity	Naturally evolved mutations in, or upstream of inhibitor (T7 gp5.7 or gp5.5) or modifier (T7 gp0.7) of host RNAP	[11]
	Retron (Sen2)	Host msDNA degradation or modification	*Salmonella enterica* ser. Typhimurium (14028s) native and heterologous expression in *E. coli* host (BW25113)	T5	dGTP depleted and phage DNA replication inhibited; viable host cells	Phage-encoded Dam methylates msDNA; prophage-encoded RecE degrades msDNA, disrupts TA interaction, and activates toxin	Genetic expression screen that induces TA-dependent cell death (*dam or recE*)	[25]
	Retron (Ec48)	Inhibition of RecBCD	*Escherichia coli* (DE147) heterologous expression in *E. coli* host (MG1655)	λ-vir, T7	RT–msDNA complex activates TM effector, disrupts membrane, and results in cell death	Phage proteins (Gam, gp5.9) inhibit RecBCD and activate Retron; unknown how this process is detected	Naturally evolved mutations in RecBCD inhibitor (λ-vir *gam;* T7 *gp5.9*)	[3]
	Retron (Se72)	Inhibition of RecBCD	*Salmonella enterica enterica sv. Heidelberg* (579083-10) heterologous expression in *E. coli* host (MG1655)	λ-vir	RT–msDNA complex activates cold-shock protein effector (unknown target), results in cell death	Phage protein (Gam) inhibits RecBCD and activates Retron	Naturally evolved mutations in, or within operon of RecBCD inhibitor (λ-vir *gam, bet*, *exo*)	[26]
	Old	Inhibition of RecBCD	*Escherichia coli* (C) native strain	λ	Old is a nuclease that degrades dsDNA, results in no viable phage and host cells	Phage protein Gam inhibits RecBCD and activates Old; unknown how this process is detected	Naturally evolved mutations in operon of RecBCD (λ *gam, bet, exo*)	[27,34]
	PrrC	Inhibition of RE	*Escherichia coli* (JM107) native and heterologous expression; *E. coli* (CTr5X) native strain	T4	PrrC is a $tRNA^{Lys}$-specific anticodon nuclease that inhibits protein synthesis and results in cell death	Phage Stp protein inhibits EcoprrI; PrrC directly monitors EcoprrI and becomes active following inhibition	Naturally evolved mutations in EcoprrI inhibitor (T4 *stp*)	[28,35–37]
	PARIS			T7				[4]

Table 2 (*continued*)

Immune system (subtype)	Effector activity	Experimental model		Mechanism of immunity	Mechanism of activation	Genetic approach and mutants	Ref.
		Bacterial host (strain)	Phage				
	Hypothesized inhibition of RE	*Escherichia coli* (B185) heterologous expression in *E. coli* host (MG1655 or DH10B)		PARIS is an ATPase (unknown target), results in cell death	Hypothesized that phage proteins that mimic DNA (Ocr) are detected and activate the system	Naturally evolved mutations in EcoKI inhibitor Ocr (T7 *gp0.3*)	

immune systems that target different phages were co-expressed and subsequently forced phages to undergo recombination-mediated genetic exchange for survival. *B. subtilis* DSR2 and *Fibrobacter* pVip7 systems were co-expressed and resulted in SPR hybrid escape phages that acquired multiple genomic fragments, one of which included the tail tube gene. Co-expression of the WT tail tube and DSR2 resulted in DSR2-dependent cell death and reduced NAD^+, and co-IP of the WT tail tube and DSR2 proteins demonstrated direct binding. Structural studies are the last step to define the DSR2 mechanisms of detection.

Questions

Numerous questions remain in this re-emerging field of bacterial immunity and mechanisms of activation in the context of phage infection. First and foremost, many studies observe that some bacterial immune systems target multiple unrelated phages, but with varying strengths and escape frequencies, leading to many different hypotheses: (i) phages harbor different PhAMPs or effectors that activate the same system, (ii) phages harbor the same PhAMP or effector, but the 'strength' or ability to activate the system differs, (iii) phages harbor different targets that affect their sensitivity toward immune activity, and/or (iv) phages harbor anti-immune genes that may be fully or partially protective. Another common observation is that different phages escape a single system through acquiring mutations in different genes, which could mean (i) phages are escaping different stages of immunity, (ii) phage components co-operate to escape, (iii) phage components have redundant function, or (iv) phages acquired passenger mutations that are not involved in escape. Additionally, studies have noted that a single escape phage can acquire multiple different mutations across its genome, supporting the previously described ideas as well as: (v) phage escape mutations have a polar effect (e.g. one mutation affects upstream or downstream gene(s)) or (vi) phage escape mutations are dominant over another mutation. Lastly, numerous studies concluded that their bacterial immune systems had unknown or hypothetical activation mechanisms and remain a rich source of new discoveries. Additional genetic screens, or biochemical pull downs, of the identified WT and mutant phage components could identify new PhAMPs and effector proteins.

Conclusion and future directions

Co-evolution of phages and bacterial immune systems has resulted in a rapidly expanding diversity of immune activation, and discovery-driven biology has provided us fundamental insights into the molecular mechanisms of phage detection and activation. This has led us to define strategies of phage-mediated activation as (i) detection of PhAMPs that directly activate bacterial immunity

(Table 1), and (ii) detection of phage-associated effector activities that indirectly activate bacterial immunity (Table 2). Of the protein effectors we discussed, their activities occur during the early stage of the phage life cycle and interfere with conserved host processes. By contrast, protein PhAMPs were typically identified as late-stage structural proteins. However, it is also possible that bacterial immune systems may also target a specific stage of the phage life cycle (e.g. directly inhibiting phage tail assembly or DNA packaging) rather than use it as an activation signal for cell death or dormancy outcomes. To date, identification of phage targets of immunity has received little attention and presents new opportunities for research and discovery. Furthermore, given the conservation of eukaryotic and prokaryotic anti-viral immunity, the field is beginning to appreciate how the discovery of new bacterial immune systems may inform us about previously unknown anti-viral immune systems in humans [51]. Therefore, it is likely that we can gain further insight into each respective immune system through identification of new phage activators and targets.

Of the studies discussed in this review, many of the future directions consist of understanding the core immune protein functions, testing the *in vivo* or *in vitro* effect of WT and mutant phage components on bacterial immunity, and clarifying the role of additional phage escape mutants. However, there are several new bacterial immune systems that have identified the stage of the life cycle that phage fails to replicate (e.g. Jumbophage Killer (Juk)), but the activator is unknown and phage escape mutants have yet to be isolated [9,10,52–58]. Other systems have an unknown mechanism of immunity [1,6], but several phage escape mutants have been isolated that may pinpoint their activating PhAMP or effector [26]. There are also several instances where escape phages have been isolated, yet they acquire mutations in genes of unknown function [26]. Altogether, there are many exciting avenues to expand our understanding of bacterial immunity. Akin to the explosion of activators of eukaryotic receptors or sensors in anti-viral innate immunity, we expect that a similar wave of characterized PhAMPs and effector activities in bacterial immunity will manifest in the next 10 years.

Data Availability

No data were used for the research described in the article.

Declaration of Competing Interest

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