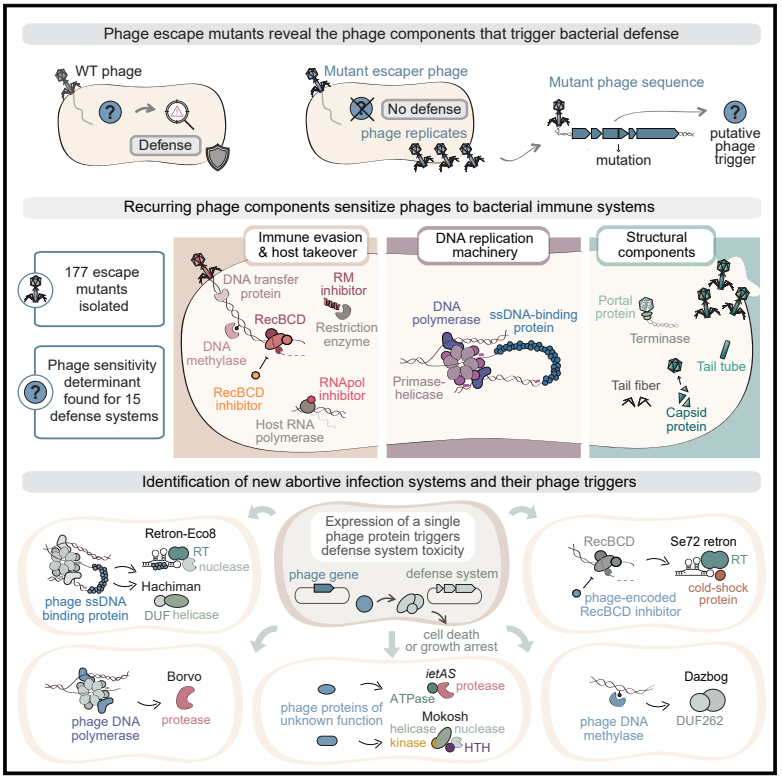
Cell

Discovery of phage determinants that confer sensitivity to bacterial immune systems

Graphical abstract



Highlights

- Phage mutants escaping bacterial immunity reveal how infection is detected
- Diverse defense systems have converged to sense the same phage components
- Discovery of the phage triggers for multiple retron defense systems
- Discovery of 5 abortive infection systems and the phage proteins that trigger them

Authors

Avigail Stokar-Avihail, Taya Fedorenko, Jens Hör, ..., Sarah Melamed, Gil Amitai, Rotem Sorek

Correspondence

rotem.sorek@weizmann.ac.il

In brief

A tour de force analysis provides fresh insights into the ways in which bacterial immune systems sense phage invaders and how the latter escape the defense mechanism.

Stokar-Avihail et al., 2023, Cell *186*, 1–14
April 27, 2023
https://doi.org/10.1016/j.cell.2023.02.029

CellPress

Please cite this article in press as: Stokar-Avihail et al., Discovery of phage determinants that confer sensitivity to bacterial immune systems, Cell (2023), https://doi.org/10.1016/j.cell.2023.02.029

Cell

CellPress

Article

Discovery of phage determinants that confer sensitivity to bacterial immune systems

Avigail Stokar-Avihail,[1] Taya Fedorenko,[1] Jens Hör,[1] Jeremy Garb,[1] Azita Leavitt,[1] Adi Millman,[1] Gabriela Shulman,[1] Nicole Wojtania,[1] Sarah Melamed,[1] Gil Amitai,[1] and Rotem Sorek[1,2,*]

[1]Department of Molecular Genetics, Weizmann Institute of Science, Rehovot 7610001, Israel

[2]Lead contact

*Correspondence: rotem.sorek@weizmann.ac.il

https://doi.org/10.1016/j.cell.2023.02.029

SUMMARY

Over the past few years, numerous anti-phage defense systems have been discovered in bacteria. Although the mechanism of defense for some of these systems is understood, a major unanswered question is how these systems sense phage infection. To systematically address this question, we isolated 177 phage mutants that escape 15 different defense systems. In many cases, these escaper phages were mutated in the gene sensed by the defense system, enabling us to map the phage determinants that confer sensitivity to bacterial immunity. Our data identify specificity determinants of diverse retron systems and reveal phage-encoded triggers for multiple abortive infection systems. We find general themes in phage sensing and demonstrate that mechanistically diverse systems have converged to sense either the core replication machinery of the phage, phage structural components, or host takeover mechanisms. Combining our data with previous findings, we formulate key principles on how bacterial immune systems sense phage invaders.

INTRODUCTION

To survive phage infections, bacteria have developed various anti-phage immune systems. The most widespread of these are restriction-modification (RM) and CRISPR-Cas, both of which cleave invader nucleic acids.[1] However, studies over the past few years have revealed a plethora of additional defense mechanisms commonly used by bacteria. These include phage restriction by prokaryotic Argonaute proteins,[2–5] production of small molecules that block phage propagation,[6–8] depletion of molecules essential for phage replication,[3,9–11] systems that use small molecule signaling to activate immune effectors,[10,12–14] retrons that involve reverse-transcription of non-coding RNAs,[15–17] and more.[1,17–23]

Although our understanding of bacterial immunity has substantially expanded by these recent discoveries, for the vast majority of the defense systems it is still unknown how they recognize invading phages, and this remains a major unanswered question in the field. Previous studies on individual defense systems, have addressed this question by examining phage mutants that escape defense.[9,13,15,24–28] Such phages sometimes evade bacterial immunity by mutations in phage components that activate the bacterial defense system; thus, escape mutants can generate valuable insights into the mechanism of defense activation.[27] For example, mutations in genes coding for phage inhibitors of the bacterial RecBCD complex enable phages to evade Ec48 retron defense, revealing that the trigger for retron immunity is phage inhibition of host RecBCD.[15] Studying mutant phages also revealed that the defense systems PrrC and PARIS trigger abortive infection when they sense phage proteins that inhibit bacterial restriction enzymes,[22,29] and the mechanism of the Lit abortive infection protein was elucidated based on the observation that T4 phages can avoid Lit activation via mutations in the activator *gol* gene.[30–32]

With the understanding that studying phage escape mutants can reveal sensitivity determinants of bacterial immune systems, we set out to systematically isolate and study phage escapers for over 50 defense systems (Figure 1). We found that the same phage protein can be sensed by different defense systems, and that central components in the phage replication cycle, including host takeover proteins, phage replication machinery and structural phage proteins, are commonly sensed by bacterial defense systems. Our study shows that bacterial immune systems have converged to sense similar phage components as a marker of infection, and lays the foundations for deciphering the mode of activation for numerous bacterial defense systems.

RESULTS

We collected a set of recently identified defense systems for which the activating phage trigger is unknown. These included four CBASS systems,[12,33] eight retron systems,[15] two SIR2-dependent systems,[3] as well as representatives of the BREX, DISARM, and Viperin systems[7,18,19] and 38 additional systems of mostly unknown function, described in Millman et al.,[1] Gao et al.,[17] and Doron et al.[20] (Figure 2). Each system was cloned

Please cite this article in press as: Stokar-Avihail et al., Discovery of phage determinants that confer sensitivity to bacterial immune systems, Cell (2023), https://doi.org/10.1016/j.cell.2023.02.029

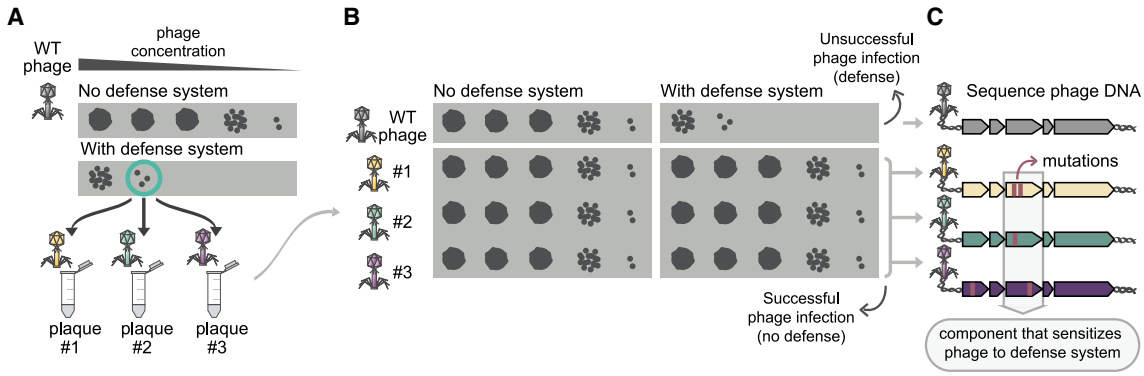


Figure 1. Pipeline for isolation and study of phages that overcome defense

(A) Defense systems cloned into *E. coli* or *B. subtilis* provide protection against phage infection, as observed using serial dilution plaque assays. Individual phage plaques that are formed on bacteria expressing the defense system are collected to screen for escaper phage mutants.

(B) The collected plaques are tested for their ability to overcome the defense system using serial dilution plaque assays.

(C) Phages that escape bacterial defense are subjected to whole genome sequencing. The genome sequences of different isolates are compared to identify loci commonly mutated in different phages, likely explaining their ability to overcome defense.

from its source organism and introduced into either *Escherichia coli* or *Bacillus subtilis*. Altogether, the collection of defense system strains examined in the current study included 62 strains representing 54 different defense systems (Figure 2; Table S1).

Bacteria expressing each of the defense systems were infected with a diverse panel of 12 *E. coli* and 14 *B. subtilis* phages (Figure 2). Despite the efficient protection that these systems provide, in many cases a small number of phage plaques still formed upon infection of bacteria expressing the system. We hypothesized that some of these plaques contain phages with a spontaneous mutation, enabling them to escape bacterial defense. These escaper phages were isolated and their ability to escape defense was verified and quantified by serial dilution plaque assays (Figures 1A and 1B). In most cases, the escaper phages generated more plaques on strains encoding the defense system compared with WT phages, whereas in some cases the escape phenotype was manifested in larger plaques of the escaping phages (Figures 3A and S1A).

Overall, we screened 199 phage-host pairs and successfully isolated 177 phages that partially or fully overcame 15 out of the 54 defense systems included in the study (Figure 3A). To identify the possible cause for phage escape from each of the defense systems, we sequenced the genomes of each of the 177 escaper phages and compared them with the sequence of the WT phage, which is sensitive to defense (Figure 1C). In 83% of the cases there was only a single mutation in the escaper phage genome; in cases where escaper phages had mutations in multiple genes, we searched for a gene that is commonly mutated in several different escaper phage isolates (Figures 1C and 3B; Table S2).

When examining the plaques formed by the escaper phages, we found that in many cases the mutant phages formed smaller plaques compared with the WT phage on control cells lacking the defense system (Figure S1D). This observation suggests that the mutation enabling phage escape from defense may come with a fitness cost. To examine this hypothesis we performed competition assays between WT and mutant phages. For this, we infected a liquid culture of bacteria lacking defense systems with a mixture of WT and mutant phages and measured the ratio between WT and mutant phages following one or two competition cycles. We found that in many cases, the mutant phage became depleted after one or two cycles (Figure S2). These results indicate that mutations that allow escape from a specific defense system also frequently result in fitness cost to the phage and explain why such mutations do not become fixed in natural settings, where each defense system is found only in the minority of bacterial hosts.[1,34,35]

The genes mutated in escaper phages mostly categorized as being involved in host takeover and immune evasion, DNA replication, or phage structural components (Figures 3B, S1B, and S1C). Below we focus on several categories of defense systems and the modes by which phages escape them.

Phage triggers for retron defense systems

Retron defense systems consist of three components: a reverse-transcriptase (RT), a non-coding RNA, and an associated effector protein.[15] The retron RT reverse-transcribes the non-coding RNA to form a chimeric RNA-DNA molecule, which, together with the RT, has been hypothesized to sense phage infection. Defense by retrons involves cell suicide or growth arrest in response to phage infection (abortive infection), which is carried out by the associated effector protein.[15,16] Based on phage escaper data, it was previously shown that retron Ec48 "guards" the cellular RecBCD complex, such that when phages inhibit RecBCD, the retron becomes active and leads the cell to growth arrest.[15] Studies on another retron, St85, demonstrated that phage proteins that degrade or modify the retron reverse-transcribed DNA activate the toxicity of the retron effector.[16] However, it is unknown how other retrons sense phages.

In the current study, we attempted to isolate defense-insensitive phages for eight retron defense systems and successfully isolated phage escapers for three of them (Figure 2). Altogether, 33 escaper phages were isolated: 10 for the Se72 retron system, 18 for Retron-Eco8, and 5 for retron Ec67 (Figure 3A; Table S2).

Please cite this article in press as: Stokar-Avihail et al., Discovery of phage determinants that confer sensitivity to bacterial immune systems, Cell (2023), https://doi.org/10.1016/j.cell.2023.02.029

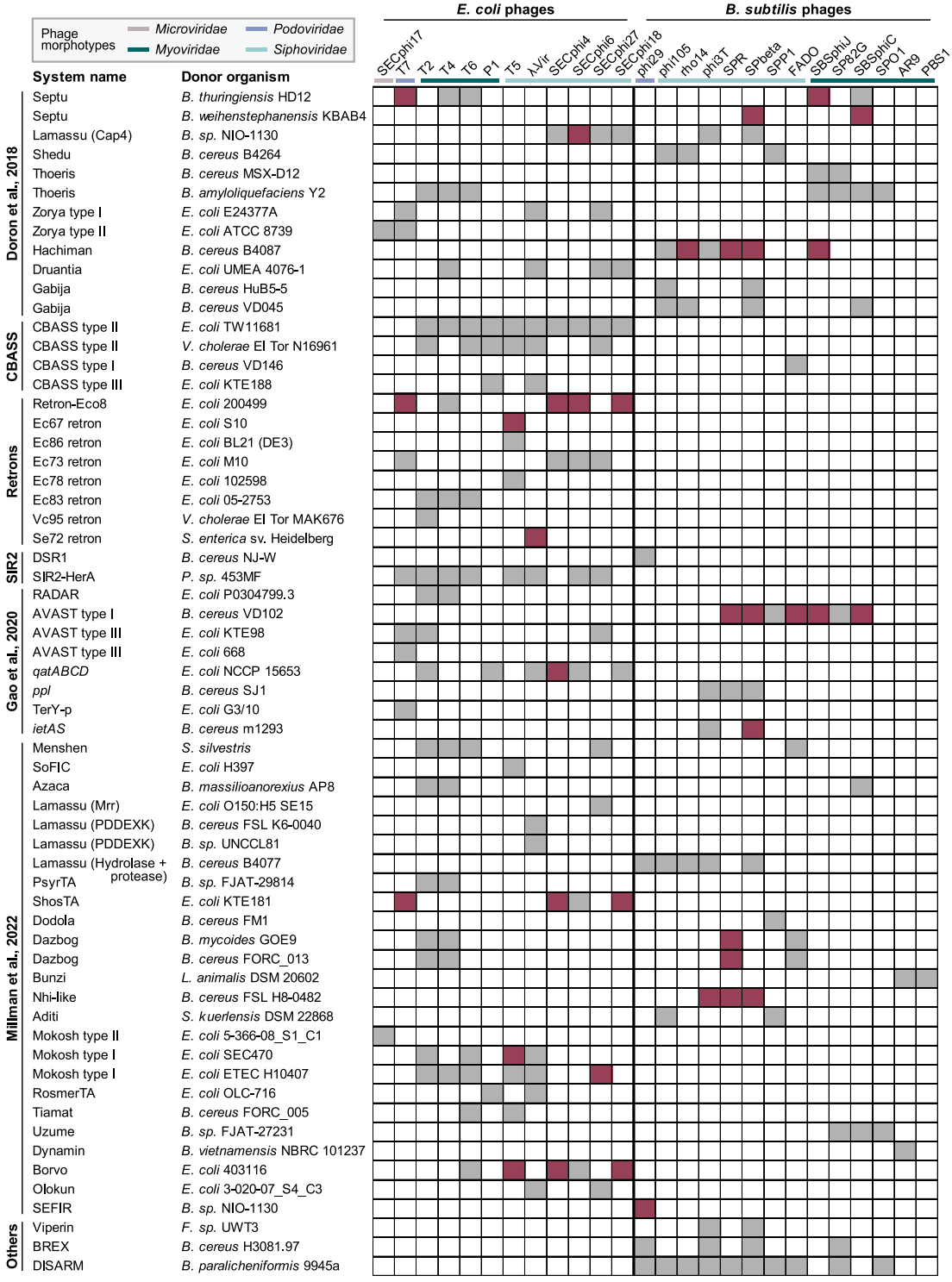


Figure 2. List of defense systems and phages included in screen

Defense systems were expressed in either *E. coli*, *B. subtilis*, or both, and subjected to an array of phages infecting the relevant host. Cases in which the system protects against a given phage are marked by a filled square. Maroon denotes cases for which at least one escaper phage was isolated, and gray denotes cases for which escaper phages were not isolated. The full names of the donor organisms for each system are provided in Table S1.

Please cite this article in press as: Stokar-Avihail et al., Discovery of phage determinants that confer sensitivity to bacterial immune systems, Cell (2023), https://doi.org/10.1016/j.cell.2023.02.029

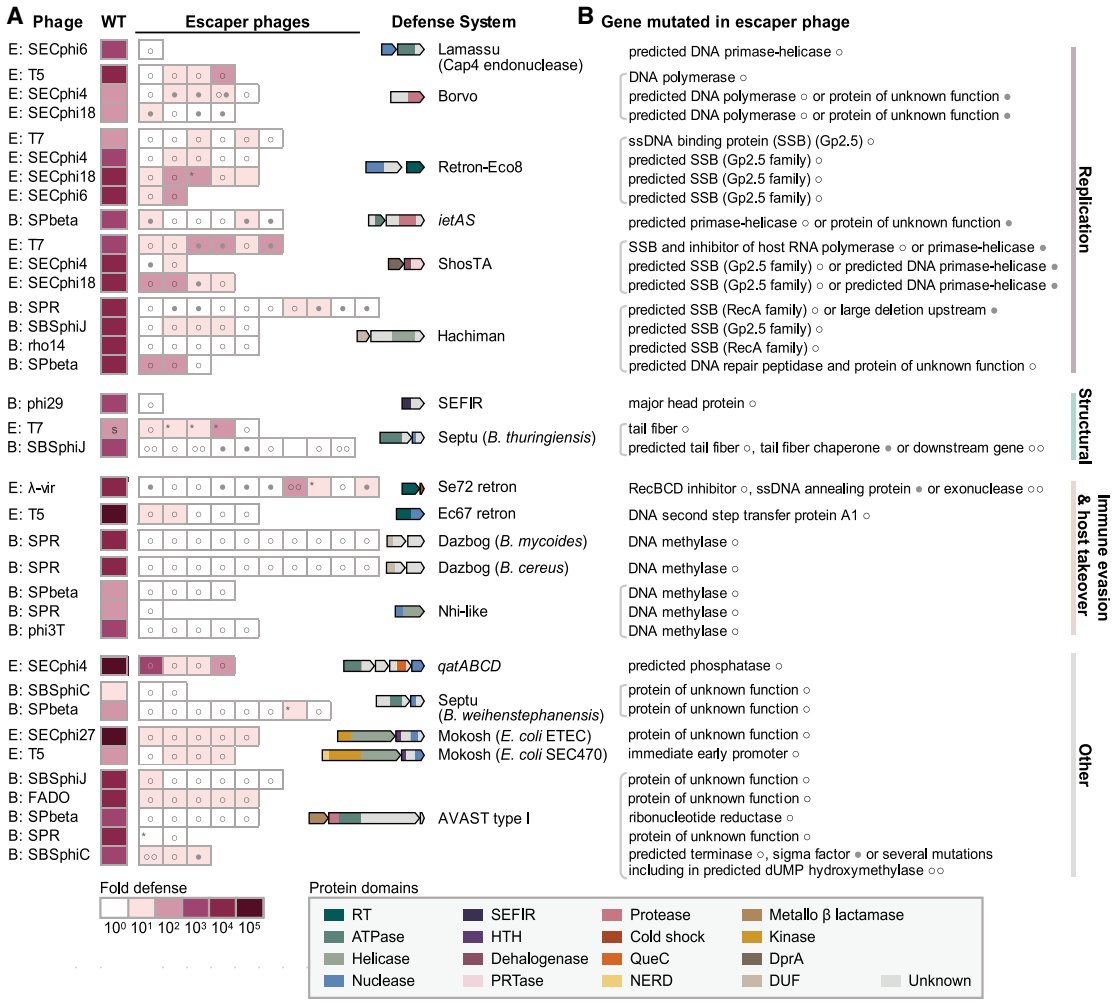


Figure 3. Phages evolve to evade diverse defense systems

(A) Anti-phage systems for which escaper phages were isolated. The isolated phage name is designated to the left, "B" and "E" mark *B. subtilis* and *E. coli* phages, respectively. Fold defense was measured using serial dilution plaque assays and calculated as described in the STAR Methods (see Figure 1B). Data represent average of two independent replicates. A designation of "s" stands for a marked reduction in plaque size on defense-containing cells compared with control cells. In these cases, escapers showed larger plaques on defense-containing cells (see Figure S1A). An asterisk on the top left of the rectangle indicates cases for which no mutation was detected in the phage sequence. Circles (empty or full) are used to specify the gene mutated in each phage isolate as detailed in (B).

(B) The identity of genes mutated in phages that escape the defense activity of each of the anti-phage systems (mutations detailed in Table S2).

See also Figures S1 and S2.

Of the ten λ-vir phages that escaped Se72 retron defense, nine contained mutations in the operon that encodes the RecBCD-inhibitor protein Gam. Mutations were found either in *gam* itself or in the two other genes in that operon, *bet* or *exo*, encoding a ssDNA annealing protein and a 5′-3′ exonuclease, respectively (Figure 3B; Table S2). This suggests that a component of the *gam-bet-exo* operon may be the trigger for Se72 defense and activate its toxic effector. To check whether either of the proteins encoded by this operon is sufficient to activate the Se72 retron, we co-expressed each of the genes together with the defense system and monitored bacterial growth. Expression of *gam* (but not *bet* or *exo*) was toxic in cells that contain the Se72 retron defense system, but not in bacteria lacking Se72 (Figure 4A). Expression of the mutated version of *gam*, found in escaper phages that overcame Se72-mediated defense, was not toxic in retron-containing cells (Figure 4A). These data indicate that the λ phage Gam protein is likely the phage component that activates Se72 retron defense. We hypothesize that mutations in *bet* or *exo* lead to escape because of a polar effect on the *gam* gene.

Because the λ phage Gam protein is an inhibitor of the host RecBCD complex, we hypothesized that, as in the case of the Ec48 retron system,[15] the Se72 retron may function by monitoring the integrity of the host RecBCD complex. To test whether perturbation of RecBCD triggers the toxic activity of the Se72 retron system, we checked if the Se72 system can be expressed

Please cite this article in press as: Stokar-Avihail et al., Discovery of phage determinants that confer sensitivity to bacterial immune systems, Cell (2023), https://doi.org/10.1016/j.cell.2023.02.029

CellPress

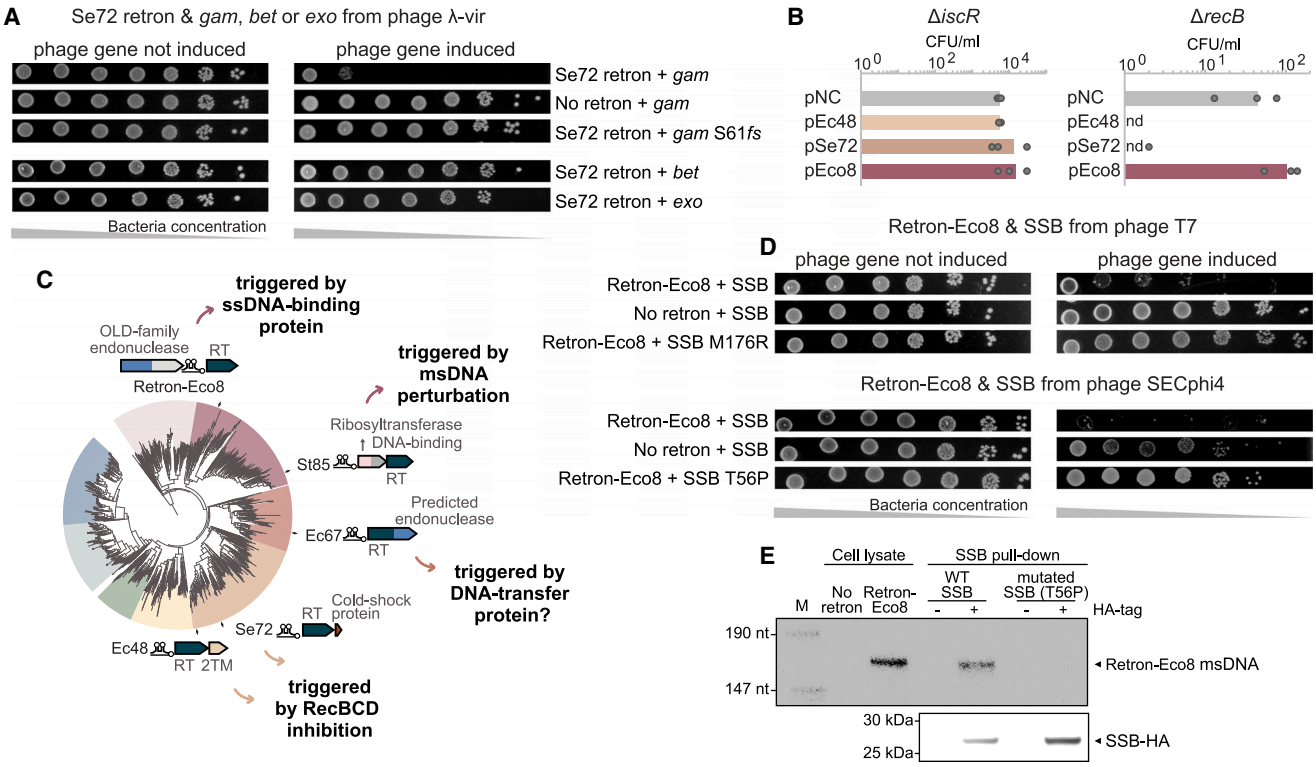


Figure 4. Phage triggers for retron defense systems

(A) Expression of a phage-encoded RecBCD inhibitor activates toxicity in a strain that contains the Se72 retron system. Bacterial viability was measured for cells encoding the Se72 defense system and a single phage gene (either *gam*, *bet* or *exo*) by plating 10-fold serial dilutions of cells in conditions that repress or induce expression of the phage gene. "No retron" is a negative control strain containing an empty vector instead of a retron. *gam*S61fs is *gam* with a frameshift mutation, found in phages that overcome Se72 (Table S2).

(B) A vector that contains the Se72 retron anti-phage system cannot be transformed into cells lacking RecB. Transformation efficiency of a plasmid containing either the Ec48, Se72 or Eco8 retron systems or an empty vector as a negative control (pNC) into an *E. coli* strain with a deletion of either *recB* or *iscR* (negative control). The number of colony-forming units (CFUs) per milliliter after transformation is presented. Bar graphs represent an average of three replicates, with individual data points overlaid. "nd," not detected, meaning that no colonies were detected (limit of detection = 1 CFU/mL).

(C) Phylogenetic analysis of ~4,800 homologs of retron reverse-transcriptases, adapted from Millman et al.[15] Selected retrons are marked, with the predicted phage-encoded activator denoted. The triggering mechanism for the St85 retron was reported previously.[16]

(D) Expression of a phage-encoded ssDNA-binding protein (SSB) activates toxicity in a strain that contains the Retron-Eco8 system. Bacterial viability was measured for cells encoding the Retron-Eco8 defense system and the WT and mutated SSB proteins from phage T7 or SECphi4. The experiment was performed as described for (A).

(E) Phage SSB binds Retron-Eco8 msDNA. HA-tagged WT or mutant SECphi4 phage SSBs were expressed with the Retron-Eco8 defense system. Presence of the Retron-Eco8 msDNA was detected using northern blotting on RNA extracted from the pulled-down SSB protein (top image). "Cell lysate" are negative and positive control samples in which RNA was extracted from a whole cell lysate without protein pull-down. "M" designates size marker. The bottom image shows a western blot of the pulled-down SSB proteins. Representative of two technical replicates.

See also Figure S3.

in cells that lack RecB. Although a control vector was transformed with high efficiency into Δ*recB* cells, a vector encoding the Se72 system appeared toxic in Δ*recB* cells (Figure 4B). The same Se72-containing vector transformed with high efficiency into a control strain deleted in an unrelated gene (Δ*iscR*), indicating that the perturbation of RecBCD activates the toxicity of the Se72 retron system. Together, our results suggest that retrons Ec48 and Se72 both guard the RecBCD complex. Interestingly, these two systems differ in their operon composition: the Ec48 system encodes an effector protein with predicted transmembrane helices, whereas the Se72 system harbors an effector with a cold-shock protein domain that has no homology to the effector of Ec48. By contrast, these two retrons share close homology in their RT proteins (Figures 4C and S3A). These data support a model in which the retron RT, possibly in combination with the non-coding RNA/DNA component, is involved in RecBCD sensing.

We have previously shown that Retron-Eco8 readily transforms into cells deleted in *recB*, which indicated that this retron is triggered via a mechanism possibly unrelated to RecBCD inhibition[15] (Figure 4B). To investigate the phage trigger for this retron, we examined 18 mutant phages that overcome the Retron-Eco8 system. These included six escape mutants of phage T7 and 12 mutants of a group of phages that are closely related to each other: SECphi4, SECphi6, and SECphi18 (Figure 3; Table S2). In all T7 escapers we found either one or two missense

Please cite this article in press as: Stokar-Avihail et al., Discovery of phage determinants that confer sensitivity to bacterial immune systems, Cell (2023), https://doi.org/10.1016/j.cell.2023.02.029

point mutations in the gene encoding the phage single-stranded DNA-binding protein (SSB), which is essential for T7 phage DNA replication.[36] The T7 SSB interacts with the phage DNA polymerase and primase-helicase to form the replication complex, where it binds and protects ssDNA intermediates during replication.[37] Intriguingly, ten of the twelve SECphi4, SECphi6 and SECphi18 phage mutants that escaped Retron-Eco8 were also mutated either in the promotor or coding sequence of a predicted SSB protein that has structural homology with the T7 SSB (Figure S3B), indicating that distantly related phages can escape Retron-Eco8 defense via mutations in their SSB protein (Figure 3B; Table S2). Co-expression of Retron-Eco8 with the SSB protein of either the T7 or the SECphi4 phages resulted in cellular toxicity, and this toxicity was alleviated when these SSB proteins harbored the point mutations found in the escaper phages (Figure 4D). These results suggest that the phage SSB protein forms the trigger for the abortive infection function of the Retron-Eco8 system.

Because the retron generates reverse-transcribed multicopy ssDNA (msDNA) that is critical for its defensive capacity,[15] we hypothesized that the phage SSB protein triggers retron defense by interacting with the msDNA. To test this hypothesis, we expressed a C-terminally tagged SSB protein from the SECphi4 phage in the presence of Retron-Eco8. We then pulled down the phage SSB, extracted the nucleic acids bound to the protein, and performed northern blotting to check for the presence of the Retron-Eco8 msDNA. We found that the WT phage SSB indeed binds Retron-Eco8 msDNA (Figure 4E). An SSB with the point mutation (T56P) found in the phage that escaped Retron-Eco8 defense did not pull down the retron msDNA. Our data, therefore, suggest that the msDNA in Retron-Eco8 directly senses the phage SSB protein, and that this sensing triggers the retron abortive infection activity.

Five T5 phage escapers were isolated for the retron Ec67 defense system (Figure 3; Table S2). All isolates had missense mutations in the T5 phage *A1* gene (either I36T, S47P, A15T, C33R, or V214M), and one of them had an additional mutation in the stop codon extending the A1 protein by 17aa (Table S2). A1 is an essential early-expressed protein required for the transfer of T5 DNA into the host, and A1 mutants display various phenotypes, including defects in host DNA degradation and host synthesis shutoff.[38] Structural modeling of the A1 protein reveals a predicted C-terminal nuclease domain and N-terminal DNA-binding domain (Figure S3C). Four of the mutations found in the T5 escapers localized to this predicted DNA-binding domain, implying that the DNA-binding activity of the A1 protein may be involved in sensitizing T5 phages to the Ec67 retron defense system. The A1 protein is toxic to *E. coli*[39]; therefore, we could not check whether expression of A1 alone activates Ec67 retron toxicity.

A common theme among retron-interacting proteins found in this and other studies, including T7 and SECphi4 SSB proteins, T5 A1, Rac prophage DNA exonuclease,[16] *E. coli* DNA methylase,[16] and RecBCD, is that they all have the capacity or predicted capacity to interact with DNA. It is possible that interaction of these proteins with the retron-generated ssDNA forms part of the basis for their sensing by the retron system, as demonstrated in the case of the SSB protein (Figure 4E).

Identifying abortive infection systems and their phage triggers

For the vast majority of anti-phage systems, it is still unknown how the system defends against infection. In the case of the defensive retrons, which provide protection by an abortive infection mechanism, our data showed that phages can escape defense via mutations in the phage protein that would normally trigger the system's toxicity. Expression of this protein alone was sufficient to trigger the toxic activity of the system (Figures 4A and 4D).[15] Based on this, we reasoned that the genes identified in our screen as mutated in escaper phages could be utilized to expose anti-phage systems, other than retrons, that function via abortive infection in response to a specific phage protein. If anti-phage defense functions via abortive infection, co-expression of the defense system with the respective activator phage protein is expected to elicit toxicity.

To identify systems potentially functioning via abortive infection, we paired each system with the phage gene found to be mutated in escaper phages isolated on that system. We then expressed these phage genes in cells that contain the respective defense system, and examined the bacterial colonies for signs of toxicity (Figures 5A–5E and S4; Table S3). Altogether, 24 pairs of defense system/phage gene were tested. In some of the cases, expression of the phage gene was not toxic in cells that contained the defense system (Figure S4A), suggesting that either the system does not elicit toxicity when activated, or that the specific phage gene, when expressed alone, is necessary but not sufficient to activate abortive infection (as recently suggested for Pycsar[13] and CBASS[28]). In several other cases, expression of the phage gene exhibited non-specific toxicity in both defense system-containing cells and in control cells lacking the system (Figure S4B). In five cases, expression of the phage gene was toxic in defense system-containing cells but not cells that lack the system, revealing a suspected abortive infection function for the systems Hachiman,[20] *ietAS*,[17] Borvo, Mokosh type I,[1] and Dazbog[1] (Figures 5A–5E). Co-expression of the defense system with the mutated version of the phage gene alleviated toxicity either fully or partially, explaining how mutant phages escaped defense (Figures 5A–5E).

Infection experiments in liquid cultures further supported the hypothesis that these systems protect via abortive infection, because introduction of phage at a high multiplicity of infection (MOI) resulted in culture collapse or growth arrest without phage propagation, whereas the cultures survived infection at low MOI (Figures 5F and S4C).[40] These results reveal an abortive infection phenotype, as well as the phage trigger, for systems whose mode of action was so far unknown.

Notably, in the cases of Borvo and Hachiman, overexpression of the mutated phage gene in the presence of the defense system was still partially toxic compared with expression of the WT phage gene (Figures 5A and 5D). It is possible that during infection, the mutated phage protein partially activates the defense system but in a manner that still allows the phage to escape defense, as shown for phages evading nucleotide depletion systems.[9] Alternatively, the toxicity we observed for the mutated phage gene could be because of its

Please cite this article in press as: Stokar-Avihail et al., Discovery of phage determinants that confer sensitivity to bacterial immune systems, Cell (2023), https://doi.org/10.1016/j.cell.2023.02.029

CellPress

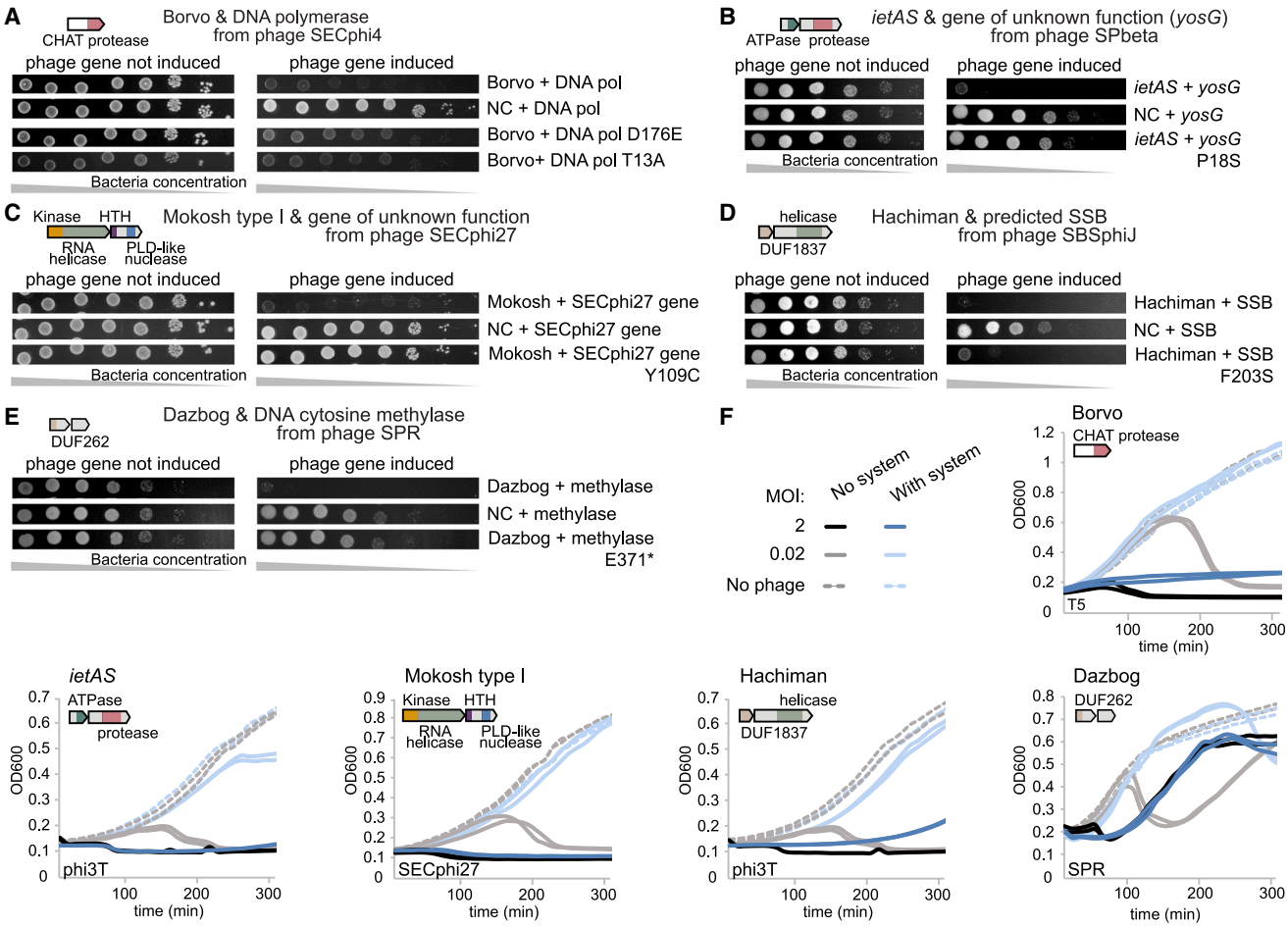


Figure 5. Identification of defense systems that function via abortive infection and the phage genes that activate them

(A–E) Expression of a single phage gene triggers toxicity in defense system-containing strains. Bacterial viability was measured by plating serial dilutions of cells encoding the specified defense system and phage gene under conditions that repress or induce expression of the phage gene. NC, is a negative control strain lacking the defense system. (A)–(E) show representative of three replicates. (A) Expression of WT or mutated DNA polymerase from phage SECphi4 in *E. coli* MG1655 with the Borvo system from *E. coli* 403116. (B) Expression of WT or mutated *yosG* from phage SPbeta in *B. subtilis* BEST7003 with the *ietAS* system from *B. cereus* m1293. (C) Expression of WT or mutated protein of unknown function (128aa) from phage SECphi27 in *E. coli* MG1655 with the Mokosh system from *E. coli* ETEC H10407. (D) Expression of WT or mutated SSB from phage SBSphiJ in *B. subtilis* BEST7003 with the Hachiman system from *B. cereus* B4087. (E) Expression of WT or mutated DNA methylase from phage SPR in *B. subtilis* BEST7003 with the Dazbog system from *B. mycoides* GOE9.

(F) Growth curves in liquid culture for E. coli MG1655 or *B. subtilis* BEST7003 bacteria that contain the specified anti-phage system ("with system") and bacteria that lack the system ("no system"), infected with *E. coli* phage T5 or SECphi27 or *B. subtilis* phage phi3T or SPR. Bacteria were infected at time 0 at a multiplicity of infection (MOI) of 0.02 or 2. Two biological replicates are presented for each experiment as individual curves. The experiments with Hachiman and *ietAS* were performed simultaneously with the same negative controls. The regrowth observed when the temperate phage SPR infects Dazbog-containing and Dazbog-lacking strains is attributed to the formation of SPR lysogens.

See also Figures S4 and S5.

overexpression, which may not represent its native expression during infection.

Although our approach reveals phage genes whose expression activates toxicity of abortive infection systems, it does not indicate whether the phage protein activates the defense system by direct binding or, alternatively, indirectly triggers the system. For example, the Dazbog system becomes toxic in cells that express a DNA methylase from phage SPR (Figure 5E). This methylase is known to methylate phage DNA at CCGG and other related motifs.[41] We found that the genome of SPR phages that escaped Dazbog became unmethylated at CCGG, showing that the escape mutation in the methylase abolished its DNA methylation function (Figure S5). It is therefore possible that Dazbog may be an abortive infection system triggered by the presence of methylated DNA, which is consistent with the growth arrest phenotype seen when Dazbog-expressing cells are infected at high MOI (Figure 5F). The first gene in the Dazbog system harbors a domain (DUF262, pfam PF03235) found in other defense systems that sense modified DNA (GmrSD and SspABCDE),[42–44] supporting the hypothesis that Dazbog activity depends on the recognition of modified DNA.

Please cite this article in press as: Stokar-Avihail et al., Discovery of phage determinants that confer sensitivity to bacterial immune systems, Cell (2023), https://doi.org/10.1016/j.cell.2023.02.029

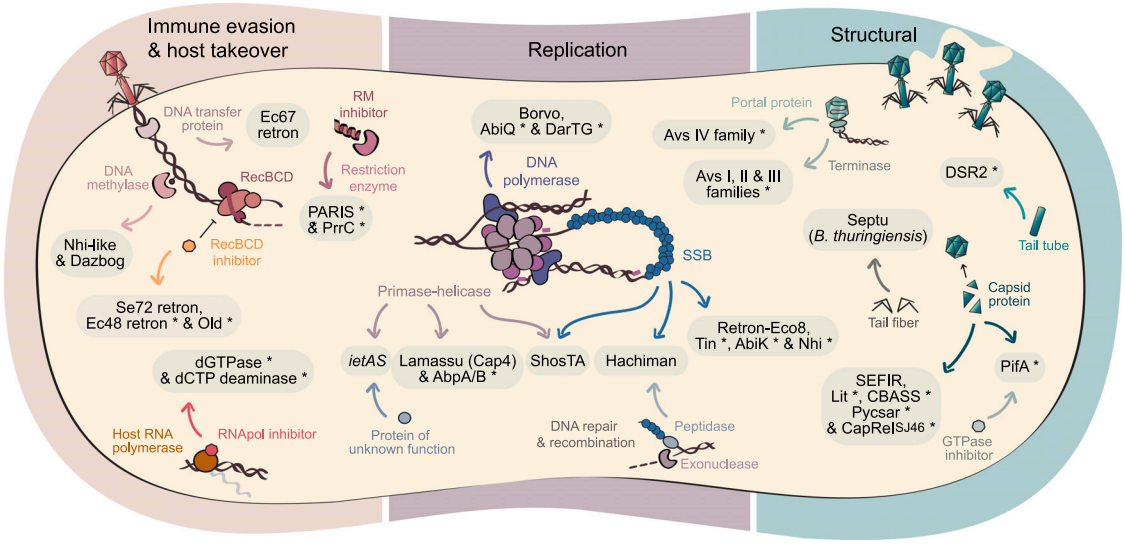


Figure 6. Recurring themes in phage escape from bacterial defense

Schematic depiction of the proteins mutated in phages that escape bacterial anti-phage systems listed in Figure 3B, as well as selected examples from previous studies (marked with an asterisk; Table S4). Proteins listed in Figure 3B that do not correspond to one of the three groups are not depicted. Phage proteins are depicted as colored shapes and text and defense systems in black text.

Recurring themes in phage escape from bacterial defense

The large number of escaper phages isolated in this study, and the richness of the defense system cohort studied, reveal common themes among the mutations that allow phage escape from bacterial defense. We identify three such main categories: components of the phage core replication machinery, phage structural proteins, and host takeover mechanisms.

Components of the phage core replication machinery

In many cases, escape mutations were detected in proteins that comprise the phage core replication machinery, including the DNA polymerase, primase-helicase, and SSB (Figures 3 and 6). Such mutations allowed phages to escape the systems Lamassu, Hachiman, *ietAS*, Retron-Eco8, ShosTA, and Borvo (Figures 3 and 6). Previous studies on the anti-phage defense systems AbiK,[45,46] AbiQ,[47] AbpA/B,[26] DarTG,[48] Tin,[49] and Nhi[24] have also reported phage escape via mutation in one of these core replication proteins. Thus, diverse defense systems may have evolved to sense or target the core determinants of phage replication. Because the phage replication machinery is essential for successful infection, it is advantageous for defense systems to converge on core components of this machinery as a trigger for infection recognition. Moreover, the essentiality of the replication complex would limit the possibility for loss-of-function escape mutations. Indeed, our phage competition assays showed severe fitness cost for T7 mutated in its SSB protein (escaper from Retron-Eco8), as well as for SECphi4 and SECphi6 phages mutated in their primase-helicase gene (escapers from ShosTA and Lamassu, respectively) (Figure S2). Another advantage of sensing the SSB protein, specifically, is that it is one of the most abundant proteins generated by the phage during infection,[50] offering ample targets for the defense systems to recognize.

In some cases, the same replication machinery gene was mutated in multiple phylogenetically distant phages to allow escape from the same defense system. For example, the T5, SECphi4 and SECphi18 phages overcame Borvo defense via a missense mutation in the phage DNA polymerase (Figure 3B; Table S2). In all cases, escape mutations occurred in the proofreading cleft of the polymerase (Figure S6). Notably, two mutations were in residues that are highly conserved across DNA polymerase proteins (T13 and D176 in the SECphi18 DNA polymerase) and were shown to play an important role in the ssDNA binding and metal binding functions of the proofreading domain.[51,52] Because there is no clear sequence homology between the DNA polymerase of T5 and the other two phages, it is possible that this system senses the general structure of the polymerase, its interaction with other proteins, or the outcome of its interaction with DNA, as a signature for infection. Additional mutants that escaped Borvo defense had mutations within or upstream of a short gene encoded immediately upstream of the phage DNA polymerase (Figures 3B and S1B). The presence of this gene within the same operon implies that its function might be connected to that of the polymerase.

Phage escape from the Hachiman and ShosTA defense systems also involved mutation in genes that participate in DNA replication and manipulation (for Hachiman, SSB proteins of the Gp2.5 and RecA families, and a DNA repair protein, and for ShosTA, SSB, and primase-helicase proteins). The fact that mutations in different DNA replication and manipulation proteins enable phage escape from defense suggests that Hachiman and ShosTA may respond to a protein-DNA complex produced as an intermediate of phage DNA replication or recombination, rather than to a specific phage protein, as suggested for the RexAB system.[53] Interestingly, the ShosTA system was previously shown to encode a toxin-antitoxin system,[54] whereby the

antitoxin shows homology to DprA, a protein that binds ssDNA and SSB proteins.[22,55] It is therefore possible that the ShosTA DprA-like protein acts as the sensor of this system, triggered by a phage replication intermediate that involves ssDNA. These hypotheses remain to be explored.

Phage structural proteins

A second major category of escape mutations involves phage structural genes (Figures 3 and 6). For example, mutations in phage capsid proteins allow various phages to overcome the defense systems SEFIR (Figure 3B; Table S2), Pycsar,[13] CBASS,[28] PifA,[56] Lit,[32] and CapRel^{SJ46}.[57] Most of these systems function via abortive infection, but at least in the case of CBASS, Pycsar, and SEFIR, co-expression of the wild-type form of the major capsid protein with the defense system did not activate toxicity of the system (Figure S4A).[13,28] This implies that the system might be triggered by a higher order structure of the capsid, or by the interaction of the capsid protein with other phage or host factors, as shown for the Lit abortive infection protein.[31] In other cases, direct interaction of the phage structural protein with the defense system results in activation of abortive infection, as recently demonstrated for the DSR2,[3] CapRel^{SJ46},[57] and Avs[58] systems. It makes biological sense for abortive infection systems to sense phage structural genes, which are typically expressed late during infection. In this way, an abortive infection system will elicit its toxic effect as a last resort when recognizing that phage infection has advanced to its late stages.

Phages that escaped *B. thuringiensis* Septu defense had mutations in their tail fiber genes (Figures 3 and S1C). Interestingly, this system provides defense against both *E. coli* and *B. subtilis* phages when expressed in either *E. coli* MG1655 *or B. subtilis* BEST7003, respectively. We isolated *E. coli* T7 phages and *B. subtilis* SBSphiJ phages that overcome Septu defense, and, although these phages are very distant from each other and infect different hosts, they both escaped the Septu system upon mutation in the phage tail fiber gene (Figures 3B and S1C). Moreover, for both phages, the mutations were located at the C-terminal tip of the tail fiber, suggesting a similar recognition mechanism despite no detectable sequence similarity between the tail fibers of these phages (Figure S1C; Table S2). It is possible that Septu may recognize the structure of the tail rather than its sequence. Such a mechanism has been found for example for the Avs proteins that detect the structures of diverse phage terminase or portal proteins sharing less than 5% sequence identity.[58] On the other hand, escape from a different variant of the Septu defense system, cloned from *B. weihenstephanensis*, occurred upon mutation in different phage genes apparently unrelated to the phage tail fiber (Figure 3B), suggesting that different variants of the same system may sense different phage components.

Host takeover mechanisms

A third category of escape mutations occur in genes involved in host takeover and immune evasion. These include genes whose protein products inhibit RecBCD, thus activating retrons Se72 and Ec48,[15] as has also been shown for the P2-encoded protein Old.[59,60] Another example are proteins that inhibit RM systems, resulting in activation of the PrrC[29] and PARIS[22] abortive infection systems. These systems are "guardians" of core defense machineries in the cell and become activated as a second line of defense if the phage tampers with the guarded system.[15] The Dazbog and Nhi-like systems form another example of how phage evasion of one system can trigger another system. In both cases, loss-of-function mutations in a phage DNA methylase enable the phage to overcome defense (Figures 3B and S5). Thus, methylation of phage DNA, which normally protects phages from cleavage by RM systems, likely sensitizes them to the Dazbog and Nhi-like systems. Phage takeover and manipulation of the host transcription machinery also seems to be the focal point for sensing by several defense systems. For example, inhibition of host transcription was shown to trigger the toxin-antitoxin system toxIN[61] and likely also the defensive deoxynucleotide depletion enzymes dCTP deaminase and dGTPase.[9]

DISCUSSION

In this study, we attempted to address the key question of how bacterial immune systems sense phage infection. By screening for phage mutants that can overcome bacterial immunity, we identified the components sensitizing phages to 15 defense systems. Although previous studies focused on individual systems, our study shows that there are unifying principles in the components mutated in phages that escape highly diverse defense systems. We demonstrate that multiple systems have evolutionarily converged to sense the same phage component as a signal for infection, and we find that the determinants sensitizing phages to bacterial immune systems fall mostly within three categories: the core replication machinery, phage structural proteins, and host immune evasion and takeover. Although 75% of the genes in a typical phage encode "hypothetical proteins" of unknown function,[62] the majority of genes mutated in escaper phages were of known functions, suggesting that defense systems tend to target core rather than accessory components of the phage.

Although the large number of phage mutants described here cannot be fully explored mechanistically in a single study, we identified specific phage proteins necessary and sufficient for activation of several defense systems. These include the Borvo and Hachiman systems that sense phage DNA polymerase and SSB, respectively, the Dazbog system that responds to phage DNA methylase activity, the *ietAS* and Mokosh systems that are triggered by phage proteins of unknown function, and two retron systems, one responding to RecBCD inhibition by phage and the other activated by phage SSB. Although we show that expression of each individual phage protein triggers the respective defense system, in most cases, our data do not reveal whether this triggering is by direct or indirect interactions of the phage protein and the defense system. Previous studies identified defense systems that are activated by direct binding to a phage protein, as shown for DSR2,[3] CapRel,[57] Avs,[58] and Retron-Eco8 (this study). On the other hand multiple defense systems are known to sense the effect of the phage protein and not the protein itself, for example, retrons Ec48 and Se72 that sense RecBCD inhibition[15] (this study), as well as PrrC that is triggered upon sensing the inhibition of a restriction enzyme by phages.[29] Further studies are necessary to elucidate

Please cite this article in press as: Stokar-Avihail et al., Discovery of phage determinants that confer sensitivity to bacterial immune systems, Cell (2023), https://doi.org/10.1016/j.cell.2023.02.029

how the phage components directly or indirectly activate the respective systems.

Some of the phage mutants we isolated may not escape by mutating the component identified by the defense system, but rather evade immunity via other means. It is possible that some mutations could allow the phage to overcome the effect of the system, as proposed for the DarTG system, which installs ADP-ribose on phage DNA in response to infection.[48] Phages mutated in their DNA polymerase could tolerate the effect of the DarTG system, and it was suggested that these mutations allow the polymerase to replicate DNA despite the DNA modifications made by DarTG.[48] Another possibility is that the mutation in the phage has a "gain-of-function" effect, generating a protein that actively inhibits the defense system. Such mutants were described in two recent studies in which mutations switched a phage anti-CBASS or anti-DarTG protein from an inactive to an active form, enabling phages to overcome defense.[28,48] Another study has identified phage mutations that allowed phages to overcome the ToxIN system by evolving a non-coding RNA acting as an antitoxin to block ToxN.[63]

Notably, despite our success in isolating escape mutants for many systems, such mutants may not easily arise in nature. We have found, in agreement with other studies, that mutant phages escaping from defense systems frequently have fitness defects and become extinct in competition assays with WT phages when infecting bacteria without the defense system[13] (Figure S2). Moreover, because most bacteria encode more than one defense system, with an average of ~6 systems per genome,[1,34] we expect that in natural settings it would be more difficult for phages to escape bacterial immunity by a single mutation. Indeed, some defense systems were found to act together to enhance anti-phage protection and limit phage escape.[64–66]

For 39 systems included in our initial screen we were unable to isolate phages that escaped defense. This highlights the limitations in our experiment, suggesting there may be additional immune-sensing strategies undetected by our method. In these cases, it is possible that the phage component sensed by the system is essential, such that mutations that escape detection by the system also render the phage inactive. Such a mechanism of detection has been found for certain Avs systems that directly sense the active site residues of the phage terminase protein.[58] In this case, mutation in the active site of the terminase reduced activation of the defense system but also rendered the terminase non-functional. It is also possible that escape may require more than one mutation event in the phage, such that the probability to achieve a combination of these mutations was too low in our experimental setup. For example, sensing of phage portal proteins by some Avs systems involves an extended interface with the phage protein, limiting the ability of the phage to escape by a single mutation.[58] In previous studies, multiple experimental evolution cycles were used to enrich for phages that escape defense, for example in the case of the CapRel^{SJ46} system.[57] However, as this method is not easy to scale up, we could not apply it on 54 defense systems. From an evolutionary point of view, it makes sense that defense systems evolved to detect phages in a manner that limits their ability to escape. Thus, it is not surprising that we were unable to isolate spontaneous phage mutants that escape many of the defense systems we studied.

The plethora of bacterial anti-phage immune systems that were recently discovered are still largely unexplored mechanistically. In addition to the direct insights derived from our study, we envision that our data and the large number of phage mutants we have collected will serve as a valuable resource for future studies aiming to solve the mechanisms of multiple defense systems. We also foresee that the general principles for phage sensing we describe in this study will hold true for other defense systems discovered and studied in the future.

Limitations of the study

Although our study has generated informative insights, it has limitations. First, we report mutations that enable phage escape from defense, and in some cases, we show that the mutated phage components are directly sensed by the respective defense systems. However, in most cases we cannot know whether the mutation identifies the phage components directly triggering the defense system, or whether the mutation allows escape from defense via an indirect mechanism. Second, in cases where defense against the WT phage is weak, it is difficult to determine whether phage escape is "real" or artifactual. This concern is increased for defense systems expressed in *E. coli*, on a multi-copy plasmid, likely generating higher expression of the system compared with its natural, genome-encoded form. To minimize such issues, we did not report escapers with weak escape phenotypes and only included cases in which at least one of the escapers isolated had an effect of more than 100-fold in its plaquing ability compared to the WT phage. In addition, for 7 of the 177 escaper phages we isolated, we could not find a mutation in their sequenced genomes, alluding to an epigenetic effect or to a limitation of our genome analysis pipeline in identifying a minority of the mutations. This implies that in some cases additional explanations would be needed to account for all of the observed phage escapers, beyond the simplified direction we have presented in the summary figure (Figure 6). In other cases, we found that seemingly unrelated genes are mutated in different phages escaping the same system. For example, SBSphiC and SPbeta phages each mutated in a different gene of unknown function, both escape Septu defense despite the mutated genes having no detectible homology. One possibility is that the two proteins may have a similar function, such that the defense system senses their activity rather than the proteins themselves, as shown for the Ec48 retron.[15] Further studies would be required to determine how such different proteins sensitize phages to the same system.

STAR★METHODS

Detailed methods are provided in the online version of this paper and include the following:

- KEY RESOURCES TABLE
- RESOURCE AVAILABILITY
  - Lead contact
  - Materials availability

SUPPLEMENTAL INFORMATION

Supplemental information can be found online at https://doi.org/10.1016/j.cell.2023.02.029.

ACKNOWLEDGMENTS

We thank the Sorek lab and A. Bernheim for fruitful discussion and comments. J.H. was funded by the Deutsche Forschungsgemeinschaft (grant 466645764). A.M. was supported by an Ariane de Rothschild Women Doctoral Program fellowship and, in part, by the Israeli Council for Higher Education via the Weizmann Data Science Research Center. R.S. was supported, in part, by the European Research Council (grant ERC-AdG GA 101018520), Israel Science Foundation (personal grant ISF 296/21 and MAPATS grant 2720/22), the Deutsche Forschungsgemeinschaft (SPP 2330, grant 464312965), the Ernest and Bonnie Beutler Research Program of Excellence in Genomic Medicine, the Minerva Foundation with funding from the Federal German Ministry for Education and Research, and the Knell Family Center for Microbiology.

AUTHOR CONTRIBUTIONS

A.S.-A. and R.S. designed the study. R.S. supervised the study. A.S.-A. performed all experiments and analyses unless otherwise indicated. A.S.-A., T.F., G.S., and N.W. isolated phages. A.S.-A. performed sequencing and computational analyses. J.H. performed northern blots. J.G. and A.S.-A. performed restriction analysis. A.M. computationally detected defense systems. A.L. and A.S.-A. performed toxicity experiments. A.L. and S.M. constructed defense strains. G.A. and A.S.-A. analyzed protein structures. The manuscript was written by A.S.-A. and R.S. All authors contributed to manuscript editing and support the conclusions.

DECLARATION OF INTERESTS

R.S. is a scientific cofounder and advisor of BiomX and Ecophage.

Received: August 12, 2022
Revised: January 9, 2023
Accepted: February 20, 2023
Published: April 7, 2023

REFERENCES

1. Millman, A., Melamed, S., Leavitt, A., Doron, S., Bernheim, A., Hör, J., Garb, J., Bechon, N., Brandis, A., Lopatina, A., et al. (2022). An expanded arsenal of immune systems that protect bacteria from phages. Cell Host Microbe *30*, 1556–1569.e5. https://doi.org/10.1016/j.chom.2022.09.017.
2. Kuzmenko, A., Oguienko, A., Esyunina, D., Yudin, D., Petrova, M., Kudinova, A., Maslova, O., Ninova, M., Ryazansky, S., Leach, D., et al. (2020). DNA targeting and interference by a bacterial Argonaute nuclease. Nature *587*, 632–637. https://doi.org/10.1038/S41586-020-2605-1.
3. Garb, J., Lopatina, A., Bernheim, A., Zaremba, M., Siksnys, V., Melamed, S., Leavitt, A., Millman, A., Amitai, G., and Sorek, R. (2022). Multiple phage resistance systems inhibit infection via SIR2-dependent NAD^+ depletion. Nat. Microbiol. *7*, 1849–1856. https://doi.org/10.1038/s41564-022-01207-8.
4. Zaremba, M., Dakineviciene, D., Golovinas, E., Zagorskaitė, E., Stankunas, E., Lopatina, A., Sorek, R., Manakova, E., Ruksenaite, A., Silanskas, A., et al. (2022). Short prokaryotic Argonautes provide defence against incoming mobile genetic elements through NAD^+ depletion. Nat. Microbiol. *7*, 1857–1869. https://doi.org/10.1038/s41564-022-01239-0.
5. Koopal, B., Potocnik, A., Mutte, S.K., Aparicio-Maldonado, C., Lindhoud, S., Vervoort, J.J.M., Brouns, S.J.J., and Swarts, D.C. (2022). Short prokaryotic Argonaute systems trigger cell death upon detection of invading DNA. Cell *185*, 1471–1486.e19. https://doi.org/10.1016/J.CELL.2022.03.012.
6. Kronheim, S., Daniel-Ivad, M., Duan, Z., Hwang, S., Wong, A.I., Mantel, I., Nodwell, J.R., and Maxwell, K.L. (2018). A chemical defence against phage infection. Nature *564*, 283–286. https://doi.org/10.1038/S41586-018-0767-X.
7. Bernheim, A., Millman, A., Ofir, G., Meitav, G., Avraham, C., Shomar, H., Rosenberg, M.M., Tal, N., Melamed, S., Amitai, G., et al. (2021). Prokaryotic viperins produce diverse antiviral molecules. Nature *589*, 120–124. https://doi.org/10.1038/s41586-020-2762-2.
8. Kever, L., Hardy, A., Luthe, T., Hünnefeld, M., Gätgens, C., Milke, L., Wiechert, J., Wittmann, J., Moraru, C., Marienhagen, J., et al. (2022). Aminoglycoside antibiotics inhibit phage infection by blocking an early step of the infection cycle. mBio *13*, e0078322. https://doi.org/10.1128/MBIO.00783-22.
9. Tal, N., Millman, A., Stokar-Avihail, A., Fedorenko, T., Leavitt, A., Melamed, S., Yirmiya, E., Avraham, C., Brandis, A., Mehlman, T., et al. (2022). Bacteria deplete deoxynucleotides to defend against bacteriophage infection. Nat. Microbiol. *7*, 1200–1209. https://doi.org/10.1038/s41564-022-01158-0.
10. Ofir, G., Herbst, E., Baroz, M., Cohen, D., Millman, A., Doron, S., Tal, N., Malheiro, D.B.A., Malitsky, S., Amitai, G., et al. (2021). Antiviral activity of bacterial TIR domains via immune signalling molecules. Nature *600*, 116–120. https://doi.org/10.1038/S41586-021-04098-7.
11. Hsueh, B.Y., Severin, G.B., Elg, C.A., Waldron, E.J., Kant, A., Wessel, A.J., Dover, J.A., Rhoades, C.R., Ridenhour, B.J., Parent, K.N., et al. (2022). Phage defence by deaminase-mediated depletion of deoxynucleotides in bacteria. Nat. Microbiol. *7*, 1210–1220. https://doi.org/10.1038/s41564-022-01162-4.
12. Cohen, D., Melamed, S., Millman, A., Shulman, G., Oppenheimer-Shaanan, Y., Kacen, A., Doron, S., Amitai, G., and Sorek, R. (2019). Cyclic GMP–AMP signalling protects bacteria against viral infection. Nature *574*, 691–695. https://doi.org/10.1038/s41586-019-1605-5.
13. Tal, N., Morehouse, B.R., Millman, A., Stokar-Avihail, A., Avraham, C., Fedorenko, T., Yirmiya, E., Herbst, E., Brandis, A., Mehlman, T., et al. (2021). Cyclic CMP and cyclic UMP mediate bacterial immunity against phages. Cell *184*, 5728–5739.e16. https://doi.org/10.1016/J.CELL.2021.09.031.
14. Whiteley, A.T., Eaglesham, J.B., de Oliveira Mann, C.C., Morehouse, B.R., Lowey, B., Nieminen, E.A., Danilchanka, O., King, D.S., Lee, A.S.Y., Mekalanos, J.J., et al. (2019). Bacterial cGAS-like enzymes synthesize diverse nucleotide signals. Nature *567*, 194–199. https://doi.org/10.1038/s41586-019-0953-5.

15. Millman, A., Bernheim, A., Stokar-Avihail, A., Fedorenko, T., Voichek, M., Leavitt, A., Oppenheimer-Shaanan, Y., and Sorek, R. (2020). Bacterial retrons function in anti-phage defense. Cell *183*, 1551–1561.e12. https://doi.org/10.1016/j.cell.2020.09.065.

16. Bobonis, J., Mitosch, K., Mateus, A., Karcher, N., Kritikos, G., Selkrig, J., Zietek, M., Monzon, V., Pfalz, B., Garcia-Santamarina, S., et al. (2022). Bacterial retrons encode phage-defending tripartite toxin–antitoxin systems. Nature *609*, 144–150. https://doi.org/10.1038/s41586-022-05091-4.

17. Gao, L., Altae-Tran, H., Böhning, F., Makarova, K.S., Segel, M., Schmid-Burgk, J.L., Koob, J., Wolf, Y.I., Koonin, E.V., and Zhang, F. (2020). Diverse enzymatic activities mediate antiviral immunity in prokaryotes. Science *369*, 1077–1084. https://doi.org/10.1126/science.aba0372.

18. Goldfarb, T., Sberro, H., Weinstock, E., Cohen, O., Doron, S., Charpak-Amikam, Y., Afik, S., Ofir, G., and Sorek, R. (2015). BREX is a novel phage resistance system widespread in microbial genomes. EMBO J. *34*, 169–183. https://doi.org/10.15252/embj.201489455.

19. Ofir, G., Melamed, S., Sberro, H., Mukamel, Z., Silverman, S., Yaakov, G., Doron, S., and Sorek, R. (2018). DISARM is a widespread bacterial defence system with broad anti-phage activities. Nat. Microbiol. *3*, 90–98. https://doi.org/10.1038/s41564-017-0051-0.

20. Doron, S., Melamed, S., Ofir, G., Leavitt, A., Lopatina, A., Keren, M., Amitai, G., and Sorek, R. (2018). Systematic discovery of antiphage defense systems in the microbial pangenome. Science *359*, eaar4120. https://doi.org/10.1126/science.aar4120.

21. Johnson, A.G., Wein, T., Mayer, M.L., Duncan-Lowey, B., Yirmiya, E., Oppenheimer-Shaanan, Y., Amitai, G., Sorek, R., and Kranzusch, P.J. (2022). Bacterial gasdermins reveal an ancient mechanism of cell death. Science *375*, 221–225. https://doi.org/10.1126/SCIENCE.ABJ8432.

22. Rousset, F., Depardieu, F., Miele, S., Dowding, J., Laval, A.L., Lieberman, E., Garry, D., Rocha, E.P.C., Bernheim, A., and Bikard, D. (2022). Phages and their satellites encode hotspots of antiviral systems. Cell Host Microbe *30*, 740–753.e5. https://doi.org/10.1016/j.chom.2022.02.018.

23. Vassallo, C.N., Doering, C.R., Littlehale, M.L., Teodoro, G.I.C., and Laub, M.T. (2022). A functional selection reveals previously undetected anti-phage defence systems in the E. coli pangenome. Nat. Microbiol. *7*, 1568–1579. https://doi.org/10.1038/s41564-022-01219-4.

24. Bari, S.M.N., Chou-Zheng, L., Howell, O., Hossain, M., Hill, C.M., Boyle, T.A., Cater, K., Dandu, V.S., Thomas, A., Aslan, B., et al. (2022). A unique mode of nucleic acid immunity performed by a multifunctional bacterial enzyme. Cell Host Microbe *30*, 570–582.e7. https://doi.org/10.1016/J.CHOM.2022.03.001.

25. Depardieu, F., Didier, J.-P., Bernheim, A., Sherlock, A., Molina, H., Duclos, B., and Bikard, D. (2016). A eukaryotic-like serine/threonine kinase protects staphylococci against phages. Cell Host Microbe *20*, 471–481. https://doi.org/10.1016/J.CHOM.2016.08.010.

26. Yasui, R., Washizaki, A., Furihata, Y., Yonesaki, T., and Otsuka, Y. (2014). AbpA and AbpB provide anti-phage activity in Escherichia coli. Genes Genet. Syst. *89*, 51–60. https://doi.org/10.1266/ggs.89.51.

27. Samson, J.E., Magadán, A.H., Sabri, M., and Moineau, S. (2013). Revenge of the phages: defeating bacterial defences. Nat. Rev. Microbiol. *11*, 675–687. https://doi.org/10.1038/nrmicro3096.

28. Huiting, E., Cao, X., Ren, J., Athukoralage, J.S., Luo, Z., Silas, S., An, N., Carion, H., Zhou, Y., Fraser, J.S., et al. (2023). Bacteriophages inhibit and evade cGAS-like immune function in bacteria. Cell *186*, 864–876. https://doi.org/10.1016/j.cell.2022.12.041.

29. Penner, M., Morad, I., Snyder, L., and Kaufmann, G. (1995). Phage T4-coded Stp: double-edged effector of coupled DNA and tRNA-restriction systems. J. Mol. Biol. *249*, 857–868. https://doi.org/10.1006/jmbi.1995.0343.

30. Yu, Y.T., and Snyder, L. (1994). Translation elongation factor Tu cleaved by a phage-exclusion system. Proc. Natl. Acad. Sci. USA *91*, 802–806. https://doi.org/10.1073/pnas.91.2.802.

31. Bingham, R., Ekunwe, S.I.N., Falk, S., Snyder, L., and Kleanthous, C. (2000). The major head protein of bacteriophage T4 binds specifically to elongation factor Tu. J. Biol. Chem. *275*, 23219–23226. https://doi.org/10.1074/jbc.M002546200.

32. Champness, W.C., and Snyder, L. (1982). The gol site: A cis-acting bacteriophage T4 regulatory region that can affect expression of all the T4 late genes. J. Mol. Biol. *155*, 395–407. https://doi.org/10.1016/0022-2836(82)90478-8.

33. Hobbs, S.J., Wein, T., Lu, A., Morehouse, B.R., Schnabel, J., Leavitt, A., Yirmiya, E., Sorek, R., and Kranzusch, P.J. (2022). Phage anti-CBASS and anti-Pycsar nucleases subvert bacterial immunity. Nature *605*, 522–526. https://doi.org/10.1038/s41586-022-04716-y.

34. Tesson, F., Hervé, A., Mordret, E., Touchon, M., D'Humières, C., Cury, J., and Bernheim, A. (2022). Systematic and quantitative view of the antiviral arsenal of prokaryotes. Nat. Commun. *13*, 2561. https://doi.org/10.1038/S41467-022-30269-9.

35. Bernheim, A., and Sorek, R. (2020). The pan-immune system of bacteria: antiviral defence as a community resource. Nat. Rev. Microbiol. *18*, 113–119. https://doi.org/10.1038/s41579-019-0278-2.

36. Kim, Y.T., and Richardson, C.C. (1993). Bacteriophage T7 gene 2.5 protein: an essential protein for DNA replication. Proc. Natl. Acad. Sci. USA *90*, 10173–10177. https://doi.org/10.1073/pnas.90.21.10173.

37. Hollis, T., Stattel, J.M., Walther, D.S., Richardson, C.C., and Ellenberger, T. (2001). Structure of the gene 2.5 protein, a single-stranded DNA binding protein encoded by bacteriophage T7. Proc. Natl. Acad. Sci. USA *98*, 9557–9562. https://doi.org/10.1073/pnas.171317698.

38. Davison, J. (2015). Pre-early functions of bacteriophage T5 and its relatives. Bacteriophage *5*, e1086500. https://doi.org/10.1080/21597081.2015.1086500.

39. Klimuk, E., Mekler, V., Lavysh, D., Serebryakova, M., Akulenko, N., and Severinov, K. (2020). Novel Escherichia coli RNA polymerase binding protein encoded by bacteriophage T5. Viruses *12*, 807. https://doi.org/10.3390/v12080807.

40. Lopatina, A., Tal, N., and Sorek, R. (2020). Abortive infection: bacterial suicide as an antiviral immune strategy. Annu. Rev. Virol. *7*, 371–384. https://doi.org/10.1146/annurev-virology-011620-040628.

41. Günthert, U., and Reiners, L. (1987). Bacillus subtilis phage SPR codes for a DNA methyltransferase with triple sequence specificity. Nucleic Acids Res. *15*, 3689–3702. https://doi.org/10.1093/nar/15.9.3689.

42. Machnicka, M.A., Kaminska, K.H., Dunin-Horkawicz, S., and Bujnicki, J.M. (2015). Phylogenomics and sequence-structure-function relationships in the GmrSD family of Type IV restriction enzymes. BMC Bioinformatics *16*, 336. https://doi.org/10.1186/s12859-015-0773-z.

43. Xiong, X., Wu, G., Wei, Y., Liu, L., Zhang, Y., Su, R., Jiang, X., Li, M., Gao, H., Tian, X., et al. (2020). SspABCD–SspE is a phosphorothioation-sensing bacterial defence system with broad anti-phage activities. Nat. Microbiol. *5*, 917–928. https://doi.org/10.1038/s41564-020-0700-6.

44. Liang, J., Wang, Z., He, X., Li, J., Zhou, X., and Deng, Z. (2007). DNA modification by sulfur: analysis of the sequence recognition specificity surrounding the modification sites. Nucleic Acids Res. *35*, 2944–2954. https://doi.org/10.1093/NAR/GKM176.

45. Bouchard, J.D., and Moineau, S. (2004). Lactococcal phage genes involved in sensitivity to AbiK and their relation to single-strand annealing proteins. J. Bacteriol. *186*, 3649–3652. https://doi.org/10.1128/JB.186.11.3649-3652.2004.

46. Wang, C., Villion, M., Semper, C., Coros, C., Moineau, S., and Zimmerly, S. (2011). A reverse transcriptase-related protein mediates phage resistance and polymerizes untemplated DNA in vitro. Nucleic Acids Res. *39*, 7620–7629. https://doi.org/10.1093/NAR/GKR397.

47. Samson, J.E., Bélanger, M., and Moineau, S. (2013). Effect of the abortive infection mechanism and type III toxin/antitoxin system AbiQ on the lytic cycle of Lactococcus lactis phages. J. Bacteriol. *195*, 3947–3956. https://doi.org/10.1128/JB.00296-13.

48. LeRoux, M., Srikant, S., Teodoro, G.I.C., Zhang, T., Littlehale, M.L., Doron, S., Badiee, M., Leung, A.K.L., Sorek, R., and Laub, M.T. (2022). The DarTG toxin-antitoxin system provides phage defence by ADP-ribosylating viral DNA. Nat. Microbiol. *7*, 1028–1040. https://doi.org/10.1038/s41564-022-01153-5.

49. Mosig, G., Yu, S., Myung, H., Haggård-Ljungquist, E., Davenport, L., Carlson, K., and Calendar, R. (1997). A novel mechanism of virus–virus interactions: bacteriophage P2 tin protein inhibits phage T4 DNA synthesis by poisoning the T4 single-stranded DNA binding protein, GP32. Virology *230*, 72–81. https://doi.org/10.1006/VIRO.1997.8464.

50. Hernandez, A.J., and Richardson, C.C. (2019). Gp2.5, the multifunctional bacteriophage T7 single-stranded DNA binding protein. Semin. Cell Dev. Biol. *86*, 92–101. https://doi.org/10.1016/j.semcdb.2018.03.018.

51. Bernad, A., Blanco, L., Lázaro, J., Martin, G., and Salas, M. (1989). A conserved 3′→ 5′ exonuclease active site in prokaryotic and eukaryotic DNA polymerases. Cell *59*, 219–228.

52. Kamtekar, S., Berman, A.J., Wang, J., Lázaro, J.M., de Vega, M., Blanco, L., Salas, M., and Steitz, T.A. (2004). Insights into strand displacement and processivity from the crystal structure of the protein-primed DNA polymerase of bacteriophage φ29. Molecular Cell *16*, 609–618.

53. Snyder, L. (1995). Phage-exclusion enzymes: a bonanza of biochemical and cell biology reagents? Mol. Microbiol. *15*, 415–420. https://doi.org/10.1111/j.1365-2958.1995.tb02255.x.

54. Kimelman, A., Levy, A., Sberro, H., Kidron, S., Leavitt, A., Amitai, G., Yoder-Himes, D.R., Wurtzel, O., Zhu, Y., Rubin, E.M., et al. (2012). A vast collection of microbial genes that are toxic to bacteria. Genome Res. *22*, 802–809. https://doi.org/10.1101/GR.133850.111.

55. Mortier-Barrière, I., Velten, M., Dupaigne, P., Mirouze, N., Piétrement, O., McGovern, S., Fichant, G., Martin, B., Noirot, P., Le Cam, E., et al. (2007). A key presynaptic role in transformation for a widespread bacterial protein: DprA conveys incoming ssDNA to RecA. Cell *130*, 824–836. https://doi.org/10.1016/j.cell.2007.07.038.

56. Molineux, I.J., Schmitt, C.K., and Condreay, J.P. (1989). Mutants of bacteriophage T7 that escape F restriction. J. Mol. Biol. *207*, 563–574. https://doi.org/10.1016/0022-2836(89)90465-8.

57. Zhang, T., Tamman, H., Coppieters 't Wallant, K., Kurata, T., LeRoux, M., Srikant, S., Brodiazhenko, T., Cepauskas, A., Talavera, A., Martens, C., et al. (2022). Direct activation of a bacterial innate immune system by a viral capsid protein. Nature *612*, 132–140. https://doi.org/10.1038/s41586-022-05444-z.

58. Gao, L.A., Wilkinson, M.E., Strecker, J., Makarova, K.S., Macrae, R.K., Koonin, E.V., and Zhang, F. (2022). Prokaryotic innate immunity through pattern recognition of conserved viral proteins. Science *377*, eabm4096. https://doi.org/10.1126/science.abm4096.

59. Myung, H., and Calendar, R. (1995). The old exonuclease of bacteriophage P2. J. Bacteriol. *177*, 497–501. https://doi.org/10.1128/JB.177.3.497-501.1995.

60. Lindahl, G., Sironi, G., Bialy, H., and Calendar, R. (1970). Bacteriophage lambda; abortive infection of bacteria lysogenic for phage P2. Proc. Natl. Acad. Sci. USA *66*, 587–594. https://doi.org/10.1073/PNAS.66.3.587.

61. Guegler, C.K., and Laub, M.T. (2021). Shutoff of host transcription triggers a toxin-antitoxin system to cleave phage RNA and abort infection. Mol. Cell *81*, 2361–2373.e9. https://doi.org/10.1016/J.MOLCEL.2021.03.027.

62. Edwards, R.A., and Rohwer, F. (2005). Viral metagenomics. Nat. Rev. Microbiol. *3*, 504–510. https://doi.org/10.1038/nrmicro1163.

63. Blower, T.R., Evans, T.J., Przybilski, R., Fineran, P.C., and Salmond, G.P.C. (2012). Viral evasion of a bacterial suicide system by RNA–based molecular mimicry enables infectious altruism. PLoS Genet. *8*, e1003023. https://doi.org/10.1371/journal.pgen.1003023.

64. Dupuis, M.È., Villion, M., Magadán, A.H., and Moineau, S. (2013). CRISPR-Cas and restriction-modification systems are compatible and increase phage resistance. Nat. Commun. *4*, 2087. https://doi.org/10.1038/NCOMMS3087.

65. Silas, S., Lucas-Elio, P., Jackson, S.A., Aroca-Crevillén, A., Hansen, L.L., Fineran, P.C., Fire, A.Z., and Sánchez-Amat, A. (2017). Type III CRISPR-Cas systems can provide redundancy to counteract viral escape from type I systems. eLife *6*, e27601. https://doi.org/10.7554/eLife.27601.

66. Maguin, P., Varble, A., Modell, J.W., and Marraffini, L.A. (2022). Cleavage of viral DNA by restriction endonucleases stimulates the type II CRISPR-Cas immune response. Mol. Cell *82*, 907–919.e7. https://doi.org/10.1016/j.molcel.2022.01.012.

67. Baba, T., Ara, T., Hasegawa, M., Takai, Y., Okumura, Y., Baba, M., Datsenko, K.A., Tomita, M., Wanner, B.L., and Mori, H. (2006). Construction of Escherichia coli K-12 in-frame, single-gene knockout mutants: the Keio collection. Mol. Syst. Biol. *2*, 2006.0008. https://doi.org/10.1038/MSB4100050.

68. Zimmermann, L., Stephens, A., Nam, S.Z., Rau, D., Kübler, J., Lozajic, M., Gabler, F., Söding, J., Lupas, A.N., and Alva, V. (2018). A completely reimplemented MPI bioinformatics toolkit with a new HHpred server at its core. J. Mol. Biol. *430*, 2237–2243. https://doi.org/10.1016/j.jmb.2017.12.007.

69. Jumper, J., Evans, R., Pritzel, A., Green, T., Figurnov, M., Ronneberger, O., Tunyasuvunakool, K., Bates, R., Žídek, A., Potapenko, A., et al. (2021). Highly accurate protein structure prediction with AlphaFold. Nature *596*, 583–589. https://doi.org/10.1038/s41586-021-03819-2.

70. Mirdita, M., Schütze, K., Moriwaki, Y., Heo, L., Ovchinnikov, S., and Steinegger, M. (2022). ColabFold: making protein folding accessible to all. Nat. Methods *19*, 679–682. https://doi.org/10.1038/s41592-022-01488-1.

71. Krissinel, E., and Henrick, K. (2004). Secondary-structure matching (SSM), a new tool for fast protein structure alignment in three dimensions. Acta Crystallogr. D Biol. Crystallogr. *60*, 2256–2268. https://doi.org/10.1107/S0907444904026460.

72. Deatherage, D.E., and Barrick, J.E. (2014). Identification of mutations in laboratory-evolved microbes from next-generation sequencing data using breseq. Methods Mol. Biol. *1151*, 165–188. https://doi.org/10.1007/978-1-4939-0554-6_12.

73. Schrödinger, L. (2015). PhysiologyMOL molecular graphics system. version 1.8. http://www.pymol.org.

74. Johnson, M., Zaretskaya, I., Raytselis, Y., Merezhuk, Y., McGinnis, S., and Madden, T.L. (2008). NCBI BLAST: a better web interface. Nucleic Acids Res. *36*, W5–W9. https://doi.org/10.1093/nar/gkn201.

75. Wilson, G.A., and Bott, K.F. (1968). Nutritional factors influencing the development of competence in the Bacillus subtilis transformation system. J. Bacteriol. *95*, 1439–1449. https://doi.org/10.1128/jb.95.4.1439-1449.1968.

76. Chung, C.T., and Miller, R.H. (1993). Preparation and storage of competent Escherichia coli Cells. Methods Enzymol. *218*, 621–627. https://doi.org/10.1016/0076-6879(93)18045-E.

77. Kropinski, A.M., Mazzocco, A., Waddell, T.E., Lingohr, E., and Johnson, R.P. (2009). Enumeration of bacteriophages by double agar overlay plaque assay. In Bacteriophages: Methods and Protocols, M.R.J. Clokie and A.M. Kropinski, eds. (Humana Press), pp. 69–76. https://doi.org/10.1007/978-1-60327-164-6_7.

78. Mazzocco, A., Waddell, T.E., Lingohr, E., and Johnson, R.P. (2009). Enumeration of bacteriophages using the small drop plaque assay system. In Bacteriophages: Methods and Protocols, M.R.J. Clokie and A.M. Kropinski, eds. (Humana Press), pp. 81–85. https://doi.org/10.1007/978-1-60327-164-6_9.

79. Baym, M., Kryazhimskiy, S., Lieberman, T.D., Chung, H., Desai, M.M., and Kishony, R.K. (2015). Inexpensive multiplexed library preparation for megabase-sized genomes. PLoS One *10*, e0128036. https://doi.org/10.1371/journal.pone.0128036.

80. Doublié, S., Tabor, S., Long, A.M., Richardson, C.C., and Ellenberger, T. (1998). Crystal structure of a bacteriophage T7 DNA replication complex at 2.2 Å resolution. Nature *391*, 251–258. https://doi.org/10.1038/34593.

81. Chen, I.-M.A., Chu, K., Palaniappan, K., Ratner, A., Huang, J., Huntemann, M., Hajek, P., Ritter, S., Varghese, N., Seshadri, R., et al. (2021). The IMG/M data management and analysis system v.6.0: new tools and advanced capabilities. Nucleic Acids Res. *49*, D751–D763. https://doi.org/10.1093/nar/gkaa939.

82. Andraos, N., Tabor, S., and Richardson, C.C. (2004). The highly processive DNA polymerase of bacteriophage T5: role of the unique N and C termini. J. Biol. Chem. *279*, 50609–50618. https://doi.org/10.1074/jbc.M408428200.

Please cite this article in press as: Stokar-Avihail et al., Discovery of phage determinants that confer sensitivity to bacterial immune systems, Cell (2023), https://doi.org/10.1016/j.cell.2023.02.029

STAR★METHODS

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Antibodies		
Rabbit primary polyclonal anti-HA	Sigma-Aldrich	Cat#H6908; RRID: AB_260070
Goat anti-Rabbit IgG HRP-conjugated	Thermo Fisher Scientific	Cat#31460; RRID: AB_228341
Pierce anti-HA magnetic beads	Thermo Fisher Scientific	Cat#88837; RRID: AB_2861399
Bacterial and virus strains		
E. coli K-12 MG1655	American Type Culture Collection (ATCC)	ATCC 47076; Genbank: NC_000913
B. subtilis BEST7003	M. Itaya	Genbank: AP012496
NEB 5-alpha Competent *E. coli*	NEW ENGLAND BioLabs	Cat#C2987H
E. coli BW25113 Δ*iscR*	Keio collection; Baba et al.[67]	Cat#JW2515-3
E. coli BW25113 Δ*recB*	Keio collection; Baba et al.[67]	Cat#JW2788-1
λ-vir	Udi Qimron	Genbank: NC_001416.1
SECphi18	Doron et al.[20]	Genbank: LT960609.1
SECphi4	Millman et al.[15]	Genbank: MT331608.1
SECphi6	Millman et al.[15]	Genbank: CADCZA020000001.1
SECphi27	Doron et al.[20]	Genbank: LT961732.1
T5	Udi Qimron	Genbank: AY543070.1
T7	Udi Qimron	Genbank: NC_001604.1
SECphi17	Doron et al.[20]	Genbank: LT960607.1
T2	German Collection of Microorganisms and Cell Cultures GmbH (DSMZ)	DSM 16352; Genbank: LC348380.1
T6	German Collection of Microorganisms and Cell Cultures GmbH (DSMZ)	DSM 4622; Genbank: MH550421.1
P1	Udi Qimron	Genbank: AF234172.1
T4	Udi Qimron	Genbank: AF158101.6
SPO1	Bacillus Genetic Stock Center	BGSC: 1P4; Genbank: NC_011421.1
SP82G	Bacillus Genetic Stock Center	BGSC: 1P5; Genbank: OM236513
SBSphiC	Bacillus Genetic Stock Center; Doron et al.[20]	BGSC: 1P46; Genbank: LT960610.1
SPbeta	Bacillus Genetic Stock Center	BGSC: 1L5; Genbank: AF020713.1
rho14	Bacillus Genetic Stock Center	BGSC: 1L15; Genbank: OM236514
SPR	Bacillus Genetic Stock Center	BGSC: 1L56; Genbank: OM236515
SPP1	Bacillus Genetic Stock Center	BGSC : 1P7; Genbank: X97918.2
FADO	Millman et al.[1]	Genbank: OM236516
phi3T	Bacillus Genetic Stock Center	BGSC: 1L1; Genbank: KY030782.1
PBS1	The Félix d'Hérelle Reference Center for Bacterial Viruses	HER: 97; Genbank: MF360957.1
phi29	German Collection of Microorganisms and Cell Cultures GmbH (DSMZ)	DSM 5546; Genbank: NC_011048.1
AR9	Konstantin Severinov	Genbank: NC_031039.1
SBSphiJ	Bacillus Genetic Stock Center; Doron et al.[20]	BGSC: 1P47; Genbank: LT960608.1
phi105	Bacillus Genetic Stock Center	BGSC: 1L11; Genbank: HM072038.1

(*Continued on next page*)

Please cite this article in press as: Stokar-Avihail et al., Discovery of phage determinants that confer sensitivity to bacterial immune systems, Cell (2023), https://doi.org/10.1016/j.cell.2023.02.029

Continued

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Critical commercial assays		
DNeasy blood and tissue kit	QIAGEN	Cat#69504
Nextera DNA Library Prep Kit Illumina	Illumina	Cat#15027865; Cat#15027866
DNase-I	Merck	Cat#11284932001
Qubit dsDNA BR Assay Kit	Thermo Fisher Scientific	Cat#Q32850
HpaII restriction enzyme	Thermo Fisher Scientific	Cat#ER0511
Deposited data		
WGS of escaper phages	This paper	ENA: PRJEB59272
Oligonucleotides		
OSM646	This paper	atcctagaagcttatcgaattcgcggccgcaagag tacaaaagcctttct
OSM647	This paper	gttgccgccgggcgtttttatggcgcgcctaagct acttccttttacatta
OSM315	This paper	ggcgcgccataaaaaac
OSM316	This paper	gcggccgcgaattc
AS330	This paper	gcctggagatccttactcgagtttggatcctcattgc tttatcctcactacggc
AS331	This paper	caaaagatcttttaagaaggagatatacatatgcgt attaaagccagcgaag
AS328	This paper	ggatccaaactcgagtaaggatctcc
AS329	This paper	atgtatatctccttcttaaaagatcttttgaattccc
AS451	This paper	ttaagcataatctggaacatcatatggatatccgga ggaacggcggcgacgtttac
JHO-0008	This paper	ggcgctataggtaactttaatactc
Recombinant DNA		
BREX, see Table S1 (BTG25)	Goldfarb et al.[18]	N/A
Viperin, see Table S1 (pVip7)	Garb et al.[3]	N/A
DISARM, see Table S1 (SG2)	Ofir et al.[19]	N/A
Septu, Lamassu, Shedu, Thoeris, Zorya, Hachiman, Druantia & Gabija, see Table S1 (SG10-SG32, SG43-SG53, SG74-SG79)	Doron et al.[20]	N/A
CBASS type I & II, see Table S1 (SG99, SG115, SG116)	Cohen et al.[12]	N/A
CBASS type III, see Table S1 (SG213)	Hobbs et al.[33]	N/A
Retrons, see Table S1 (SG186, AA10-AA20)	Millman et al.[15]	N/A
DSR1 & SIR2-HerA, see Table S1 (SG154, SG197)	Garb et al.[3]	N/A
RADAR, AVAST, *qatABCD, ppl*, TerY-p & *ietAS*, see Table S1 (SG82, SG131, SG135, SG167, SG181, SG204, SG205, SG209)	This study, Genscript Corp.	N/A
Menshen, SoFIC, Azaca, Lamassu, PsyrTA, Dodola, Dazbog, Bunzi, Nhi-like, Aditi, Mokosh, RosmerTA, Tiamat, Uzume, Dynamin, Borvo, Olokun, Mokosh, SEFIR & ShosTA, see Table S1 (SG34, SG63, SG94, SG108, SG123-SG130, SG133-SG134, SG138-SG150, SG158, SG165, SG173-SG178, SG196, SG199, SG202, SG208, SG210)	Millman et al.[1]	N/A
Phage genes, see Table S3	This study, Genscript Corp.	N/A
Software and algorithms		
NovoAlign (Novocraft) v3.06.05	Novocraft	http://www.novocraft.com/documentation/novoalign-2/novoalign-ngs-quick-start-tutorial/s
HHpred server	Zimmermann et al.[68]	https://toolkit.tuebingen.mpg.de/tools/hhpred

(*Continued on next page*)

Please cite this article in press as: Stokar-Avihail et al., Discovery of phage determinants that confer sensitivity to bacterial immune systems, Cell (2023), https://doi.org/10.1016/j.cell.2023.02.029

Continued

REAGENT or RESOURCE	SOURCE	IDENTIFIER
AlphaFold2 within ColabFold	Jumper et al.[69]; Mirdita et al.[70]	https://colab.research.google.com/github/sokrypton/ColabFold/blob/main/AlphaFold2.ipynb
PDBeFold	Krissinel and Henrick[71]	https://www.ebi.ac.uk/msd-srv/ssm/
Breseq (version 0.29.0 and 0.34.1)	Deatherage and Barrick[72]	https://barricklab.org/twiki/bin/view/Lab/ToolsBacterialGenomeResequencing
PyMOL program v. 2.5.1	Schrödinger[73]	N/A
BLASTp on the NCBI BLAST web interface	Johnson et al.[74]	https://blast.ncbi.nlm.nih.gov/Blast.cgi

RESOURCE AVAILABILITY

Lead contact

Further information and requests for resources and reagents should be directed to the lead contact, Rotem Sorek (rotem.sorek@weizmann.ac.il).

Materials availability

This study did not generate new unique reagents.

Data and code availability

- Phage sequencing data was deposited in the European Nucleotide Archive (ENA) under accession number PRJEB59272.
- This paper does not report original code.
- Any additional information required to reanalyze the data reported in this paper is available from the lead contact upon request.

EXPERIMENTAL MODEL AND SUBJECT DETAILS

Bacterial strains and phages

Defense systems were expressed in either *E. coli* MG1655 (ATCC 47076) or *B. subtilis* BEST7003 (obtained from M. Itaya at Keio University, Japan). *E. coli* BW25113 knockout strains (Δ*recB* and Δ*iscR*) from the Keio collection[67] were obtained from the Weizmann institute bacteriology repository (#JW2788-1 (*RecB*), #JW2515-3 (*iscR*)). Bacteria were grown in MMB (LB supplemented with 0.1 mM $MnCl_2$ and 5 mM $MgCl_2$) at 37°C shaking at 200 r.p.m, unless specified otherwise, and the appropriate antibiotics were added. For genes expressed in *E. coli*, ampicillin (100 μg/ml) or kanamycin (50 μg/ml)) was used to ensure maintenance of plasmids. For genes expressed in *B. subtilis*, spectinomycin (100 μg/ml) or chloramphenicol (5 μg/ml) was used to ensure the presence of an integrated antibiotics resistance cassette in the *B. subtilis* genome.

The phages used in this study are listed in the key resources table and Table S5. Infection was performed in MMB with or without agar (0.3% or 0.5% as detailed in Table S5). For all *Bacillus* phages, MMB was supplemented with 5 mM $CaCl_2$ for all plaque assays and infection in liquid culture.

METHOD DETAILS

Plasmid and strain construction

Defense systems included in this study are listed in the key resources table and Table S1. For defense-system-expressing strains that were constructed previously, the reference study describing the details regarding the construction of the strain is provided (Table S1). For the defense systems reported by Gao et al. including RADAR, AVAST, *qatABCD*, *Ppl*, TerY-p and *ietAS*, a homolog of the system from the specified donor organism (Table S1) was cloned into the pSG1 shuttle vector[20] together with their native promoters between the AscI and NotI sites of the multiple cloning site. The sequences of these systems are provided in Table S1. DNA sequences of the RADAR, AVAST, *qatABCD*, PpI and TerY-p systems were synthesized and cloned by Genscript Corp. The *ietAS* system was amplified from genomic DNA of the donor strain using KAPA HiFi HotStart ReadyMix (Kapa Biosystems KK2601) with primers OSM646 and OSM647 (key resources table), and cloned using the NEBuilder HiFI DNA Assembly cloning kit (NEB E5520S) into the pSG1 vector (amplified with primers OSM315 and OSM316 (key resources table)).

Three of the systems were cloned by Doron et al.[20] for expression in *B. subtilis* BEST7003 (Lamassu from *Bacillus* sp. NIO-1130, Septu from *B. thuringiensis*, and Thoeris from *B. amyloliquefaciens*). In this study, we used the pSG1 shuttle vectors expressing these three systems constructed by Doron et al.[20] for expression of the systems in *E. coli* MG1655 as well. Expression of these systems in *E. coli* provided defense against several different *E. coli* phages, which were therefore included in our screen (Figure 2).

Please cite this article in press as: Stokar-Avihail et al., Discovery of phage determinants that confer sensitivity to bacterial immune systems, Cell (2023), https://doi.org/10.1016/j.cell.2023.02.029

Defense-system-containing vectors were transformed into either electrocompetent *E. coli* MG1655 or using MC medium into *B. subtilis* BEST7003.[75] From an overnight culture of *B. subtilis*, 60 μl were diluted in 2700 μl ultra-pure water, 30 μl 1M $MgSO_4$ and 300 μl 10X MC medium (80 mM K_2HPO_4, 2% glucose, 30 mM trisodium citrate, 22 μg/ml ferric ammonium citrate, 0.2% sodium glutamate, 30 mM KH_2PO_4 and 0.1% casein hydrolysate (Merck Millipore 102245)). The bacteria were incubated at 37°C, 200 r.p.m until reaching an OD_{600} of 0.6. Then 300 μl was transferred to a new 15 ml tube and 300 ng of plasmid DNA was added. The tube was incubated for another 3 hours (37°C, 200 r.p.m), and then plated on LB agar plates supplemented with 100 μg/ml spectinomycin and incubated overnight at 30°C. The resulting transformants were screened on starch plates for an amylase-deficient phenotype to identify integration of the defense system into the *B. subtilis amyE* locus.

As negative control strains lacking an inserted defense system, an empty pSG1 plasmid was used without any system inserted in the multiple cloning site. For the *E. coli* negative control (NC) strain, the empty plasmid was transformed into *E. coli* MG1655. For the *B. subtilis* NC strain, the empty plasmid was transformed into *B. subtilis* BEST7003 and the spectinomycin-resistance gene was integrated into the *amyE* locus.

The majority of the phage genes expressed in this study were synthesized and cloned by Genscript Corp. A list of all phage genes expressed in this study is provided in Table S3. For expression in *E. coli*, the phage gene was cloned under an arabinose-inducible P_{BAD} promoter, by replacing the RFP ORF in either pBbS8k-RFP or pBbE8k-RFP (Addgene Plasmids #35276 and #35270). For expression in *B. subtilis*, the phage gene was cloned under an Isopropyl β-D-1-thiogalactopyranoside (IPTG) inducible promoter ($P_{hy\text{-}spank}$) by replacing the sfGFP ORF in the *Bacillus* integration vector pSG-*thrC*-$P_{hy\text{-}spank}$-sfGFP[3] for genomic integration in the *B. subtilis thrC* locus. The SECphi4 56aa protein of unknown function was the only gene not synthesized by Genscript Corp. The gene encoding this protein was amplified from genomic DNA of the SECphi4 phage using KAPA HiFi HotStart ReadyMix (Kapa Biosystems KK2601) with primers AS330+AS331 (key resources table), and cloned using the NEBuilder HiFI DNA Assembly kit into the pBbE8k-RFP vector (amplified with primers AS328+AS329; key resources table).

Vectors expressing each of the phage genes were transformed into defense system-containing cells or negative control cells lacking the system (for all cases besides the predicted tail fiber gene of phage SBSphiJ, see note below). Transformation to *E. coli* strains expressing defense systems was performed using either chemical transformation in TSS medium (LB supplemented with 10% (w/v) PEG 8000, 0.6% (w/v) $MgCl_2$*$6H_20$, and 5% (v/v) DMSO)[76] or by electroporation into electrocompetent cells expressing the required defense system. Following transformation, the cells were recovered in LB media supplemented with 1% glucose to repress leaky expression from the P_{BAD} promoter, and then plated on LB agar plates supplemented with 50 μg/ml kanamycin + 100 μg/ml ampicillin + 1% glucose. Transformation to *B. subtilis* strains expressing defense systems was performed using MC medium as described above, and incubated overnight at 30°C on LB agar plates supplemented with 5 μg/ml chloramphenicol and 100 μg/ml spectinomycin. For expression of the SBSphiJ predicted tail fiber gene, we found that the *B. thuringiensis* HD12 Septu defense system inhibited transformation when expressed in *B. subtilis* BEST7003, and we therefore first cloned the phage gene into the bacterial genome at the *thrC* locus, and then introduced the Septu defense system into the *amyE* locus.

For pulldown experiments of the SECphi4 phage SSB protein, a C-terminal HA tag was added to the gene expressed on the pBbS8k plasmid using primers AS451 and AS328, followed by KLD treatment (NEB cat #M0554S) and transformation to NEB 5-alpha competent *E. coli* (NEB cat #C2987H). The pBbS8k-SSB-HA plasmid was then transformed into *E. coli* MG1655 cells expressing a mutated version of the Retron-Eco8 defense system, with a point mutation in the OLD-family endonuclease gene (K36A) on the pSG1 plasmid.[15]

Isolation of mutant phages that overcome defense

To screen for mutant phages that escape defense, phages were plated on bacteria expressing each of the defense systems (Table S1) using the double-layer plaque assay[77] (Figure 1A). For this, 100 μl of bacterial cells grown in MMB to an OD_{600} of 0.3 were mixed with 100 μl phage lysate. After 10 minutes at room temperature, 5 ml pre-melted 0.3% or 0.5% MMB agar was added (Table S5), and the mixture was poured onto MMB 1.1% agar plates. The double layer plates were incubated overnight at 37°C or room temperature (Table S2) and single plaques were picked into 90 μl phage buffer (50 mM Tris pH 7.4, 100 mM MgCl2, 10 mM NaCl)). In addition to picking single plaques, the entire top layer was scraped into 2 ml phage buffer to enrich for phages that escape defense. Phages were left for 1 hour at room temperature during which the phages were mixed several times by vortex to release them from the agar into the phage buffer. The phages were centrifuged at 3200 g for 10 min to remove agar and bacterial cells, and the supernatant was transferred to a new tube.

In order to test the collected phages for their ability to escape from the defense system they formed plaques on, the small drop plaque assay was used[78] (Figure 1B). 300 μl of an overnight culture of bacteria expressing each defense system or a negative control strain lacking the defense system, were mixed with 30 ml melted 0.3% or 0.5% MMB agar (Table S5). The mixture was poured onto a 10-cm square plate and left to dry for 1 hour at room temperature. Ten-fold serial dilutions in phage buffer were performed for the ancestor phages (WT phage used for the original double layer plaque assay) and the phages collected from plaques formed on the defense system strain. 10 μl drops of each phage were plated on the bacterial layer. The plates were incubated overnight at 37°C or room temperature (Table S2). Plaque-forming units (PFUs) observed after overnight incubation were counted and used to calculate the fold defense using the ratio between phage PFUs obtained on negative control cells and PFUs obtained on defense-containing cells. When individual plaques could not be deciphered, a faint lysis zone across the drop area was considered to be 10 plaques. To minimize the risk of including escaper phages that may be artifactual, we disregarded cases for which the

Please cite this article in press as: Stokar-Avihail et al., Discovery of phage determinants that confer sensitivity to bacterial immune systems, Cell (2023), https://doi.org/10.1016/j.cell.2023.02.029

supporting data was weak. Specifically, we removed systems for which only one escaper phage was identified with weak effect (less than 100-fold infection difference between escaper and WT), or cases where the escape effect was solely manifested in enlarged plaque sizes but not in more numerous plaques.

Amplification of mutant phages

Isolated phages for which there was decreased defense compared to the ancestor phage were propagated to obtain a high titter phage stock for sequencing. For this, a single plaque formed on the defense strain in the small drop plaque assay was picked into a liquid culture of defense-system-expressing cells grown in 1 ml MMB to an OD_{600} of 0.3. The phages were incubated with the bacteria at 37°C 200 r.p.m shaking for 3 hours, and then an additional 9 ml of bacterial culture grown to OD_{600} 0.3 in MMB was added, and incubated for another 3 hours (37°C 200 r.p.m). The lysate was then centrifuged at 3200 g for 10 min and the supernatant was filtered through a 0.2 μM filter to get rid of remaining bacteria. Phage titer was then measured using the small drop plaque assay on the negative control strain and in cases where the titer was less than 10^7 PFU/ml, the phage titer was raised using either another round of propagation in liquid culture or with a plate lysate.[77]

For plate lysates, 10^3-10^5 PFUs formed on defense-system-expressing cells using a double-layer plaque assay were scraped into 5 ml of phage buffer. After 1 hour at room temperature, the phages were centrifuged at 3200 g for 10 min, and the supernatant was filtered through a 0.2 μM filter.

Sequencing and genome analysis of phage mutants

DNA was extracted from 500 μl of a high titer phage lysate (> 10^7 PFU/ml). The phage lysate was treated with DNase-I (Merck cat #11284932001) added to a final concentration of 20 μg/ml and incubated at 37°C for 1 hour to remove bacterial DNA. DNA was then extracted using the QIAGEN DNeasy blood and tissue kit (cat #69504) starting from the Proteinase-K treatment step to lyse the phages. Libraries were prepared for Illumina sequencing using a modified Nextera protocol.[79] Reads were aligned to the phage reference genomes (NCBI accession numbers provided in the key resources table) and mutations compared to the reference genome were identified using Breseq (version 0.29.0) with default parameters.[72] Only mutations that occurred in the isolated mutants, but not in the ancestor phage, were considered. Silent mutations within protein coding regions were disregarded as well. Mutations marked by Breseq under "marginal evidence" were reported if the mutation was supported by more than 20% of the aligned reads, and the percent of the mutated reads for such cases is provided in Table S2.

Bacterial growth upon induction of phage genes

For each of the defense systems, at least one phage sensitivity component identified in our screen was chosen for cloning. A plasmid containing each of the phage genes under an inducible promoter ($P_{hy\text{-}spank}$ or P_{BAD}) (Table S3) was transformed into defense system-containing bacteria or negative control cells lacking the defense system as explained above. After overnight incubation, 8 to 16 of the freshly transformed colonies were then streaked on LB agar plates with or without added inducer (1mM IPTG or 0.3% arabinose; Table S3). Bacterial growth was examined after overnight incubation at 37°C for *E. coli* or 30°C for *B. subtilis*.

The toxicity of the phage genes was measured quantitatively by counting the number of bacterial colony-forming units (CFUs) per ml formed after inducing expression of the phage gene overnight. For genes expressed in *B. subtilis*, two loopfuls of bacteria were taken from 3 different streaked colonies that grew without added inducer and inoculated directly in 90 μl phosphate-buffered saline (PBS) (Thermo Fisher Scientific 14200075). For genes expressed in *E. coli*, 3 different streaked colonies that grew without added inducer were inoculated in MMB supplemented with 100 μg/ml ampicillin + 50 μg/ml kanamycin + 1% glucose and grown to stationary phase at 37°C 200 r.p.m. Then, ten-fold serial dilutions of the bacteria in PBS were performed and 5 μl drops were spotted on LB agar plates with or without added inducer, either IPTG or arabinose, for expression of the phage gene. Inducer levels used for each gene are detailed in Table S3. Since some phage genes were toxic upon overexpression at maximal inducer levels (1mM IPTG or 0.3% arabinose), the level of inducer was lowered to reach a point where expression was not toxic in control cells. Plates were incubated overnight at 37°C for *E. coli* or 30°C for *B. subtilis*, and bacterial colonies imaged and counted.

For both bacterial colony streaking and CFU experiments, non-induced plates were supplemented with 1% glucose to inhibit leaky expression of phage genes expressed under the P_{BAD} promoter.

Transformation efficiency

Strains from the *E. coli* Keio collection (Δ*recB* and Δ*iscR*) were used as recipients for chemical transformation in TSS media.[76] The gene *iscR* (iron-sulfur cluster transcriptional regulator) is an *E. coli* gene unrelated to RecBCD, and thus the Δ*iscR* strain was used as a negative control. An overnight culture of the recipient bacteria grown in MMB with 50 μg/ml kanamycin was diluted 1:100 into 5 ml of fresh media and grown at 37°C 200 r.p.m to OD_{600}=0.2. Bacteria were then centrifuged at 3200 g for 10 minutes, after which the bacterial pellet was resuspended in 220 μl cold TSS media and put on ice. 50 μl of the bacteria were transferred to a new Eppendorf tube, and 100 ng of the vector expressing a retron defense system (pEc48, pSe72 or pEco8) or a negative control empty vector (pNC) was added. The bacteria were incubated with the vector on ice for 5 minutes, then at room temperature for 5 minutes, and again on ice for 5 minutes. 950 μl MMB was added and the bacteria were incubated at 37°C 200 r.p.m for 1 hour. Ten-fold serial dilutions of the bacteria in PBS were then plated on LB agar plates supplemented with 100 μg/ml ampicillin. Transformation efficiency was assessed by counting single colonies formed after overnight incubation at 37°C.

Please cite this article in press as: Stokar-Avihail et al., Discovery of phage determinants that confer sensitivity to bacterial immune systems, Cell (2023), https://doi.org/10.1016/j.cell.2023.02.029

Fitness assay of mutant phages

To examine the fitness of mutant escaper phages compared to their respective ancestor phages, each mutant and WT phage pair were mixed in a target ratio of approximately 1:1. Overnight cultures of host bacteria (*E. coli* MG1655 or *B. subtilis* BEST7003) were diluted 1:100 in MMB medium. Cells were incubated at 37°C while shaking at 200 r.p.m until reaching an OD_{600}=0.3. Bacteria were infected with the phage lysate at a final MOI of ~0.01, and incubated for 3 hours at 37°C while shaking at 200 r.p.m. Samples were centrifuged at 4000 g for 5 min to pellet bacteria, and the cleared phage lysate was transferred to a new tube. Phage titer was then checked using the small drop plaque assay on the host strain as described above. This phage lysate was subsequently used for the infection of a fresh culture of bacteria at an MOI of ~0.01 (second cycle of competition). For each of the experiments, 500 μl of the phage lysates from the original phage 1:1 mixture, after one cycle of competition and after two cycles of competition, were taken for DNA extraction and sequencing as detailed above. Sequenced reads were aligned to the WT phage genome sequence (accessions provided in the key resources table) and the proportion of WT vs. mutated reads was calculated for each mutant, based on the reads aligning at the position of the expected mutation. To detect single base substitutions, read alignment was performed using NovoAlign (Novocraft) v3.06.05 with default parameters, discarding reads that were non-uniquely mapped. To detect insertion mutations, read alignment was performed using Breseq (version 0.34.1) with default parameters.[72]

Detection of msDNA binding to phage SSB protein

Cultures of *E. coli* MG1655 bacteria expressing the Retron-Eco8-K36A defense system from its native promoter[15] and either WT or mutant C-terminally HA-tagged SSB from phage SECphi4 were grown overnight at 37°C in MMB supplemented with 100 μg/ml ampicillin + 50 μg/ml kanamycin + 1% glucose. For cell lysate controls, cultures of *E. coli* MG1655 bacteria expressing the WT Retron-Eco8 or a negative control strain (containing the empty pSG1 vector) were used and grown at 37°C in MMB supplemented with 100 μg/ml ampicillin only. Overnight cultures were diluted 1:100 in 100 ml fresh MMB supplemented with antibiotics and 0.5% glucose was added to strains containing the SECphi4-SSB to avoid toxicity due to leaky expression. The samples were incubated at 37°C and 200 r.p.m until they reached OD_{600}=0.6. Then, 0.2% arabinose was added to induce expression of the SSB protein and the incubation was continued for 1 hour at 37°C and 200 r.p.m. Each of the samples was split into two 50 ml tubes serving as technical replicates and centrifuged at 3200 g for 10 min. The supernatant was discarded and the cell pellets stored at -80°C. The cell pellets were thawed on ice and then resuspended in 700 μl cold TBS-T buffer (Bio-Lab cat# 208923) with protease inhibitor (Roche cat # 05892791001) and transferred into 2 ml tubes containing glass beads (lysing matrix B; MP Biomedicals cat # 116911050-CF). Cells were lysed at 4°C in a FastPrep-24 bead beater for 40 seconds at 6 m/s, followed by centrifugation at 4°C and 12,000 g for 10 min to remove the beads and cell debris. For cell lysate samples, 40 μl of the supernatant was mixed with 1 ml TRIzol (Thermo Scientific cat #15596018) and RNA was extracted as detailed below. For samples expressing the SECphi4 SSB, HA-tagged proteins were pulled down using anti-HA magnetic beads (Thermo Scientific cat #88837). For each sample, 25 μl magnetic beads were washed with 1 ml of cold TBS-T buffer. Then, 700 μl of the supernatant was transferred onto the anti-HA beads and incubated rotating at 4°C for 1 hour. The samples were transferred to a magnetic stand and washed three times with 500 μl cold TBS-T buffer. From the last wash, 400 μl of each sample was transferred to a new tube for RNA extraction, and the remaining 100 μl was used for protein analysis, to verify successful pulldown of the SSB-HA protein (see below).

RNA extraction, gel electrophoresis and Northern blotting

For RNA extraction from the SSB-HA proteins bound to the magnetic beads, the 400 μl of TBS-T buffer was removed from the samples on a magnetic stand and the samples were resuspended in 1 ml TRIzol. To each sample, 200 μl chloroform was added and mixed by inversion. After 5 min of incubation at room temperature, the samples were centrifuged for 15 min at 16,900 g and 4°C. Then, 500 μl of the top phase was transferred to new Eppendorf tubes. To each sample, 1 μl GlycoBlue co-precipitant (Thermo Scientific cat #AM9515) and 500 μl cold isopropanol were added. The samples were mixed by inversion and incubated overnight at -20°C for precipitation. Samples were centrifuged for 30 min at 4°C and 16,900 g and the supernatant was discarded. Pellets were washed in 400 μl cold 70% ethanol and centrifuged for 15 min at 16,900 g and 4°C. The 70% ethanol was discarded and the tubes were left open at room temperature until dry. The pellets were then dissolved in 35 μl ultra-pure water. RNA extracted from cell lysates or from the HA-tagged SECphi4-SSB protein was separated in a 6% polyacrylamide gel containing 6.7 M urea in 1x TBE for 2 h at 300 V. For cell lysates, 5 μg RNA was loaded and for SECphi4-SSB samples, 5 μl of each sample was loaded on the gel. For northern blotting, the gel was transferred to a Hybond-N+ membrane (Cytiva cat# GERPN203B) for 1 hour at 50 V in 1x TBE. The RNA was UV-crosslinked to the membrane at 254 nm and hybridized overnight at 42°C in hybridization buffer (Carl Roth cat# A981.1) with a radioactively labeled DNA oligonucleotide (JHO-0008; key resources table) specific to the reverse-transcribed DNA part of the Eco8 msDNA. The membrane was washed for 15 min at 42°C with 5x SSC, 1x SSC and 0.5x SSC, all containing 0.1% SDS. Next, the membrane was dried, wrapped in plastic foil and exposed to a phosphor screen, followed by imaging using a phosphorimager after 24 hours of exposure.

Protein gel electrophoresis and Western-blotting

For protein analysis, the 100 μl of TBS-T buffer was removed from the samples on a magnetic stand and the samples were resuspended in 35 μl 1x Bolt LDS Sample Buffer (Thermo Scientific cat #B0007) supplemented with 50 mM DTT. The samples were boiled at 95°C for 3 min and 10 μl of each sample were separated by 4-12% Bis-Tris SDS-PAGE (Thermo Scientific cat# NW04122BOX) in 1x MES buffer (Thermo Scientific cat# B0002) for 22 min at 200 V. For Western-blotting, the gel was transferred to a nitrocellulose membrane (Invitrogen cat # LC2001) for 1 hour at 10 V in 1x transfer buffer (Thermo Scientific cat# BT00061) and probed with a

primary rabbit anti-HA antibody (Sigma Aldrich cat #H6908; 1:3000 dilution in TBS-T with 3% BSA). Visualization of the primary antibody was performed using HRP-conjugated goat anti-rabbit secondary antibody (Thermo Scientific cat #31460; 1:10,000 dilution in TBS-T) and incubation with ECL solution (Merck Millipore cat# WBLUF0500).

Phage-infection dynamics in liquid medium

Overnight cultures of bacteria (*E. coli* MG1655 or *B. subtilis* BEST7003 expressing a defense system; Table S1) or a negative control strain (containing the empty pSG1 vector) were diluted either 1:100 in MMB medium supplemented with ampicillin (100 μg/ml) for *E. coli* strains, or 1:50 in MMB medium supplemented with spectinomycin (100 μg/ml) for *B. subtilis* strains. The diluted cultures were incubated at 37 °C 200 r.p.m for 100 min until they reached an OD_{600}=0.3, after which 180 μl of the culture was transferred into a 96-well plate containing 20 μl of either phage buffer (for uninfected control) or phage lysate for a final MOI of 2 or 0.02. Plates were incubated at either 37 °C (for phage T5), 25°C (for phages SECphi27 and phi3T) or 30°C (for phage SPR) with shaking in a TECAN Infinite200 plate reader and OD_{600} was measured every 10 min. Infections were performed in duplicate from overnight cultures prepared from two separate colonies. To measure phage replication, infection was performed as detailed above, at an MOI of 2, and the small drop plaque assay[78] was used to quantify phage titer before and after infection. For this, 10 μl of the infected culture were sampled either 110, 150 or 165 min post infection, serially diluted in phage buffer, and plated on a lawn of either *E. coli* MG1655 or *B. subtilis* BEST7003. Plates were incubated overnight at 37 °C or 25°C for *E. coli* and *B. subtilis*, respectively, and PFU/ml was calculated. The sample for time 0 was produced by mixing 20 μl of the phage used for infection in 180 μl MMB medium. Infections for phage quantification were performed in triplicate in three separate cultures.

Phage DNA restriction assay

High concentration phage DNA was prepared by first precipitating phages from a large volume, followed by DNA extraction using phenol-chloroform-isoamylalcohol. For this, 20 ml phage lysate was mixed with 4 ml 5M NaCl and 2 gr PEG8000. The samples were left to rotate overnight at 4°C, and then centrifuged at 11,000 g for 10 min at 4°C. The supernatant was discarded and the pellet was resuspended in 400 μl phage buffer (5mM Tris pH 7.4, 100 mM MgCl2, 10 mM NaCl). The samples were then treated with DNase-I (Merck cat #11284932001) added to a final concentration of 40 μg/ml and incubated at 37°C for 1 hour to remove bacterial DNA. Phages were then lysed by the addition of 200 μg/ml Proteinase-K (Bio-Lab cat #1673238300), 0.5% SDS (Bio-Lab cat #19812323) and 20 mM EDTA, incubated at 55°C for 1 hour. The lysate was then mixed with 1 volume of phenol-chloroform-isoamylalcohol (Merck cat #P2069), and centrifuged at maximal speed in a table top centrifuge at 4°C for 15 min. The upper aqueous fraction was transferred to a new tube. To precipitate the DNA, 1 volume of isopropanol and 1/30 of the volume of NaAc 3M pH 6.5 were added to the collected aqueous fraction and mixed by inversion followed by incubation at -80°C for 25 min. Then the samples were centrifuged at maximal speed in a table top centrifuge at 4°C for 30 min, and the pellet was washed twice with cold 75% ethanol. The pellets were dried at 37°C for 5 min and resuspended in 50 μl ultra-pure water. DNA concentration was measured using a Qubit dsDNA BR Assay Kit (Thermo Fisher cat # Q32850). For the restriction assay, 300 ng phage DNA was mixed with 14 μl lysis buffer (50 mM Tris pH 7.5, 150 mM KCl, 1mM MgCl2, 1mM MnCl2, 1mM CaCl2) and 1 μl HpaII restriction enzyme (Thermo Fisher cat #ER0511). The reaction was incubated for 2 hours at 37°C and then loaded into a 0.7% agarose gel for analysis of cleavage pattern.

Phage protein structure prediction and alignment

The structures for the SECphi4 phage putative SSB and T5 phage A1 proteins (accessions in GenBank: QJI52528.1, AAS77051.1, respectively) were predicted using AlphaFold2[69] within ColabFold[70] with default parameters. For the SECphi4 SSB protein, the best ranking AlphaFold2 predicted structure was aligned to the T7 phage SSB (PDB: 1JE5, chain A) using the "cealign" method of the alignment plugin within the PyMOL program v. 2.5.1.[73]

For the T5 phage A1 protein, the amino acid sequence was first submitted to HHpred,[68] which revealed a putative nuclease domain at the C-terminus of the A1 protein but no known domain at the A1 N-terminus. The predicted structure of the A1 N-terminal domain (residues 1-60) was extracted from the best ranking AlphaFold2 predicted structure of the protein and submitted to PDBeFold,[71] a protein structure comparison web service to identify structural homologs, retrieving a high confidence hit to the DNA-binding domains of the *Caulobacter crescentus* GcrA (PDB: 5yiv, chain D). To visualize the alignment, the crystal structure of the GcrA DNA-binding domain was aligned to the AlphaFold2 predicted structure of the T5 phage A1 protein using the alignment plugin within the PyMOL program with the "cealign" method.

The structures for the SECphi4 and T5 phage DNA polymerase proteins (accessions in GenBank: QJI52526.1, AAS77168.1, respectively) were predicted using AlphaFold2[69] within ColabFold[70] with default parameters, and the best ranking AlphaFold2 predicted structure out of five predictions was chosen. The domains of the proteins were predicted using HHpred[68] (3′–5′ exonuclease domain, pfam accession PF01612.23, and polymerase domain, pfam accession PF00476.23). The putative location of the active site was marked by adding the predicted metals to the AlphaFold2 structure. The alphafold2 predicted structures were aligned to the crystal structure of the T7 DNA polymerase (PDB: 1T7P)[80] using the alignment plugin within the PyMOL program with the "cealign" method, and the location of the metals in the T7 DNA polymerase active sites were overlaid on the SECphi4 and T5 predicted DNA polymerase structures to identify the active site pockets.

Please cite this article in press as: Stokar-Avihail et al., Discovery of phage determinants that confer sensitivity to bacterial immune systems, Cell (2023), https://doi.org/10.1016/j.cell.2023.02.029

Sequence alignment of retron RTs

The protein sequences of the Ec48 and Se72 retron RTs (Gene IDs in the IMG database[81]: 2642317602 and 2633939248, respectively) were aligned to each other using BLASTp on the NCBI BLAST web interface[74] with default parameters.

QUANTIFICATION AND STATISTICAL ANALYSIS

The average of three triplicates is shown throughout with individual points overlaid, unless stated otherwise. Statistical details of experiments can be found in the figure legends.

Supplemental figures

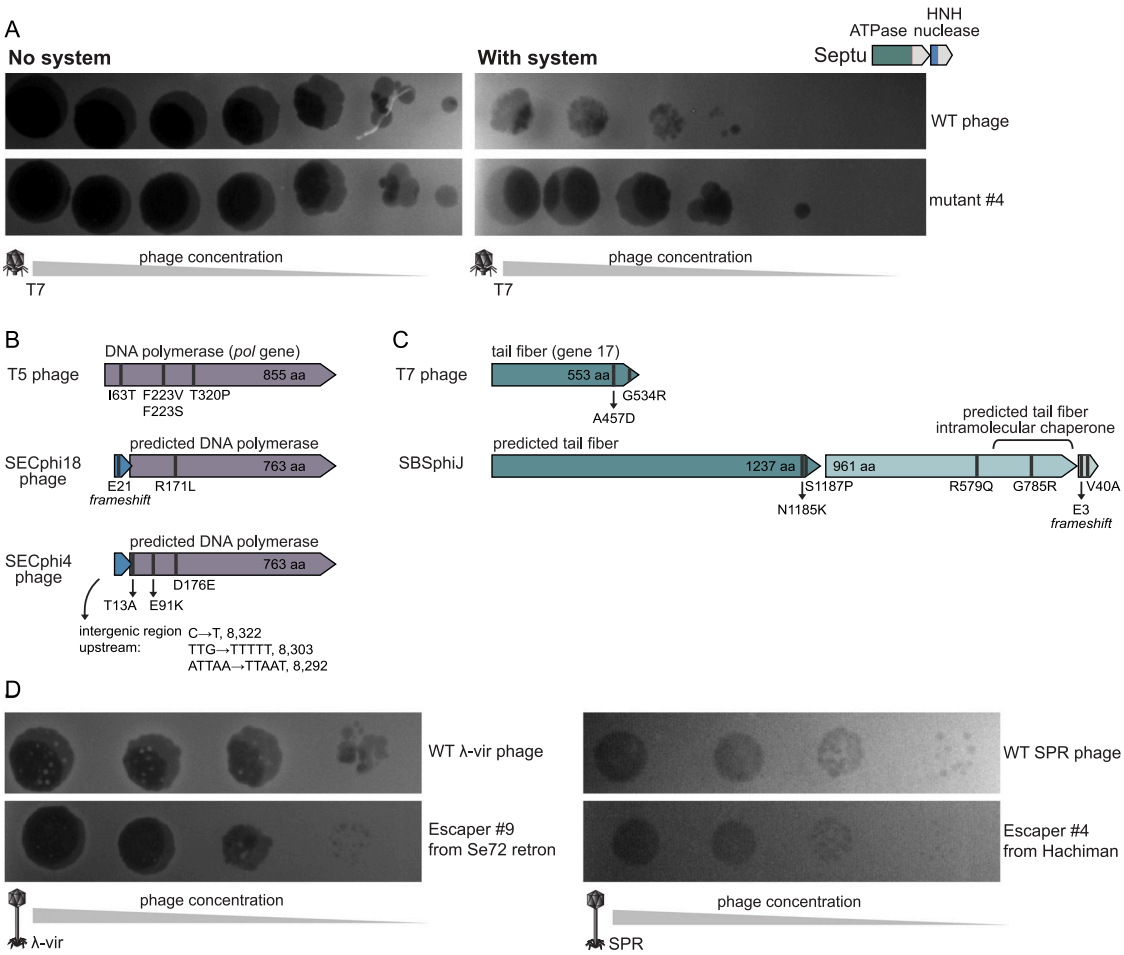


Figure S1. Additional analyses of escaper phages, related to Figure 3

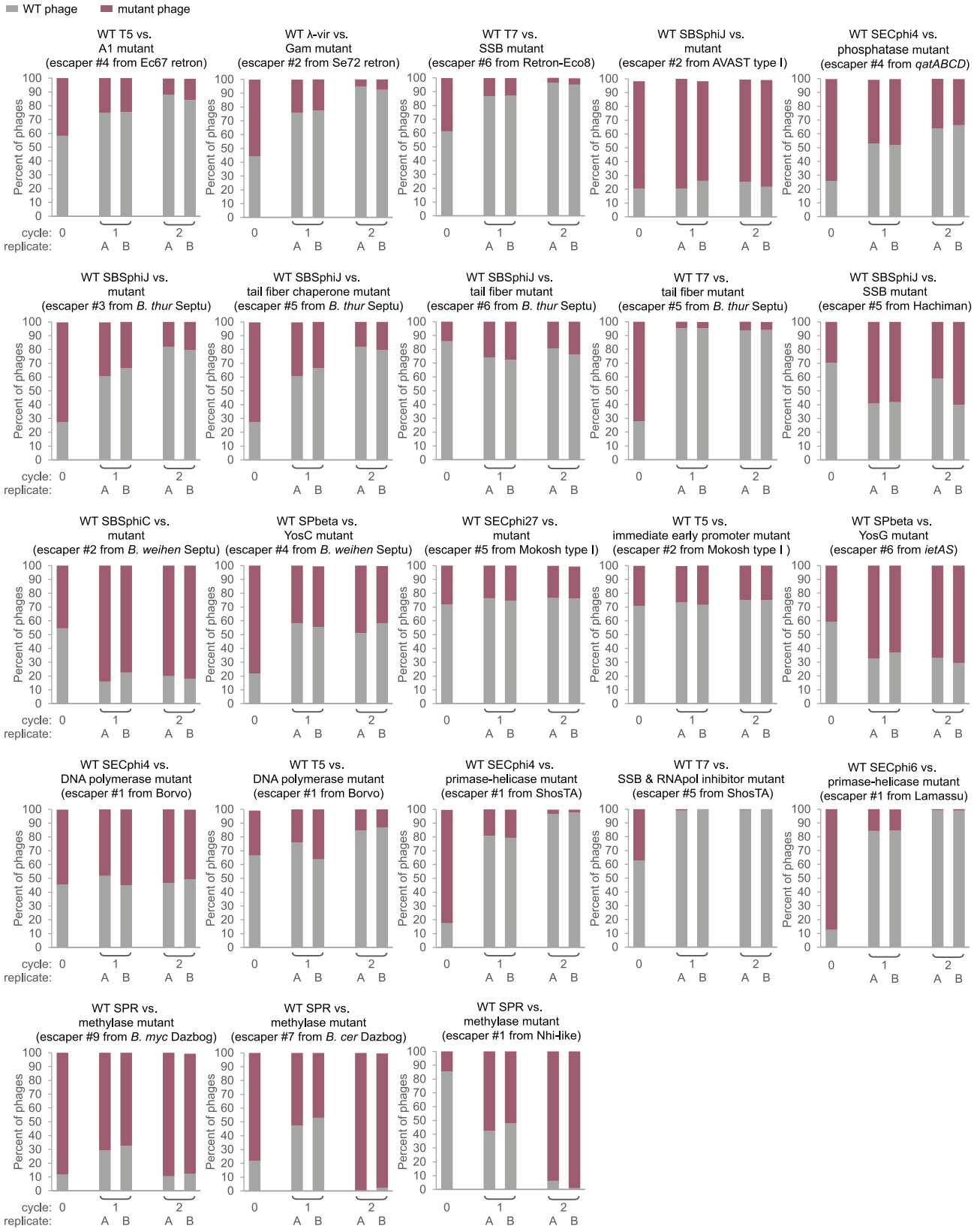
(A) Escaper phages overcome reduction in plaque size. An example of a case in which protection against the WT phage manifests in decreased plaque size and the escaping phages form large plaques even when infecting bacteria with the defense system. Serial dilution plaque assays are presented for WT and mutant T7 phages infecting *E. coli* MG1655 expressing the Septu defense system from *B. thuringiensis* HD12.

(B and C) The putative function of genes of unknown function that are mutated in certain escaper phages can be inferred based on their operon associations.

(B) Location of mutations found in phages that escape from Borvo defense. Escaper phages harbor a mutation either in the gene encoding for the phage DNA polymerase or in a short gene immediately upstream.

(C) Location of mutations found in phages that escape from *B. thuringiensis* HD12 Septu defense. 10 out of 14 isolated escaper phages harbor a mutation either in the gene encoding the phage tail fiber or in the two genes immediately downstream to the phage tail fiber gene, one of which contains a predicted tail fiber intramolecular chaperone. The full list of mutations is detailed in Table S2.

(D) Escaper phage mutations come with a fitness cost. Examples of two cases in which the mutant phages formed smaller plaques compared with the WT phage on control cells lacking the defense system, suggesting the mutations may come with a fitness cost to the phage. Serial dilution plaque assays are presented for WT and mutant λ-Vir phages infecting the *E. coli* MG1655 negative control strain (left), and SPR phages infecting the *B. subtilis* BEST7003 negative control strain (right).



(legend on next page)

Figure S2. Competition assays between WT and mutant phages on control cells lacking a defense system, related to Figure 3

The indicated mutant phage and its corresponding WT phage were mixed in a target ratio of approximately 1:1, and added to a culture of bacteria for infection at an MOI of 0.01. Competition was performed in two cycles, and the phages were sequenced before and after each cycle to quantify the fraction of WT and mutant phages from the total phage mixture (y axis). The x axis represents the competition cycle, with 0 representing the mixture before first competition. "A" and "B" represent two different replicates performed on two separate bacterial cultures, starting from the same initial phage mixture. "*B. thur*" is *Bacillus thuringiensis* HD12, "*Bacillus weihen*" is *Bacillus weihenstephanensis* KBAB4, "*B. myc*" is *Bacillus mycoides* GOE9, and "*B. cer*" is *Bacillus cereus* FORC_013.

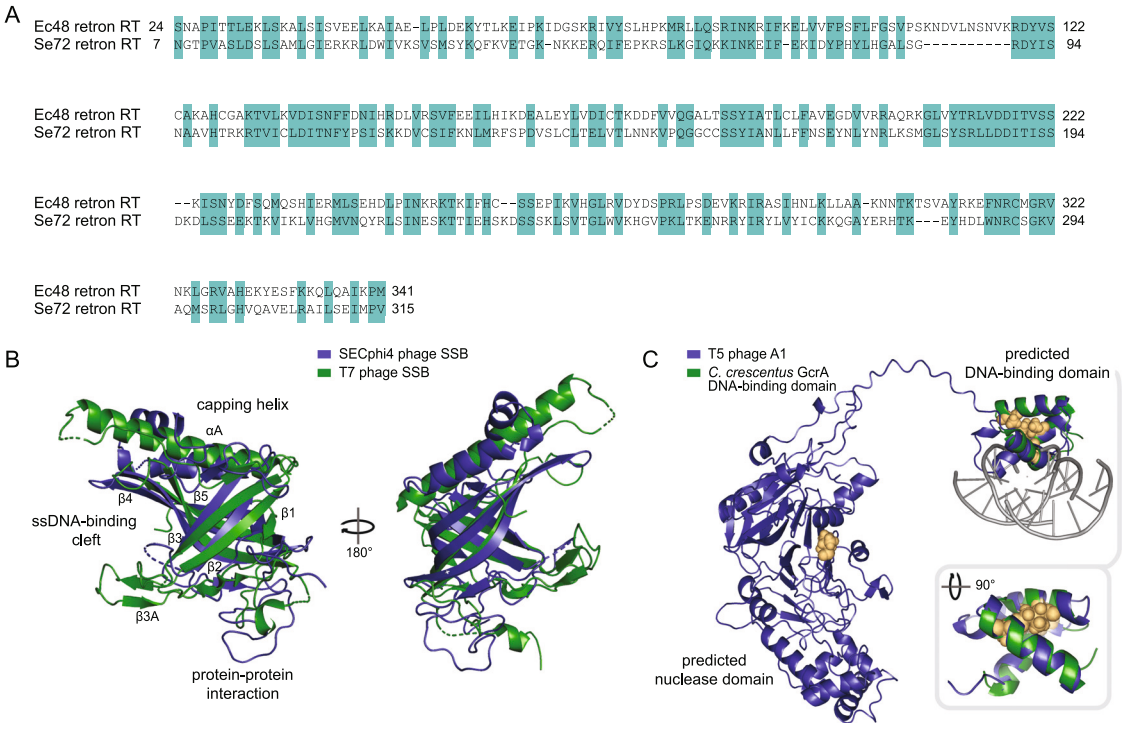


Figure S3. Analysis of components mutated in phages that escape retron defense systems, related to Figure 4

(A) Amino acid sequence homology between the reverse-transcriptase (RT) proteins of retron Ec48 and retron Se72. Identical and similar amino acids are shaded in turquoise.

(B) Structural homology between the T7 phage single-stranded DNA-binding protein (SSB) and a predicted SSB from phage SECphi4. The AlphaFold2-predicted protein structure of the SECphi4 phage putative SSB (residues 1–186) is presented in blue, and the crystal structure of the T7 phage SSB (PDB: 1JE5, residues 1–196) is in green. Flexible C-terminal residues are not displayed for both proteins. Secondary structure elements of the ssDNA-binding OB-fold of the T7 SSB are annotated based on Hollis et al.[37]

(C) AlphaFold2-predicted protein structure of phage T5 A1 protein (blue). The N-terminal domain (residues 1–60) of the predicted A1 protein structure show structural homology to the *Caulobacter crescentus* GcrA DNA-binding domain (PDB: 5yiv, chain D (green), bound to dsDNA [gray]). Amino acid residues mutated in T5 phages that overcome Ec67 retron-mediated defense are displayed as yellow spheres.

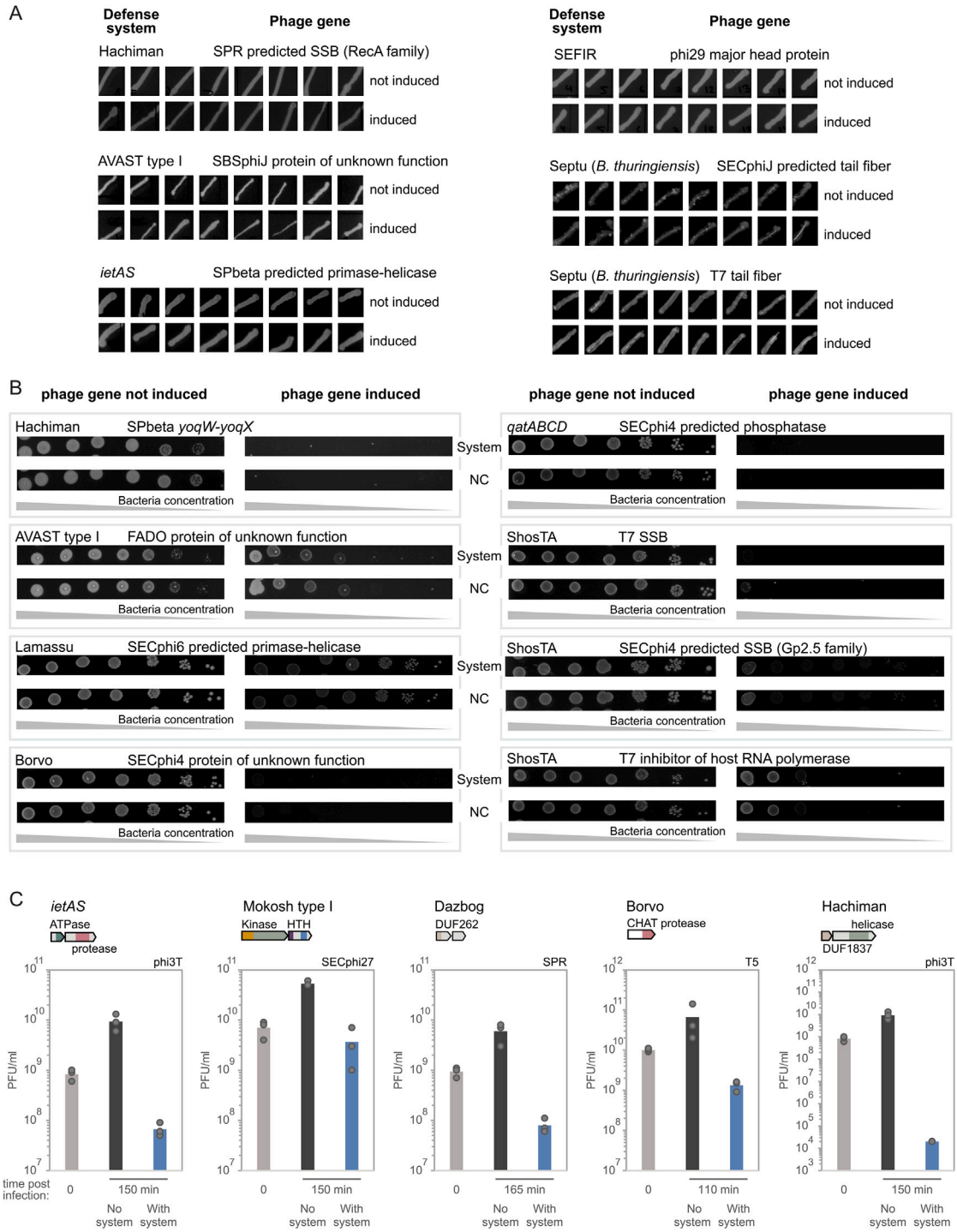


Figure S4. Expression of single phage genes in defense system-containing strains to identify defense systems that act via abortive infection and the phage protein that activates them, related to Figure 5

Bacterial viability was measured for cells encoding a defense system upon expression of single phage genes presented in Figure 3B. Bacterial growth was assessed by plating bacteria in conditions that repress or induce expression of the phage gene (with either arabinose or IPTG; see STAR Methods).

(A) Cases in which expression of the phage gene was not toxic in cells that contained the defense system. Eight different bacterial colonies were streaked to assess toxicity upon expression of the phage gene in defense system-containing bacteria.

(B) Cases in which expression of the phage gene exhibited non-specific toxicity in both defense system-containing cells and in control cells lacking the system (NC). Measurement of the toxicity of the phage gene was performed by plating 10-fold serial dilutions of the bacteria. Images are representatives of three replicates.

(legend continued on next page)

(C) Phages do not replicate on specific defense system strains. Replication of phage phi3T, SECphi27, SPR, or T5 in the presence or absence of the specified defense system. Phage lysates were collected before infection (time 0) and 110–165 min post infection of liquid cultures at an initial MOI of 2, and phage titer was quantified by plating serial dilution of the lysates on a control strain lacking the defense system. Bar graphs represent an average of three replicates, with individual data points overlaid. The two experiments with the phi3T phage were performed simultaneously with the same negative controls; thus, time 0 and no system data are identical for the Hachiman and *ietAS* panels.

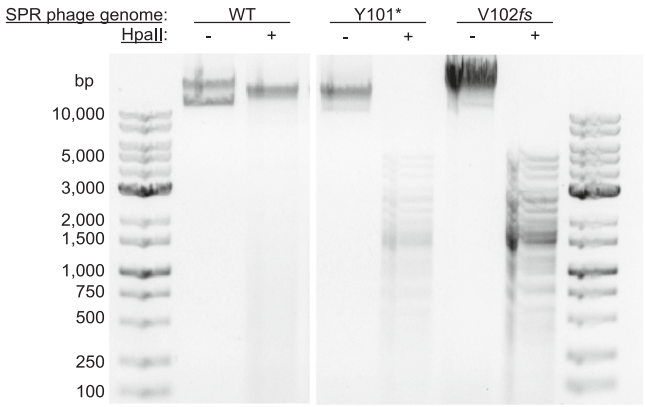


Figure S5. The genome of SPR escaper phages that overcome the Dazbog anti-phage system are unmethylated at CCGG, related to Figure 5E

Restriction analysis of WT or mutated SPR phage DNA incubated for 2 h with the HpaII restriction enzyme, which cleaves unmethylated DNA at CCGG motifs. Y101* and V102*fs* are mutations in the SPR DNA cytosine methylase that are found in phages that overcome Dazbog defense (Table S2). DNA bands were resolved by agarose gel electrophoresis in a 0.7% agarose gel. The genome of WT SPR phages is methylated at CCGG and is thus protected from cleavage by HpaII, whereas the genomes of mutant phages that escape Dazbog defense are restricted.

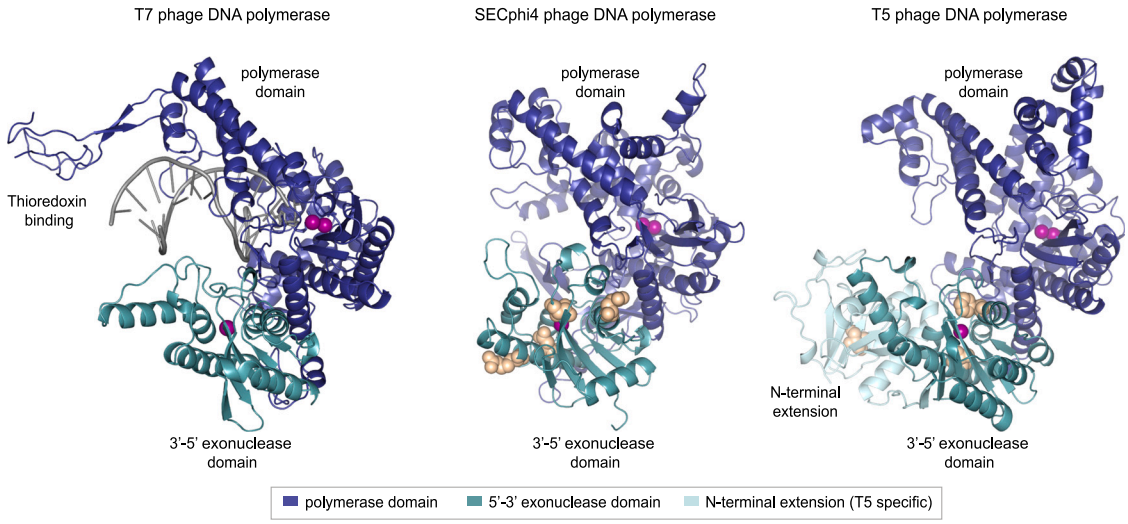


Figure S6. Mutations in the proofreading domain of phage DNA polymerase proteins enable escape from Borvo defense, related to Figure 3

AlphaFold2-predicted protein structures of the T5 and SECphi4 phage DNA polymerases are presented, as well as the crystal structure of the T7 phage DNA polymerase (PDB: 1T7P).[80] Amino acid residues mutated in the DNA polymerase protein of phages T5 (I63, F223, T320), SECphi4 (T13, E91, D176) and SECphi18 (R171) that overcome Borvo-mediated defense are displayed as yellow spheres. The SECphi18 phage DNA polymerase is highly similar to that of SECphi4, thus SECphi18 mutations are marked on the SECphi4 DNA polymerase structure. The polymerase and proofreading (3′-5′ exonuclease) domains are colored in blue and cyan, respectively. T5 phage DNA polymerase contains an N-terminal extension (marked in light blue) that is absent in other DNA polymerases from the RNA Pol I family. This N-terminal extension is not part of the conserved 3′-5′ exonuclease, but might play a role in proofreading activity in T5, because its truncation reduces exonuclease activity.[82] Metals found in the active site of the polymerase and exonuclease domains are marked as magenta spheres based on their location in the T7 DNA polymerase crystal structure and are mapped onto the SECphi4 and T5 DNA polymerase predicted structures to mark their corresponding active sites.