The bacterial immune system: identifying evolved defense adaptations

Ellinor O. Alseth[a,b,c] & Sam P. Brown[a,b]#

[a]Center for Microbial Dynamics and Infection, Georgia Institute of Technology, Atlanta, Georgia, USA

[b]School of Biological Sciences, Georgia Institute of Technology, Atlanta, Georgia, USA

[c]Centre for New Antibacterial Strategies, Department of Pharmacy, UiT– The Arctic University of Norway, Tromsø, Norway

sam.brown@biology.gatech.edu

"Evolutionary adaptation is a special and onerous concept that should not be used unnecessarily, and an effect should not be called a function unless it is clearly produced by design and not by chance." – George Williams(1)

Abstract

The last few years have witnessed a rapid expansion of reported bacterial defense mechanisms. Alongside established mechanisms of defense against molecular parasites (e.g. CRISPR-Cas, restriction-modification), hundreds of novel defenses are being described each year, contributing to an ever-expanding 'bacterial immune system'. Terms like 'defense' and 'immune' are often used as shorthand for an observed anti-infection phenotype, but they can also be read as implying an evolutionary adaptation with a specific anti-infection function. Despite the field's rapid progress, there is currently no widely agreed framework for identifying bacterial defense adaptations. The question then emerges: when is a defense mechanism an evolved adaptation? Here, we leverage prior debates in evolutionary biology over adaptation to propose four main 'evidence' criteria, spanning bioinformatic comparative tests, experimental fitness assays, evolutionary theory, and their integration. Together, these criteria can strengthen the case that a defense mechanism is an evolved defensive adaptation. We highlight how these criteria are met for the most established model systems such as CRISPR-Cas, and how they also provide a clear research agenda for other newly identified defense mechanisms. Beyond bacterial immunity, these criteria offer a research roadmap to address functional controversies across microbiology.

Importance

The rapid growth of the bacterial defense literature is an exciting frontier in microbiology. But it also blurs a critical distinction: functional terms like “defense” and “immune” are used either to refer to an experimentally demonstrated protection phenotype or to the stronger evolutionary claim that a system evolved because it provides protection (a defense adaptation). This distinction matters in practice, because traits that are directly shaped by selection for defense are more likely to yield robust, predictable protection across contexts—key for phage therapy, synthetic biology, and microbial engineering. Here we offer an integrative framework to drive the identification of evolutionary adaptations. We synthesize four complementary evidence criteria spanning comparative bioinformatics, experimental fitness assays, evolutionary hypothesis development, and integration across approaches. Using CRISPR–Cas and surface modification as worked examples, we outline a practical research agenda for strengthening adaptation claims across the rapidly growing bacterial immunity field.

Introduction

All around us, bacteria are challenged by mobile genetic elements (MGEs), whose interactions with hosts can span the parasite—mutualist continuum. MGEs (bacteriophages (phages), plasmids, etc.) have coevolved with their bacterial hosts for billions of years, shaping the course of evolution and diversification in the microbial world and beyond(2–4). Phages alone have been estimated to be responsible for as much as 40% of daily bacterial deaths(5), posing a major evolutionary driver for their hosts(6). Beyond phage-induced death, diverse MGEs can also impose costs that range from marginal losses of fecundity through to complete growth arrest(7,

8). In light of these substantial mortality and fecundity costs caused by parasitism, it is natural to expect that bacteria will carry a diverse arsenal of defensive adaptations to limit the costs of parasite infection.

Ten years ago, the list of defined defense mechanisms in bacteria was short. Restriction-modification (RM)(9), abortive infection(10), and clustered regularly interspaced short palindromic repeats-CRISPR associated (CRISPR-Cas)(11–13) were and still are the best known mechanisms through which bacteria can defend themselves against their molecular parasites. Since the discovery of CRISPR-Cas as a defense mechanism however, the molecular, genomic, and bioinformatic tools available to researchers have resulted in an explosion of newly discovered defense mechanisms(14–26) (Fig. 1). Combined, these defenses have come to be known as the 'bacterial immune system'(27–31), potentially echoing the complex suite of coordinated defensive tools of the vertebrate adaptive and innate immune systems(32, 33). Over the last few years alone, over 250 new phage defense mechanisms have in rapid succession been discovered via a combination of computational and experimental approaches(19). Additionally, bioinformaticians have—through computational prediction—suggested the existence of over 7000 potential defense genes(23).

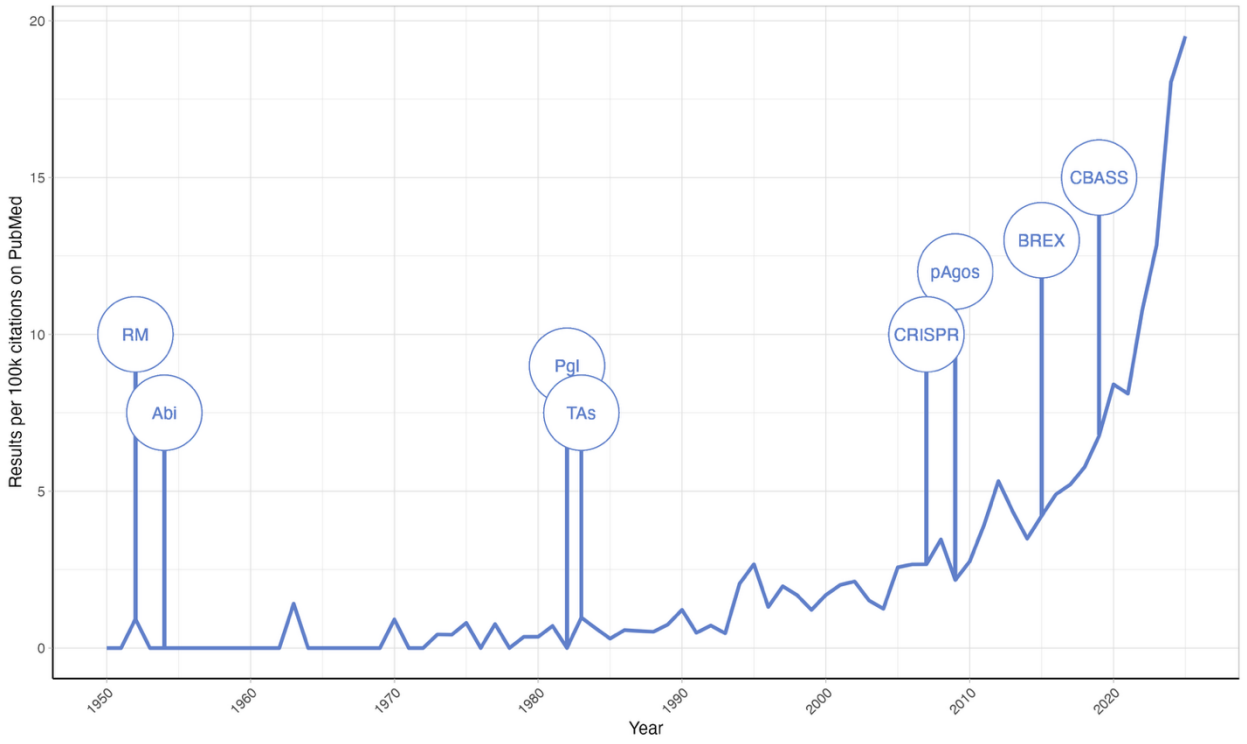

Fig. 1. The bacterial defense literature is rapidly expanding. Hits per 100,000 citations (proportion for each search by year) when searching for 'phage defense' on PubMed, using the 'PubMed by Year' tool(34). Highlighted through the timeline are restriction modification (RM)(9), abortive infection (Abi)(10), Phage growth limitation (Pgl)(35), toxin-antitoxin systems (TA)(36), CRISPR-Cas(11–13), prokaryotic Argonaute proteins (pAgos)(37), bacteriophage exclusion (BREX), and cyclic oligonucleotide-based anti-phage signalling system (CBASS)(22). Note that proportional measures of research activity underplay the absolute increase in the defense literature, given the increasing volume of publications per year (see Fig. S1 for increase in raw paper counts).

The exploding number of defense mechanisms (Fig. 1) raises a fundamental question: how do we know that a mechanism of interest is truly the product of natural selection for the adaptive role

of anti-parasite defense? To guide this discussion, we distinguish between a measurable phenotype (e.g. a gene reducing phage replication in defined lab conditions) and its evolutionary origin as an adaptation shaped by natural selection. From a practical standpoint, understanding whether a trait evolved specifically to limit MGE activity can inform predictions about its stability and context-specific effectiveness—critical applied concerns for fields such as phage therapy, synthetic biology, and microbial engineering. In this perspective, we outline a general and iterative approach combining experiment, bioinformatics, and evolutionary theory to provide the most robust support for claims of evolutionary adaptation, applicable to defense mechanisms and the study of adaptation in biology in general. Due to most defenses being described as having anti-phage functions, this perspective will also largely focus on bacteria-phage dynamics unless otherwise specified.

Four criteria to identify adaptations

The identification and study of adaptive function or 'design' has a central yet controversial place in evolutionary biology. It is widely accepted that natural selection is a driver of the appearance of biological design, capable of producing complex adaptations with close fit between form and function - with the vertebrate eye being a canonical example(38, 39). Yet the observation of a measurable phenotype (e.g. the ability of a protein to bind a specific substrate) does not ensure that this phenotype is an adaptation shaped by natural selection. Recognition of this imperfect mapping between measurable phenotype and evolutionary adaptation has led to repeated debate over decades. For example, Williams described adaptation as an 'onerous concept'(1), meaning that any claim of adaptation carries a substantial burden of proof, as it asserts historical

shaping by natural selection, and not merely a present-day effect. Relatedly, Gould and Lewontin critiqued what they called adaptationist 'just-so' storytelling(40, 41): post hoc narratives that explain a phenotype via prior selection but without sufficient independent evidence or evaluation of alternate hypotheses. More recently, the same issues have led to heated controversy over the evolutionary relevance of molecular phenotypes in the human genome(42–45) (discussed in more detail below). We pose these classic cautions here because the current explosion of defense systems makes it especially easy to move from 'this locus affects phage' to 'this locus evolved for defense' without clearly stating assumptions or evaluating competing hypotheses. More broadly, this tension reflects a classic distinction in biological explanation between mechanistic ('how') and evolutionary ('why') questions. Mayr's proximate/ultimate framing(46) and Tinbergen's 'four questions'(47) provide a useful context for keeping these explanatory goals distinct, while still treating them as complementary.

By reviewing this history of debate over the identification of evolutionary adaptations, we identify a best-practice iterative approach, combining layers of experimental, comparative (bioinformatic), and conceptual/theoretical evidence(48). This approach identifies four main criteria (Fig. 2) to help winnow a broad menu of defense mechanisms towards a list of well-supported defense adaptations. Here, we briefly sketch these criteria, before applying them to some of the more well-studied defense mechanisms.

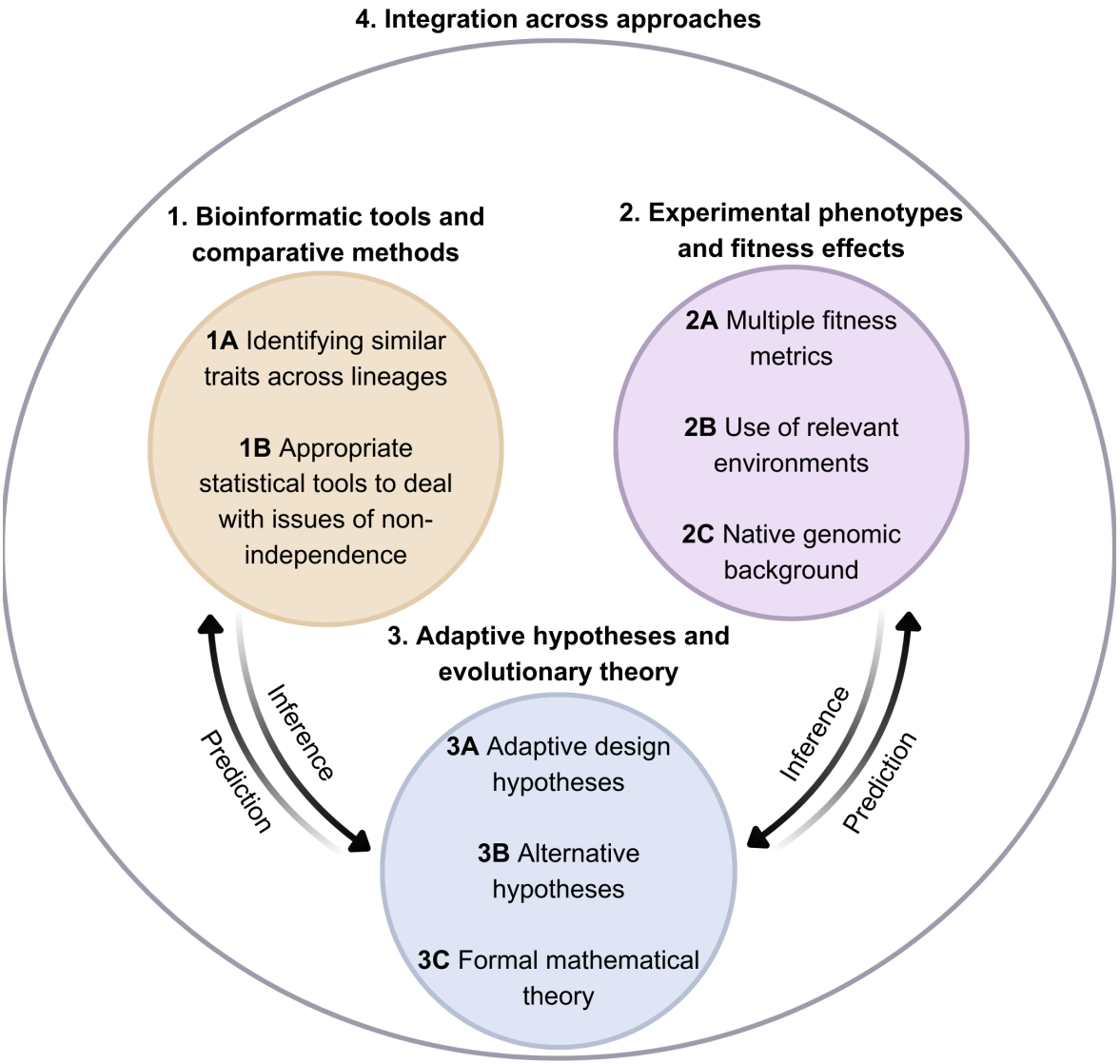

Fig. 2. A workflow for evaluating defense mechanisms in an evolutionary context. Starting from an initial defense mechanism discovery (typically bioinformatic (1) or experimental (2)), we flag the importance of explicitly identifying alternate defense and non-defense evolutionary hypotheses (3), and using these hypotheses to drive additional experimental and bioinformatic tests. Arrows highlight the importance of integration (4) by flagging how, for example, bioinformatic discoveries (1) can drive experimental predictions (2) by inspiring new testable hypotheses (3). Similarly, experimental data (2) can drive bioinformatic predictions (1), mediated

by new functional hypotheses and associated conceptual models (3). Mechanisms can cycle through these steps multiple times as new data accumulates, both within and across multiple studies (4). The criteria sub-components are discussed in the main text. We underline that while our third criterion is inclusive of formal (e.g. mathematically explicit, 3C) theory, mathematical modelling is not necessary for criterion 3.

1. Bioinformatic tools and comparative analyses

The observation of similar traits in distinct lineages in response to similar environmental challenges lends support to an adaptive interpretation of the trait(48, 49). Returning to the eye, the hypothesis that this trait is a product of natural selection is further supported by the presence of distinct, independently evolved 'camera eye' solutions in distinct taxa (vertebrates and cephalopods), and their loss in taxa that live in the dark(50). Evaluating adaptive hypotheses using comparative methods can therefore provide valuable evidence, but also faces a number of challenges, principally in the identification of functional similarities (homologues—similarity due to common ancestry, and analogues—similarity due to convergence) across lineages (1A), and the use of appropriate statistical analysis (1B).

2. Experimental phenotypes and fitness effects

Any adaptive trait that is the product of natural selection must confer a fitness advantage under relevant environmental conditions. Demonstrating that phenotype expression correlates with fitness can therefore provide evidence of adaptation(51). Notes of caution must be sounded however, as misspecification of the experimental environment or timescale of an assay can

generate false negative or positive associations between a trait of interest and specific measures of fitness(51). These concerns can be mitigated by conducting experimental assays using a range of fitness metrics (2A), natural environments (2B), and native genomic backgrounds (2C).

3. Adaptive hypotheses and evolutionary theory

Many studies report a phenotype affecting bacteria–phage interactions (e.g. reduced phage replication) and describe this using functional language ('defense,' 'immunity,' 'anti-phage'). In mechanistic and molecular contexts, such terms are often used as shorthand for an experimentally demonstrated effect in a particular assay or environment, without implying why the trait exists. In evolutionary contexts, however, the same terms are commonly taken to imply an adaptation claim—that the trait evolved *because* it confers defense. This split in default interpretation mirrors the classic proximate–evolutionary distinction(46) and Tinbergen's framing of complementary questions(47).

Even when authors intend only an operational, assay-specific statement, this functional 'defense' phrasing can subtly invite adaptive 'what it is for' interpretations unless the intended level of inference is stated explicitly. Considering these conflicting readings of the same terminology, we underline the importance of making any implicit adaptive hypotheses explicit (3A), examining alternative adaptive and non-adaptive hypotheses (3B), and encourage the use of formal mathematical modelling approaches to sharpen inferences and generate testable predictions (3C). We emphasize that mechanistic demonstrations are foundational; our goal is simply to be explicit about when the claim shifts from *what it may do* to *why it evolved*.

To underline the importance of an evolutionary perspective when dealing with functional questions on a molecular scale, the 'Encyclopedia of DNA elements' (ENCODE) debate is instructive(44, 45). Based on vast volumes of expression and protein-binding data, The ENCODE project concluded that ~ 80% of the human genome is functionally involved in regulatory control of coding DNA(42, 43). This result was a striking departure from prevailing views that most of the human genome is 'junk DNA', and resulted in heated critiques from evolutionary biologists(44, 45). In short, these critiques revolved around a failure to assess simple alternative and non-adaptive hypotheses (e.g. some proteins will bind promiscuously to a broad range of DNA sites), and therefore a failure to avoid the trap of 'pan-adaptationism'(45). A recurring concern with a naïve adaptationist approach is that adaptive hypotheses (e.g. 'the eye is a camera'; 'non-coding region *x* is a regulatory element') are generated in light of a phenotype of interest, and then supported by existence of the phenotype itself(48). Without other lines of evidence, this approach can result in hypotheses being tuned to limited phenotypic observations, and the pitfall of 'just so' storytelling(40). This is why we underline the importance of other experimental and bioinformatic lines of evidence, together with their integration.

4. Integration across approaches

We finally highlight the importance of an iterative integration of the broad approaches outlined above(48). For example, phenotypic data on bacteria/phage interactions (criterion 2) can generate adaptive and non-adaptive hypotheses (criterion 3) which then deliver predictions for both experimental (criterion 2) and bioinformatic tests (criterion 1); which in turn will drive

revisions to functional hypotheses (criterion 3), and so on. We note that this integration criterion is not simply a test for the presence of criteria 1-3 above, it is an additional test for their integration and agreement, so that different research avenues provide a complementary and coherent picture of adaptation (Fig. 2). Typically, the deepest level of integration develops over time across multiple studies, with confidence in an existing conclusion increasing (or decreasing) in light of new data. This iterative process can be seen as a form of Bayesian updating(48) and is consistent with the principle of abductive reasoning(52). We see this level of multi-study integration of multiple and distinct lines of evidence as an important benchmark response to Williams' recognition of adaptation as an onerous concept(1).

The bacterial immune system

With so many newly proposed defense mechanisms and the inevitability of more to come, robust ways of categorising them are essential. A number of existing classification schemes exist, focusing on genetic and molecular similarities, host fate, and mode of action(30, 31, 53). Here, we propose a simple functional classification to aid our subsequent discussion of adaptation (Fig. 3), contrasting individual cell-level defense mechanisms that promote individual survival of a cell (Fig. 3A) and defense mechanisms that result in individual cell death while promoting group survival (Fig. 3B) (see supplemental appendix for more detail on cell- vs group-level defenses).

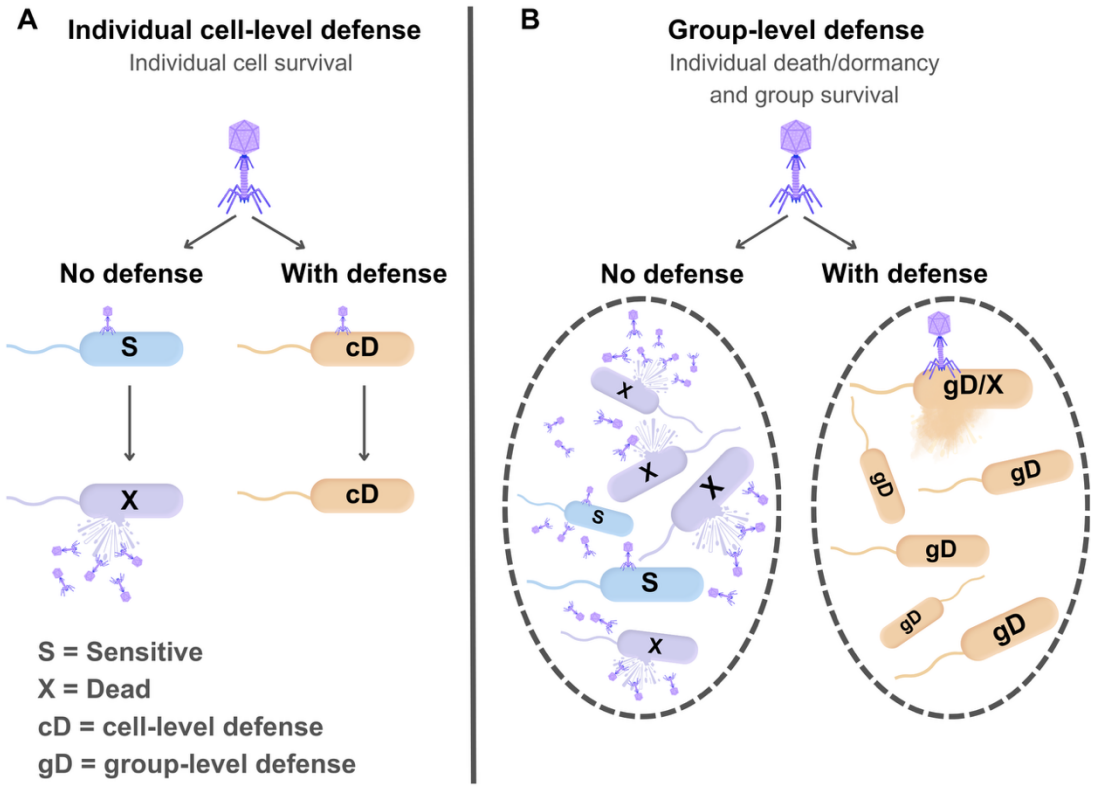

Fig. 3. Individual cell-level versus group-level defenses. **A** Individual cell-level defenses protect cells from phage or MGE mediated death as well as potentially protecting neighboring cells by limiting phage amplification (e.g. CRISPR-Cas, RM). **B** Group-level defenses promote the persistence of groups of cells (demarcated by dotted lines) by limiting phage amplification in initially infected focal cells, often via rapid cell death (e.g. abortive infection). See the appendix for further discussion on cell- vs group-level defenses.

In bacteria, the majority of sequenced genomes (78%) encode at least two defense mechanisms, with some containing dozens(30). Defenses are usually found together on so-called 'defense islands', and many a phage defense mechanism has been discovered through guilt-by-association/co-localization approaches(54) focusing on the uncharted genomic sea that immediately surrounds these expanding 'islands'. In this section we have attempted to give a

brief overview of a rapidly growing field, and consequently have omitted many emerging mechanisms. For an up-to-date overview of defense mechanisms, we recommend using the online tools 'PADLOC'(55) and 'DefenseFinder'(19). As the list of proposed defense mechanisms grows, our key questions remain: are all these systems truly evolved adaptations to enhance host defense? What evidence is appropriate to make such a claim?

Reviewing the evidence and challenges to the field

Fig. 2 summarizes a general workflow to identify, test, and refine adaptive hypotheses in biology. To provide a model case in the context of bacterial defense mechanisms specifically, we first apply our criteria to CRISPR-Cas (Box 1), highlighting a defense mechanism that generally addresses all criteria discussed above.

While we hold up CRISPR-Cas as a successful model of adaptive reasoning (Box 1), we have by no means reached the last word on this topic. The comparative bioinformatic observation of widespread CRISPR-Cas carriage(31) still stands in some tension with the experimental observation that the acquisition of CRISPR spacers is rarely observed in the lab. One possible explanation is that prior experiments have tended to miss the mark on the 'relevant environment' criterion 2B(56–61), or perhaps CRISPR-Cas is less often a decisive defensive mechanism in nature(62).

Looking beyond CRISPR-Cas, an initial survey of defense mechanisms highlights that while adaptive claims for some defense mechanisms are relatively well supported, our approach flags

potential conflicts between criteria for some mechanisms (Box 2). In the following sections we discuss common issues raised by our analysis, referring back to specific criteria outlined in Figure 2 throughout.

[Box 1]

CRISPR-Cas as a model defense

1. Bioinformatic tools and comparative analyses

1A and B) Identifying and analysing similar traits across lineages using appropriate tools. Comparative analyses of CRISPR-Cas systems face a substantial challenge: among all the complexity of CRISPR-Cas architectures, there are no universally present orthologues that can serve as the basis for a common phylogeny. The *cas1* gene (important for spacer insertion) is among the most prevalent genes, but is still absent in many lineages(63). In contrast, the critical destructive nuclease genes are represented by multiple analogous variants (e.g. *cas9* or *cas13*, or multi-part variants in Class 1 CRISPR-Cas systems(63)). Given this challenge, comparative genomicists have developed new, innovative methods combining phylogenetic analyses of common (though not ubiquitous) *Cas* genes (notably *cas1*) with analyses of shared architecture and gene content, e.g. using network-based tools of shared gene content(63, 64). These approaches have produced increasingly robust classification schemes to help understand the diversity of CRISPR-Cas systems across the bacterial and archaeal phylogeny, and have also shed

light on potential pathways to CRISPR-Cas evolution, based on the co-option of multiple mobile genetic element genes(63).

2. Experimental phenotypes and fitness effects:

2A) Use of multiple fitness metrics. The fitness benefits and costs provided by CRISPR-Cas have been evaluated via a broad range of direct and indirect measures. Direct measures of fitness effects of CRISPR-Cas include the utilisation of time-shift and cross-infection assays to experimentally verify trends in phage resistance as exemplified by Laanto *et al*.(65). Indirect 'proxy' measures of fitness include measurements of spacer diversity and acquisition of novel spacers, as spacer carriage implies a potential future benefit of immunity to a matching phage or MGE. The implied fitness benefits of spacer carriage can be further parsed by matching spacers to locally prevalent phage genomes. Sequenced spacers tend to match sympatric rather than allopatric phages, indicating evolutionary matching of defenses to locally prevalent MGEs(66, 67).

2B) Use of relevant environments. The fitness consequences of CRISPR-Cas carriage have been experimentally evaluated under a broad range of potentially relevant conditions, including variation in force of infection(58), nutrient availability(56, 61), and interspecific competition(57). Alseth *et al*., for example, highlighted the role of interspecific competition in increasing the costs of resistance via surface receptor modification, and therefore promoting CRISPR-based phage immunity in a community context(57). Fitness costs of CRISPR carriage have also been established through studies on the risk of autoimmunity(68), restriction of horizontal gene transfer (HGT)(69), and other genetic conflicts(70).

2C) Use of native genomic backgrounds. While CRISPR-Cas genetic engineering tools are deployed in a variety of genetic backgrounds, basic research into the intrinsic defensive functions of CRISPR-Cas is typically carried out in native genomic backgrounds. For example, some of the earliest work on CRISPR was done using *Streptococcus thermophilus* and its native CRISPR-Cas immune system(71); a model organism that is still used today(60). Other bacteria commonly used for studying CRISPR-Cas in its native genomic background are *Pseudomonas aeruginosa*(56, 57), *Pectobacterium atrosepticum*(72), *Sulfolobus solfataricus*(73), and more(74).

3. Functional hypotheses and evolutionary theory:

3A) Adaptive design hypothesis CRISPR-Cas has a clear adaptive rationale, featuring multiple components working in tandem to perform complex tasks associated with learning, memory, recognition, and killing of parasite threats(75). Figure 4 maps these coordinated functional tasks onto molecular components which have been intensively characterized both alone and in combination(75). These functional tasks are centred around two largely ubiquitous (they are in most, but not all CRISPR systems) proteins: Cas1 and Cas2, which are involved in spacer acquisition(76). Beyond these two proteins, there is extensive variation in Cas genes between CRISPR-Cas sub-types (15). Additionally, it is worth noting how the target spacer that is integrated is not random, as most CRISPR systems have a short sequence (the protospacer adjacent motif (PAM)) located next to the protospacer and that plays an important role in spacer acquisition and interference(76). These highly conserved features are shared across most CRISPR-Cas systems, with some variation based on type (i.e. type I vs type II systems, and the sub-types therein)(63).

3B) Alternative hypotheses. Alternate functional hypotheses for CRISPR-Cas have also been investigated, and CRISPR systems do show remarkable functional versatility in addition to their role in adaptive defense(63, 64). For example, alternate functions involving regulatory control of gene expression have been proposed for a sub-set of CRISPR-Cas systems(77), and some may have lost their immune function completely(77–79).

3C) Formal mathematical theory. The detailed knowledge of CRISPR-Cas components and their coordination (Fig. 4) provides a valuable springboard for the application of mathematical models in an attempt to capture the logical structure of a biological process, and then derive predictions on system (CRISPR-Cas) behaviour in different contexts(80–83). For example, Gurney *et al*. used population dynamical modelling to evaluate conditions that could favour the evolution of CRISPR-based phage immunity, when simpler surface-based 'defense' mechanisms are available (e.g. loss of or change to surface factors that also function as phage receptors)(84). They found that CRISPR-Cas may be selected for over surface modification when phages have limited abilities to undergo continued co-evolutionary escape from CRISPR-based immunity, and/or when surface factor modification is sufficiently costly(84).

4. Integration across approaches:

While the CRISPR field continues to be dominated by mechanistic molecular characterisation of specific CRISPR-Cas systems(63), a growing thread of evolution-informed research links design concepts/theory with experimental and bioinformatic data(56, 60, 85). Collectively this work

generally buttresses the adaptive defense hypothesis, via mutually reinforcing theoretical, experimental and bioinformatic lines of evidence.

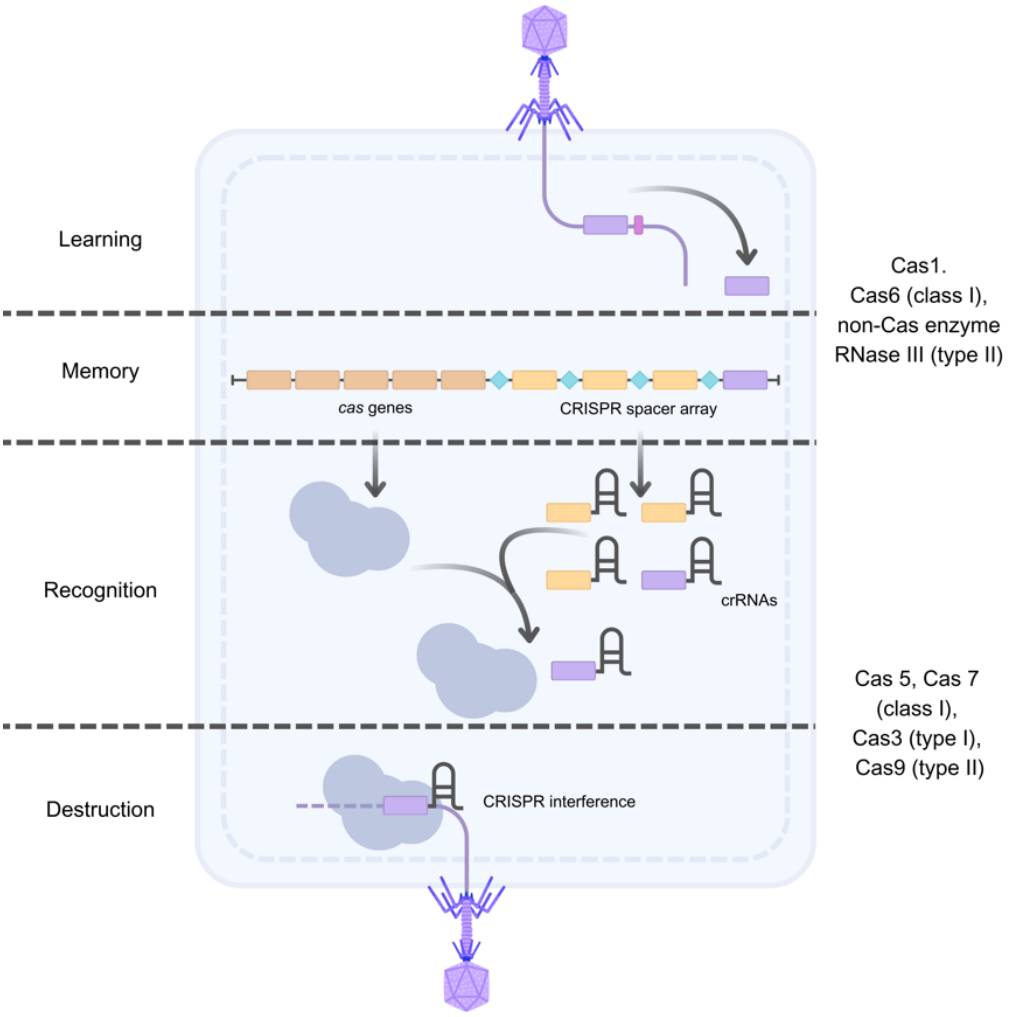

Fig. 4. The CRISPR-Cas adaptive design hypothesis showcases the complex coordination of multiple component parts. CRISPR-Cas has a clear and established design rationale, featuring complex and coordinated components for the learning, memory, recognition, and destruction of invading phages. In short, CRISPR-Cas is activated upon infection, after which a small sequence of parasite DNA can be incorporated into the bacterial genome as a so-called 'spacer'(71, 75). Once a spacer has been acquired and incorporated in the CRISPR array, subsequent infection by

the same or other genetic parasites with a sequence that matches a spacer will trigger the immune response(71, 75). The gene lists on the right provide a brief sample of genes associated with each functional sub-task.

[end Box 1]

[Box 2]

Surface modification – defense adaptation or structural trade-off?

To highlight the importance of our functional hypothesis and evolutionary criterion 3, we review a more challenging and borderline case, namely the widespread occurrence of reduced phage susceptibility mediated by the modification or masking of a cell envelope feature ('phage receptor')(86). Mutations leading to modifications or loss of phage-binding surface factors are routinely reported to confer fitness advantages when challenged with phage (supporting experimental criterion 2) and are consistently reported for genetically unrelated receptors across phylogenetically distinct bacterial systems(87, 88) (supporting criterion 1).

Given this volume of supportive data, why the challenge? First, consider criterion 3A: what exactly is the hypothesised adaptive mechanism of defense? A strong adaptive hypothesis should state, in concrete mechanistic terms, what the trait of interest does and how this benefits the carrier. The surface modification mechanism is very simple – the reduction of a pre-existing vulnerability, like removing or boarding a doorway in the face of an external threat. Note that in contrast to CRISPR-Cas (Box 1), surface modification provides no active processes of defense, and

374 while simple traits can be adaptations, the complex coordination of multiple component parts
375 (the vertebrate eye; CRISPR-Cas, Fig. 4) can offer supporting evidence for the organising force of
376 natural selection(89, 90).
377
378 Next, we turn to criterion 3B: what are the alternative hypotheses? In the context of surface
379 modification, it is broadly accepted that the varied surface factors in question (pili(91),
380 flagella(92), LPS(87), capsular polysaccharides(93), and more(87)) have important and
381 environment-specific adaptive roles (e.g. nutrient uptake(94), motility(92) or antibiotic
382 resistance(95)), and a secondary cost of generating vulnerability to phage infection(96). In
383 environments where phage pressure is high and specific nutrient uptake is non-essential
384 (common in laboratory experiments using rich media), the trade-off between the costs and
385 benefits of receptor expression can readily tip towards loss(56, 57). Therefore, and despite
386 satisfying Criteria 1 and 2, surface modification can also be understood as the result of a
387 cost/benefit trade-off governing receptor expression.
388
389 We note here that surface modification is not solely a mutational process. The expression level
390 of surface factors is often under regulatory control, and the case has been made that these
391 regulatory systems serve to mitigate risks of infection, by limiting phage receptor expression in
392 phage-prone environments(97). Intriguingly, one example of surface factor regulatory control is
393 itself encoded by a prophage, and has been implicated in limiting super-infection(98). This
394 example illustrates our discussion of 'agency' or 'who protects who from whom' in the main text
395 (criterion 3A). In light of the multi-functional roles and regulation of surface factors, we view

surface modification as a trait where the relative importance of defense versus other functions likely varies across environments and lineages.

Beyond the specifics of surface modification, we stress the importance of developing and assessing multiple alternate adaptive and non-adaptive hypotheses(99), keeping in mind the potential for 'spandrels'(40); phenotypes that are byproducts of other adaptive traits. For any given trait – what are the alternative functions that may be the focus of selection, and what non-adaptive processes could give rise to the trait in question? We note that evidence based primarily on an inability of parasites to bind to or reproduce within a cell may simply be a consequence of damaged cellular machinery, rather than an active and selectively tailored process of adaptive defense. We further note that alternate functions can still play a critical role in the derivation of novel adaptive roles, via the process of exaptation(100).

[end Box 2]

Challenges raised by bioinformatic tools and comparative methods (criterion 1).

Bioinformatic tools are a major driver behind the discovery of novel defense mechanisms(101, 102). Much of the progress is based on tools that allow large-scale database mining, focusing on varied principles of proximity to known defensive genes (e.g. sequence similarity, gene co-occurrence(102), or physical distance/co-localization/'guilt by association'(101)). In addition to discovery tasks, bioinformatic tools also provide novel means to conduct hypothesis tests on biological data. When addressing hypotheses across multiple lineages, this raises core challenges within the field of phylogenetic comparative methods:

Identifying similar traits across lineages (1A). In order to conduct a comparative analysis, we first need to identify functionally similar traits that are shared across multiple lineages. Today, this is increasingly led by bioinformatic tools (e.g. 'reciprocal best hit' via BLAST, and hidden Markov models (HMM)) to identify orthologous genes). What is more challenging is to identify traits that serve the same function but are not the product of homologous gene evolution (analogous traits, see vertebrate/cephalopod eye discussion above), as this requires information on similarity of function.

To identify analogous features, we must pay attention to functional characterization. Clear examples of functional convergence here are RM(103, 104) and toxin-antitoxin (TA) systems. Focusing on the latter, TA systems converge on a general toxin versus anti-toxin functional design(105, 106), while the molecular and genetic details of the toxin and anti-toxin can vary widely (from proteins to small RNAs(107)). The identification of new functional analogies across other defense mechanisms is an important challenge for the field. To help address this challenge, a number of new tools are emerging that look beyond sequence identity. Notably, AlphaFold 3(108) offers a path towards the identification of functional convergence in diverse proteins, via the identification of structural similarities among non-homologous proteins(109). This detection of structural similarities is particularly helpful, as defense mechanisms can have low sequence identities due to being under particularly strong selection from MGE infection.

Appropriate statistical tools to deal with issues of non-independence (1B). Whenever dealing with data that is distributed across lineages (species, strains, etc.), it is important to address the potential confounding role of variable shared inheritance (where possible, as the mobile nature of many defenses(110, 111) does need to be considered). Critically, lineage-specific data are not statistically independent measurements due to variable patterns of co-ancestry. In response to this challenge, a rich body of phylogenetically grounded tools for comparative analysis have been developed over the years(112–114), and a number of primers and tools exist to introduce relevant methods (e.g. phytools(115)).

Challenges raised by experimental phenotypes and fitness effects (criterion 2).

The recent discovery of RADAR(23) provides an impressive example of the power of coupling bioinformatic and experimental screening tools. An initial bioinformatic screen of ~620 million bacterial protein-coding genes used 'guilt by association' methods to deliver 48 priority candidates for experimental investigation. Heterologous expression in *E. coli* revealed that 29 of the 48 genes provided an anti-phage phenotype, including the structurally novel RADAR system(116). High-throughput experimental screening tools can also be applied for the initial discovery of novel defense mechanisms, such as Tn-seq, which was used in the discovery of MADS(117). Here, we focus on the enduring importance of experimental methods for further testing of adaptive hypotheses for defined defense mechanisms, flagging important challenges to address:

Use of multiple fitness metrics (2A). Once a defense mechanism has been identified, an important line of experimental evidence for a defensive adaptation is a positive correlation between expression of the phenotype, and fitness. For individual defense mechanisms, current measures of fitness effects largely focus on quantifying phage or bacterial density, in the presence versus absence of the defense mechanism and phage/MGE. The observation of a measured reduction in phage density is indicative of defense, yet can also be the result of a non-specific deficit in cellular functioning, that in turn limits phage reproduction. How to distinguish the two remains a challenge, which is why taking multiple metrics into account enhances our ability to discriminate. This is especially true in the case of group-beneficial defenses (e.g. abortive infection), where a common diagnostic is a reduction in bacterial density given phage attack(53). While reduction in bacterial density could indicate cellular death to protect kin, it could also be a simple result of phage-induced cell death and phage proliferation.

Overall, we highlight the importance of measuring both phage/MGE and bacterial dynamics as metrics of fitness. A robust defense adaptation will likely have minimal or modest costs on growth in the absence of molecular predators, and clear benefits in their presence (i.e. reduced phage amplification, increased bacterial survival/ growth). In contrast, a non-specific cellular deficit will often show strong dependency on the growth environment(56), flagging the importance of assessing bacteria and phage/MGE dynamics across relevant environmental conditions (2B).

Beyond these general demographic measures of fitness, we underline the importance of leveraging alternate adaptive and non-adaptive hypotheses (criteria 3A, B) to deliver additional

experimental predictions on phenotypic 'fitness proxies'. For example, CRISPR spacer acquisition is a potential 'fitness proxy', implying the ability to resist a defined phage/MGE, and therefore gain demographic benefits. The use of this kind of proxy methodology has shed light on how CRISPR-Cas enables bacteria to track and match locally prevalent phage genomes over evolutionary time(65, 67).

The menu of demographic and phenotypic fitness measures provides opportunities for greater resolution among alternate adaptive and non-adaptive hypotheses. The same experimental approaches can also provide invaluable mechanistic insights, flagging the continued dialogue between mechanistic and evolutionary questions. For example, the characterization of escape phages (i.e. phages that have evolved to overcome the defense in question) can provide us with critical information on counter-defense mechanisms such as anti-CRISPRs(118, 119), which in turn drive evolutionary questions on the nature and drivers of co-evolutionary dynamics.

Use of relevant environments (2B). Given that fitness costs and benefits can be environment dependent, the choice of environmental conditions is of critical concern when separating adaptive and non-adaptive hypotheses. In practice, most experimental assays on defense mechanisms are conducted in convenient lab growth media, that tend to be nutrient rich. This choice can modulate the importance of different defenses, both through direct regulatory impacts and indirect selective mechanisms, with one example being the common enrichment of surface modification mutants in rich media(56). We note that choice of environment extends beyond the biochemical components of growth media, and includes dimensions such as solid

versus liquid culturing conditions(120, 121), multiplicity of infection, MGE/phage diversity, and intra- and/or interspecific competition(57, 60). While assessing bacterial and phage dynamics across diverse environments is a powerful approach, it is difficult to scale across many defense mechanisms. When deciding which environments to test a defense mechanism in, we recommend that such a choice should be motivated by the specific set of alternate hypotheses under consideration (criterion 3B). Additionally, when the choice of a single test environment is necessary, we flag that new techniques allow the design of defined lab growth media that directly mimic the biochemical properties of the growth conditions of interest, including human infection environments(122).

Use of native genomic backgrounds (2C). As far as we can tell, only CRISPR-Cas (Box 1), RM(9), Pgl(35, 123), BREX(124), Long-A pAgos(125, 126), SiAgo-like pAgos(127), CBASS(128), AbiA-AbiZ systems(129), RexAB(130), and DarTG(131) of the defenses mentioned here (Appendix) have been tested in their native genetic background, as most defense mechanisms are tested through cloning the mechanism in question into model strains of *E. coli* or *B. subtilis* (often on a plasmid or under the control of a foreign promoter) before assessing effects of defense on phage density(23, 132, 133). Consequently, few defenses have yet been evaluated in native genomic contexts, providing many avenues for further research.

We stress however that screening in *E. coli* and similar laboratory model species is a powerful starting point for hypothesis testing. Largely unique to microbiology, this ability to isolate the effect of a candidate gene on a controlled phage-susceptible background can overcome the

challenge of multiple redundant defense systems in wild-type strains(124). Yet while tractable and controlled backgrounds are an important tool, they also present taxonomic and mechanistic biases, likely due to host compatibility constraints(23). Additionally, assays often rely on convenient sets of phages (e.g. *E. coli* T-even coliphages) that give clear, reproducible readouts. These may not represent the phage/MGE threats present in the native ecology of the defense mechanism, so follow-up tests against ecologically relevant phage panels can be important.

Two recent studies(134, 135) underscore the importance of considering the native host background: By knocking out eight defense mechanisms encoded by their native host, David *et al*. found that only the loss of two (RM and PD-T4-3(136)) had an effect on phage infectivity, while the other defenses had no detectable effect(134). Meanwhile, Paoli *et al*. used RNA-seq data to quantify defense-system expression across abiotic and biotic conditions, and found that genetic context can affect defense expression(135). Considering this concern, we underline validation in a native host background as an important step. When testing in non-native backgrounds, initial screens should also consider downstream effects of cloning on bacterial growth rate, as any genetic changes that affect growth might also influence bacteria-phage dynamics(137–139) in ways that are simple artefacts of the experimental genomic manipulation.

Finally, we note that many defense mechanisms can move and function across diverse bacterial hosts via horizontal gene transfer (HGT)(110, 111). This mobility may favor 'portable' mechanisms whose phenotypic effects are robust across multiple genomic backgrounds. At the same time, HGT does not imply background-independence: even mobile genes experience host-

specific epistasis and regulatory context, and the degree to which adaptive improvements translate across hosts can depend on how strongly fitness effects align across genomic backgrounds. Recent work explicitly frames this problem in terms of cross-host fitness-landscape alignment for horizontally transferred genes, providing a useful conceptual link between portability and background dependence(140).

Challenges in the development of adaptive hypotheses (criterion 3)

Criterion 3 addresses the logic of inference required to support an evolutionary (selected-effect) claim. Because the relevant alternative hypotheses and discriminating tests depend on ecological context and on the system under study, the goal is not a single universal assay but a transparent procedure for making assumptions explicit and sharpening testable predictions.

Adaptive design hypotheses (3A). Reviewing the current discussions of adaptive 'defense' hypotheses flags three major and inter-connected challenges, namely group selection, benefits of defense, and agency. Across these challenges, we note that there is a large opportunity to leverage existing theory (largely from the field of sociobiology, e.g.(141)) to drive novel testable hypotheses (3C).

Group and kin selection. An increasing number of bacterial defense mechanisms are proposed to function on the group level (Fig. 3B), where an individual cell sacrifices itself to halt the development of viable phage/MGE progeny, thus protecting neighbouring cells from infection. From an evolutionary perspective, this is an example of an 'altruistic' behaviour, where an

individual pays a substantial cost (death or dormancy) to confer benefits to others (escape from infection). The conditions favoring the evolution of altruism (a strong form of cooperation(142)) are narrow compared to traits that provide direct benefits to individuals, and require high levels of population structuring (high relatedness or assortment) so that benefits of the altruistic behaviour are more likely to go to carriers of the altruistic gene(143). This is far from an impossible barrier: microbial life histories can often generate high relatedness via a combination of single-cell dispersal followed by local clonal expansion(144–146). We further note that analogous mechanisms of group-protective defenses against parasitism have been reported in social insects(147). So, in order to develop a complete adaptive hypothesis for group-level defenses (Appendix), the role of population structure and social evolution must be taken into account.

Benefits of defense. For an individually beneficial trait, the trait will be favored when benefits b exceed costs c ($b > c$). For a group-level trait, the cost/benefit threshold is further restricted by Hamilton's rule: $rb > c$, where r is relatedness between actor and recipients(143). When applying either inequality to defense mechanisms, a core assumption is that benefits exist, i.e. $b > 0$, and while defense against a lethal parasite such as a lytic phage has evident adaptive benefit, defense against MGEs that do not kill the cell is less clear-cut. These MGEs can potentially confer beneficial traits such as antibiotic resistance, blurring the ecological line between costly parasite and beneficial mutualist. For instance, the presence of CRISPR-Cas is negatively associated with antibiotic resistance due to CRISPR-Cas limiting the acquisition of potentially beneficial MGEs(69, 70, 148, 149). This negative association implies that defenses can become costly liabilities,

dependent on changes to environmental and MGE contexts, but for most defense mechanisms this remains to be formally assessed.

Agency. The simplest adaptive interpretation of the cell-level or group-level defenses in Fig. 3 assumes that the cell (or group) is protecting itself from external parasite attack. Yet, genomic inspection has increasingly revealed that defense mechanisms are commonly found on MGEs themselves(150–152). This positioning raises the question of evolutionary agency, or 'who defends whom from what'(152, 153). When considering defense against a lytic phage for instance, there is a common interest in cellular defense, shared by both chromosomal and MGE genes. In other scenarios this common interest can break down, however, for example when foreign MGEs potentially carry beneficial genes, or where group-level defense (Fig. 3B) entails cell death, such as for abortive infection(152). The widespread observation of abortive infection and other group-beneficial mechanisms on MGEs connects to the kin-selection topic introduced above, as the very process of local HGT can increase the relatedness parameter *r* for genes on MGEs, compared to chromosomal loci(154). Increased relatedness at mobile loci can then increase the likelihood that the condition $rb > c$ is met, supporting MGE investment in costly, group-level defenses.

Alternative hypotheses (3B). A common problem is that alternative explanations are not considered when evaluating a defense mechanism, as discussed in Box 2 in the context of surface modifications. As a further example, while recent data illustrates that the production of aminoglycoside antibiotics can produce clear benefits by blocking phage replication(155), we

note that this anti-phage role is potentially a secondary effect of antibiotic production in response to interspecific bacterial competition. A related class of examples comes from phase variation and antigenic variation. Reversible switching of surface structures can strongly alter phage adsorption and infectivity, producing a clear anti-phage phenotype. However, these switching mechanisms are often discussed as evolving under multiple selective pressures (e.g. immune evasion, colonization, or niche switching), with altered phage susceptibility arising as a collateral consequence. In such cases, 'defense' may describe a real phenotype, but the primary evolved adaptation may lie elsewhere, illustrating the importance of explicitly evaluating alternative adaptive roles and potential 'spandrel'/byproduct explanations(40).

Additionally, systems often labelled as 'defense' may be maintained because they confer benefits to MGEs in conflicts with other MGEs, and not always in defensive roles. For example, when carried on plasmids, CRISPR–Cas can promote plasmid establishment by targeting resident competing plasmids following entry into a new host cell—an 'attack' role from the perspective of the invading element—while toxin–antitoxin systems can likewise enhance invasion success(156). These cases highlight that a clear anti-phage/anti-MGE phenotype need not imply selection for host-level defense: with the same molecular machinery taking on 'defensive' versus 'offensive' roles depending on context.

For any given trait, we recommend considering both alternative adaptive hypotheses (i.e. alternate evolved functions) and non-adaptive hypotheses, including the potential for experimental or bioinformatic artefacts. Toxin-antitoxin (TA) systems provide an example where

multiple alternate adaptive hypotheses are already present in the literature, spanning defense, persister phenotypes, MGE competition, and addiction(7, 107, 157).

Formal mathematical theory (3C). While we underline that a conceptual model and associated testable hypotheses are a necessary step in our proposed iterative process (Fig. 2), formal (math-based) theory is not required. That said, mathematical biology tools (e.g. optimization, game theory, population genetic modelling, adaptive dynamics) can help test the logic of adaptive claims—in some cases even rejecting hypotheses on purely logical grounds(158). In addition, mathematical models can provide quantitative predictions on measurable aspects of adaptive design that flow from the logical assumptions of the model, and which can be tested using experimental and bioinformatics approaches discussed above. Overall, there is a general lack of formal theoretical modelling for most defense mechanisms, presenting an opportunity to the field. Outside of CRISPR-Cas studies (Box 1), we are only aware of formal theory on restriction-modification systems(84) and abortive infection(159).

Conclusion

Evolutionary biologists have grappled with the concept of evolutionary adaptation for over half a century. By leveraging this history of debate, we summarize an inter-related set of criteria that can provide both a diagnostic overview (Box 1, 2) and a multi-pronged research agenda (Fig. 2) for studies of microbial adaptation. While applicable to any debate over microbial adaptation, we apply these criteria to the rapidly growing 'bacterial immune system' field, which has seen an unprecedented expansion in recent years (Fig. 1). We hope these criteria may inspire

experimental design, highlight research gaps, and spark discussion on defining anti-phage/anti-MGE defense adaptation. Our approach emphasizes benchmark cases of integrated theoretical, experimental, and bioinformatic evidence, alongside cases where evidence is limited or indeed contradictory. In some cases, we suggest that while passive defensive functions exist, they are potentially either secondary effects of other primary adaptive functions (e.g. aminoglycoside production), or the result of trade-offs modifying a distinct adaptation (e.g. surface modification). Overall, we emphasize that the majority of proposed mechanisms are so new it is only natural that they do not meet all the criteria we have put forth. In this fast-moving context, our perspective flags future research needs to truly establish and define the 'bacterial immune system' and which defenses solidly qualify to be part of it.

Acknowledgements

The authors would like to thank Iris Irby, Alice Maestri, Meaghan Castledine, Mike Brockhurst, Paul Hoskisson, John Bruce, and Daan Swarts for careful reading and feedback on the manuscript.

References

1. Williams GC. 1966. Adaptation and natural selection; a critique of some current evolutionary thought. Princeton, N.J., Princeton University Press. Retrieved 1 June 2023.

2. Buckling A, Rainey PB. 2002. The role of parasites in sympatric and allopatric host diversification. 6915. Nature 420:496–499.

3. Forterre P, Prangishvili D. 2009. The Great Billion-year War between Ribosome- and Capsid-encoding Organisms (Cells and Viruses) as the Major Source of Evolutionary Novelties. Ann N Y Acad Sci 1178:65–77.

4. Morran LT, Schmidt OG, Gelarden IA, Parrish RC, Lively CM. 2011. Running with the Red Queen: Host-Parasite Coevolution Selects for Biparental Sex. Science 333:216–218.

5. Suttle CA. 2007. Marine viruses — major players in the global ecosystem. 10. Nat Rev Microbiol 5:801–812.

6. Buckling A, Rainey PB. 2002. Antagonistic coevolution between a bacterium and a bacteriophage. Proc Biol Sci 269:931–936.

7. Page R, Peti W. 2016. Toxin-antitoxin systems in bacterial growth arrest and persistence. Nat Chem Biol 12:208–214.

8. San Millan A, MacLean RC. 2017. Fitness Costs of Plasmids: a Limit to Plasmid Transmission. Microbiol Spectr 5:10.1128/microbiolspec.mtbp-0016–2017.

9. Luria SE, Human ML. 1952. A nonhereditary, host-induced variation of bacterial viruses. J Bacteriol 64:557–569.

10. Gross SR. 1954. Abortive infection of a strain of Escherichia coli by coliphage T21. J Bacteriol 68:36–42.

11. Bolotin A, Quinquis B, Sorokin A, Ehrlich SD. 2005. Clustered regularly interspaced short palindrome repeats (CRISPRs) have spacers of extrachromosomal origin. Microbiology 151:2551–2561.

12. Mojica FJM, Díez-Villaseñor C, García-Martínez J, Soria E. 2005. Intervening Sequences of Regularly Spaced Prokaryotic Repeats Derive from Foreign Genetic Elements. J Mol Evol 60:174–182.

13. Pourcel C, Salvignol G, Vergnaud G. 2005. CRISPR elements in Yersinia pestis acquire new repeats by preferential uptake of bacteriophage DNA, and provide additional tools for evolutionary studies. Microbiology 151:653–663.

14. Abby SS, Néron B, Ménager H, Touchon M, Rocha EPC. 2014. MacSyFinder: A Program to Mine Genomes for Molecular Systems with an Application to CRISPR-Cas Systems. PLOS ONE 9:e110726.

15. Blower TR, Short FL, Rao F, Mizuguchi K, Pei XY, Fineran PC, Luisi BF, Salmond GPC. 2012. Identification and classification of bacterial Type III toxin-antitoxin systems encoded in chromosomal and plasmid genomes. Nucleic Acids Res 40:6158–6173.

16. Makarova KS, Wolf YI, Snir S, Koonin EV. 2011. Defense islands in bacterial and archaeal genomes and prediction of novel defense systems. J Bacteriol 193:6039–6056.

17. Makarova KS, Wolf YI, Koonin EV. 2013. Comparative genomics of defense systems in archaea and bacteria. Nucleic Acids Res 41:4360–4377.

714 18. Payne LJ, Meaden S, Mestre MR, Palmer C, Toro N, Fineran PC, Jackson SA. 2022. PADLOC: a
715 web server for the identification of antiviral defence systems in microbial genomes.
716 Nucleic Acids Res 50:W541–W550.

717 19. Tesson F, Hervé A, Mordret E, Touchon M, d'Humières C, Cury J, Bernheim A. 2022.
718 Systematic and quantitative view of the antiviral arsenal of prokaryotes. 1. Nat Commun
719 13:2561.

720 20. Zheng Y, Posfai J, Morgan RD, Vincze T, Roberts RJ. 2009. Using shotgun sequence data to
721 find active restriction enzyme genes. Nucleic Acids Res 37:e1.

722 21. Cheng R, Huang F, Wu H, Lu X, Yan Y, Yu B, Wang X, Zhu B. 2021. A nucleotide-sensing
723 endonuclease from the Gabija bacterial defense system. Nucleic Acids Res 49:5216–5229.

724 22. Cohen D, Melamed S, Millman A, Shulman G, Oppenheimer-Shaanan Y, Kacen A, Doron S,
725 Amitai G, Sorek R. 2019. Cyclic GMP-AMP signalling protects bacteria against viral
726 infection. Nature 574:691–695.

727 23. Gao L, Altae-Tran H, Böhning F, Makarova KS, Segel M, Schmid-Burgk JL, Koob J, Wolf YI,
728 Koonin EV, Zhang F. 2020. Diverse enzymatic activities mediate antiviral immunity in
729 prokaryotes. Science 369:1077–1084.

730 24. Goldfarb T, Sberro H, Weinstock E, Cohen O, Doron S, Charpak-Amikam Y, Afik S, Ofir G,
731 Sorek R. 2015. BREX is a novel phage resistance system widespread in microbial
732 genomes. EMBO J 34:169–183.

25. Xu T, Yao F, Zhou X, Deng Z, You D. 2010. A novel host-specific restriction system associated with DNA backbone S-modification in Salmonella. Nucleic Acids Res 38:7133–7141.

26. Zhang T, Tamman H, Coppieters ’t Wallant K, Kurata T, LeRoux M, Srikant S, Brodiazhenko T, Cepauskas A, Talavera A, Martens C, Atkinson GC, Hauryliuk V, Garcia-Pino A, Laub MT. 2022. Direct activation of a bacterial innate immune system by a viral capsid protein. Nature 1–9.

27. Hampton HG, Watson BNJ, Fineran PC. 2020. The arms race between bacteria and their phage foes. 7790. Nature 577:327–336.

28. Rostøl JT, Marraffini L. 2019. (Ph)ighting Phages: How Bacteria Resist Their Parasites. Cell Host Microbe 25:184–194.

29. Bernheim A, Sorek R. 2020. The pan-immune system of bacteria: antiviral defence as a community resource. 2. Nat Rev Microbiol 18:113–119.

30. Mayo-Muñoz D, Pinilla-Redondo R, Birkholz N, Fineran PC. 2023. A host of armor: Prokaryotic immune strategies against mobile genetic elements. Cell Rep 42.

31. Georjon H, Bernheim A. 2023. The highly diverse antiphage defence systems of bacteria. Nat Rev Microbiol 1–15.

32. Ledvina HE, Whiteley AT. 2024. Conservation and similarity of bacterial and eukaryotic innate immunity. Nat Rev Microbiol 1–15.

33. Barthes K, Rousset F, Wein T. 2025. Viral effectors trigger innate immunity across the tree of life. Philos Trans R Soc B Biol Sci 380:20240077.

34. Sperr E. 2016. PubMed by Year. https://esperr.github.io/pubmed-by-year/. Retrieved 25 March 2024.

35. Chinenova TA, Mkrtumian NM, Lomovskaia ND. 1982. Genetic characteristics of a new phage resistance trait in Streptomyces coelicolor A3(2). Genetika 18:1945–1952.

36. Ogura T, Hiraga S. 1983. Mini-F plasmid genes that couple host cell division to plasmid proliferation. Proc Natl Acad Sci 80:4784–4788.

37. Makarova KS, Wolf YI, van der Oost J, Koonin EV. 2009. Prokaryotic homologs of Argonaute proteins are predicted to function as key components of a novel system of defense against mobile genetic elements. Biol Direct 4:29.

38. Darwin C. 1859. On the Origin of Species by Means of Natural Selection, or the Preservation of Favoured Races in the Struggle for Life. John Murray, London.

39. Lamb TD. 2011. Evolution of the Eye. Sci Am. https://www.scientificamerican.com/article/evolution-of-the-eye/. Retrieved 12 March 2024.

40. Gould SJ, Lewontin RC. 1979. The spandrels of San Marco and the Panglossian paradigm: a critique of the adaptationist programme. Proc R Soc Lond B Biol Sci 205:581–598.

769 41. Queller DC. 1995. The Spaniels of St. Marx and the Panglossian Paradox: A Critique of a
770 Rhetorical Programme. Q Rev Biol 70:485–489.

771 42. 2004. The ENCODE (ENCyclopedia Of DNA Elements) Project. Science 306:636–640.

772 43. Dunham I, Kundaje A, Aldred SF, Collins PJ, Davis CA, Doyle F, Epstein CB, Frietze S, Harrow
773 J, Kaul R, Khatun J, Lajoie BR, Landt SG, Lee B-K, Pauli F, Rosenbloom KR, Sabo P, Safi A,
774 Sanyal A, Shoresh N, Simon JM, Song L, Trinklein ND, Altshuler RC, Birney E, Brown JB,
775 Cheng C, Djebali S, Dong X, Dunham I, Ernst J, Furey TS, Gerstein M, Giardine B, Greven
776 M, Hardison RC, Harris RS, Herrero J, Hoffman MM, Iyer S, Kellis M, Khatun J, Kheradpour
777 P, Kundaje A, Lassmann T, Li Q, Lin X, Marinov GK, Merkel A, Mortazavi A, Parker SCJ,
778 Reddy TE, Rozowsky J, Schlesinger F, Thurman RE, Wang J, Ward LD, Whitfield TW, Wilder
779 SP, Wu W, Xi HS, Yip KY, Zhuang J, Bernstein BE, Birney E, Dunham I, Green ED, Gunter C,
780 Snyder M, Pazin MJ, Lowdon RF, Dillon LAL, Adams LB, Kelly CJ, Zhang J, Wexler JR, Green
781 ED, Good PJ, Feingold EA, Bernstein BE, Birney E, Crawford GE, Dekker J, Elnitski L,
782 Farnham PJ, Gerstein M, Giddings MC, Gingeras TR, Green ED, Guigó R, Hardison RC,
783 Hubbard TJ, Kellis M, Kent WJ, Lieb JD, Margulies EH, Myers RM, Snyder M,
784 Stamatoyannopoulos JA, Tenenbaum SA, Weng Z, White KP, Wold B, Khatun J, Yu Y,
785 Wrobel J, Risk BA, Gunawardena HP, Kuiper HC, Maier CW, Xie L, Chen X, Giddings MC,
786 Bernstein BE, Epstein CB, Shoresh N, Ernst J, Kheradpour P, Mikkelsen TS, Gillespie S,
787 Goren A, Ram O, Zhang X, Wang L, Issner R, Coyne MJ, Durham T, Ku M, Truong T, Ward
788 LD, Altshuler RC, Eaton ML, Kellis M, Djebali S, Davis CA, Merkel A, Dobin A, Lassmann T,
789 Mortazavi A, Tanzer A, Lagarde J, Lin W, Schlesinger F, Xue C, Marinov GK, Khatun J,

Williams BA, Zaleski C, Rozowsky J, Röder M, Kokocinski F, Abdelhamid RF, Alioto T, Antoshechkin I, Baer MT, Batut P, Bell I, Bell K, Chakrabortty S, Chen X, Chrast J, Curado J, Derrien T, Drenkow J, Dumais E, Dumais J, Duttagupta R, Fastuca M, Fejes-Toth K, Ferreira P, Foissac S, Fullwood MJ, Gao H, Gonzalez D, Gordon A, Gunawardena HP, Howald C, Jha S, Johnson R, Kapranov P, King B, Kingswood C, Li G, Luo OJ, Park E, Preall JB, Presaud K, Ribeca P, Risk BA, Robyr D, Ruan X, Sammeth M, Sandhu KS, Schaeffer L, See L-H, Shahab A, Skancke J, Suzuki AM, Takahashi H, Tilgner H, Trout D, Walters N, Wang H, Wrobel J, Yu Y, Hayashizaki Y, Harrow J, Gerstein M, Hubbard TJ, Reymond A, Antonarakis SE, Hannon GJ, Giddings MC, Ruan Y, Wold B, Carninci P, Guigó R, Gingeras TR, Rosenbloom KR, Sloan CA, Learned K, Malladi VS, Wong MC, Barber GP, Cline MS, Dreszer TR, Heitner SG, Karolchik D, Kent WJ, Kirkup VM, Meyer LR, Long JC, Maddren M, Raney BJ, Furey TS, Song L, Grasfeder LL, Giresi PG, Lee B-K, Battenhouse A, Sheffield NC, Simon JM, Showers KA, Safi A, London D, Bhinge AA, Shestak C, Schaner MR, Ki Kim S, Zhang ZZ, Mieczkowski PA, Mieczkowska JO, Liu Z, McDaniell RM, Ni Y, Rashid NU, Kim MJ, Adar S, Zhang Z, Wang T, Winter D, Keefe D, Birney E, Iyer VR, Lieb JD, Crawford GE, Li G, Sandhu KS, Zheng M, Wang P, Luo OJ, Shahab A, Fullwood MJ, Ruan X, Ruan Y, Myers RM, Pauli F, Williams BA, Gertz J, Marinov GK, Reddy TE, Vielmetter J, Partridge E, Trout D, Varley KE, Gasper C, The ENCODE Project Consortium, Overall coordination (data analysis coordination), Data production leads (data production), Lead analysts (data analysis), Writing group, NHGRI project management (scientific management), Principal investigators (steering committee), Boise State University and University of North Carolina at Chapel Hill Proteomics groups (data production and analysis), Broad Institute

812 Group (data production and analysis), Cold Spring Harbor U of G Center for Genomic
813 Regulation, Barcelona, RIKEN, Sanger Institute, University of Lausanne, Genome Institute
814 of Singapore group (data production and analysis), Data coordination center at UC Santa
815 Cruz (production data coordination), Duke University E University of Texas, Austin,
816 University of North Carolina-Chapel Hill group (data production and analysis), Genome
817 Institute of Singapore group (data production and analysis), HudsonAlpha Institute C UC
818 Irvine, Stanford group (data production and analysis). 2012. An integrated encyclopedia
819 of DNA elements in the human genome. 7414. Nature 489:57–74.

820 44. Graur D, Zheng Y, Price N, Azevedo RBR, Zufall RA, Elhaik E. 2013. On the Immortality of
821 Television Sets: "Function" in the Human Genome According to the Evolution-Free
822 Gospel of ENCODE. Genome Biol Evol 5:578–590.

823 45. Doolittle WF. 2013. Is junk DNA bunk? A critique of ENCODE. Proc Natl Acad Sci 110:5294–
824 5300.

825 46. Mayr E. 1961. Cause and Effect in Biology. Science 134:1501–1506.

826 47. Nesse RM. 2018. Tinbergen's four questions. Evol Med Public Health 2019:2.

827 48. Olson ME, Arroyo-Santos A. 2015. How to Study Adaptation (and Why To Do It That Way). Q
828 Rev Biol 90:167–191.

829 49. Losos JB. 2011. Convergence, Adaptation, and Constraint. Evolution 65:1827–1840.

50. Porter ML, Sumner-Rooney L. 2018. Evolution in the Dark: Unifying our Understanding of Eye Loss. Integr Comp Biol 58:367–371.

51. Williams GC. 1992. Gaia, Nature Worship and Biocentric Fallacies. Q Rev Biol 67:479–486.

52. Griffiths PE. 1996. The Historical Turn in the Study of Adaptation. Br J Philos Sci 47:511–532.

53. Aframian N, Eldar A. 2023. Abortive infection antiphage defense systems: separating mechanism and phenotype. Trends Microbiol 31:1003–1012.

54. Makarova KS, Timinskas A, Wolf YI, Gussow AB, Siksnys V, Venclovas Č, Koonin EV. 2020. Evolutionary and functional classification of the CARF domain superfamily, key sensors in prokaryotic antivirus defense. Nucleic Acids Res 48:8828–8847.

55. Payne LJ, Todeschini TC, Wu Y, Perry BJ, Ronson CW, Fineran PC, Nobrega FL, Jackson SA. 2021. Identification and classification of antiviral defence systems in bacteria and archaea with PADLOC reveals new system types. Nucleic Acids Res 49:10868–10878.

56. Westra ER, van Houte S, Oyesiku-Blakemore S, Makin B, Broniewski JM, Best A, Bondy-Denomy J, Davidson A, Boots M, Buckling A. 2015. Parasite Exposure Drives Selective Evolution of Constitutive versus Inducible Defense. Curr Biol 25:1043–1049.

57. Alseth EO, Pursey E, Luján AM, McLeod I, Rollie C, Westra ER. 2019. Bacterial biodiversity drives the evolution of CRISPR-based phage resistance. Nature 574:549–552.

847 58. Meaden S, Capria L, Alseth E, Gandon S, Biswas A, Lenzi L, van Houte S, Westra ER. 2020.
848 Phage gene expression and host responses lead to infection-dependent costs of CRISPR
849 immunity. ISME J 1–10.

850 59. Blazanin M, Turner PE. 2021. Community context matters for bacteria-phage ecology and
851 evolution. ISME J 1–10.

852 60. Guillemet M, Chabas H, Nicot A, Gatchich F, Ortega-Abboud E, Buus C, Hindhede L,
853 Rousseau GM, Bataillon T, Moineau S, Gandon S. 2022. Competition and coevolution
854 drive the evolution and the diversification of CRISPR immunity. Nat Ecol Evol 1–9.

855 61. Elliott JFK, Cozens K, Cai Y, Waugh G, Watson BN, Westra E, Taylor TB. 2025. Phage
856 susceptibility to a minimal, modular synthetic CRISPR-Cas system in Pseudomonas
857 aeruginosa is nutrient dependent. Philos Trans R Soc B Biol Sci 380:20240473.

858 62. Westra E, Levin B. 2020. It is unclear how important CRISPR-Cas systems are for protecting
859 natural populations of bacteria against infections by mobile genetic elements. Proc Natl
860 Acad Sci 117:27777–27785.

861 63. Makarova KS, Wolf YI, Iranzo J, Shmakov SA, Alkhnbashi OS, Brouns SJJ, Charpentier E,
862 Cheng D, Haft DH, Horvath P, Moineau S, Mojica FJM, Scott D, Shah SA, Siksnys V, Terns
863 MP, Venclovas Č, White MF, Yakunin AF, Yan W, Zhang F, Garrett RA, Backofen R, van der
864 Oost J, Barrangou R, Koonin EV. 2020. Evolutionary classification of CRISPR–Cas systems:
865 a burst of class 2 and derived variants. 2. Nat Rev Microbiol 18:67–83.

866 64. Koonin EV, Makarova KS. 2022. Evolutionary plasticity and functional versatility of CRISPR
867 systems. PLoS Biol 20:e3001481.

868 65. Laanto E, Hoikkala V, Ravantti J, Sundberg L-R. 2017. Long-term genomic coevolution of
869 host-parasite interaction in the natural environment. Nat Commun 8:111.

870 66. Westra ER, Dowling AJ, Broniewski JM, van Houte S. 2016. Evolution and Ecology of CRISPR.
871 Annu Rev Ecol Evol Syst 47:307–331.

872 67. Kunin V, He S, Warnecke F, Peterson SB, Garcia Martin H, Haynes M, Ivanova N, Blackall LL,
873 Breitbart M, Rohwer F, McMahon KD, Hugenholtz P. 2008. A bacterial metapopulation
874 adapts locally to phage predation despite global dispersal. Genome Res 18:293–297.

875 68. Stern A, Keren L, Wurtzel O, Amitai G, Sorek R. 2010. Self-targeting by CRISPR: gene
876 regulation or autoimmunity? Trends Genet 26:335–340.

877 69. Wheatley RM, MacLean RC. 2020. CRISPR-Cas systems restrict horizontal gene transfer in
878 Pseudomonas aeruginosa. ISME J 1–14.

879 70. Palmer KL, Gilmore MS. 2010. Multidrug-Resistant Enterococci Lack CRISPR-cas. mBio
880 1:e00227-10.

881 71. Barrangou R, Fremaux C, Deveau H, Richards M, Boyaval P, Moineau S, Romero DA, Horvath
882 P. 2007. CRISPR Provides Acquired Resistance Against Viruses in Prokaryotes. Science
883 315:1709–1712.

884 72. Richter C, Dy RL, McKenzie RE, Watson BNJ, Taylor C, Chang JT, McNeil MB, Staals RHJ,
885 Fineran PC. 2014. Priming in the Type I-F CRISPR-Cas system triggers strand-independent
886 spacer acquisition, bi-directionally from the primed protospacer. Nucleic Acids Res
887 42:8516–8526.

888 73. Erdmann S, Garrett RA. 2012. Selective and hyperactive uptake of foreign DNA by adaptive
889 immune systems of an archaeon via two distinct mechanisms. Mol Microbiol 85:1044–
890 1056.

891 74. Jackson SA, McKenzie RE, Fagerlund RD, Kieper SN, Fineran PC, Brouns SJJ. 2017. CRISPR-
892 Cas: Adapting to change. Science 356:eaal5056.

893 75. McGinn J, Marraffini LA. 2019. Molecular mechanisms of CRISPR–Cas spacer acquisition.
894 Nat Rev Microbiol 17:7–12.

895 76. Hille F, Charpentier E. 2016. CRISPR-Cas: biology, mechanisms and relevance. Philos Trans R
896 Soc B Biol Sci 371:20150496.

897 77. Westra ER, Buckling A, Fineran PC. 2014. CRISPR–Cas systems: beyond adaptive immunity.
898 Nat Rev Microbiol 12:317–326.

899 78. Pougach K, Semenova E, Bogdanova E, Datsenko KA, Djordjevic M, Wanner BL, Severinov K.
900 2010. Transcription, processing and function of CRISPR cassettes in Escherichia coli. Mol
901 Microbiol 77:1367–1379.

79. Touchon M, Charpentier S, Clermont O, Rocha EPC, Denamur E, Branger C. 2011. CRISPR Distribution within the Escherichia coli Species Is Not Suggestive of Immunity-Associated Diversifying Selection. J Bacteriol 193:2460–2467.

80. Childs LM, Held NL, Young MJ, Whitaker RJ, Weitz JS. 2012. MULTISCALE MODEL OF CRISPR-INDUCED COEVOLUTIONARY DYNAMICS: DIVERSIFICATION AT THE INTERFACE OF LAMARCK AND DARWIN. Evolution 66:2015–2029.

81. Childs LM, England WE, Young MJ, Weitz JS, Whitaker RJ. 2014. CRISPR-Induced Distributed Immunity in Microbial Populations. PLOS ONE 9:e101710.

82. Gandon S, Vale PF. 2014. The evolution of resistance against good and bad infections. J Evol Biol 27:303–312.

83. Weissman JL, Laljani RMR, Fagan WF, Johnson PLF. 2019. Visualization and prediction of CRISPR incidence in microbial trait-space to identify drivers of antiviral immune strategy. 10. ISME J 13:2589–2602.

84. Gurney J, Pleška M, Levin BR. 2019. Why put up with immunity when there is resistance: an excursion into the population and evolutionary dynamics of restriction–modification and CRISPR-Cas. Philos Trans R Soc B Biol Sci 374:20180096.

85. Weissman JL, Alseth EO, Meaden S, Westra ER, Fuhrman JA. 2021. Immune lag is a major cost of prokaryotic adaptive immunity during viral outbreaks. Proc R Soc B Biol Sci 288:20211555.

86. van Houte S, Buckling A, Westra ER. 2016. Evolutionary Ecology of Prokaryotic Immune Mechanisms. Microbiol Mol Biol Rev 80:745–763.

87. Bertozzi Silva J, Storms Z, Sauvageau D. 2016. Host receptors for bacteriophage adsorption. FEMS Microbiol Lett 363:fnw002.

88. Dowah ASA, Clokie MRJ. 2018. Review of the nature, diversity and structure of bacteriophage receptor binding proteins that target Gram-positive bacteria. Biophys Rev 10:535.

89. Ridley M. 1993. Evolution. Boston : Blackwell Scientific. http://archive.org/details/evolution00ridl. Retrieved 13 March 2024.

90. Poulin R. 1995. “Adaptive” changes in the behaviour of parasitized animals: A critical review. Int J Parasitol 25:1371–1383.

91. Shin H, Lee J-H, Kim H, Choi Y, Heu S, Ryu S. 2012. Receptor diversity and host interaction of bacteriophages infecting Salmonella enterica serovar Typhimurium. PloS One 7:e43392.

92. Guerrero-Ferreira RC, Viollier PH, Ely B, Poindexter JS, Georgieva M, Jensen GJ, Wright ER. 2011. Alternative mechanism for bacteriophage adsorption to the motile bacterium Caulobacter crescentus. Proc Natl Acad Sci 108:9963–9968.

93. Fehmel F, Feige U, Niemann H, Stirm S. 1975. Escherichia coli capsule bacteriophages. VII. Bacteriophage 29-host capsular polysaccharide interactions. J Virol 16:591–601.

94. Lenski RE, Levin BR. 1985. Constraints on the Coevolution of Bacteria and Virulent Phage: A Model, Some Experiments, and Predictions for Natural Communities. Am Nat 125:585–602.

95. Gordillo Altamirano F, Forsyth JH, Patwa R, Kostoulias X, Trim M, Subedi D, Archer SK, Morris FC, Oliveira C, Kielty L, Korneev D, O'Bryan MK, Lithgow TJ, Peleg AY, Barr JJ. 2021. Bacteriophage-resistant Acinetobacter baumannii are resensitized to antimicrobials. Nat Microbiol 1–5.

96. Mangalea MR, Duerkop BA. 2020. Fitness Trade-Offs Resulting from Bacteriophage Resistance Potentiate Synergistic Antibacterial Strategies. Infect Immun 88.

97. Fernández L, Rodríguez A, García P. 2018. Phage or foe: an insight into the impact of viral predation on microbial communities. ISME J 12:1171–1179.

98. Shah M, Taylor VL, Bona D, Tsao Y, Stanley SY, Pimentel-Elardo SM, McCallum M, Bondy-Denomy J, Howell PL, Nodwell JR, Davidson AR, Moraes TF, Maxwell KL. 2021. A phage-encoded anti-activator inhibits quorum sensing in Pseudomonas aeruginosa. Mol Cell 81:571-583.e6.

99. Platt JR. 1964. Strong Inference. Science 146:347–353.

100. Gould SJ, Vrba ES. 1982. Exaptation—a Missing Term in the Science of Form. Paleobiology 8:4–15.

101. Burroughs AM, Aravind L. 2020. Identification of Uncharacterized Components of Prokaryotic Immune Systems and Their Diverse Eukaryotic Reformulations. J Bacteriol 202:e00365-20.

102. Kupczok A, Gavriilidou A, Paulitz E, Guerrero-García L, Baumdicker F. 2025. Gene co-occurrence and its association with phage infectivity in bacterial pangenomes. Philos Trans R Soc B Biol Sci 380:20240070.

103. Bujnicki JM. 2001. Understanding the evolution of restriction-modification systems: clues from sequence and structure comparisons. Acta Biochim Pol 48:935–967.

104. Oliveira PH, Touchon M, Rocha EPC. 2016. Regulation of genetic flux between bacteria by restriction–modification systems. Proc Natl Acad Sci 113:5658–5663.

105. Koonin EV, Makarova KS, Wolf YI. 2017. Evolutionary Genomics of Defense Systems in Archaea and Bacteria. Annu Rev Microbiol 71:233–261.

106. Atkinson GC, Tenson T, Hauryliuk V. 2011. The RelA/SpoT homolog (RSH) superfamily: distribution and functional evolution of ppGpp synthetases and hydrolases across the tree of life. PloS One 6:e23479.

107. Jurėnas D, Fraikin N, Goormaghtigh F, Van Melderen L. 2022. Biology and evolution of bacterial toxin–antitoxin systems. 6. Nat Rev Microbiol 20:335–350.

108. Abramson J, Adler J, Dunger J, Evans R, Green T, Pritzel A, Ronneberger O, Willmore L, Ballard AJ, Bambrick J, Bodenstein SW, Evans DA, Hung C-C, O'Neill M, Reiman D,

Tunyasuvunakool K, Wu Z, Žemgulytė A, Arvaniti E, Beattie C, Bertolli O, Bridgland A, Cherepanov A, Congreve M, Cowen-Rivers AI, Cowie A, Figurnov M, Fuchs FB, Gladman H, Jain R, Khan YA, Low CMR, Perlin K, Potapenko A, Savy P, Singh S, Stecula A, Thillaisundaram A, Tong C, Yakneen S, Zhong ED, Zielinski M, Žídek A, Bapst V, Kohli P, Jaderberg M, Hassabis D, Jumper JM. 2024. Accurate structure prediction of biomolecular interactions with AlphaFold 3. Nature 630:493–500.

109. Barrio-Hernandez I, Yeo J, Jänes J, Mirdita M, Gilchrist CLM, Wein T, Varadi M, Velankar S, Beltrao P, Steinegger M. 2023. Clustering predicted structures at the scale of the known protein universe. Nature 622:637–645.

110. Hussain FA, Dubert J, Elsherbini J, Murphy M, VanInsberghe D, Arevalo P, Kauffman K, Rodino-Janeiro BK, Gavin H, Gomez A, Lopatina A, Le Roux F, Polz MF. 2021. Rapid evolutionary turnover of mobile genetic elements drives bacterial resistance to phages. Science 374:488–492.

111. Beamud B, Benz F, Bikard D. 2024. Going viral: The role of mobile genetic elements in bacterial immunity. Cell Host Microbe 32:804–819.

112. Felsenstein J. 1985. Phylogenies and the Comparative Method. Am Nat 125:1–15.

113. Grafen A. 1989. The phylogenetic regression. Philos Trans R Soc Lond B Biol Sci 326:119–157.

114. Harvey PH, Pagel MD. 1992. The comparative method in evolutionary biology. J Classif 9:169–172.

115. Revell LJ. 2012. phytools: an R package for phylogenetic comparative biology (and other things). Methods Ecol Evol 3:217–223.

116. Duncan-Lowey B, Tal N, Johnson AG, Rawson S, Mayer ML, Doron S, Millman A, Melamed S, Fedorenko T, Kacen A, Brandis A, Mehlman T, Amitai G, Sorek R, Kranzusch PJ. 2023. Cryo-EM structure of the RADAR supramolecular anti-phage defense complex. Cell 186:987-998.e15.

117. Maestri A, Pons BJ, Pursey E, Chong CE, Gandon S, Custodio R, Olina A, Agapov A, Chisnall MAW, Grasso A, Paterson S, Szczelkun MD, Baker KS, Houte S van, Chevallereau A, Westra ER. 2024. The bacterial defense system MADS interacts with CRISPR-Cas to limit phage infection and escape. Cell Host Microbe 32:1412-1426.e11.

118. Borges AL, Zhang JY, Rollins MF, Osuna BA, Wiedenheft B, Bondy-Denomy J. 2018. Bacteriophage Cooperation Suppresses CRISPR-Cas3 and Cas9 Immunity. Cell 174:917-925.e10.

119. Landsberger M, Gandon S, Meaden S, Rollie C, Chevallereau A, Chabas H, Buckling A, Westra ER, van Houte S. 2018. Anti-CRISPR Phages Cooperate to Overcome CRISPR-Cas Immunity. Cell 174:908-916.e12.

120. Mason G, Footer MJ, Rojas ER. 2023. Mechanosensation induces persistent bacterial growth during bacteriophage predation. mBio 14:e02766-22.

121. Alseth EO, Roush C, Irby I, Kopylov M, Bobe D, Diggs MW, Nguyen K, Xu H, Schmidt-Krey I, Bryksin AV, Rather PN. 2025. Mystique, a broad host range Acinetobacter phage, reveals

the impact of culturing conditions on phage isolation and infectivity. PLOS Pathog 21:e1012986.

122. Lewin GR, Kapur A, Cornforth DM, Duncan RP, Diggle FL, Moustafa DA, Harrison SA, Skaar EP, Chazin WJ, Goldberg JB, Bomberger JM, Whiteley M. 2023. Application of a quantitative framework to improve the accuracy of a bacterial infection model. Proc Natl Acad Sci 120:e2221542120.

123. Hoskisson PA, Sumby P, Smith MCM. 2015. The phage growth limitation system in *Streptomyces coelicolor* A(3)2 is a toxin/antitoxin system, comprising enzymes with DNA methyltransferase, protein kinase and ATPase activity. Virology 477:100–109.

124. Gordeeva J, Morozova N, Sierro N, Isaev A, Sinkunas T, Tsvetkova K, Matlashov M, Truncaitė L, Morgan RD, Ivanov NV, Siksnys V, Zeng L, Severinov K. 2019. BREX system of Escherichia coli distinguishes self from non-self by methylation of a specific DNA site. Nucleic Acids Res 47:253–265.

125. Swarts DC, Jore MM, Westra ER, Zhu Y, Janssen JH, Snijders AP, Wang Y, Patel DJ, Berenguer J, Brouns SJJ, van der Oost J. 2014. DNA-guided DNA interference by a prokaryotic Argonaute. 7491. Nature 507:258–261.

126. Jolly SM, Gainetdinov I, Jouravleva K, Zhang H, Strittmatter L, Bailey SM, Hendricks GM, Dhabaria A, Ueberheide B, Zamore PD. 2020. Thermus thermophilus Argonaute Functions in the Completion of DNA Replication. Cell 182:1545-1559.e18.

127. Zeng Z, Chen Y, Pinilla-Redondo R, Shah SA, Zhao F, Wang C, Hu Z, Wu C, Zhang C, Whitaker RJ, She Q, Han W. 2022. A short prokaryotic Argonaute activates membrane effector to confer antiviral defense. Cell Host Microbe 30:930-943.e6.

128. Huiting E, Cao X, Ren J, Athukoralage JS, Luo Z, Silas S, An N, Carion H, Zhou Y, Fraser JS, Feng Y, Bondy-Denomy J. 2023. Bacteriophages inhibit and evade cGAS-like immune function in bacteria. Cell https://doi.org/10.1016/j.cell.2022.12.041.

129. Chopin M-C, Chopin A, Bidnenko E. 2005. Phage abortive infection in lactococci: variations on a theme. Curr Opin Microbiol 8:473–479.

130. Parma DH, Snyder M, Sobolevski S, Nawroz M, Brody E, Gold L. 1992. The Rex system of bacteriophage lambda: tolerance and altruistic cell death. Genes Dev 6:497–510.

131. Patel KM, Seed KD. 2023. Sporadic phage defense in epidemic Vibrio cholerae mediated by the toxin-antitoxin system DarTG is countered by a phage-encoded antitoxin mimic. bioRxiv https://doi.org/10.1101/2023.12.14.571748.

132. Doron S, Melamed S, Ofir G, Leavitt A, Lopatina A, Keren M, Amitai G, Sorek R. 2018. Systematic discovery of antiphage defense systems in the microbial pangenome. Science 359:eaar4120.

133. Millman A, Bernheim A, Stokar-Avihail A, Fedorenko T, Voichek M, Leavitt A, Oppenheimer-Shaanan Y, Sorek R. 2020. Bacterial Retrons Function In Anti-Phage Defense. Cell 183:1551-1561.e12.

134. David E, Plantady C, Poissonnier S, Elliott JFK, Kenck E, Le Boulch J, Gutierrez A, Chevallereau A. 2025. Systematic functional assessment of anti-phage systems in their native host. Philos Trans R Soc B Biol Sci 380:20240067.

135. Paoli L, Laruelle B, Lavenir R, Loubat A, Tesson F, Bernheim A. Environment and physiology shape antiphage system expression.

136. Vassallo CN, Doering CR, Littlehale ML, Teodoro GIC, Laub MT. 2022. A functional selection reveals previously undetected anti-phage defence systems in the E. coli pangenome. Nat Microbiol 1–12.

137. Hadas H, Einav M, Fishov I, Zaritsky A. 1997. Bacteriophage T4 Development Depends on the Physiology of its Host Escherichia Coli. Microbiology 143:179–185.

138. Šivec K, Podgornik A. 2020. Determination of bacteriophage growth parameters under cultivating conditions. Appl Microbiol Biotechnol 104:8949–8960.

139. Attrill EL, Łapińska U, Westra ER, Harding SV, Pagliara S. 2023. Slow growing bacteria survive bacteriophage in isolation. 1. ISME Commun 3:1–9.

140. Kosterlitz O, Grassi N, Werner B, McGee RS, Top EM, Kerr B. 2023. Evolutionary "Crowdsourcing": Alignment of Fitness Landscapes Allows for Cross-species Adaptation of a Horizontally Transferred Gene. Mol Biol Evol 40.

141. Frank SA. 1998. Foundations of social evolution. Princeton University Press, Princeton, NJ.

142. West SA, Griffin AS, Gardner A. 2007. Social semantics: altruism, cooperation, mutualism, strong reciprocity and group selection. J Evol Biol 20:415–432.

143. Hamilton WD. 1964. The genetical evolution of social behaviour. I. J Theor Biol 7:1–16.

144. Simonet C, McNally L. 2021. Kin selection explains the evolution of cooperation in the gut microbiota. Proc Natl Acad Sci 118:e2016046118.

145. Raymond B, West SA, Griffin AS, Bonsall MB. 2012. The Dynamics of Cooperative Bacterial Virulence in the Field. Science 337:85–88.

146. Smith J, Strassmann JE, Queller DC. 2016. Fine-scale spatial ecology drives kin selection relatedness among cooperating amoebae. Evolution 70:848–859.

147. Heinze J, Walter B. 2010. Moribund Ants Leave Their Nests to Die in Social Isolation. Curr Biol 20:249–252.

148. Huo W, Price VJ, Sharifi A, Zhang MQ, Palmer KL. 2023. Enterococcus faecalis Strains with Compromised CRISPR-Cas Defense Emerge under Antibiotic Selection for a CRISPR-Targeted Plasmid. Appl Environ Microbiol 0:e00124-23.

149. Pursey E, Dimitriu T, Paganelli FL, Westra ER, van Houte S. 2022. CRISPR-Cas is associated with fewer antibiotic resistance genes in bacterial pathogens. Philos Trans R Soc B Biol Sci 377:20200464.

150. Rousset F, Depardieu F, Miele S, Dowding J, Laval A-L, Lieberman E, Garry D, Rocha EPC, Bernheim A, Bikard D. 2022. Phages and their satellites encode hotspots of antiviral systems. Cell Host Microbe 30:740-753.e5.

151. Pfeifer E, Sousa JM, Touchon M, Rocha EP. 2022. When bacteria are phage playgrounds: interactions between viruses, cells, and mobile genetic elements. Curr Opin Microbiol 70:102230.

152. Rocha EPC, Bikard D. 2022. Microbial defenses against mobile genetic elements and viruses: Who defends whom from what? PLOS Biol 20:e3001514.

153. Koonin EV, Makarova KS, Wolf YI, Krupovic M. 2020. Evolutionary entanglement of mobile genetic elements and host defence systems: guns for hire. Nat Rev Genet 21:119–131.

154. Nogueira T, Rankin DJ, Touchon M, Taddei F, Brown SP, Rocha EPC. 2009. Horizontal Gene Transfer of the Secretome Drives the Evolution of Bacterial Cooperation and Virulence. Curr Biol 19:1683–1691.

155. Kever L, Hardy A, Luthe T, Hünnefeld M, Gätgens C, Milke L, Wiechert J, Wittmann J, Moraru C, Marienhagen J, Frunzke J. 2022. Aminoglycoside Antibiotics Inhibit Phage Infection by Blocking an Early Step of the Infection Cycle. mBio 13:e00783-22.

156. Sünderhauf D, Ringger JR, Payne LJ, Pinilla-Redondo R, Gaze WH, Brown SP, Houte S van. 2026. CRISPR-Cas is beneficial in plasmid competition, but limited by competitor toxin–antitoxin activity when horizontally transferred. PLOS Biol 24:e3003658.

157. Kelly A, Arrowsmith TJ, Went SC, Blower TR. 2023. Toxin–antitoxin systems as mediators of phage defence and the implications for abortive infection. Curr Opin Microbiol 73:102293.

158. Servedio MR, Brandvain Y, Dhole S, Fitzpatrick CL, Goldberg EE, Stern CA, Cleve JV, Yeh DJ. 2014. Not Just a Theory—The Utility of Mathematical Models in Evolutionary Biology. PLOS Biol 12:e1002017.

159. Iranzo J, Lobkovsky AE, Wolf YI, Koonin EV. 2015. Immunity, suicide or both? Ecological determinants for the combined evolution of anti-pathogen defense systems. BMC Evol Biol 15:43.

Appendix

Individual cell-level defenses

Cell-level defenses include some of the 'biggest names' of the bacterial immune system, such as RM and CRISPR-Cas which both function by targeting and degrading parasitic nucleic acids. This category also contains largely phage-targeting mechanisms including prokaryotic viperins (pVips) (1, 2), BREX(3), and DISARM(4). Additionally, there are some defenses that mainly but not exclusively target plasmids, like Wadjet(5) and long-A prokaryotic Argonaute proteins (pAgos)(6, 7). Further, some secondary metabolites have been found to limit the synthesis of invasive DNA and RNA without killing the host (anthracyclines and aminoglycosides produced by *Streptomyces*(8–10)). There are exceptions (such as CRISPR Type III, which results in abortive infection(11)) and uncertainties of course, and surviving the onslaught of molecular parasites is neither straightforward nor guaranteed(12, 13) but is more likely if the bacterium being targeted has one or more of these systems present in its genome.

Group-level defenses

Group-level defenses differ in that they do not necessarily enhance protection of a focal individual cell, but instead limit parasite reproduction in a way that may potentially protect the broader bacterial group. The most well established class of group-level defenses are 'abortive infection' systems, for example toxin-antitoxin systems such as avcID(14), CapRel(15), ToxIN(16), RnlAB(17), DarTG(18) and RosmerTA(19). Other abortive infection systems include CBASS(20), Pycsar(20, 21), Thoeris(22, 23), DSRs(24), SEFIRs(25), long-B and short pAgos(6, 26), AbiA-AbiZ(27), retrons(28), Lamassu(25, 29), PARIS(30, 31), Gabija(32, 33), Avs(30, 34, 35), Stk2(36),

Zorya(37), bacterial gasdermins(38), and RexAB(39, 40). Additionally, there are defenses involving deoxynucleotide depleting defense enzymes (dCTP, dGTPase)(41), and cell poisoning mechanisms (RADAR(34)). It is important to note that some of these defense 'families' might have sub-types facilitating cell survival, but for the sake of clarity they will remain in this category.

Increase in phage defense literature (raw counts)

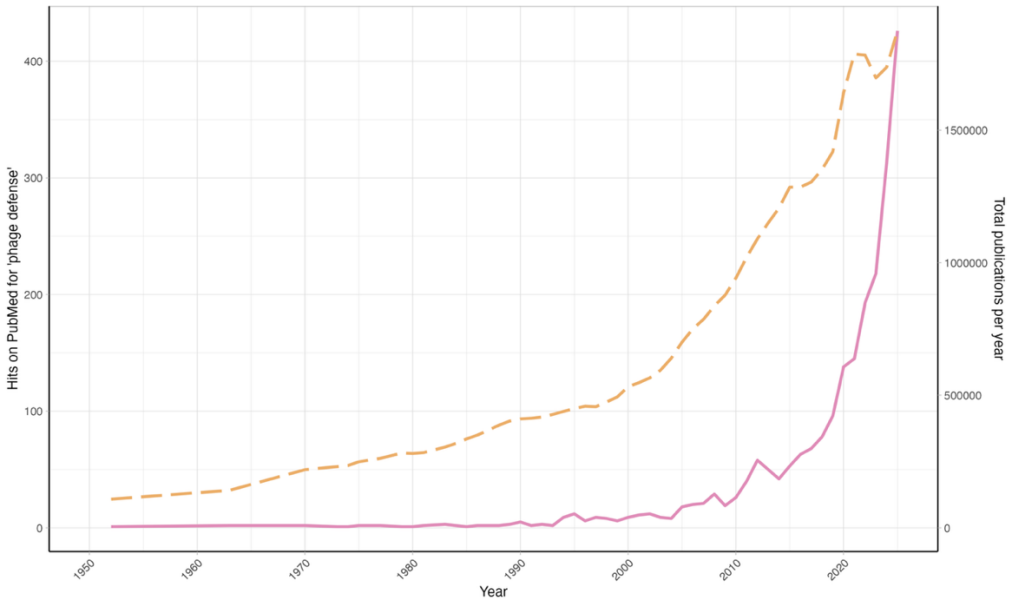

Fig. S1. The bacterial defense literature is rapidly expanding (raw counts). Total Pubmed hits per year for 'phage defense' (red line, left y axis) and total publications per year (yellow dashed line, right y axis).

References

1. Bernheim A, Millman A, Ofir G, Meitav G, Avraham C, Shomar H, Rosenberg MM, Tal N, Melamed S, Amitai G, Sorek R. 2021. Prokaryotic viperins produce diverse antiviral molecules. 7840. Nature 589:120–124.

2. Shomar H, Georjon H, Feng Y, Olympio B, Guillaume M, Tesson F, Cury J, Wu F, Bernheim A. 2024. Viperin immunity evolved across the tree of life through serial innovations on a conserved scaffold. Nat Ecol Evol 1–13.

3. Goldfarb T, Sberro H, Weinstock E, Cohen O, Doron S, Charpak-Amikam Y, Afik S, Ofir G, Sorek R. 2015. BREX is a novel phage resistance system widespread in microbial genomes. EMBO J 34:169–183.

4. Ofir G, Melamed S, Sberro H, Mukamel Z, Silverman S, Yaakov G, Doron S, Sorek R. 2018. DISARM is a widespread bacterial defence system with broad anti-phage activities. Nat Microbiol 3:90–98.

5. Deep A, Gu Y, Gao Y-Q, Ego KM, Herzik MA, Zhou H, Corbett KD. 2022. The SMC-family Wadjet complex protects bacteria from plasmid transformation by recognition and cleavage of closed-circular DNA. Mol Cell 82:4145-4159.e7.

6. Swarts DC, Jore MM, Westra ER, Zhu Y, Janssen JH, Snijders AP, Wang Y, Patel DJ, Berenguer J, Brouns SJJ, van der Oost J. 2014. DNA-guided DNA interference by a prokaryotic Argonaute. 7491. Nature 507:258–261.

7. Bobadilla Ugarte P, Barendse P, Swarts DC. 2023. Argonaute proteins confer immunity in all domains of life. Curr Opin Microbiol 74:102313.

8. Kronheim S, Daniel-Ivad M, Duan Z, Hwang S, Wong AI, Mantel I, Nodwell JR, Maxwell KL. 2018. A chemical defence against phage infection. 7735. Nature 564:283–286.

9. Kever L, Hardy A, Luthe T, Hünnefeld M, Gätgens C, Milke L, Wiechert J, Wittmann J, Moraru C, Marienhagen J, Frunzke J. 2022. Aminoglycoside Antibiotics Inhibit Phage Infection by Blocking an Early Step of the Infection Cycle. mBio 13:e00783-22.

10. Hardy A, Kever L, Frunzke J. 2023. Antiphage small molecules produced by bacteria – beyond protein-mediated defenses. Trends Microbiol 31:92–106.

11. Mayo-Muñoz D, Smith LM, Garcia-Doval C, Malone LM, Harding KR, Jackson SA, Hampton HG, Fagerlund RD, Gumy LF, Fineran PC. 2022. Type III CRISPR-Cas provides resistance against nucleus-forming jumbo phages via abortive infection. Mol Cell 82:4471-4486.e9.

12. Georjon H, Bernheim A. 2023. The highly diverse antiphage defence systems of bacteria. Nat Rev Microbiol 1–15.

13. Aframian N, Eldar A. 2023. Abortive infection antiphage defense systems: separating mechanism and phenotype. Trends Microbiol 31:1003–1012.

14. Hsueh BY, Ferrell MJ, Sanath-Kumar R, Bedore AM, Waters CM. 2023. Replication cycle timing determines phage sensitivity to a cytidine deaminase toxin/antitoxin bacterial defense system. PLOS Pathog 19:e1011195.

15. Zhang T, Tamman H, Coppieters ’t Wallant K, Kurata T, LeRoux M, Srikant S, Brodiazhenko T, Cepauskas A, Talavera A, Martens C, Atkinson GC, Hauryliuk V, Garcia-Pino A, Laub MT. 2022. Direct activation of a bacterial innate immune system by a viral capsid protein. Nature 1–9.

16. Guegler CK, Laub MT. 2021. Shutoff of host transcription triggers a toxin-antitoxin system to cleave phage RNA and abort infection. Mol Cell 81:2361-2373.e9.

17. Koga M, Otsuka Y, Lemire S, Yonesaki T. 2011. Escherichia coli rnlA and rnlB Compose a Novel Toxin–Antitoxin System. Genetics 187:123–130.

18. LeRoux M, Srikant S, Teodoro GIC, Zhang T, Littlehale ML, Doron S, Badiee M, Leung AKL, Sorek R, Laub MT. 2022. The DarTG toxin-antitoxin system provides phage defence by ADP-ribosylating viral DNA. 7. Nat Microbiol 7:1028–1040.

19. Ernits K, Saha CK, Brodiazhenko T, Chouhan B, Shenoy A, Buttress JA, Duque-Pedraza JJ, Bojar V, Nakamoto JA, Kurata T, Egorov AA, Shyrokova L, Johansson MJO, Mets T, Rustamova A, Džigurski J, Tenson T, Garcia-Pino A, Strahl H, Elofsson A, Hauryliuk V, Atkinson GC. 2023. The structural basis of hyperpromiscuity in a core combinatorial network of type II toxin–antitoxin and related phage defense systems. Proc Natl Acad Sci 120:e2305393120.

20. Cohen D, Melamed S, Millman A, Shulman G, Oppenheimer-Shaanan Y, Kacen A, Doron S, Amitai G, Sorek R. 2019. Cyclic GMP-AMP signalling protects bacteria against viral infection. Nature 574:691–695.

21. Tal N, Morehouse BR, Millman A, Stokar-Avihail A, Avraham C, Fedorenko T, Yirmiya E, Herbst E, Brandis A, Mehlman T, Oppenheimer-Shaanan Y, Keszei AFA, Shao S, Amitai G, Kranzusch PJ, Sorek R. 2021. Cyclic CMP and cyclic UMP mediate bacterial immunity against phages. Cell 184:5728-5739.e16.

22. Ka D, Oh H, Park E, Kim J-H, Bae E. 2020. Structural and functional evidence of bacterial antiphage protection by Thoeris defense system via NAD+ degradation. 1. Nat Commun 11:2816.

23. Ofir G, Herbst E, Baroz M, Cohen D, Millman A, Doron S, Tal N, Malheiro DBA, Malitsky S, Amitai G, Sorek R. 2021. Antiviral activity of bacterial TIR domains via immune signalling molecules. 7887. Nature 600:116–120.

24. Garb J, Lopatina A, Bernheim A, Zaremba M, Siksnys V, Melamed S, Leavitt A, Millman A, Amitai G, Sorek R. 2022. Multiple phage resistance systems inhibit infection via SIR2-dependent NAD+ depletion. Nat Microbiol 1–8.

25. Millman A, Melamed S, Leavitt A, Doron S, Bernheim A, Hör J, Garb J, Bechon N, Brandis A, Lopatina A, Ofir G, Hochhauser D, Stokar-Avihail A, Tal N, Sharir S, Voichek M, Erez Z, Ferrer JLM, Dar D, Kacen A, Amitai G, Sorek R. 2022. An expanded arsenal of immune systems that protect bacteria from phages. Cell Host Microbe 30:1556-1569.e5.

26. Koopal B, Potocnik A, Mutte SK, Aparicio-Maldonado C, Lindhoud S, Vervoort JJM, Brouns SJJ, Swarts DC. 2022. Short prokaryotic Argonaute systems trigger cell death upon detection of invading DNA. Cell 0.

27. Durmaz E, Klaenhammer TR. 2007. Abortive Phage Resistance Mechanism AbiZ Speeds the Lysis Clock To Cause Premature Lysis of Phage-Infected Lactococcus lactis. J Bacteriol 189:1417–1425.

28. Millman A, Bernheim A, Stokar-Avihail A, Fedorenko T, Voichek M, Leavitt A, Oppenheimer-Shaanan Y, Sorek R. 2020. Bacterial Retrons Function In Anti-Phage Defense. Cell 183:1551-1561.e12.

29. Jaskólska M, Adams DW, Blokesch M. 2022. Two defence systems eliminate plasmids from seventh pandemic Vibrio cholerae. 7905. Nature 604:323–329.

30. Rousset F, Depardieu F, Miele S, Dowding J, Laval A-L, Lieberman E, Garry D, Rocha EPC, Bernheim A, Bikard D. 2022. Phages and their satellites encode hotspots of antiviral systems. Cell Host Microbe 30:740-753.e5.

31. Belukhina S, Saudemont B, Depardieu F, Lorthios T, P. Maviza T, Livenskyi A, Serebryakova M, Aleksandrova M, Ukholkina E, Burmistrova N, Sergiev P, Libiad M, Dubrac S, Barras F, Motorin Y, Marchand V, Hagelueken G, Isaev A, Bikard D, Rouillon C. 2025. Specificity and mechanism of tRNA cleavage by the AriB Toprim nuclease of the PARIS bacterial immune system. Philos Trans R Soc B Biol Sci 380:20240074.

32. Cheng R, Huang F, Wu H, Lu X, Yan Y, Yu B, Wang X, Zhu B. 2021. A nucleotide-sensing endonuclease from the Gabija bacterial defense system. Nucleic Acids Res 49:5216–5229.

33. Cheng R, Huang F, Lu X, Yan Y, Yu B, Wang X, Zhu B. 2023. Prokaryotic Gabija complex senses and executes nucleotide depletion and DNA cleavage for antiviral defense. Cell Host Microbe 31:1331-1344.e5.

34. Gao L, Altae-Tran H, Böhning F, Makarova KS, Segel M, Schmid-Burgk JL, Koob J, Wolf YI, Koonin EV, Zhang F. 2020. Diverse enzymatic activities mediate antiviral immunity in prokaryotes. Science 369:1077–1084.

35. Gao LA, Wilkinson ME, Strecker J, Makarova KS, Macrae RK, Koonin EV, Zhang F. 2022. Prokaryotic innate immunity through pattern recognition of conserved viral proteins. Science 377:eabm4096.

36. Depardieu F, Didier J-P, Bernheim A, Sherlock A, Molina H, Duclos B, Bikard D. 2016. A Eukaryotic-like Serine/Threonine Kinase Protects Staphylococci against Phages. Cell Host Microbe 20:471–481.

37. Doron S, Melamed S, Ofir G, Leavitt A, Lopatina A, Keren M, Amitai G, Sorek R. 2018. Systematic discovery of antiphage defense systems in the microbial pangenome. Science 359:eaar4120.

38. Johnson AG, Wein T, Mayer ML, Duncan-Lowey B, Yirmiya E, Oppenheimer-Shaanan Y, Amitai G, Sorek R, Kranzusch PJ. 2022. Bacterial gasdermins reveal an ancient mechanism of cell death. Science 375:221–225.

39. Snyder L, McWilliams K. 1989. The rex genes of bacteriophage lambda can inhibit cell function without phage superinfection. Gene 81:17–24.

40. Parma DH, Snyder M, Sobolevski S, Nawroz M, Brody E, Gold L. 1992. The Rex system of bacteriophage lambda: tolerance and altruistic cell death. Genes Dev 6:497–510.

41. Tal N, Millman A, Stokar-Avihail A, Fedorenko T, Leavitt A, Melamed S, Yirmiya E, Avraham C, Brandis A, Mehlman T, Amitai G, Sorek R. 2022. Bacteria deplete deoxynucleotides to defend against bacteriophage infection. 8. Nat Microbiol 7:1200–1209.