Multi-layered genome defences in bacteria

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Abstract

Bacteria have evolved a variety of defence mechanisms to protect against mobile genetic elements, including restriction-modification systems and CRISPR-Cas. In recent years, dozens of previously unknown defence systems have been discovered. Notably, diverse defence systems often coexist within the same genome, and some co-occur at frequencies significantly higher than would be expected by chance, implying potential synergistic interactions. Recent studies have provided evidence of defence mechanisms that enhance or complement one another. Here, we review the interactions between defence systems at the mechanistic, regulatory, ecological, and evolutionary levels.

Introduction

Almost all bacterial genomes contain mobile genetic elements (MGEs), including phages, plasmids, and transposons. Such MGEs play important roles in bacterial evolution, by mediating movement of genetic material within or between genomes, thus driving horizontal gene transfer (HGT). Although MGE-mediated HGT can accelerate adaptation through spreading ecologically beneficial genes, gaining an MGE can also impose heavy fitness costs upon the host bacterial cell, including in the case of phages the lethal cost of cellular lysis. Consequently, bacterial genomes have evolved myriad defence systems (DSs) that target MGEs or MGE effects upon the cell. However, DSs are a double-edged sword, because although they can help bacteria survive infection by parasitic MGEs, they also limit the spread of potentially beneficial traits within a population via HGT [1]. As such, the interplay between MGEs and DSs is likely to play an important role in shaping bacterial genome evolution.

We are currently in a period of fast discovery of novel DSs driven by the rapid increase of bacterial genomic data and the development of new bioinformatics tools (see below). It is now evident that a large arsenal of bacterial defences exists, exhibiting high diversity in genomic architecture and complexity, mechanisms of action, and evolutionary origin. Based on their mode of action, prokaryotic DSs can be grouped into three main categories (Figure 1). Firstly, defences such as Restriction Modification (RM) and CRISPR-Cas [2] degrade or modify the nucleic acids of the invading MGEs [2–8]. Secondly, systems like Thoeris [9] block MGE infection by inducing dormancy that can lead to cell death before the MGE spreads. This mechanism is called Abortive infection (Abi) and can be achieved through depletion of essential molecules, such as ATP [10] and NAD^+ [9,11–13], disruption of the bacterial membrane [14–17] or inhibition of translation [18]. Finally, DSs like the prokaryotic viperins [19] inhibit MGE replication by nucleotide depletion or modification, or synthesis of other small inhibitory molecules [19–21].

As well as the rapid discovery of novel DSs, we are also learning about their genomic organisation. A key finding is that DSs are often clustered together in regions of the bacterial genome called “defence islands” [22,23]. Indeed, it is this clustering that has enabled DS discovery: bioinformatic tools have been developed that systematically identify novel defence genes based on their genomic vicinity with known DSs, leading to the discovery of dozens of previously-unknown DSs [24–26]. The analysis of defence islands, making use of conserved gene boundaries [27–31] or transposon mutagenesis [31,32] has thus been a fruitful method for detecting new DSs, some of which share ancestry with eukaryotic immune systems [33]. Defence islands may themselves be encoded upon MGEs, such as integrative conjugative elements (ICEs), transposons, and prophages, enabling HGT of DSs [27,28,31,34–36] and DS cointegration to form defence hotspots [28,37]. However, while DS co-occurrence has fuelled discovery of novel DSs and their mechanisms (reviewed in [38,39]), we know relatively little of why DSs co-occur in the first place, and if and how these co-occurring systems interact with one another. Here, we review our current understanding of DS interactions, their (co)regulation and evolution.

Defence prevalence and co-occurrence

Bacterial genomes contain, on average, 5-6 DSs per genome, with the majority (78%) encoding more than two DSs [40,41]. The most common DSs found in prokaryotic genomes are RM systems (83%), followed by CRISPR-Cas (38%), with the prevalence of most other systems falling below 20% [40]. Studies investigating the DS content of prokaryotic genomes have found that certain sets of DSs are more conserved in certain bacterial genera, suggesting that

synergisms between DSs may be advantageous for bacterial survival in phage diverse environments [42,43]. Analysis of the co-occurrence and non-co-occurrence patterns among DSs might point to valuable insights on DS-DS interactions. Indeed, it has been hypothesised that defence islands may form due to synergy between DSs promoting co-localisation and parallel mobilisation similar to the evolutionary forces that result in aggregation of antibiotic resistance and pathogenicity genes [37,42,44]. It has been observed that DSs sharing phage sensing strategies are found to co-occur more often than expected by chance, forming a multi-layered defence [42]. For example, the anti-RM/BREX protein Ocr (overcome classical restriction) can be detected by the DSs' PARIS, Gabija and Zorya Type II, which act as a second line of defence [27,42]. Additionally, experimental data have shown that systems such as RM and CRISPR-Cas work together to increase phage defence [45,46]. However, studies so far have suggested that whilst certain sets of prokaryotic DSs do co-occur, this does not necessarily correlate with a synergistic defence response [42,43]. Therefore, it is likely that there is some functional redundancy within DSs and/or that the selection of defence system combinations is a response to an organism's environment, in line with broader pangenome theory [34,43,47,48]. Apparent discrepancies between co-occurrence and phenotypic synergisms may also reflect a lack of statistical power in studies to date and/or inherent biases in publicly available datasets.

Costs and benefits of multi-layered defences

Whilst various studies have tried to elucidate the conditions that favour one DS over another (see e.g., [49–52]), less attention has been paid to the question why many bacteria carry a whole arsenal of multiple DSs. Carrying DSs can impose substantial fitness costs on their hosts due to metabolic burden, potential for autoimmunity due to self-targeting, selfish behaviour of DSs, such as those forming toxin-antitoxin systems, and genetic conflicts between DSs and the rest of the genome [53–57]. Having multiple DSs may increase the costs cumulatively, and investing in multiple DSs may result in reduced performance in other activities like growth and reproduction [58]. In addition, there may be genetic conflict between the different DSs that co-exist in the same genome, including epigenetic conflict where DNA modifications introduced by one DS cause autoimmunity by another DS [59]. Obviously, for selection to favour bacteria with multiple DSs, the benefits need to outweigh these costs. Recently, several potential benefits of carrying multiple DSs have been put forward.

First, the most widely explored benefit of carrying multiple DSs is that it increases the levels and durability of resistance. For example, the co-existence and simultaneous action of RM and CRISPR-Cas reduces the frequency of phage escape and increases the rate of CRISPR immunity acquisition [45,46,60]. In the case of type VI CRISPR-Cas systems, which induce a dormancy response [61], co-occurrence with RM not only increases the ability to clear phage infections but also the recovery from the dormancy response [62]. In other cases, simultaneous DS activity can lead to synergy through complementary action. For example, the co-occurrence of Type I BREX and Type IV RM reduces the success of epigenetic mutants that can overcome BREX, because unmodified phages are restricted by BREX whereas modified phages are restricted by the Type IV RM [32]. In other cases, synergy may emerge through sequential action of different DSs. For example, phage-mediated inhibition of RecBCD innate immunity triggers retron-mediated Abi [17]. In this case, the second layer of defence safeguards the primary layer, ensuring that programmed cell death is not activated unless phages by-pass the first layer of defence, as recently explored mathematically in [63]. A second reason why

bacteria may need multiple DSs is to provide a division of labour, with each defence specialising on a subset of MGEs (Figure 1). For example, Wadjet cleaves closed-circular DNA substrates and protects bacteria from acquiring small plasmids [64–66], whereas Abi systems are frequently triggered through pattern recognition of conserved proteins associated with phages [18,67,68]. Finally, different DSs may be active under different environmental conditions [69] (Figure 1). This is supported by the idea that selection for different types of defences strongly depends on ecological variables [70], and that expression of defences can be controlled by different environmental cues [71].

Consequently, selection for multiple DSs is likely to depend on the environmental conditions, such as the force of infection, the diversity of MGEs, as well as the wider biotic and abiotic environment. For example, within complex microbial communities, cells may interact with multiple MGEs, some beneficial and some harmful, while facing increased competition (from other community members) for resources. Such communities may impose additional selection pressures, leading to the effects of certain DSs being enhanced or dampened. [72]. Moreover, increased phage diversity makes it difficult for one system to be effective against all, while increased phage abundance necessitates an economically optimized immune response; both scenarios may promote the evolution of multi-layered defence. Phage diversity can also impose a trade-off for phages (host infectivity vs inter-viral competition). This may lead to adoption of novel strategies, for example by increasing the selective advantage gained from infecting resistant cells, which may lead to the evolution of anti-DS systems and thus increase the benefits of having multi-layered DSs [73]. Challenging environmental conditions can dictate investment into DSs. For example, limited nutrient levels can raise fitness costs associated with multiple DSs, environmental niche can determine the number of maintained DSs, or rapid turnover of environment may maximise diversity [22,74]. Exclusion of foreign genetic material may not always be beneficial to the cell, potentially creating conflict between the host and DSs. For example, as HGT allows bacteria to adapt to environmental challenges, DSs can act as barriers against the acquisition of beneficial DNA. In such cases, certain DSs may negatively affect host fitness. MGEs may also use DSs for MGE-MGE conflict by hijacking DSs to defend against competitors [27,75]. Therefore, fitness interests of host and individual DS may not always align, which may result in selection of multi-layered DS [27,76].

Regulation of defence activity

Regulation of DSs activity may minimize costs and maximize benefits of DSs and can occur both at the transcriptional and post-translational level.

Transcriptional and post-transcriptional regulation

Given that DSs are not commonly found in isolation, we have little understanding on how these systems are regulated to facilitate a coordinated (and potentially layered) response to infection by MGEs. There are features of collective DSs that are suggestive of coordinated expression; such as co-localisation on ‘islands’, or clustering within single operons [32]. This organisation will require transcriptional regulation at a global level, or through dedicated regulators of islands and operons. Coordinated regulation would further suggest the potential for an organised prokaryotic immune system [31].

Multiple global inputs have been demonstrated to regulate defence responses [77]. If cell density is very high, a population might be particularly vulnerable to phages. In turn, quorum sensing, used to monitor population density, has been shown to regulate multiple defences

including CRISPR-Cas [78], dCTP deaminase and Lamassu [79] at the transcriptional level (Figure 2). Stress responses and cell metabolic status also regulate defence, either suppressing or inducing CRISPR-Cas [80–82] (Figure 2). Post-transcriptional methods of regulating defence are also beginning to emerge, such as Rsm/Csr mediated binding of transcripts and suppression of type I and III CRISPR-Cas in *Serratia* [83].

Defence islands have also been found to carry their own regulatory elements. The defence island of plasmid pEFER contains an operon encoding both a BREX system and a GmrSD type IV restriction homologue, BrxU [32]. A recent study identified a WYL-domain protein, BrxR, negatively regulating operon expression [84] (Figure 2). Homologues of BrxR were also identified controlling BREX in *Acinetobacter* [85] and CBASS [86], and searches identified BrxR associated with a wide variety of other defences [84]. This is the first example of a predominantly defence-associated regulator, and the presence of the WYL domain suggests control via the detection of nucleic acids [87]. Understanding how defence island regulatory elements integrate with global regulatory inputs is essential for understanding the spread and maintenance of horizontally acquired DSs.

Post-translational regulation

The mechanisms used by DSs to detect viral infection are diverse and can broadly be divided into Direct and Indirect detection. Direct detection involves sensing of early signals of infection, including phage DNA (e.g. by the RM, CRISPR and DISARM systems [26]); DNA replication machinery (e.g. detection of phage SSB by Retron-Eco8 and phage primase-helicase by Lamassu [67]); or a specific phage RNA by type I CBASS [88]. Systems with these sensing mechanisms typically constitute the frontline anti-MGE defences and are amongst the most widespread DSs in bacteria [40,89]. Later signals include direct detection of phage structural proteins (for example by PYCSAR [13] and CBASS [90]), or detection of phage anti-defence proteins, such as Ocr by the PARIS defence system [27]. Indirect signals of infection can provide a second line of defence. For example, the detection of DNA degradation products, arising from either frontline defences or damage to host DNA incurred from viral attack, results in activation of the RloC nuclease which degrades tRNA [91]. Inhibition of host RNA polymerase and altered cellular transcription can result in the activation of toxins that have an RNA antitoxin – a notable example being the dCTP deaminase defence enzyme [92]. DSs commonly also act to deplete specific nucleotides [10,20], to slow down cell metabolism and viral replication kinetics. As infection progresses, perturbation of the nucleotide pools and depletion of ATP may also act as an activation signal for defence. Some second line DSs are activated on detection of inhibited frontline defences – for example the Ec48 Retron is activated on encountering phage-inhibited RecBCD [17]. Thus, the activation of DSs is highly varied, allowing for the possibility of synergistic action and control of timing.

Evolution of novel defences and defence combinations

Evolution of phage and other MGEs to overcome bacterial defence is likely an important driver of both the acquisition and loss of DSs from bacterial genomes, as well as the evolution of novel DSs. In the short term, MGEs may evolve to overcome DSs through epigenetic modifications or point mutations in genes whose products are recognised by the bacterial DSs, such as phage structural proteins or RecBCD inhibitors [67,93]. However, given that point mutations are often costly to the MGE, more sophisticated low-cost counter-defence mechanisms may evolve over longer timescales to specifically block bacterial DS functions,

which in turn may favour bacteria that acquired additional DSs. Carrying additional DSs not only "renews" the levels of protection against MGE infection but can also interfere with the deployment of counter-defence genes. Specifically, infection studies with phage that encode an anti-CRISPR (*acr*) counter-defence gene showed that bacteria that carry both MADS and CRISPR-Cas immune systems were less susceptible to the emergence and spread of phages that overcome MADS, compared to bacteria that carry only MADS [31]. Synergy between MADS and CRISPR-Cas emerged in this case because the ancestral Acr-encoding phage were unable to infect bacteria due to MADS activity, whereas rare MADS escaper phages were unable to amplify on CRISPR-immune bacteria because their density was below the critical density that supports cooperation and amplification of phage with Acr [94,95].

As detailed above, most defence-dedicated systems are found among accessory genes, implying frequent DS transmission between bacteria [22,24,96]. The association between defence and HGT led to the 'pan-immune' hypothesis, which posits that microbial communities possess a dispersed, shared immune system that community members draw on for protection [34]. While such an immune system provides protection against parasitic MGEs, several recent studies have shown that many DSs are themselves encoded in MGEs such as prophages and conjugative elements [27,76,96,97]. Carriage by MGEs enables DSs to transfer efficiently between cells by transduction or conjugation, and as different MGEs come and go, DSs will likewise be re-shuffled and rapidly turned over, resulting in different complements between closely related strains [98]. Access to the defence arsenal is obstructed not only by generic factors that restrict HGT (e.g., sequence length is known to be a major barrier, especially during transduction [96]), but also by MGE-MGE interactions and the presence of resident DSs. After being transferred to a new cell, DS combinations are then subject to natural selection arising from genetic context (e.g., metabolic burden, self-targeting) and environmental conditions (e.g., force of phage infection, nutrient availability), often resulting in loss from most recipient cells, but occasionally resulting in powerful new multi-layer defence.

Besides the variability from DS gain and loss, another level of variability is represented by gene swapping among DSs [99]. DSs are often modular, and different DSs sometimes use the same domains for signalling, regulation or as effectors. For example, nucleases cleave DNA or RNA, which can cause cell death if the chromosome is targeted. DNA methyltransferases can provide protection against such auto-immunity, but also influence gene expression more widely [100]. ATPases (helicases, AAA+ ATPases, ABC transporter families, etc.) manipulate DNA structure and can also sense infection, activating effectors. NTPases, deaminases and cyclases modulate nucleotide pools to deplete resources or signal infection while Toll/interleukin-1 (IL-1) receptor (TIR) and Silent information regulator 2 (Sir2 or sirtuins) proteins deplete NAD+ for programmed cell death [5,10,11,13,101,102]. Many domains identified have unknown functions and/or targets. The evolution of new defences could arise by shuffling and novel combinations of such modules. There is clear evidence for exchange within systems (reviewed in [99]) In practice, for instance, Type I RM DNA specificity subunits can complement in trans (e.g., from a plasmid [103]), and undergo dynamic genetic rearrangements that facilitate phase variation within otherwise clonal bacterial populations [104]. The exchange of protein modules driven by MGEs such as transposons and between-host signalling systems may form the basis for the evolution of complex defence systems, as has been proposed for the adaptation and interference modules of CRISPR-Cas immune systems [105].

Conclusion and outlook

The co-occurrence of multiple and layered DSs within a single bacterium is likely to have arisen through a co-evolutionary arms race between bacteria, phages and other MGEs, played out along different cost-benefit axes. The apparent benefit against evolvable counter-defence mechanisms and phenotypic diversity of MGEs will be offset by both additional metabolic costs and antagonistic interactions that may prevent uptake of potentially beneficial MGEs. The exact compositions of required molecular machineries needed to coordinate layered DSs will thus be strongly affected by prevailing environmental conditions and the individual mechanisms of the combined DSs. Understanding the combinatorial problem of multi-layered defence will provide insight into bacterial evolution, the viability of phage therapies, and our own immune systems.

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Declaration of interest

AM and EW are inventors on patent GB2303034.9.

References

1. Puigbò P, Makarova KS, Kristensen DM, Wolf YI, Koonin EV: **Reconstruction of the evolution of microbial defense systems**. *BMC Evol Biol* 2017, **17**:94.

2. Makarova KS, Wolf YI, Iranzo J, Shmakov SA, Alkhnbashi OS, Brouns SJJ, Charpentier E, Cheng D, Haft DH, Horvath P, et al.: **Evolutionary classification of CRISPR-Cas systems: a burst of class 2 and derived variants**. *Nat Rev Microbiol* 2020, **18**:67–83.

3. Kuzmenko A, Oguienko A, Esyunina D, Yudin D, Petrova M, Kudinova A, Maslova O, Ninova M, Ryazansky S, Leach D, et al.: **DNA targeting and interference by a bacterial Argonaute nuclease**. *Nature* 2020, **587**:632–637.

4. Tock MR, Dryden DTF: **The biology of restriction and anti-restriction**. *Curr Opin Microbiol* 2005, **8**:466–472.

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

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

7. Xiong X, Wu G, Wei Y, Liu L, Zhang Y, Su R, Jiang X, Li M, Gao H, Tian X, et al.: **SspABCD-SspE is a phosphorothioation-sensing bacterial defence system with broad anti-phage activities**. *Nat Microbiol* 2020, **5**:917–928.

8. LeGault KN, Barth ZK, DePaola P, Seed KD: **A phage parasite deploys a nicking nuclease effector to inhibit viral host replication**. *Nucleic Acids Res* 2022, **50**:8401–8417.

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

10. Rousset F, Yirmiya E, Nesher S, Brandis A, Mehlman T, Itkin M, Malitsky S, Millman A, Melamed S, Sorek R: **A conserved family of immune effectors cleaves cellular ATP upon viral infection**. *Cell* 2023, **186**:3619-3631.e13.

11. Garb J, Lopatina A, Bernheim A, Zaremba M, Siksnys V, Melamed S, Leavitt A, Millman A, Amitai G, Sorek R: **Multiple phage resistance systems inhibit infection via SIR2-dependent NAD+ depletion**. *Nat Microbiol* 2022, **7**:1849–1856.

12. Koopal B, Potocnik A, Mutte SK, Aparicio-Maldonado C, Lindhoud S, Vervoort JJM, Brouns SJJ, Swarts DC: **Short prokaryotic Argonaute systems trigger cell death upon detection of invading DNA**. *Cell* 2022, **185**:1471-1486.e19.

13. Tal N, Morehouse BR, Millman A, Stokar-Avihail A, Avraham C, Fedorenko T, Yirmiya E, Herbst E, Brandis A, Mehlman T, et al.: **Cyclic CMP and cyclic UMP mediate bacterial immunity against phages**. *Cell* 2021, **184**:5728-5739.e16.

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

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

16. Duncan-Lowey B, McNamara-Bordewick NK, Tal N, Sorek R, Kranzusch PJ: **Effector-mediated membrane disruption controls cell death in CBASS antiphage defense**. *Mol Cell* 2021, **81**:5039-5051.e5.

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

18. Zhang T, Tamman H, Coppieters ’t Wallant K, Kurata T, LeRoux M, Srikant S, Brodiazhenko T, Cepauskas A, Talavera A, Martens C, et al.: **Direct activation of a bacterial innate immune system by a viral capsid protein**. *Nature* 2022, **612**:132–140.

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

20. Tal N, Millman A, Stokar-Avihail A, Fedorenko T, Leavitt A, Melamed S, Yirmiya E, Avraham C, Brandis A, Mehlman T, et al.: **Bacteria deplete deoxynucleotides to defend against bacteriophage infection**. *Nat Microbiol* 2022, **7**:1200–1209.

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

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

23. Raleigh EA: **Organization and function of the mcrBC genes of Escherichia coli K-12**. *Mol Microbiol* 1992, **6**:1079–1086.

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

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

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

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

This paper elegantly shows that the fitness advantage provided by defence systems is contingent to the ecological context: defence systems carried by phage satellites, which parasite their helper phages for their own replication, hence imposing a fitness costs to their helpers most of the times, can actually provide fitness advantage to their helper phages when helpers are involved in competition with other phages.

28. Johnson MC, Laderman E, Huiting E, Zhang C, Davidson A, Bondy-Denomy J: **Core defense hotspots within Pseudomonas aeruginosa are a consistent and rich source of anti-phage defense systems**. *Nucleic Acids Res* 2023, **51**:4995–5005.

29. Fillol-Salom A, Rostøl JT, Ojiogu AD, Chen J, Douce G, Humphrey S, Penadés JR: **Bacteriophages benefit from mobilizing pathogenicity islands encoding immune systems against competitors**. *Cell* 2022, **185**:3248-3262.e20.

30. Dedrick RM, Jacobs-Sera D, Bustamante CAG, Garlena RA, Mavrich TN, Pope WH, Reyes JCC, Russell DA, Adair T, Alvey R, et al.: **Prophage-mediated defence against viral attack and viral counter-defence**. *Nat Microbiol* 2017, **2**:16251.

31. Maestri A, Pursey E, Chong C, Pons BJ, Gandon S, Custodio R, Chisnall M, Grasso A, Paterson S, Baker K, et al.: **Bacterial defences interact synergistically by disrupting phage cooperation**. 2023, doi:10.1101/2023.03.30.534895.

32. Picton DM, Luyten YA, Morgan RD, Nelson A, Smith DL, Dryden DTF, Hinton JCD, Blower TR: **The phage defence island of a multidrug resistant plasmid uses both BREX and type IV restriction for complementary protection from viruses**. *Nucleic Acids Res* 2021, **49**:11257–11273.

33. Wein T, Sorek R: **Bacterial origins of human cell-autonomous innate immune mechanisms**. *Nat Rev Immunol* 2022, **22**:629–638.

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

35. Novick RP, Ram G: **The Floating (Pathogenicity) Island: A Genomic Dessert**. *Trends Genet* 2016, **32**:114–126.

36. Patel PH, Maxwell KL: **Prophages provide a rich source of antiphage defense systems**. *Current Opinion in Microbiology* 2023, **73**:102321.

37. Hochhauser D, Millman A, Sorek R: **The defense island repertoire of the Escherichia coli pan-genome**. *PLoS Genet* 2023, **19**:e1010694.

38. Mayo-Muñoz D, Pinilla-Redondo R, Birkholz N, Fineran PC: **A host of armor: Prokaryotic immune strategies against mobile genetic elements**. *Cell Rep* 2023, **42**:112672.

39. Georjon H, Bernheim A: **The highly diverse antiphage defence systems of bacteria**. *Nat Rev Microbiol* 2023, **21**:686–700.

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

This paper introduces the bioinformatic tool DefenseFinder, which screens prokaryotic genomes for DSs. Together with PADLOC these tools are widely used to describe DSs distribution and co-occurrences across microbial genomes.

41. Millman A, Melamed S, Leavitt A, Doron S, Bernheim A, Hör J, Garb J, Bechon N, Brandis A, Lopatina A, et al.: **An expanded arsenal of immune systems that protect bacteria from phages**. *Cell Host & Microbe* 2022, **30**:1556-1569.e5.

42. Wu Y, Hurk A van den, Aparicio-Maldonado C, Kushwaha SK, King CM, Ou Y, Todeschini TC, Clokie MRJ, Millard AD, Gençay YE, et al.: **Defence systems provide synergistic anti-phage activity in E. coli**. 2022, doi:10.1101/2022.08.21.504612. *

A first comprehensive study of DSs present in *E. coli* which identifies patterns of co-occurrence and mutual exclusion. A synergistic anti-phage activity was demonstrated for several pairs of co-occurring DSs.

43. Costa AR, Berg DF van den, Esser JQ, Muralidharan A, Bossche H van den, Bonilla BE, Steen BA van der, Haagsma AC, Fluit AC, Nobrega FL, et al.: **Accumulation of defense systems in phage resistant strains of Pseudomonas aeruginosa**. 2023, doi:10.1101/2022.08.12.503731. *

Here, Costa *et al.* analyse a panel of *Pseudomonas aeruginosa* clinical isolates and shows that a set of DSs largely determines phages resistance pattern. The strains carrying a larger number of defence systems demonstrate a higher phage resistance.

44. Partridge SR, Kwong SM, Firth N, Jensen SO: **Mobile genetic elements associated with antimicrobial resistance**. *Clin Microbiol Rev* 2018, **31**:e00088-17.

45. Dupuis M-È, Villion M, Magadán AH, Moineau S: **CRISPR-Cas and restriction-modification systems are compatible and increase phage resistance**. *Nat Commun* 2013, **4**:2087.

46. Maguin P, Varble A, Modell JW, Marraffini LA: **Cleavage of viral DNA by restriction endonucleases stimulates the type II CRISPR-Cas immune response**. *Mol Cell* 2022, **82**:907-919.e7. *

Maguin and co-authors demonstrate one of the first examples of synergy between DSs. The results show that viral DNA cleavage by RM promotes spacer acquisition by CRISPR-Cas naturally co-occurring in the *Staphylococcus aureus* strain, which leads to higher protection level.

47. Domingo-Sananes MR, McInerney JO: **Mechanisms that shape microbial pangenomes**. *Trends Microbiol* 2021, **29**:493–503.

48. McInerney JO, McNally A, O'Connell MJ: **Why prokaryotes have pangenomes**. *Nat Microbiol* 2017, **2**:17040.

49. Weinberger AD, Wolf YI, Lobkovsky AE, Gilmore MS, Koonin EV: **Viral diversity threshold for adaptive immunity in prokaryotes**. *mBio* 2012, **3**:e00456-00412.

50. Gurney J, Pleška M, Levin BR: **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 Lond B Biol Sci* 2019, **374**:20180096.

51. Westra ER, Levin BR: **It is unclear how important CRISPR-Cas systems are for protecting natural populations of bacteria against infections by mobile genetic elements**. *Proc Natl Acad Sci U S A* 2020, **117**:27777–27785.

52. Westra ER, van Houte S, Oyesiku-Blakemore S, Makin B, Broniewski JM, Best A, Bondy-Denomy J, Davidson A, Boots M, Buckling A: **Parasite exposure drives selective evolution of constitutive versus inducible defense**. *Curr Biol* 2015, **25**:1043–1049.

53. Zaayman M, Wheatley RM: **Fitness costs of CRISPR-Cas systems in bacteria**. *Microbiology (Reading)* 2022, **168**.

54. Vale PF, Lafforgue G, Gatchitch F, Gardan R, Moineau S, Gandon S: **Costs of CRISPR-Cas-mediated resistance in Streptococcus thermophilus**. *Proc Biol Sci* 2015, **282**:20151270.

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

56. Bernheim A, Calvo-Villamañán A, Basier C, Cui L, Rocha EPC, Touchon M, Bikard D: **Inhibition of NHEJ repair by type II-A CRISPR-Cas systems in bacteria**. *Nat Commun* 2017, **8**:2094.

57. Birkholz N, Jackson SA, Fagerlund RD, Fineran PC: **A mobile restriction-modification system provides phage defence and resolves an epigenetic conflict with an antagonistic endonuclease**. *Nucleic Acids Res* 2022, **50**:3348–3361.

58. Liu Z-L, Hu E-Z, Niu D-K: **Investigating the relationship between CRISPR-Cas content and growth rate in bacteria**. *Microbiol Spectr* 2023, **11**:e0340922.

59. Heitman J, Model P: **Site-specific methylases induce the SOS DNA repair response in Escherichia coli.** *J Bacteriol* 1987, **169**:3243–3250.

60. Hynes AP, Villion M, Moineau S: **Adaptation in bacterial CRISPR-Cas immunity can be driven by defective phages**. *Nat Commun* 2014, **5**:4399.

61. Meeske AJ, Nakandakari-Higa S, Marraffini LA: **Cas13-induced cellular dormancy prevents the rise of CRISPR-resistant bacteriophage**. *Nature* 2019, **570**:241–245.

62. Williams MC, Reker AE, Margolis SR, Liao J, Wiedmann M, Rojas ER, Meeske AJ: **Restriction endonuclease cleavage of phage DNA enables resuscitation from Cas13-induced bacterial dormancy**. *Nat Microbiol* 2023, **8**:400–409.

63. Arias CF, Acosta FJ, Bertocchini F, Herrero MA, Fernández-Arias C: **The coordination of anti-phage immunity mechanisms in bacterial cells**. *Nat Commun* 2022, **13**:7412.

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

65. Panas MW, Jain P, Yang H, Mitra S, Biswas D, Wattam AR, Letvin NL, Jacobs WR: **Noncanonical SMC protein in Mycobacterium smegmatis restricts maintenance of Mycobacterium fortuitum plasmids**. *Proc Natl Acad Sci U S A* 2014, **111**:13264–13271.

66. Liu HW, Roisné-Hamelin F, Beckert B, Li Y, Myasnikov A, Gruber S: **DNA-measuring Wadjet SMC ATPases restrict smaller circular plasmids by DNA cleavage**. *Mol Cell* 2022, **82**:4727-4740.e6.

67. Stokar-Avihail A, Fedorenko T, Hör J, Garb J, Leavitt A, Millman A, Shulman G, Wojtania N, Melamed S, Amitai G, et al.: **Discovery of phage determinants that confer sensitivity to bacterial immune systems**. *Cell* 2023, **186**:1863-1876.e16.

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

69. de Freitas Almeida GM, Hoikkala V, Ravantti J, Rantanen N, Sundberg L-R: **Mucin induces CRISPR-Cas defense in an opportunistic pathogen**. *Nat Commun* 2022, **13**:3653.

70. Chevallereau A, Pons BJ, van Houte S, Westra ER: **Interactions between bacterial and phage communities in natural environments**. *Nat Rev Microbiol* 2022, **20**:49–62.

71. Smith LM, Jackson SA, Malone LM, Ussher JE, Gardner PP, Fineran PC: **The Rcs stress response inversely controls surface and CRISPR-Cas adaptive immunity to discriminate plasmids and phages**. *Nat Microbiol* 2021, **6**:162–172.

72. van Houte S, Ekroth AKE, Broniewski JM, Chabas H, Ashby B, Bondy-Denomy J, Gandon S, Boots M, Paterson S, Buckling A, et al.: **The diversity-generating benefits of a prokaryotic adaptive immune system**. *Nature* 2016, **532**:385–388.

73. Srikant S, Guegler CK, Laub MT: **The evolution of a counter-defense mechanism in a virus constrains its host range**. *Elife* 2022, **11**:e79549.

74. Somerville V, Schowing T, Chabas H, Schmidt RS, von Ah U, Bruggmann R, Engel P: **Extensive diversity and rapid turnover of phage defense repertoires in cheese-associated bacterial communities**. *Microbiome* 2022, **10**:137.

75. Owen SV, Wenner N, Dulberger CL, Rodwell EV, Bowers-Barnard A, Quinones-Olvera N, Rigden DJ, Rubin EJ, Garner EC, Baym M, et al.: **Prophages encode phage-defense systems with cognate self-immunity**. *Cell Host Microbe* 2021, **29**:1620-1633.e8.

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

77. Tesson F, Bernheim A: **Synergy and regulation of antiphage systems: toward the existence of a bacterial immune system?** *Curr Opin Microbiol* 2023, **71**:102238.

78. Patterson AG, Jackson SA, Taylor C, Evans GB, Salmond GPC, Przybilski R, Staals RHJ, Fineran PC: **Quorum Sensing Controls Adaptive Immunity through the Regulation of Multiple CRISPR-Cas Systems**. *Mol Cell* 2016, **64**:1102–1108.

79. O'Hara BJ, Alam M, Ng W-L: **The Vibrio cholerae Seventh Pandemic Islands act in tandem to defend against a circulating phage**. *PLoS Genet* 2022, **18**:e1010250.

80. Borges AL, Castro B, Govindarajan S, Solvik T, Escalante V, Bondy-Denomy J: **Bacterial alginate regulators and phage homologs repress CRISPR-Cas immunity**. *Nat Microbiol* 2020, **5**:679–687.

81. Lucas-Elío P, Molina-Quintero LR, Xu H, Sánchez-Amat A: **A histidine kinase and a response regulator provide phage resistance to Marinomonas mediterranea via CRISPR-Cas regulation**. *Sci Rep* 2021, **11**:20564.

82. Patterson AG, Yevstigneyeva MS, Fineran PC: **Regulation of CRISPR-Cas adaptive immune systems**. *Curr Opin Microbiol* 2017, **37**:1–7.

83. Campa AR, Smith LM, Hampton HG, Sharma S, Jackson SA, Bischler T, Sharma CM, Fineran PC: **The Rsm (Csr) post-transcriptional regulatory pathway coordinately controls multiple CRISPR-Cas immune systems**. *Nucleic Acids Res* 2021, **49**:9508–9525.

84. Picton DM, Harling-Lee JD, Duffner SJ, Went SC, Morgan RD, Hinton JCD, Blower TR: **A widespread family of WYL-domain transcriptional regulators co-localizes with diverse phage defence systems and islands**. *Nucleic Acids Res* 2022, **50**:5191–5207. *

This study determined the structure and a mode of action of a transcriptional repressor BrxR, involved in regulation of BREX system. The paper also shows that BrxR is a member of a large family widespread across prokaryotes and associated with defence islands. This is the first example of a transcriptional factor specialised in regulation of DSs.

85. Luyten YA, Hausman DE, Young JC, Doyle LA, Higashi KM, Ubilla-Rodriguez NC, Lambert AR, Arroyo CS, Forsberg KJ, Morgan RD, et al.: **Identification and characterization of the WYL BrxR protein and its gene as separable regulatory elements of a BREX phage restriction system**. *Nucleic Acids Res* 2022, **50**:5171–5190.

86. Blankenchip CL, Nguyen JV, Lau RK, Ye Q, Gu Y, Corbett KD: **Control of bacterial immune signaling by a WYL domain transcription factor**. *Nucleic Acids Res* 2022, **50**:5239–5250.

87. Keller LM, Weber-Ban E: **An emerging class of nucleic acid-sensing regulators in bacteria: WYL domain-containing proteins**. *Curr Opin Microbiol* 2023, **74**:102296.

88. Banh DV, Roberts CG, Amador AM, Brady SF, Marraffini LA: **Bacterial cGAS senses a viral RNA to initiate immunity**. 2023, doi:10.1101/2023.03.07.531596.

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

This paper introduces the bioinformatic tool PADLOC, which searches for defence systems in prokaryotic genomes. Together with DefenseFinder these tools are widely used to describe DSs distribution and co-occurrences across microbial genomes.

90. Huiting E, Cao X, Ren J, Athukoralage JS, Luo Z, Silas S, An N, Carion H, Zhou Y, Fraser JS, et al.: **Bacteriophages inhibit and evade cGAS-like immune function in bacteria**. *Cell* 2023, **186**:864-876.e21.

91. Klaiman D, Steinfels-Kohn E, Krutkina E, Davidov E, Kaufmann G: **The wobble nucleotide-excising anticodon nuclease RloC is governed by the zinc-hook and DNA-dependent ATPase of its Rad50-like region**. *Nucleic Acids Res* 2012, **40**:8568–8578.

92. Hsueh BY, Severin GB, Elg CA, Waldron EJ, Kant A, Wessel AJ, Dover JA, Rhoades CR, Ridenhour BJ, Parent KN, et al.: **Phage defence by deaminase-mediated depletion of deoxynucleotides in bacteria**. *Nat Microbiol* 2022, **7**:1210–1220.

93. Piel D, Bruto M, Labreuche Y, Blanquart F, Goudenège D, Barcia-Cruz R, Chenivesse S, Le Panse S, James A, Dubert J, et al.: **Phage-host coevolution in natural populations**. *Nat Microbiol* 2022, **7**:1075–1086.

94. Landsberger M, Gandon S, Meaden S, Rollie C, Chevallereau A, Chabas H, Buckling A, Westra ER, van Houte S: **Anti-CRISPR phages cooperate to overcome CRISPR-Cas immunity**. *Cell* 2018, **174**:908-916.e12.

95. Borges AL, Zhang JY, Rollins MF, Osuna BA, Wiedenheft B, Bondy-Denomy J: **Bacteriophage cooperation suppresses CRISPR-Cas3 and Cas9 immunity**. *Cell* 2018, **174**:917-925.e10.

96. Vassallo CN, Doering CR, Littlehale ML, Teodoro GIC, Laub MT: **A functional selection reveals previously undetected anti-phage defence systems in the E. coli pangenome**. *Nat Microbiol* 2022, **7**:1568–1579.

97. León LM, Park AE, Borges AL, Zhang JY, Bondy-Denomy J: **Mobile element warfare via CRISPR and anti-CRISPR in Pseudomonas aeruginosa**. *Nucleic Acids Res* 2021, **49**:2114–2125.

98. Hussain FA, Dubert J, Elsherbini J, Murphy M, VanInsberghe D, Arevalo P, Kauffman K, Rodino-Janeiro BK, Gavin H, Gomez A, et al.: **Rapid evolutionary turnover of mobile genetic elements drives bacterial resistance to phages**. *Science* 2021, **374**:488–492.

99. Mariano G, Blower TR: **Conserved domains can be found across distinct phage defence systems**. *Mol Microbiol* 2023, **120**:45–53.

100. Anton BP, Roberts RJ: **Beyond restriction modification: epigenomic roles of DNA methylation in prokaryotes**. *Annu Rev Microbiol* 2021, **75**:129–149.

101. Chi H, Hoikkala V, Grüschow S, Graham S, Shirran S, White MF: **Antiviral type III CRISPR signalling via conjugation of ATP and SAM**. *Nature* 2023, **622**:826–833.

102. Zaremba M, Dakineviciene D, Golovinas E, Zagorskaitė E, Stankunas E, Lopatina A, Sorek R, Manakova E, Ruksenaite A, Silanskas A, et al.: **Short prokaryotic Argonautes provide defence against incoming mobile genetic elements through NAD+ depletion**. *Nat Microbiol* 2022, **7**:1857–1869.

103. Madsen A, Westphal C, Josephsen J: **Characterization of a novel plasmid-encoded HsdS subunit, S.LlaW12I, from Lactococcus lactis W12**. *Plasmid* 2000, **44**:196–200.

104. De Ste Croix M, Vacca I, Kwun MJ, Ralph JD, Bentley SD, Haigh R, Croucher NJ, Oggioni MR: **Phase-variable methylation and epigenetic regulation by type I restriction-modification systems**. *FEMS Microbiol Rev* 2017, **41**:S3–S15.

105. Koonin EV, Makarova KS: **Origins and evolution of CRISPR-Cas systems**. *Philos Trans R Soc Lond B Biol Sci* 2019, **374**:20180087.

Figure 1. Overview of multi-layered defence. The three main modes of action of DSs are shown in the frames: targeting viral nucleic acids, abortive infection or dormancy, inhibition of phage by small molecules. A combination of diverse DSs protects the host from wide range of MGEs. Environmental factors such as presence of nutrients or antibiotics favour certain types of DSs. Created with BioRender.com.

Figure 2. Mechanisms of transcriptional and post-translational regulation of DS. Mechanisms of regulation are shown in bold text. Defence systems are shown in grey italicised text and regulation mechanisms which activate respective systems are indicated by grey dashed arrows. Created with BioRender.com.

Figure 3. Mobility of defence systems and modules. Left: Acquisition of new systems from MGE's in plasmids and prophages provides new defence diversity, whilst module switching between systems develops variability. Right: A small selection of shared domains have been highlighted between different DS's. Many DS's share similar domains, demonstrating the versatility of this module exchange, and some systems have very diverse variants. For example, CBASS and PYCSAR are known to utilise a conserved sensor domain linked to variable effectors, such as REases or NADases. Some domains have also adapted their target to fit different systems, an example of this are nucleases. MGE-targeted nucleases target the invading DNA whilst protecting the self, whereas host-targeted nucleases often lead to abortive infection or growth arrest by targeting the host DNA or RNA. Created with BioRender.com.