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Multi-layered genome defences in bacteria

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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 (DSs) have been discovered. Notably, diverse DSs 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 DSs at the mechanistic, regulatory, ecological and evolutionary levels.

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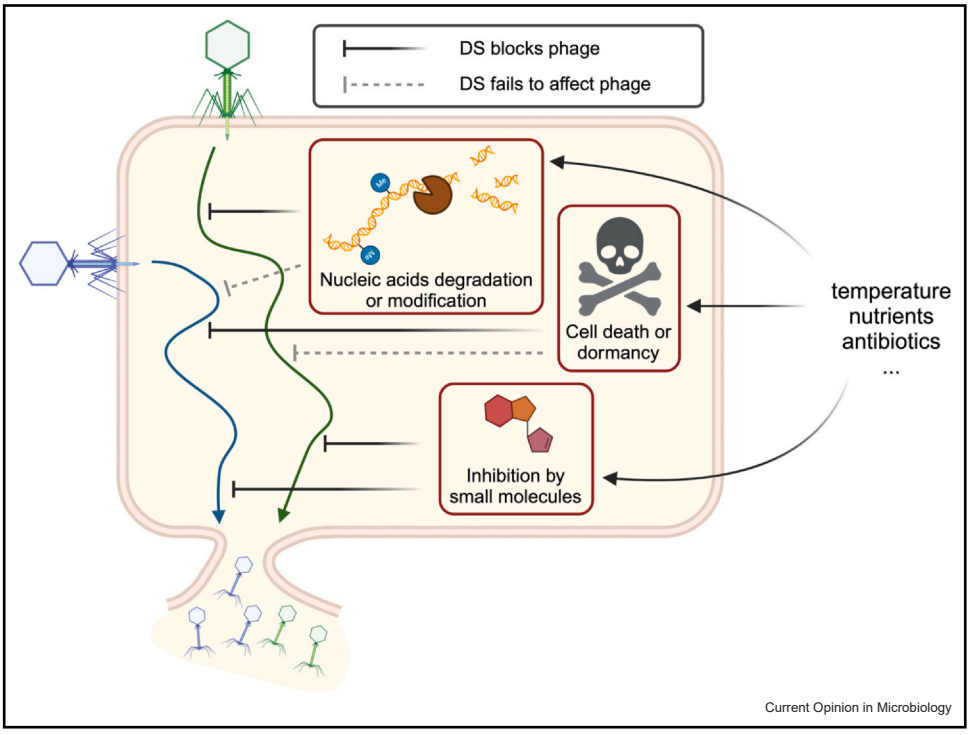
Introduction

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

Figure 1



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

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 such as 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 adenosine triphosphate [10] and Nicotinamide adenine dinucleotide [9,11–13], disruption of the bacterial membrane [14–17] or inhibition of translation [18]. Finally, DSs such as 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, 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/Bacteriophage Exclusion (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 DS 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 such as growth and reproduction [58]. In addition, there may be genetic conflict between the different DSs that coexist 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 coexistence 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 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 bypass 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 optimised 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 the 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 DS activity may minimise costs and maximise 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].

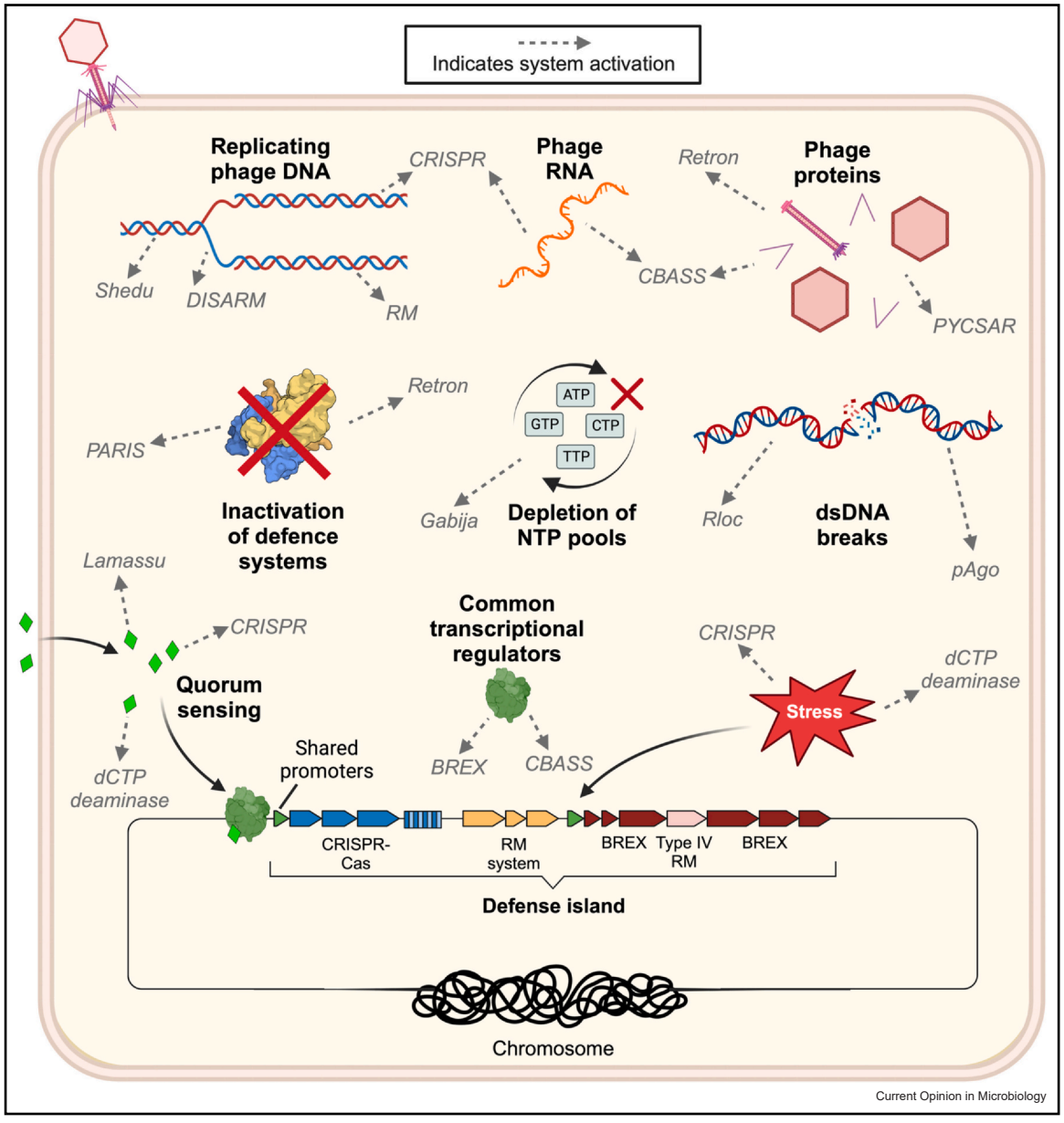
Multiple global inputs have been demonstrated to regulate defence responses [77]. If cell density is very high, a population might be particularly vulnerable to phages. In turn, quorum sensing, used to monitor population density, has been shown to regulate multiple defences, including CRISPR–Cas [78], Deoxycytidine triphosphate deaminase and Lamassu [79] at the transcriptional level (Figure 2). Stress responses and cell metabolic status also regulate defence, by 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 domain name, not an abbreviation-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 domain name, not an abbreviation 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 defense island system associated with Restriction Modification 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 Cyclic oligonucleotide-Based Anti-phage Signaling System (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 (e.g. by pyrimidine cyclase system for antiphage resistance [13] and CBASS [90]), or detection of phage anti-defence proteins, such as Ocr by the PARIS DS [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

Figure 2



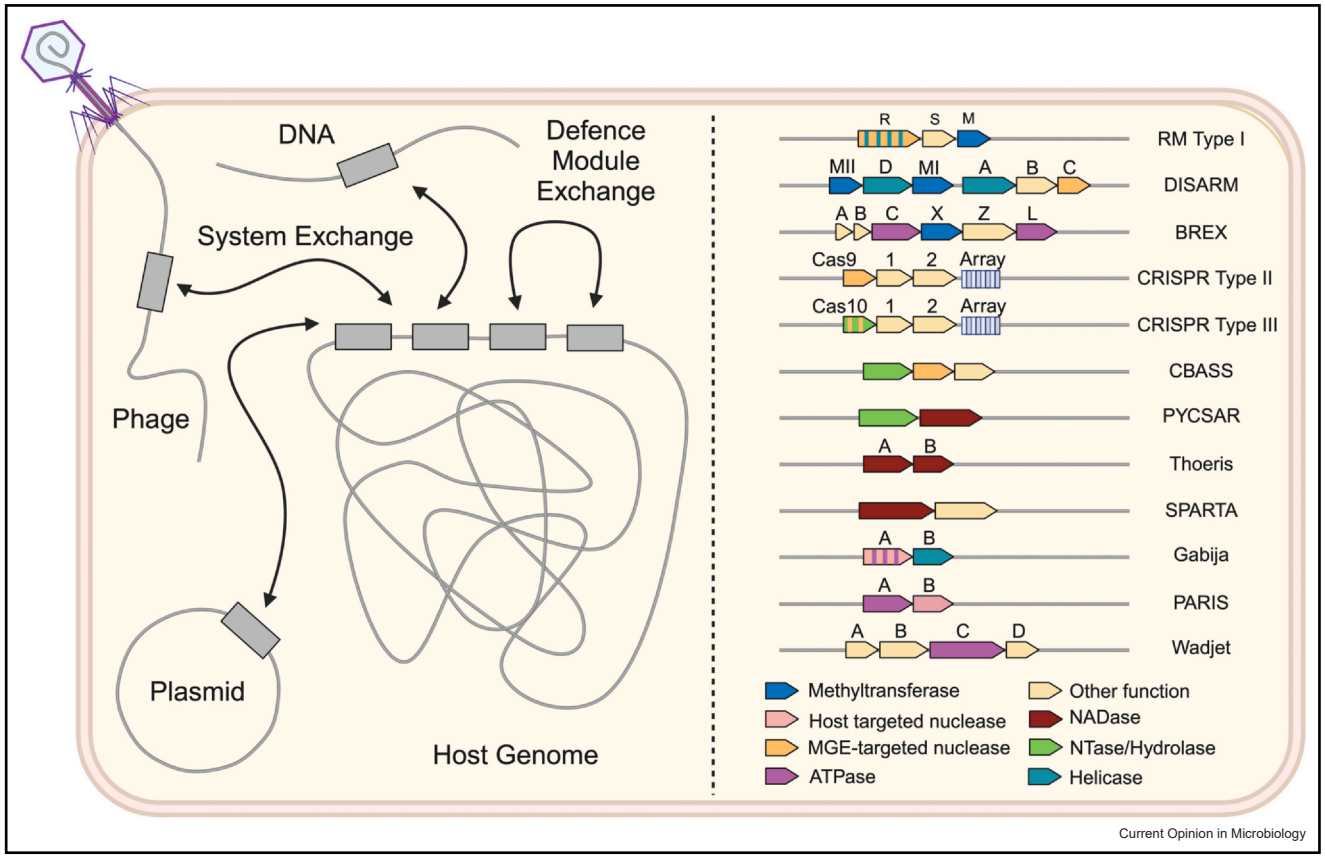
Mechanisms of transcriptional and post-translational regulation of DS. Mechanisms of regulation are shown in bold text. DSs are shown in grey italicised text and regulation mechanisms that activate respective systems are indicated by grey dashed arrows. Created with BioRender.com.

that 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 Deoxycytidine triphosphate 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 adenosine triphosphate 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,

Figure 3



Mobility of DSs and modules. Left: Acquisition of new systems from MGEs in plasmids and prophages provides new defence diversity, whilst modules switching between systems develop variability. Right: A small selection of shared domains have been highlighted between different DSs. Many DSs 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 Abi or growth arrest by targeting the host DNA or RNA. Created with BioRender.com. CBASS, Cyclic oligonucleotide-Based Anti-phage Signaling System. PYCSAR. pyrimidine cyclase system for antiphage resistance.

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 encodes an anti-CRISPR (*acr*) counter-defence gene showed that bacteria that carry both MADS and clustered regularly interspaced short palindromic repeats (CRISPR)-Cas (CRISPR-associated proteins) immune systems were less susceptible to the emergence and spread of phages that overcome Methylation Associated Defence System (MADS), compared with bacteria that carry only MADS [31]. Synergy between MADS and CRISPR–Cas emerged in this case because the ancestral Acr-encoding phages 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 reshuffled 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 (Figure 3).

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 (Figure 3). For example, nucleases cleave DNA or RNA, which can cause cell death if the chromosome is targeted. DNA methyltransferases can provide protection against such autoimmunity, 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. nucleoside triphosphatase, deaminases and cyclases modulate nucleotide pools to deplete resources or signal infection, while Toll/interleukin-1 receptor and Silent information regulator- 2 (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 DSs, 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.

Data Availability

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

Declaration of Competing Interest

AM and EW are inventors on patent GB2303034.9.

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This paper elegantly shows that the fitness advantage provided by DSs is contingent to the ecological context: DSs 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.

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