The pan-immune system of bacteria

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The pan-immune system of bacteria: anti-phage defense as a community resource

Aude Bernheim and Rotem Sorek

Department of Molecular Genetics, Weizmann Institute of Science, Rehovot 76100, Israel

Abstract:

Prokaryotes and their viruses are engaged in a constant arms race leading to the development of anti-phage defense mechanisms. Recent studies have revealed that the immune arsenal of bacteria and archaea is much more diverse than previously envisioned. These discoveries have led to seemingly contradictory observations: on one hand, individual microbes often encode multiple distinct defense systems, some of which are acquired by horizontal gene transfer, alluding to their fitness benefit. Conversely, defense systems are frequently lost from prokaryotic genomes on short evolutionary time scales, suggesting that they impose a fitness cost. Here, we present the "pan immune system" model, in which we suggest that while a single strain cannot carry all possible defense systems due to their burden, it can employ horizontal gene transfer to access immune mechanisms encoded by closely related strains. Thus, the "effective" prokaryotic immune system is not the one encoded by the genome of a single microbe but rather by its pan genome, comprising the sum of all immune systems available for a microbe to horizontally acquire and use.

Bacteriophages (phages), which are viruses that infect prokaryotes, are the most abundant viruses on the planet. The majority of free living prokaryotic species are thought to be infected by phages, as evidenced by the widespread presence of prophages (dormant phages) in sequenced bacterial and archaeal genomes[1,2]. It was estimated that phages developed shortly after the emergence of bacteria billions of years ago[3], and hence the arms race between prokaryotes and phages is considered almost as old as bacteria themselves.

Facing the abundance and diversity of phages, bacteria and archaea have developed multiple lines of defense that can collectively be referred to as the prokaryotic "immune system". Early research on prokaryotic defense systems mainly focused on restriction-modification (R-M) and abortive infection systems, while in the past decade the focus shifted to CRISPR-Cas systems. In recent years it has been recognized that prokaryotic immunity is much more complex than previously perceived, with evidence for chemical defense[4], intracellular signaling regulating defense outcome[5,6] , as well as the discovery of a large number of new defense systems whose mechanisms are still unknown[7] .

Individual bacterial and archaeal species can encode multiple different defense systems, and it was shown that such systems can be horizontally acquired and lost on short evolutionary time scales[8,9]. In this Perspective we discuss the immune system of prokaryotes from evolutionary and ecological points of view. We begin by reviewing the major types of known anti-phage systems as well as evasion strategies employed by phages (topics that will be covered only briefly as they were recently reviewed elsewhere[9–14]). We then discuss the necessity for encoding several lines of defense on one hand, and the burdens of anti-phage defense systems on the other hand, leading to rapid gain and loss of such systems in microbial genomes. We present the "pan-immune system" model to explain why closely related species encode different sets of defense systems, and conclude by discussing the implications on the evolution of anti-defense strategies in phages.

Diverse anti-phage defense systems encoded by bacteria

Anti-phage defense systems can roughly be divided into those that target phage nucleic acids (e.g., R-M, CRISPR-Cas), abortive infection systems that lead the microbe to commit suicide once infected, and other types of systems (Figure 1). Of these, the most abundant and elaborate systems are those that target nucleic acids[15–17], presumably because nucleic acid is usually the first phage component to penetrate the bacterial cell upon infection (Figure 1a-b).

R-M (restriction modification) collectively refers to systems that cleave or degrade DNA through recognition of specific sequence motifs on the phage genome. These sequence motifs are modified in the host self DNA, usually by methylation, to prevent the host genome from being targeted (with the exception of type IV R-M systems, which target modified phage DNA while the host genome remains unaltered). R-M systems are classified into four types[18] and are present in more than 74% of prokaryotic genomes[15]. On average, a genome encodes two R-M systems[15]. DNA modification as a strategy to discriminate between self and non-self DNA is not limited to methylation. For example, the *dnd* defense system modifies the host DNA backbone to include a sulfur group[19], and the *dpd* system utilizes a multi-enzyme pathway to modify guanine residues into 7-deazaguanine derivatives in the host DNA[20]. The BREX[21] (Bacteriophage Exclusion) and DISARM[22] (Defense Islands System Associated with R-M) systems also function through methylation of host DNA, although the mechanisms of phage DNA targeting in these systems are still unknown. All of these defense systems constitute part of the innate immunity of prokaryotes.

A large fraction of bacteria and archaea encode CRISPR-Cas[17], a family of adaptive immune systems that also function through recognition and degradation of phage nucleic acids. The CRISPR-Cas immune

memory is formed through acquisition of short phage-derived DNA sequences that are incorporated as CRISPR "spacers" within the host genome[23]. These sequences are then transcribed and processed into CRISPR RNAs (crRNAs) that guide the CRISPR machinery , through sequence complementarity, to target the phage nucleic acids[17]. CRISPR-Cas systems are diverse, comprising of two classes, six types and more than 20 subtypes[24,25] that differ in the composition of the interference machinery, their mechanisms of targeting and the nucleic acid targeted (i.e., DNA or RNA). In most cases, both spacer acquisition and interference necessitate the occurrence of a short sequence motif named PAM (Protospacer Adjacent Motif) next to the sequence matched by the spacer in the targeted molecule [26].

Operons that include prokaryotic argonautes (pAgos) have also been hypothesized to provide defense. Present in 9% and 32% of bacterial and archaeal genomes, respectively [27], their frequent localization in defense islands (regions in the bacterial genome in which defense systems are concentrated, see Box 1) as well as their protective activity against plasmids[28], suggest that they are involved in anti-phage defense.

Another common strategy of defense against phages is abortive infection (Abi). Abi systems allow the bacterial cell, once infected, to kill itself or to arrest its metabolism before the phage reproductive cycle is completed, thus preventing the phage from spreading and killing the surrounding bacterial community. Abi systems have been detected in a wide variety of organisms[8], but given their high diversity, it is challenging to assess their abundance in nature. These systems are usually triggered by a specific component that could be a phage protein, nucleic acid, or a cellular state caused by phage infection. For example, the *E. coli* Lit Abi is activated upon sensing a unique substrate formed by the Gol peptide of phage T4 when bound to the ribosomal elongation factor EF-Tu. Once active, the Lit protein cleaves EF-Tu thus inhibiting translation and ultimately killing the cell[29]. Another example is the PrrC gene in *E. coli*, which cleaves bacterial $tRNA^{Lys}$ molecules when it senses that the phage suppresses bacterial R-M systems[30]. In *Lactococci*, many Abi genes (around 20) have been described: For example AbiZ accelerates lysis before phage assembly[31] while AbiB leads to non-specific degradation of mRNAs[32]. In *Staphylococci*[33], the serine threonine kinase STK2 protein is activated when exposed to the phage protein PacK, leading to phosphorylation of proteins involved in multiple cellular pathways and eventual cell death[33]. Toxin-antitoxin systems, representing a large family of two-gene modules each comprising a toxin and an immunity component, were also shown to execute Abi in some cases, although their general role in defense against phages is still disputed[10,34].

Recent studies have revealed the existence of many additional families of anti-phage defense systems in prokaryotes. An effort to map microbial defense islands (Box 1) has resulted in the discovery of nine new defense systems that are widespread in bacterial and archaeal genomes[7]. These systems were named after protective deities from world mythologies including Hachiman, Thoeris, Zorya, Gabija and Shedu, and their molecular mechanisms of action are yet to be deciphered. Finally, species of *Streptomyces* produce small molecules called Doxorubicin and Daunorubicin that act as DNA intercalants, and were recently shown to specifically block phage DNA replication but not the replication of bacterial DNA[4].

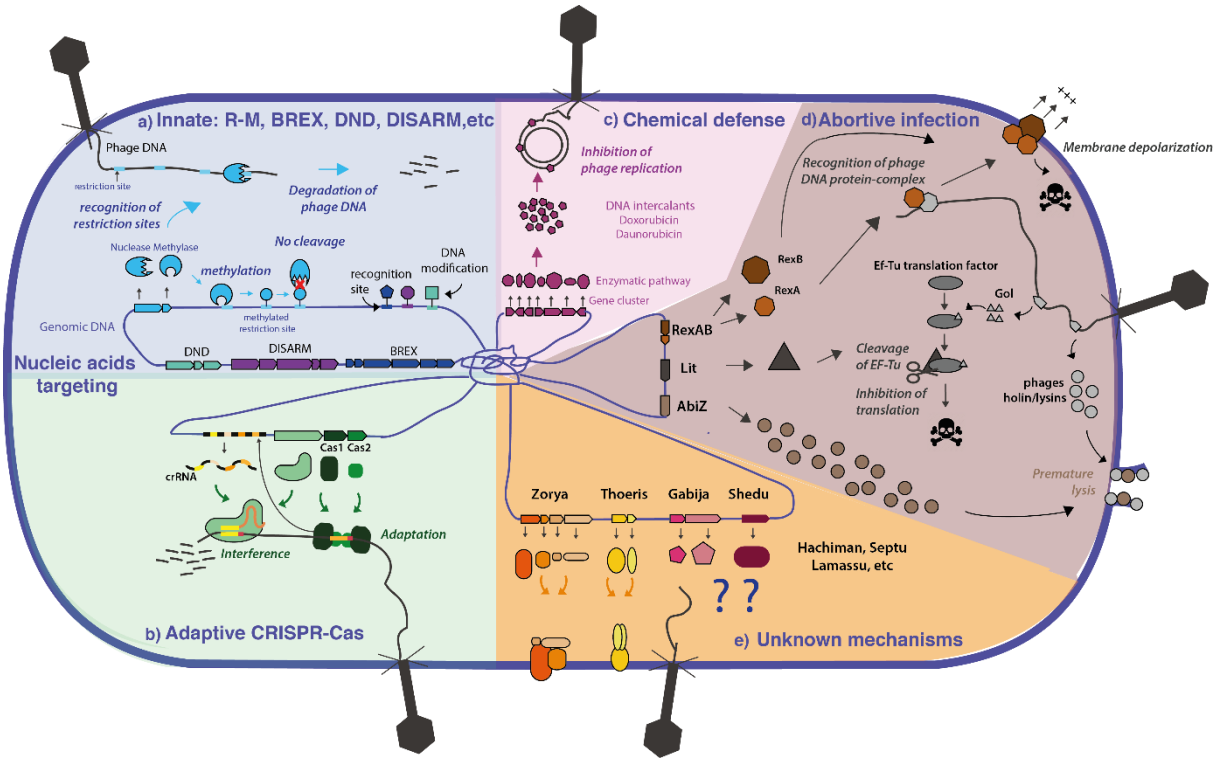

Figure 1: Anti-phage defense systems

(a-b) Defense systems that target nucleic acids encompass both innate and adaptive immunity. (a) Restriction modification (R-M) and other related systems modify specific sequence motifs in the host genome and cleave or degrade unmodified foreign DNA. (b) CRISPR-Cas systems work in two main phases: adaptation, where a complex of Cas proteins guides the acquisition of new phage-derived spacers; and interference, where Cas proteins complexed with a spacer-derived RNA (crRNA) target and degrade phage nucleic acids. (c) Chemical defense has been described in *Streptomyces*, in which bacteria produce a small anti-phage molecule that intercalates into phage DNA and inhibits its replication. (d) Abortive infection mechanisms are diverse. The *E. coli* protein RexA recognizes a specific DNA-protein complex formed by the Lambda phage, and activates RexB, an ion channel that depolarizes the membrane leading to cell death. Upon expression of the T4 phage protein Gol, the *E. coli* Lit protein inhibits translation through cleavage of the Ef-Tu elongation factor. In concert with phage-encoded holins and lysins of phage Phi31, AbiZ from *Lactococcus lactis* accelerates lysis before phage assembly is completed. (e) Multiple systems have recently been demonstrated to hold anti-phage roles, but their mechanisms remain unknown.

<u>Why do microbes need multiple defense systems?</u>

Analysis of sequenced prokaryotic genomes demonstrates that they can concomitantly harbor multiple different defense systems. As shown in Figure 2, a single strain can encode diverse defense strategies mixing Abi, R-M and CRISPR-Cas. Many bacteria and archaea encode multiple defense systems of the same kind: for example, *Helicobacter pylori* F30 encodes three type I R-M, eleven type II R-M, one type III R-M and one type IV R-M systems[15]. In total, it was estimated that up to 10% of some prokaryotic genomes is dedicated to defense systems[8]. These observations raise a basic question – what is the benefit for a single microbe to encode so many different lines of defense?

One obvious answer is that some defense systems can protect only from a specific type of phage. For example, the GmrSD type IV R-M system only targets phages such as T4, whose genomes are modified to include glucosylated hydroxymethylcytosine[35]. Cas9, on the other hand, cannot cleave the DNA of phage T4 due to its heavily modified cytosine residues [36]. The Thoeris defense system seems to protect only against phages from the Myoviridae family[7]. Therefore, for a microbe to be protected against a wide variety of phage types, it should encode a broad defense arsenal that can overcome the multiple types of phages that can infect it.

There are benefits for a prokaryote to encode multiple defense systems even if these systems overlap in the range of phages that they target. This is because phages can develop resistance to defense (reviewed in [12–14,18]). First, phage genomes can evolve to eliminate specific sequences such as motifs targeted by restriction enzymes[18] or PAM sequences that are essential for CRISPR-Cas defense[37]. Secondly, phages often encode anti-defense proteins[12], including anti-CRISPR and anti-restriction proteins. These proteins are either injected to the cell together with the phage DNA[18], or expressed early upon infection, and inhibit the bacterial defense systems. Anti-CRISPRs are typically short proteins that bind the CRISPR-Cas complex and prevent it from working properly[13,38]. Recent discoveries report on anti-CRISPRs working as enzymes that can cleave the crRNA or add an acetyl group to a PAM-sensing residue in the Cas effector [39,40]. Similarly, anti-restriction proteins inhibit restriction enzymes: for example, the T4 IPI (Internal Protein I) inhibits type IV R-M systems[41], while the DarA/DarB proteins of phage P1 bind the restriction sites on the phage genome and mask them from cleavage by the type I R-M system of *E. coli* [42]. Faced by phages that encode counter-defense mechanisms, bacteria and archaea cannot rely on a single defense system and thus need to present several lines of defense as a bet-hedging strategy of survival.

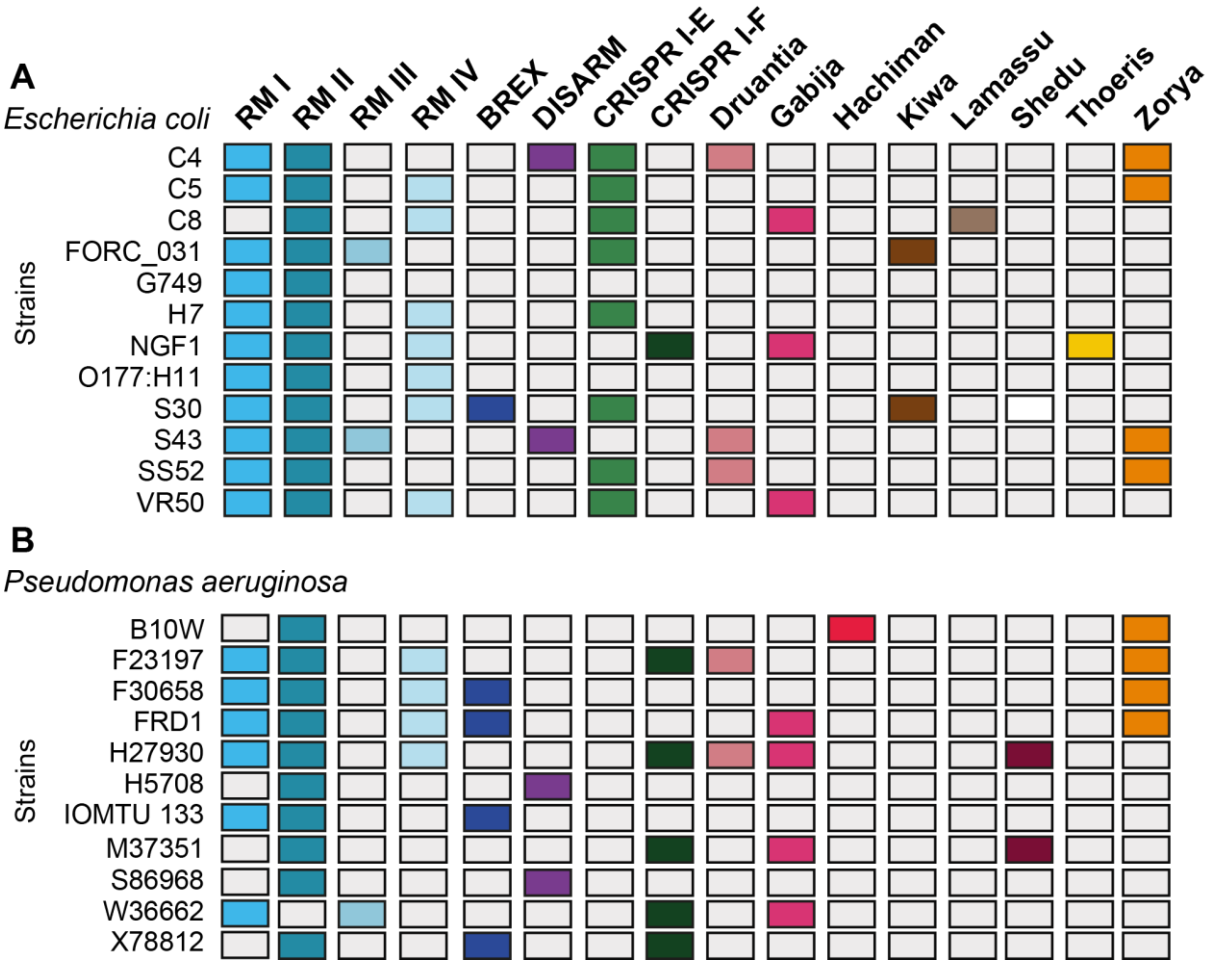

Figure 2: Closely related bacterial strains encode diverse defense systems. Each line represents a different strain of either (A) *E. coli* or (B) *P. aeruginosa.* Each column corresponds to a different defense system (grey=absence, color=presence). CRISPR-Cas systems were detected using CRISPR-Cas Finder[43], R-M systems using HHsearch with HMM profiles from ref [44], BREX by the presence of PglZ gene, and DISARM as described in ref [22]. Other defense systems were detected as described in ref [7].

Rapid gain and loss of defense systems in prokaryotic genomes

Due to the selective advantage that defense systems provide, they are frequently gained by bacteria and archaea through horizontal gene transfer (HGT)[8,9]. Multiple studies based on phylogenetic analyses and comparative genomics have confirmed the high rate of transfer of defense systems[8,15,24,45,46] . For example, only ∼4% of R- M systems are found in the core-genomes of prokaryotic species suggesting recent transfer events [15]. In another example, an analysis of phylogenetic trees of Cas proteins and CRISPR repeats showed weak consistency with the species tree, demonstrating the dominance of horizontal transfer for the spread of CRISPR-Cas loci[24]. Both CRISPR-Cas and R-M systems have been detected on mobile genetic elements such as plasmids, transposons and phages, partially explaining their mode of HGT [15,47–49]. In addition, genomic analyses have shown that defense systems tend to be concentrated in "defense islands" - regions of the prokaryotic chromosome that are also enriched with mobile elements presumably responsible for the genetic mobilization of the islands between bacteria (Box 1)[50].

Given their selective advantage in the arms race against phages, one might expect that defense systems, once acquired (either through direct evolution or via HGT), would accumulate in prokaryotic genomes and be selected for. Surprisingly, this is not the case, as defense systems are known to be frequently lost from microbial genomes over short evolutionary time scales, suggesting that they can impose selective disadvantages in the absence of phage pressure[8,9]. A major drawback of defense systems is autoimmunity: CRISPR-Cas, for example, can make mistakes in the process of spacer acquisition and acquire spacers from the chromosome instead of from the invading phage[51,52] . This directs the CRISPR-Cas interference machinery to attack the chromosome, resulting in cell death [51,53,54], or in survival through pseudogenization and eventual deletion of the CRISPR-Cas locus[51,52,54]. Similarly, R-M systems can also rarely target the chromosome, cleaving self-DNA at a low but measurable rate and inflicting a fitness cost[55]. Unwanted activity of Abi systems can also lead to dormancy or cell death [56]. In addition to autoimmunity, defense systems can also impose an energy burden on the cell: some R-M systems require the consumption of one ATP molecule per base pair for translocation of the restriction enzyme along the DNA [9,57].

As a result of these costs, there is a selective pressure for bacteria and archaea to get rid of defense systems under conditions when there is no selection pressure exerted by phages. Indeed, competition studies between strains encoding defense systems such as CRISPR-Cas or the Lit Abi, and cognate defense-lacking strains have demonstrated the existence of a fitness cost in the absence of phage infection[56,58]. An experimental study in *Staphylococcus epidermidis* showed that the loss of CRISPR-Cas systems by large deletions has little or no fitness cost[59]. Another study demonstrated that inactivation of CRISPR-Cas systems in *S. pneumoniae* is even advantageous under specific conditions[60].

The frequent gain and loss of defense systems over short time scales leads to a highly variable pattern of presence and absence of systems in microbial genomes. Even in closely related strains with otherwise similar genomes, the composition of defense systems can dramatically vary, as demonstrated in Figure 2. Defense systems appear to be in a state of constant genetic flux, constituting the second most dynamic class of genes after mobile genetic elements (MGE) in terms of rates of gain and loss in microbial genomes[61,62].

BOX 1

Defense islands in microbial genomes

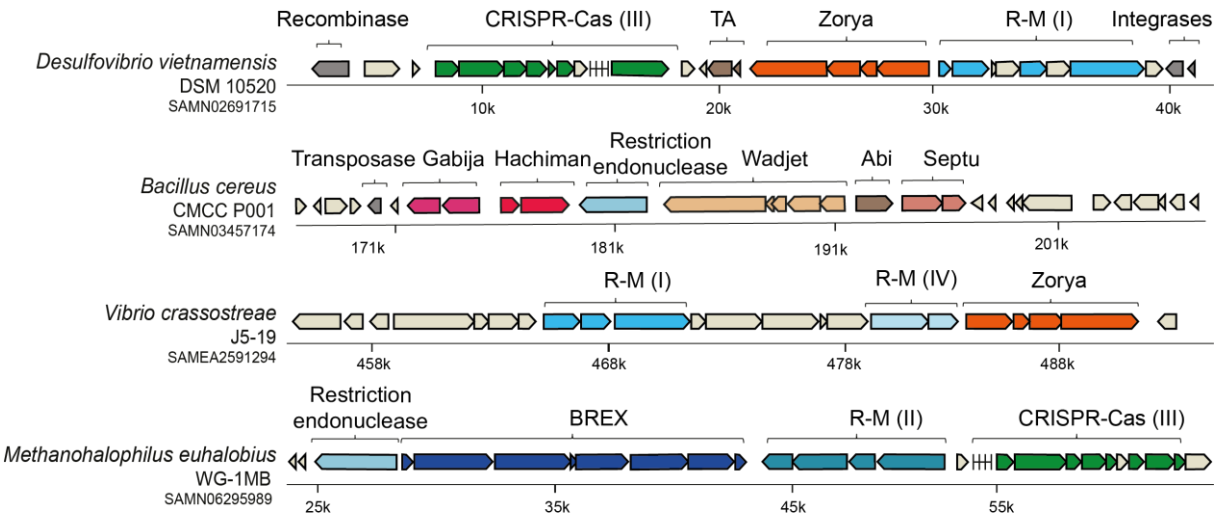

Figure 3: Examples of defense islands

Defense islands of different microorganisms are displayed. Different colors represent different defense systems. Beige represents genes of non-defense functions or of unknown function.

Anti-phage defense systems tend to cluster on microbial chromosomes in regions denoted as defense islands[50]. Defense islands typically comprise diverse defense systems (Figure 3). They are also enriched with genes typical of mobile genetic elements such as transposases, recombinases and conjugation genes [46,50]. Some defense islands were predicted to encode more than 100 defense genes [8].

The origin and the mechanism of formation of defense islands are currently unknown but could reflect different effects. First, co-localization of defense genes with mobile genes could facilitate horizontal transfer of multiple defense systems from one microbe to another in a single transfer event. Alternatively, such islands can be hotspots for integration of horizontally acquired genes[63], with defense systems clustering in defense islands through the "garbage and pile effect"[8], in which high rates of acquisition and loss are not strongly deleterious. In addition, such co-localization of defense genes could reflect functional links between the defense systems, including possible co-regulation or positive epistasis.

The phenomenon of defense islands in prokaryotic genomes allows the prediction of novel defense systems through a "guilt by association" approach. In this approach, protein families with unknown functions that are enriched in defense islands, can be predicted to constitute new defense systems. This methodology has led to the discovery of individual defense systems such as BREX or DISARM[21,22], and its application in a systematic manner recently revealed nine new anti-phages systems that are widespread in bacteria and archaea[7].

The microbial pan-immune system as a shared resource

Given the costs inflicted by anti-phage systems, it is probable that no single bacterial or archaeal strain can encode, in the long term, all possible defense systems without suffering serious competitive disadvantages. On the other hand, the access to a diverse set of defense mechanisms is essential in order to combat the enormous genetic and functional diversity of phages. We propose that these seemingly contradictory requirements can be reconciled when considering the available arsenal of immune systems as a resource shared by a population of bacteria or archaea rather than by individual cells.

In the example shown in Figure 2, none of the strains encodes all defense systems. However, if these strains are mixed as part of a population, the pan-genome of this population would encode an "immune potential" that encompasses all of the depicted systems. As these systems can be readily available by HGT, given the high rate of HGT of defense systems, the population in effect harbors an accessible reservoir of immune systems that can be acquired by population members. When the population is subjected to phage attack, this diversity ensures that at least some population members would encode the appropriate defense system, and these members would survive and form the basis for the perpetuation of the population (Figure 4). We thus hypothesize that some of the selection for defense systems occurs at the group level.

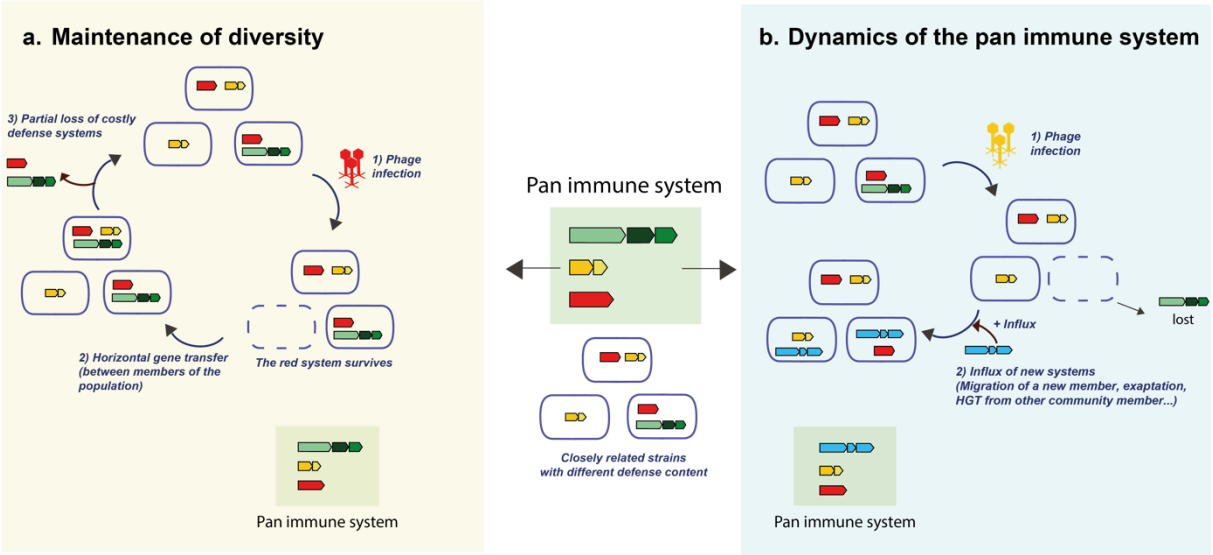

Figure 4: The pan-immune system model
Closely related strains within a population encode a diverse set of anti-phage systems constituting the pan-immune system. **A.** Maintenance of diversity of the pan-immune system. Phage infection results in bacterial selection for those encoding a specific (red) defense system that can overcome that phage. (1) In the absence of phage pressure over a period of time, the population can acquire a diverse set of defense systems, through horizontal gene transfer (HGT) (2), while some cells lose defense systems due to their selective cost. (3) The cycle continues, resulting in a population that together constitutes the immune potential of the population. **B.** Dynamic changes to the pan immune system composition. Phage infection results in selection for members encoding a specific (in this case yellow) defense system that can overcome that phage (1). In some cases this can result in the loss of immune systems (green). Conversely, new systems can be introduced into the population through horizontal gene transfer from a more distantly related strain, migration of a new member in the population or emergence of a new system through mutation or exaptation (2).

In a sense, this pan-immune system model aligns well with previous observations and mathematical models of distributed immunity that specifically focused on CRISPR-Cas systems. Studies on CRISPR-Cas have shown that spacer diversity in the population is essential to overcome phage infections[64–66].

In co-evolution experiments between *P. aeruginosa* and *S. thermophilus* and their respective phages, bacterial populations in which different strains encoded different sets of spacers overcame phage infection and resulted in phage extinction, while populations comprised of homogenous sets of spacers allowed phage propagation[66]. This, because no single phage could accumulate enough mutations to overcome the diversity of spacers encoded by the population as a whole[66]. In the context of CRISPR-Cas, mathematical models that explored the parameters leading to the emergence of a distributed immunity depicted two key parameters[64] : 1) The cost of generating a new allele (in this case a new spacer) should be small; and 2) fitness constraints of evolving escape mutations for phages is enhanced by the fact that an escape mutant will be resistant only to one allele (one spacer in the case of the CRISPR model) [64]. Beyond the specific case of CRISPR-Cas, the same conditions also fit the broader context of the microbial pan-immune system model, which can be viewed as satisfying the two parameters mentioned above: 1. Given the high rate of HGT of defense systems (which can be considered as acquisition of alleles of defense), the cost of acquiring a new allele via HGT is expected to be relatively small; and 2) due to the diversity of molecular mechanisms among different defense systems, the emergence of one phage mutation that allows escape from a specific defense system is not expected to abolish defense by others systems. As group selection occurs within closely related kin, we expect the pan immune system model to be mainly relevant among populations of similar, related strains that differ in their defense content thus allowing for selection at the group level.

Implications for phage anti-defense strategies

It is well documented that individual phages have well-defined host ranges, such that they can infect some, but rarely all, strains of the same species[67]. This is often attributed to the diversity of surface molecules among the infected microbial strains, since these are used by phages as specific receptors[68]. However, given the diversity of defense systems observed in different strains of the same species, it is clear that the host range of any given phage would depend on its ability to overcome multiple defense systems. This predicts that phages would need to encode many different counter-defense mechanisms in order to have a broad host range.

This prediction may help reconcile the puzzle of dispensable genes in phage genomes. As phage genomes are under strong selection, one might expect that most of their genes are essential. However, serial mutational analyses showed that as much as 79% of genes in phage T4 and 63% of genes in phage T7 are not essential for successful infection of the *E. coli* laboratory strain[69,70]. We predict that many of these genes would turn out to encode anti-defense proteins that target defense systems not present in the *E. coli* host strain used in these studies. In a sense, we would therefore expect that the set of anti-defense genes cumulatively encoded by strains of a phage species should mirror the set of defense systems encoded by its host pan-genome.

Conclusions and outlook

Apart from exploring the existence of numerous anti-defense genes in phages, the pan immune system model raises several interesting research avenues. Are there limitations to the co-occurrence of defense systems within a single genome? Both positive and negative epistasis (dependency and incompatibility) have been demonstrated to occur between DNA repair pathways and CRISPR-Cas systems[71,72], underlying potential requirements of a specific genetic background to allow compatibility of a CRISPR-Cas subtype in a given species[73]. Beyond CRISPR-Cas systems, it would be interesting to understand the influence of the core genome of a species on the composition of its pan-immune system. Similarly, is this composition influenced by environmental conditions, past infections, or other events in the life history of the microbe?

If the immune potential of a species encompasses many diverse defense systems, does epistasis exist between these systems? It has been shown that CRISPR-Cas and R-M systems can work synergistically[74,75]. Is this true for other defense systems? Within CRISPR-Cas systems, other forms of epistasis have been observed. One example of this is that of functional redundancy through using the same spacers with different interference modules to limit emergence of phage escape mutants[76]. Another is the coupling of "nucleic acids targeting" strategies and "dormancy/death" in type III CRISPR-Cas systems in which a non-specific nuclease is activated upon failure to fully restrict phage DNA[5,6,77]. Given the newly revealed diversity of defense systems, the study of interactions between defense systems promises to unravel novel understanding of the complexity of prokaryotic immune defense.

Beyond fundamental questions regarding prokaryotic biology, understanding the pan-immune system could have implications in the treatment of bacterial infections by phages. Given the rise of antibiotic resistance, phage therapy, the use of bacteriophages to kill pathogenic bacteria, has re-emerged as a promising therapeutic possibility[78,79]. The main strategy consists of using a cocktail of phages to limit the emergence of bacterial resistance to phages. Such cocktails of phages should be studied in light of the pan-immune system of target species to ensure that the chosen phages will be equipped to overcome the set of defense systems potentially encoded by the population.

Acknowledgements

We thank Shany Doron and Adi Millman for their help in the analysis presented in Figures 2 and 3, Maya Voichek and Nitzan Tal for their helpful comments on the figures in this study, Jean Cury and the Sorek lab members for comments on earlier versions of this manuscript. A.B is the recipient of an EMBO Long Term Fellowship (EMBO ALTF 186-2018). R.S was supported, in part, by the Israel Science Foundation (personal grant 1360/16), the European Research Council (grant ERC-CoG 681203), the German Research Council (DFG) priority program SPP 2002 (grant SO 1611/1-1), the Ernest and Bonnie Beutler Research Program of Excellence in Genomic Medicine, and the Knell Family Center for Microbiology.

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