Many defense systems in microbial genomes, but which is defending whom from what?

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Abstract: Prokaryotes have numerous mobile genetic elements (MGE) that mediate horizontal gene transfer between cells. These elements can be costly, even deadly, and cells use numerous defense systems to filter, control or inactivate them. Surprisingly, many phages, conjugative plasmids, and their parasites, phage satellites or mobilizable plasmids, encode defense systems homologous to those of bacteria. They constitute a significant fraction of the systems found in bacterial genomes. As components of MGEs, they have presumably evolved to provide them, not the cell, adaptive functions that may be defensive, offensive, or both. This sheds new light on the role, effect, and fate of the so called "cellular defense systems", whereby they are not merely microbial defensive weapons in a two-partner arms race, but tools of intragenomic conflict between multiple genetic elements with divergent interests. It also raises many intriguing questions.

Introduction: mobile genetic elements drive gene flow at a (sometimes hefty) cost

Horizontal gene transfer (HGT) allows Bacteria and Archaea to rapidly match novel ecological challenges and opportunities. HGT is most frequently mediated by self-mobilizable mobile genetic elements (MGE) like bacteriophages (phages) and conjugative elements that are present in most genomes, often in multiple copies. These elements can autonomously transfer themselves from one cell to another using viral particles or conjugative pilus, processes that also contribute to the exchange of chromosomal DNA. Besides their ability to drive HGT, many MGEs encode traits adaptive to the host genome. For example, key virulence factors in human pathogens are encoded in prophages and antibiotic resistance genes are often transferred by conjugative elements [1, 2]. By increasing the host fitness, these traits contribute to increase the frequency of MGEs in communities, *i.e.*, they directly contribute to MGE fitness.

Conjugative elements and phages have their own molecular parasites that take advantage of their mechanisms of horizontal transmission to transfer between cells. For example, viral particles produced by phages can be hijacked by phage satellites [3] and conjugative pili can be used by so-called mobilizable elements. The latter are at least as abundant as conjugative plasmids, and possibly much more [4]. Recent data suggests that satellite phages are also very common [5]. Many other MGEs lack known mechanisms of horizontal transmission and may transfer between cells by exploiting phages and conjugative elements [6]. Importantly,

the presence of a MGE affects the frequency of other MGEs in the cell. This is the case for mobilizable plasmids and phage satellites that co-transfer in synchrony with self-mobilizable elements. It is also the case of phages that use the conjugative pilus as a receptor for cell infection [7] and of plasmids capable of retro-transfer [8], a process by which a plasmid in the recipient cell uses the incoming pilus to transfer to the donor cell. Finally, MGE infection may spur the transfer of other elements. Phage infection favors the transfer of SXT-like integrative conjugative elements (ICE) [9] and conjugation-induced SOS response activates MGEs in the recipient cells [10]. The cellular genome thus harbors a cosmos of MGEs establishing complex interactions among each other and with the host cell.

The association between the host and its MGEs lays on a gradient from pure parasitism to intimate mutualism because vertical and horizontal transmission of MGEs impose fitness costs to the cell that may eventually be compensated by the accessory traits encoded by them. The replication of virulent phages implicates cell death, and they are at the edge of maximal virulence in this gradient. The fitness effects of the remaining MGEs are more diverse and vary with the physiological state of the cell and the presence of competing MGEs. Temperate phages provide striking examples of such ambiguity. Their integration in the genome can provide novel adaptive traits [11], but their subsequent excision from the genome usually ends in host death [12]. MGEs that are parasites of other MGEs impact the fitness of the latter. If this impact is very high and the parasitized MGE is deleterious to bacteria, then the parasite of the parasite may end up benefiting the host cell. For example, some satellites can abolish phage transmission resulting in cell death by release of viral particles exclusively packaged with the satellite genome [13]. Although this process still ends in cell death, the inhibitory effect of the satellite on phage reproduction blocks its epidemic growth thereby protecting the microbial population. Since most genomes contain MGEs, and virulent phages are extremely abundant in the environment [4, 14, 15], the fate of cells often hangs in the outcome of their interaction with MGEs and that of MGEs among themselves.

The wavering nature of interactions between MGEs and the host led to the evolution of defense mechanisms to filter, control or inactivate these elements [16, 17]. Some defenses are part of core cellular systems and provide protection from MGEs as part of a broader set of cellular functions. For example, RecBCD is a powerful exonuclease involved in the repair of double strand breaks by homologous recombination. It degrades linear double stranded DNA until it meets a Chi site beyond which DNA is cut and RecA is loaded. Phages lacking Chi sites are rapidly degraded by the enzyme [18]. Yet, phages can overcome this cell defense by either blocking the host RecBCD enzymes or by evolving chi sites that trick RecBCD in recognizing them as self [19]. In response to phage-encoded anti-RecBCD systems, retrons that induce cell death when the RecBCD function is compromised have a protective anti-anti-RecBCD function [20]. Contrary to RecBCD, many defense systems are not involved in core cellular processes. Instead, they are specialized in providing innate or adaptive immunity. Restriction-modification (R-M) systems, by far the most abundant [21], provide excellent illustrations of the evolutionary processes resulting in the evolution of defense and counter-defense systems (Figure 1). They imprint epigenetically the cellular genome and inactivate (restrict) infecting MGEs lacking the adequate DNA modifications. As a response, some phages counteract the activity of R-M systems by either producing anti-restriction proteins or by extensively modifying their DNA [22, 23]. Anti-restriction functions

can in turn be recognized by anti-anti-restriction systems that provide a second layer of resistance when R-M fails [24, 25]. As a complement, phages with extensive modifications in their DNA can be recognized by specific anti-methylation restriction systems [26]. Some host-phage interactions can be very complex, revealing long successions of tit-for-tat strategies. For example, phage T4 encodes an anti-restriction system that can be recognized by an anti-anti-restriction system inducing cell death in *E. coli* by tRNA cleaving, which can be repaired by T4 using a pair of proteins that constitute an anti-anti-anti-restriction system [27].

The function and evolution of defense and counter-defense systems are often studied in the light of the antagonistic interaction between one host and one MGE, often a virulent phage. But the reality is much more complex, and interesting. Genomes have many different MGEs [4, 14] and results are piling up to show that many of the systems found in bacterial genomes and once thought to be dedicated to the defense of the cell are actually encoded in these MGEs. This includes systems encoded in temperate phages [28-31], satellites [24, 32, 33], conjugative elements [9, 34, 35] or mobilizable plasmids [21]. It has also been pointed out that some components of MGEs, such as site-specific nucleases, are often shuttled between MGEs and defense systems [36]. These observations raise intriguing questions concerning the role, function and evolution of the co-called cellular defense systems (Figure 1.I).

Why are there so many defense systems in each genome?

Most bacterial genomes encode several R-Ms, but often also CRISPR-Cas, retrons, and many other defense systems [37]. For example, the two first sequenced genomes of *Helicobacter pylori* encoded a total of more than 20 putative R-M systems [38], and genomes with multiple CRISPR arrays and Cas systems are frequent [39]. The fast pace of discovery of novel defense and counter-defense systems suggests they may account for a significant number of the unknown function genes in genomes. The abundance of defense systems could allow cells to be protected from a broad range of MGEs, counteracting the latter's tendency to evolve counter-defenses. Yet, defense systems can be costly [40], because of production costs when they are required at high concentration [41], because their activity can be energetically costly [42], or because they may be incompatible with other cellular mechanisms [43]. They can also kill the cell by auto-immunity [44]. Hence, the number of defense systems in a genome is expected to depend on the balance between these costs and advantages of extensive protection against MGEs.

The observations that genomes have many MGEs and that these encode many defense systems provide an alternative or complementary explanation for why genomes contain so many such systems: they acquire the systems when they are infected by the MGEs. This does not exclude selection for a multiplicity of systems by each cell, but it brings to the fore that to understand their frequency in cellular genomes one must also account for the infectivity of MGEs. This means that the multiplicity of systems in cellular genomes might be a consequence of the high transmissibility and abundance of MGEs, not (only) the result of natural selection for protection of the cell.

Why are defense systems very diverse within species?

Defense systems tend to be diverse across strains of a species [16]. This could be explained by several factors. First, the MGEs may differ between habitats and locally adapted populations may need to select for different systems. Second, genetic diversity favors the emergence of resistance in populations [45] and antagonistic co-evolution between MGEs and their hosts could be result in a diverse repertoire of defenses in populations. For example, individuals with rare alleles are favored by negative frequency dependent selection because most antagonists lack the response mechanisms to tackle them. As these individuals rise in frequency in the population, antagonists with the ability to tackle them also rise in frequency, thereby decreasing the advantage of the initial clone. This may result in diversification of the population into many different systems or in the rapid cyclic turn-over of a few defense systems [46]. In addition, the presence of various systems providing immunity against different phages within a single community of bacteria is expected to provide better defense at the population level without requiring each individual genome to encode a very large number of defense systems, in what has been described as distributed (pan) immunity [37].

The existence of multiple different MGEs across strains of a species contributes to explain why defense systems are so diverse: they are brought by different MGEs. To understand why different MGEs carry different defenses systems it will be necessary to dissect the complex networks of interactions between the host and its multiple MGEs.

How is immunity gained?

The repertoire of defenses in genomes can vary as the result of dedicated molecular mechanisms of variation. Many systems include mechanisms driving their own diversification, e.g., CRISPR arrays can acquire spacers to target novel elements [17]. Some R-M systems are also capable of rapidly change their sequence specificity [47]. These mechanisms allow the host to fine-tune its defenses very rapidly. Yet, the available evidence suggests that HGT and gene loss have key roles in the diversification of defense repertoires at the species level [48]. Accordingly, pseudogenes of defense systems have been observed for many R-M [38] and CRISPR-Cas systems [49].

The abundance of defense systems in MGEs suggests a very straightforward mechanism for the acquisition of novel mechanisms of immunity: systems are transferred across strains by the MGEs encoding them. Mechanisms of transfer of MGEs between cells are well-known and their epidemiological patterns are being described in detail. Furthermore, MGEs are gained at high rates because of their infectiousness, and they are frequently lost from populations because of their cost. The genetic linkage between the defense systems and the MGEs thereby contributes to explain the acquisition of novel defense systems. It may also offer some clues on how entirely novel defense strategies emerge. The recent discovery of many anti-phage systems shows that they frequently consist in an assemblage of protein domains also implicated in other cellular processes such as nucleases, kinases, deaminases, proteases, or ATPases [36]. For instance, the Stk2 defense kinase is part of a family of kinases whose members are implicated in various cellular process such as the control of the cell cycle or the exit of dormancy [50]. The anti-phage Viperins are close homologues to GTP

cyclases involved in other functions [51]. The cooption of proteins, or protein domains, with other functions, and the creation of novel assemblages leading to genetic innovation by recombination and mutation is likely facilitated by the horizontal transfer of defense systems across genetic backgrounds [52]. While the probability of a functional innovation resulting from the co-option of each these systems is low, their very frequent transfer and rapid evolution may result in such a high rate of novel combinations of domains that some will eventually result in adaptive novel defense systems. MGEs will then eventually capture such innovations for their own use and spread them across species.

Defending whom from what?

Experimental verification of the function of defense systems usually involves testing the success of infection by virulent phages. As a result, the role of defense systems tends to be discussed in the light of phage-bacteria interactions. It does seem reasonable to assume that systems present in a microbial genome for a long time are protecting the cell from MGEs, and especially against virulent phages given their lethality for the cell. Yet, systems encoded in MGEs are more likely to be selected because they benefit the MGE. Sometimes the two objectives, cell defense and MGE fitness, coincide. Defense systems encoded in P4-like satellites were shown experimentally to protect the cell from several phages [53]. In this case the satellite and the cell have the same interest in preventing infection by phages that can kill the cell and cannot be exploited by the satellite to propagate. But sometimes, the interests of the MGE and the cell are not so well aligned. This is exemplified by expensive exclusion systems encoded by conjugative systems to fend off closely related plasmids [54]. For example, the surface exclusion system of plasmid F prevents infection by similar plasmids thanks to the production of thousands of copies of an outer membrane protein that accounts for a large part of the plasmid carrier cost [55]. A plasmid encoding an expensive defense system against another plasmid is engaging in an antagonistic interaction whose cost to the cell may be much larger than the expected reward. Temperate phages encoding defense systems against virulent phages seem very common [29, 31, 56]. While they may provide temporary relief to a cell, they may also have little long-term impact in bacterial fitness when the victorious phage is eventually induced and lyses the cell. Finally, phages encoding defense or anti-defense systems against satellites are engaging in an interaction with their parasites in a way that resembles their own interaction with the cell (but with their own position reversed as they are now the host) [33]. Such prophage-encoded defense systems could be highly deleterious to the cell because they remove a protective satellite and favor a phage that will eventually kill the host.

The existence of defense systems in MGEs and the interactions between them raise two key questions. Who is encoding the system? The identification of the defense system as MGE-associated depends on the precise delimitation of the latter, which may be difficult both computationally and experimentally. Genomes encode many defective MGEs and it may also be unclear if a defense system is part of a functional MGE, is being co-opted by the cell, or is non-functional. Such distinctions may be key to understand their role. Which genetic elements are being targeted by the defense system? While many systems are effective against virulent phages, it is often unclear which other elements are being targeted, i.e. which target elements lead to the selection for the conservation of the defense system. Systems encoded by MGEs may be targeting other competing MGE that are not costly to the

cell. They may even be targeting elements that are adaptive to the cell, or targeting the cell itself (*e.g.*, addictive systems or anti-defense systems), thereby lowering bacterial fitness. The role of the defense system in the MGE can thus be intimately associated with the positive or negative fitness effect of the MGE to the cell. Knowing which genetic elements are being targeted in nature will require a better understanding of the ecological contexts where such systems are selected for.

How do defense systems affect gene flow?

Selection for cell defense systems depends on a trade-off between costs and benefits [40]. In addition to the costs of production and the cost of auto-immunity, there is an evolutionary cost to restricting adaptive gene flow, including allelic recombination and acquisition of novel genes by HGT. For example, epidemic *V. cholerae* strains depend on a prophage for a key virulence factor (the cholera toxin). When they are infected by SXT-like conjugative elements carrying defense systems they are hampered in their ability to acquire the toxin [9]. More generally, a computational analysis of ca. 80 species showed that gene flow is decreased when strains of a species have incompatible R-M systems [57]. Hence, when R-M systems diversify within populations, their DNA exchanges become more frequent between strains with similar systems (Figure 1.II). Beyond R-M, other defense systems including BREX, DISARM, CRISPR or Wadjet might also restrict gene flow. As a rule, one would expect that very generic systems, like R-M, would have an important effect on restricting gene flow, whereas more targeted systems, like CRISPR-Cas, would tend to affect gene flow driven by a few particularly deleterious elements. Accordingly, the impact of CRISPR-Cas in restricting gene flow has remained controversial [58, 59]. The effect of defense systems on gene flow is however not always straightforward. Transduction, the transfer of bacterial DNA in viral particles, is favored by the existence of CRISPR-Cas systems in recipient cells when they target the phage DNA. In this case, bacterial DNA in viral particles is transferred into the cell whereas the phage DNA is excluded, resulting in cells that receive exogenous cellular DNA while being protected from phages [60].

The negative impact of defense systems on gene flow has been regarded as a costly by-product of selection for protection of the cell. But defense systems in MGEs may be selected because they block HGT to prevent the cell from acquiring competitor MGEs. The resulting sexual isolation is advantageous for the MGE but can be deleterious to the cell. Further work is needed to quantify the impact of different systems in gene flow and how they affect the evolvability of microbes.

Is it defense, attack, or something else?

While many systems have been called defensive relative to their ability to defend bacteria, they may be attack systems when part of MGEs. A striking example is provided by phage-satellite interactions. The reproduction of virulent phages of the ICP1 family in *Vibrio cholerae* is abolished by the PLE satellite elements [13]. In response, ICP1 phages have evolved the ability to encode a CRISPR-Cas system or specific nucleases that allow to eliminate the satellite [53]. In this context they could be regarded as attack systems, since their success results in cell death. Some systems may even have multiple roles. R-M systems contribute to the stabilization of plasmids in the cell by acting as poison-antidote addictive

systems [61]. In such cases, loss of the plasmid and its R-M system prevents further expression of the latter. Since endonucleases have longer half-lives than methylases, this eventually results in genomes that are restricted because they are insufficiently methylated. R-Ms are thus part of the offensive arsenal of plasmids that allows them to be maintained in cells. Yet, these R-M systems can also protect the consortium (cell and plasmid) from infection by other MGEs, thereby acting as cell defense systems. Plasmids also frequently encode toxin-antitoxin systems that behave as addiction systems [62], some of which are implicated in phage defense. Homologs of cell defense systems encoded in MGEs can thus be offensive tools with positive side-effects in cell defense. The relative contribution of such systems to the different types of ecological interactions, defense or offense, remains to be explored.

Are MGEs at the origin of "defense islands"?

It was observed a decade ago that defense systems are often clustered in a few loci in microbial chromosomes [63]. This characteristic was leveraged into a systematic method to discover novel systems by co-localization with known ones [64]. Interestingly, recent data has revealed that anti-defense systems, both anti-R-M and anti-CRISPR-Cas, tend to cluster in bacterial genomes, often in recognizable MGEs [65]. The clustering of defense systems, and that of anti-defense systems, could be selected to facilitate their co-regulation and interaction for a more effective function against MGEs. For the moment, there is very little evidence available of that.

The presence of such systems in MGEs provides a simple explanation for their co-localization in bacterial genomes (Figure 1.III). Genes acquired by HGT, and MGEs in particular, tend to integrate a small number of chromosome hotspots [6]. These elements may degenerate by the accumulation of mutations, deletions, and insertions. Chromosome hotspots are thus littered with remnants of previous events of transfer. As MGEs are integrated and eventually degrade in the hotspot, some genes may remain functional because they are adaptive for the cell [52]. Since MGEs often carry defense and anti-defense systems, the rapid turnover of the former in hotspots may be accompanied by selection for the conservation of the latter. As rounds of MGE integration/degradation succeed in natural history, the remnant defense systems form clusters in the chromosome. The clustering of these systems may facilitate the evolution of functional interactions between them or co-regulation of gene expression. For example, it has been observed that type I and type III CRISPR-Cas systems provide two integrated levels of defense against phages [66]. These systems are often co-localized [39], but the functional advantages of their co-localization in the genome are yet unclear.

Outlook

MGEs of Bacteria and Archaea encode many accessory functions that can have adaptive value for the host. They are also units of selection that prosper if they manage to increase their population size by horizontal or vertical transmission. To understand the roles of defense and/or counter-defense systems, given their abundance in MGEs, one must attain a better understanding of the complex networks of interactions between these semi-autonomous agents. Their study will shed novel light on the function, evolution, and ecology

of defense systems. In terms of function, many novel molecular mechanisms of interference may yet remain to be discovered. From the evolutionary point of view, these observations suggests that the existence of many defense systems in genomes may be more directly related to selection for survival and reproduction of the MGEs than of the cell. In terms of ecology, given the impact of MGEs in adaptation and regulation of bacterial populations, a better understanding of the defense systems may be key to understand and manipulate microbial population dynamics.

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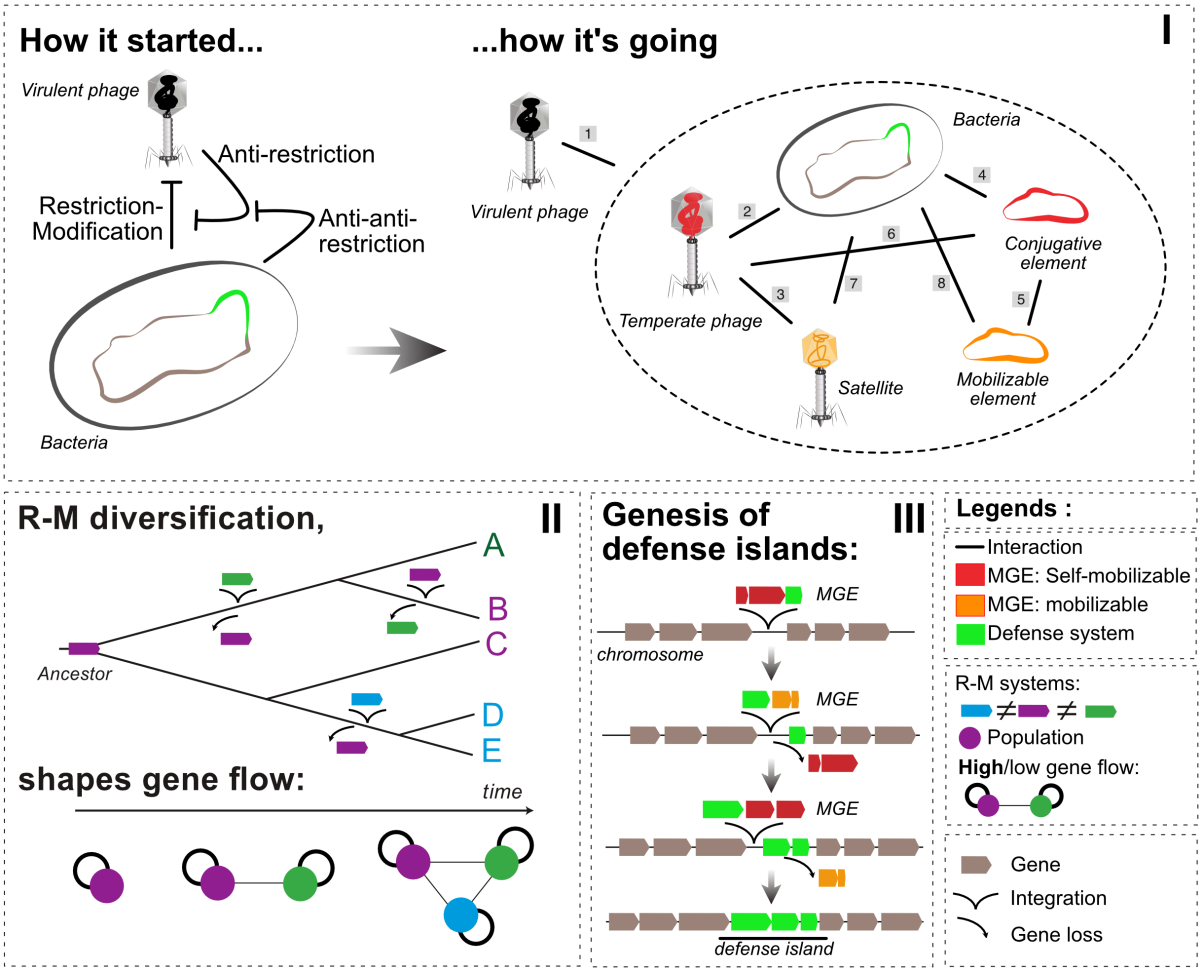

Figure 1. **I**. Defense and anti-defense systems are often studied in the context of the interaction between one host and one MGE, usually a virulent phage (left). Yet, the presence of numerous MGEs in populations and their ability to encode their own systems renders the picture more complex (right). Virulent phages establish antagonistic interactions with the other MGEs and the cell (1). But the associations between the other MGEs and the cell can be more diverse (2-7). Temperate phages and conjugative plasmids exploit their cellular host (2,4) and can be exploited by other MGEs (3, 5). Plasmids often encode systems that are effective barriers to phages, e.g. R-M (6). Phages are a threat to plasmids when they kill the host cell (6). Satellites may benefit the host by diminishing phage infection (7). Most of these interactions (2-8) can at times be beneficial to both partners, e.g. when a conjugative plasmid provides a nosocomial bacterium with antibiotic resistance. **II**. Diversification of R-M systems changes gene flow within species. As the diversity of systems increases, cells preferentially exchange genes with those carrying the same R-M. Hence, diversification of R-M systems may result in the fragmentation of gene flow in populations. **III**. MGEs tend to integrate the chromosome at a few hotspots and may become inactivated by mutations resulting in the loss of genes that are not adaptive to the host. The succession of MGE integration and co-option of their defense systems in the hotspots may result in clusters (or islands) of defense systems.