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# Going viral: the role of mobile genetic elements in bacterial immunity

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## Summary

Bacteriophages and other mobile genetic elements (MGEs) pose a significant threat to bacteria, subjecting them to constant attacks. In response, bacteria have evolved a sophisticated immune system that employs diverse defensive strategies and mechanisms. Remarkably, a growing body of evidence suggests that most of these defenses are encoded by MGEs themselves. This realization challenges our traditional understanding of bacterial immunity and raises intriguing questions about the evolutionary forces at play. Our review provides a comprehensive overview of the latest findings on the main families of MGEs and the defense systems they encode. We also highlight how a vast diversity of defense systems remains to be discovered and their mechanism of mobility understood. Altogether, the composition and distribution of defense systems in bacterial genomes only makes sense in the light of the ecological and evolutionary interactions of a complex network of MGEs.

## In Brief

Bacteria deploy a broad range of defense systems to protect themselves against viruses and other mobile genetic elements. Recent work is revealing how most defense systems are carried by mobile elements themselves, leading us to rethink the evolutionary forces that drive bacterial immunity.

## 1. Introduction

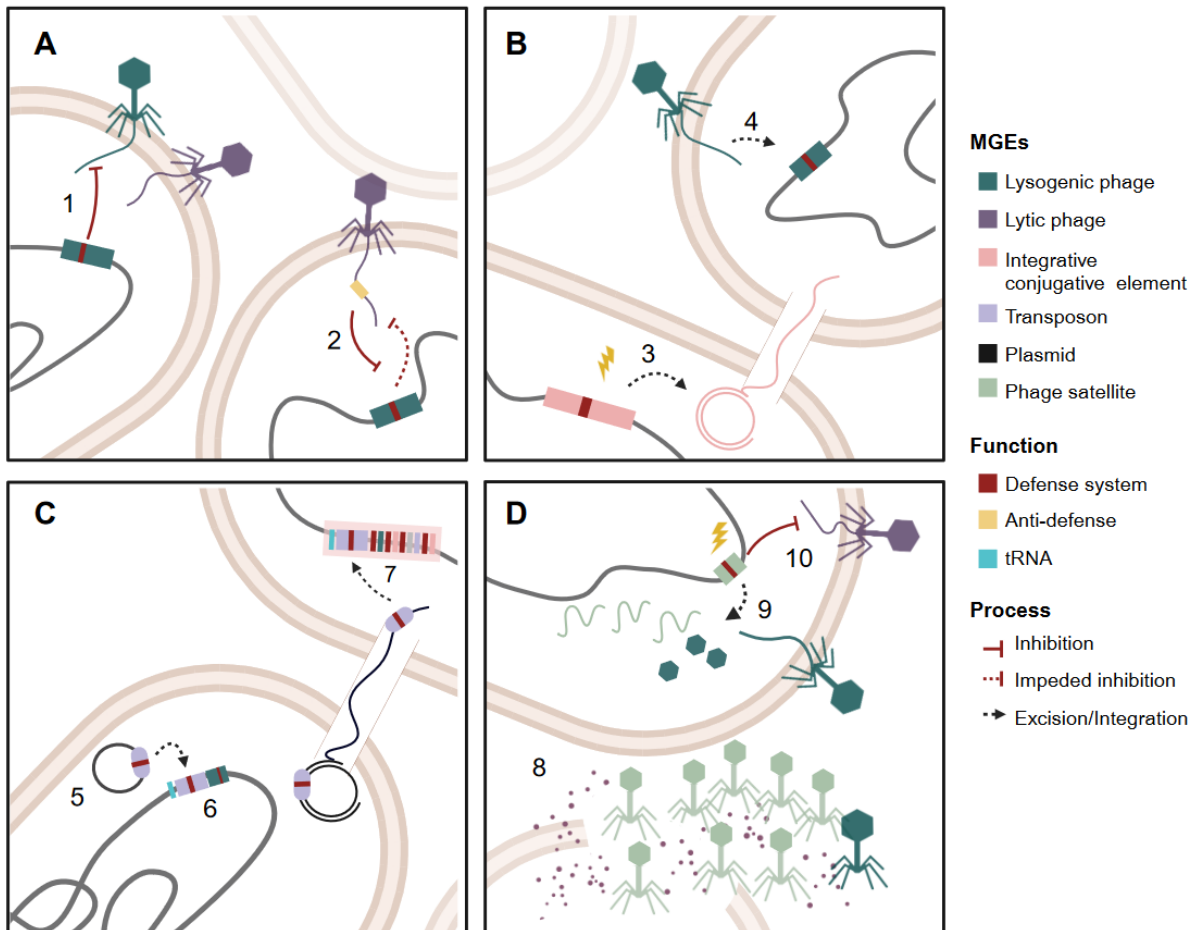
Nearly three decades after the publication of the first complete bacterial genomes<sup>1,2</sup>, we now have access to millions of sequences which revealed the remarkable plasticity of bacterial genomes. A striking example is *Escherichia coli*, where less than half of the genes carried by a given strain are shared by the entire species, while the rest represent accessory genes<sup>3,4</sup>. Most of the accessory genome of bacteria is concentrated in specific locations or genomic islands, which are clusters of genes likely acquired by horizontal gene transfer (HGT). Depending on the functions of the encoded proteins, genomic islands were traditionally identified as pathogenicity, metabolic and resistance islands<sup>5</sup>.

In 2011, Makarova and colleagues discovered that defense systems against bacterial viruses (phages) also tend to cluster in genomes and coined the term defense island<sup>6</sup>. This finding has led to the bioinformatic prediction of candidate defense systems based on their co-localization with known defenses, such as restriction-modification (RM), CRISPR-Cas, and abortive infection (Abi) systems, using a “guilt-by-association” approach<sup>6-9</sup>. Subsequent experimental validation of their anti-phage activity has led to the identification of dozens of new defense systems, unveiling a bacterial immune repertoire that is far more diverse than initially thought<sup>7-11</sup>.

The molecular characterization of some of these novel defense systems is currently underway and has started to unveil a wide array of strategies to counteract phage infection, either by targeting phage processes directly, or by targeting the host machinery and leading to cell death or dormancy<sup>12</sup>. These include the non-specific degradation of DNA or RNA, the specific inactivation of tRNAs, the degradation of key metabolites such as NADH or ATP, the formation of pores in the membrane, the inhibition of phage protein assembly, and more<sup>12-14</sup>. Many systems and mechanisms show parallels to eukaryotic immunity and likely share a common evolutionary origin<sup>15</sup>. Furthermore, some of these novel systems show great potential for clinical and biotechnological applications, similar to RM and CRISPR-Cas systems<sup>16-18</sup>.

Recent work has not only expanded the diversity of defense systems and mechanisms, but also the number of mobile genetic elements (MGEs) known to encode defense systems<sup>19-25</sup>. In fact, the discovery of treasure troves of defense systems carried by MGEs has contributed to the expansion of the current list of known defense systems to more than 150<sup>19,20,25,26</sup>. Remarkably, we are increasingly coming to the realization that the majority of bacterial defense systems protecting their hosts against phages and other MGEs are in fact encoded by MGEs themselves<sup>20,27,28</sup> (**Figure 1**). This realization forces us to rethink our initial concept of bacterial immunity, as these defenses have presumably evolved for the benefit of the MGEs that carry them, which might not always be aligned with the interest of the host bacterium<sup>29</sup>. The resulting defensive actions of MGEs against other MGEs complicates our ability to untangle the ecological and evolutionary dynamics shaping the bacterial immune repertoire.

In this review, we focus on the role of MGEs in bacterial defense, both as potential threats and carriers of defense genes (**Figure 1**). We discuss the diversity of MGEs that encode defense systems, assessing their overall contribution to the bacterial defense arsenal. We further discuss the intricate role played by MGE-encoded defenses in the complex conflicts that arise between multiple MGEs and between MGEs and their bacterial hosts.



**Figure 1. MGEs as targets and allies in bacterial immunity.** A population of bacteria with its multiple defense systems is shown. Defense systems protect against viruses and other MGEs, with a significant fraction of the defense systems encoded by MGEs themselves.

**A.** Defense systems only protect against a narrow set of MGEs for two main reasons: (1) their intrinsic mechanism of action, exemplified by a RM system which blocks a phage only if it carries a specific DNA motif; (2) anti-defense strategies employed by MGEs, as shown by the action of an anti-restriction protein, in yellow.

**B.** Defense systems are often carried by active MGEs, such as integrative conjugative elements, which can be excised under certain environmental conditions and transferred by conjugation (3), or lysogenic phages, which can integrate into the host chromosome (4). The integration and excision of MGEs thereby contributes to the rapid loss and gain of defense systems in bacteria.

**C.** Defense-encoding MGEs such as lysogenic phages and transposons, drive the acquisition of defense systems in chromosomal hotspots (e.g. downstream of tRNA loci, in blue) (5). These chromosomal hotspots can be occupied by a single or few defense cassettes (6) or defense islands, assemblages of multiple defense systems, and MGE components (7).

**D.** MGEs are selfish entities with their own evolutionary interest, which is not always aligned with that of their host. Phage satellites will eventually lyse their host in the presence of a helper phage which is required for the satellite to move between cells (8). Defenses encoded by phage satellites do not block their helper phages (9) but block lytic phages (10) or other satellites.

## 2. The highly diverse and plastic defense repertoire of bacteria

### 2.1 Bacteria require diverse defense systems to counteract MGEs

MGE is an umbrella-term for any genetic element that can move within or between genomes by HGT (**Figure 1**). Due to their role in gene flux, MGEs are often discussed in the light of bacterial adaptation<sup>30</sup>, but they can also be costly to their hosts. For example, integrons and transposons are linked with chromosomal instability and gene disruption<sup>31-33</sup>. The expression of genes encoded by conjugative elements can impose a metabolic burden<sup>34,35</sup>, which is higher in multi-copy elements, that can also result in genetic conflicts<sup>36</sup>. Among MGEs, phages are likely the most harmful, contributing to a mean of 10-20% daily bacterial mortality<sup>37</sup>, and up to 200% during bacterial blooms<sup>38</sup>. This mortality comes not only from lytic phages, but also from the activation of prophages residing within the host chromosome. For example, most viruses in the gut are lysogenic, and they are constantly reactivated under conditions such as antibiotic treatment<sup>39</sup>.

The fitness cost of MGEs has led to the evolution of multiple bacterial strategies for protection, including the modification of extracellular receptors and a diversity of intracellular defense strategies (**Figure 2**). On average, bacteria encode five defense systems, with RM and CRISPR-Cas systems being the most prevalent, occurring in 83% and 39% of bacterial genomes, respectively. In contrast, the remaining defense systems are present in less than 17% of bacterial genomes, with 35% of these systems being extremely rare (occurring in less than 1% of genomes)<sup>40</sup>. However, their prevalence can vary significantly between bacterial clades. For instance, the Wadjet system, which specifically degrades plasmids, is highly frequent in Actinobacteria (42%), but rare in other phyla (7%)<sup>41</sup>.

Defense systems are not only diverse but also highly dynamic, representing the functional category with the fastest rate of acquisition and loss in bacteria<sup>42</sup>. This can be mostly explained by the prominent role of HGT in the evolution of defense genes<sup>43</sup>. For instance, only 5% of type I RM systems are shared by members of the same species, with the rest being part of the accessory genome<sup>44</sup>. This rapid evolution typically results in the presence of different combinations of defense systems among closely-related bacteria within a species. For example, 90% of the accessory genome of nearly-clonal strains of *Vibrio* are defense elements, and their variation explains patterns of phage susceptibility<sup>22,45</sup>.

The diversity and plasticity of bacterial immunity suggests that multiple systems are needed to counteract the sheer diversity of MGEs. Due to their specific mechanisms of action, defense systems can only block a small fraction of potential invaders (**Figure 1A**). For instance, RM systems recognize specific sequence motifs on foreign DNA, while Wadjet targets plasmids<sup>8</sup>, and systems such as CapRel or DSR2 sense specific phage capsid proteins<sup>46,47</sup> (**Figure 2**). The level of protection provided by these defenses can be highly variable. For example, the Gabija system, which encodes a DNA-nicking endonuclease and an NTPase, protects *Bacillus subtilis* against four SP $\beta$ -like phages, with protection ranging from 10- to 1,000-fold, but has no effect on 32 other phages tested, including 7 SP $\beta$ -like phages<sup>48</sup>. Interestingly, the

differential sensitivity between closely-related SP $\beta$ -like phages led to the discovery of several anti-defense proteins which inhibit Gabija<sup>48</sup>. In fact, the presence of inhibitors of defense systems in MGEs is common and contributes to the specificity of defense systems<sup>49,50</sup>.

This ongoing arm-race between MGEs and bacteria results in narrower individual defenses, increasing the need for bacteria to counteract with multiple, and diverse defense systems (**Figure 1A**). For example, clinical *Pseudomonas* strains can accumulate up to 19 defense systems, together conferring resistance to every phage tested<sup>51</sup>. This raises the question of whether defense systems found within a genome tend to act independently or together as one immune system. Defenses found to co-occur in bacterial genomes can sometimes exhibit synergistic activity such as the one described between RM and CRISPR-Cas, where RM promote the acquisition of novel spacers by CRISPR-Cas through the induction of double-stranded DNA breaks in foreign DNA<sup>52</sup>. Another recently described example is that of the tmn system, a system with unknown mechanism, which presents synergistic defense with systems harboring an ABC ATPase such as Gabija, Septu I, or PrrC<sup>53</sup>. Complementary systems can also provide multi-layered defenses<sup>54</sup>. For example, the PARIS abortive infection system provides a second line of defense against phages encoding an anti-restriction protein, which inhibits the first line of defense of RM systems<sup>19</sup>.

Altogether, MGEs pose a significant threat to bacteria, leading to the evolution of defense systems and counter-defenses. The diversity of MGEs, combined with the fact that defenses typically protect only against a narrow range of elements, pushes bacteria to accumulate defense systems which can work in synergy.

## 2.2 Fitness costs limit the accumulation of defense systems

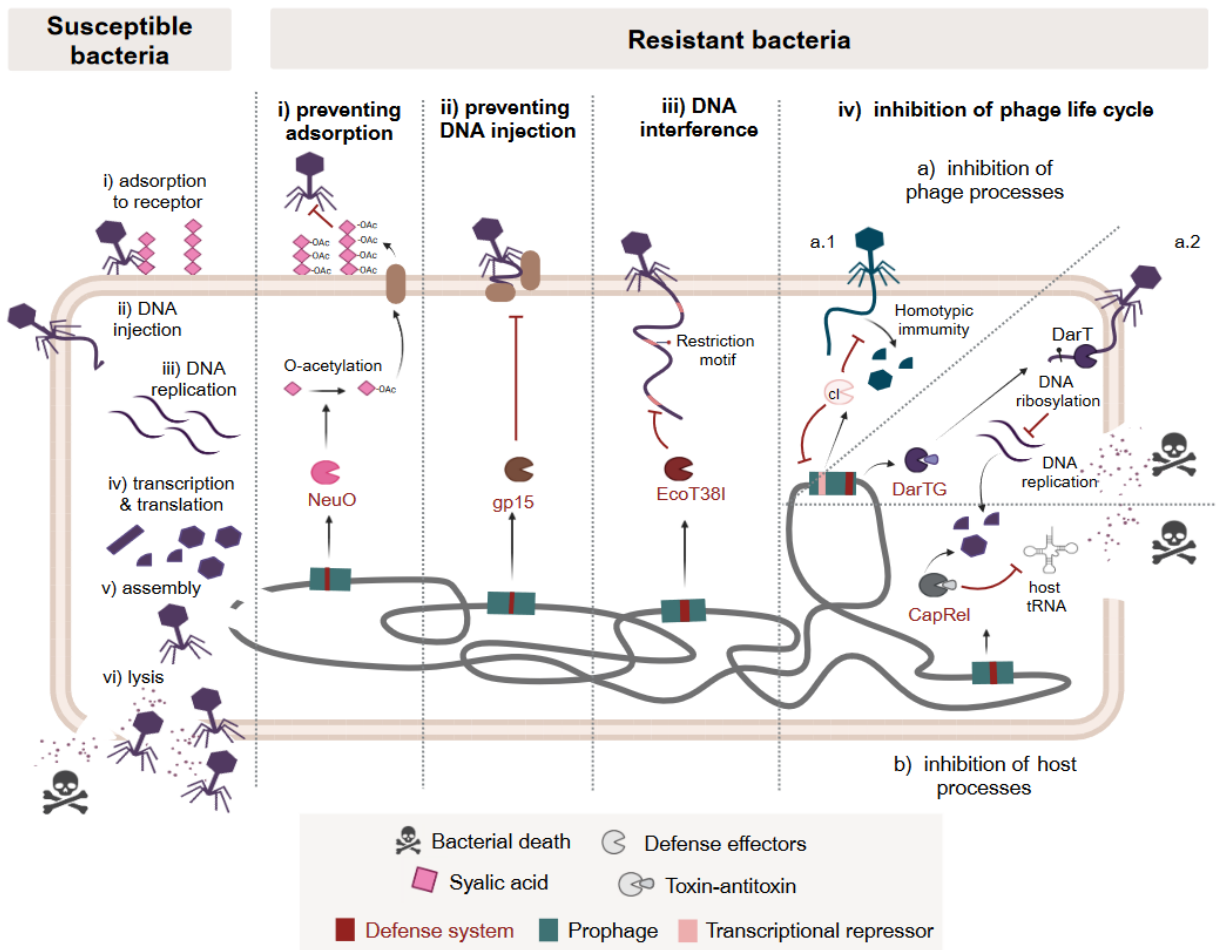
The accumulation of multiple complementary systems increases both the range and potency of phage resistance<sup>45,51,53</sup>. This accumulation is however limited by their fitness costs. In the absence of threatening MGEs, defenses can be metabolically costly, or result in toxicity due to self-targeting<sup>55-57</sup>. For example, RM systems of type I-III can methylate host motifs, which may be subsequently targeted by horizontally-acquired RM type IV systems, leading to a genetic conflict that can be lethal to the cell if left unresolved<sup>58</sup>. There is also an opportunity cost to defense systems, which by blocking HGT can also limit the ability of bacteria to adapt to new environments<sup>29</sup>. For instance, the presence of CRISPR-Cas, RM, and Gabija systems is associated with a reduced accessory genome<sup>59</sup>. In fact, defense genes are often disrupted by insertion-sequences (ISs), suggesting selection of defense inactivation under certain conditions<sup>60,61</sup>.

Overall, the cost of carrying too many defense systems at once makes the evolution of general resistance in bacteria unlikely. Instead, bacteria appear to benefit from the rapid evolution of the

immune repertoire, by losing and gaining systems from the “defense pool” of related strains, an evolutionary model known as the “pan-immune system”<sup>57</sup>.

## 2.3 MGEs account for the rapid gain and loss of defense systems

The “pan-immune” scenario is enabled by the tight association of defense systems with MGEs. Besides genes needed for their own replication and spread, MGEs often carry “cargo genes”, accessory genes that can increase the fitness of their bacterial host. Notably, some of these accessory genes are defense systems, creating a symbiotic relationship that protects both the host and MGE from other invading MGEs<sup>19–22,25,62,63</sup>. This association allows the rapid acquisition of defense systems by conjugation or transduction, but also their rapid loss due to the intrinsic dynamics and cost of MGEs. This dynamic exchange of MGEs is regulated by a combination of MGE-, host-, and environmental-specific factors<sup>39,64–67</sup>. Stress signals, such as the SOS response resulting from DNA damage promote the induction of prophages and the transfer of conjugative elements<sup>68,69</sup>. Interestingly, single stranded DNA entering the cell by conjugation can also trigger the SOS response and thus resident mobile elements<sup>70</sup>. HGT events occurring in response to stresses can then provide surrounding bacteria with defense systems that could help them face the threat<sup>24</sup>.



**Figure 2. Defense strategies employed by temperate phages.** Here, we describe the main immune strategies through the lens of prophage-encoded defense systems for which we provide arbitrarily chosen examples. In susceptible bacteria, phage infection results in cell lysis (phage lytic cycle, left panel) or in lysogeny (phage lysogenic cycle). In resistant bacteria, lysogens can provide resistance to other phages due to the prophage-encoded defense elements, which operate through various mechanisms (right panels): i) Preventing adsorption: *Escherichia* CUS-3 prophage encodes NeuO, an acetyltransferase that modifies polysialic acid, the composing unit of the K1 capsule, resulting in phage adsorption inhibition<sup>71</sup>. ii) Preventing DNA injection: *Escherichia* HK97 prophage encodes gp15, a superinfection exclusion protein that affects DNA injection by interfering with the phage tail tape measure protein-PtsG complex<sup>72</sup>. iii) DNA interference: *Escherichia* P2 prophage encodes EcoT38I, an RM system that recognizes a specific sequence motif on the infecting phage, leading to the degradation of its DNA<sup>73</sup>. iv. Inhibition of phage life cycle. a) Inhibition of phage processes: (1) In homotypic immunity a resident prophage will block the lytic cycle of related phages through the action of its repressor. (2) Defenses can target heterologous phages, e.g. *E. coli* C7 prophage encodes DarTG, a toxin-antitoxin (TA) system where the DarT protein ADP-ribosylates thymidine on viral ssDNA, preventing phage replication<sup>74</sup>. b) Inhibition of host processes. *Salmonella* phage SJ46 encodes CapRel, a TA system that pyrophosphorylates the host tRNA, thereby inhibiting host translation and phage protein production<sup>46</sup>. In both cases (iv.a2 and iv.b) bacteria die without producing mature viral particles.

### 3. Here, there and everywhere: the diversity of MGEs carrying defense systems

The discovery that MGEs encode defense systems against phages dates to the late 1980s- early 1990s<sup>75</sup>, as illustrated by the description of the pif exclusion system of the F plasmid<sup>76</sup>, the Lit system of the cryptic *E. coli* prophage e14<sup>77</sup>, or the RexAB exclusion system of phage lambda<sup>78</sup>. However, recent high-throughput genomic analyses and experimental work have revealed that this phenomenon is much more common than initially thought. Interestingly, the localization of defense systems within MGEs is not random, enabling to define MGE defense hotspots<sup>78</sup>: positions harboring a diversity of systems in between two conserved genes of a family of MGE, or between a conserved gene and the boundary of the MGE (**Figure 3**). In the following subsections, we will explore the main families of MGEs found to carry defense systems and their unique features. Of note, the majority of the defense systems have been tested exclusively against phages, which limits our appreciation of defenses against other types of MGEs.

#### 3.1 Phages and defense systems

Prophages are ubiquitous in bacteria, being present in nearly half of bacterial genomes with some encoding up to 15 prophages<sup>79</sup>. During their lysogenic phase, some prophages maintain the expression of accessory genes called morons<sup>80</sup>, many of which function as defense systems<sup>81</sup>. Due to the size constraint of viral capsids, phages can only carry a small number of accessory genes, which represent less than 10% of their genome<sup>19,82</sup>. The location of accessory genes within prophages is not random. For example, the comparison of 12 MP22-like phages of *P. aeruginosa* revealed 5 major positions of genetic diversity, including a locus that seems dedicated to anti-CRISPR genes<sup>62</sup>. More recently, the study of these diversity hotspots in other prophages has led to the identification of numerous defense systems and phage defense hotspots<sup>19,25,81</sup>.

*E. coli* P2-like phages carry diverse defense systems in-between the genes *gpQ* and *gpA*, as well as at the Z/fun locus<sup>83</sup> (**Figure 3A**). Specifically, the investigation of the *gpQ-gpA* locus revealed over 160 unique genetic arrangements including known defense systems such as RM or retrons<sup>19</sup>, but also 10 novel systems recently found to provide phage defense through unknown mechanisms, with likely a lot more to discover<sup>19,20</sup> (**Figure 3A**).

*Mycobacterium* N prophages harbor a defense hotspot between the lysis and integrase genes, with some defense systems expressed up to ten-fold more than the lytic cycle repressor<sup>81</sup>. Interestingly, the location of defense hotspots in *Vibrio* and *Bacillus* prophages is downstream of transcriptional regulators<sup>19</sup>, which may allow the regulation of their expression during lysogeny. This is also the case for the defense protein BstA, a prophage-encoded defense system found in several enterobacteria prophages<sup>84</sup>, and the Tha protein in *Staphylococcus aureus* prophages<sup>85</sup>. Remarkably, the Tha system is

part of a non-contiguous operon with a cognate inhibitor, a genetic organization which ensures that it provides defense against other phages during lysogeny but avoids self-targeting during the phage's own lytic cycle<sup>85</sup>.

### 3.2 Phage satellites and defense systems

Phage satellites, parasites of phages, lack structural components of virions and exploit other phages, called helper phages, for their own transmission (**Figure 1D**). They typically have a small genome (6-18 kilobases; kb), which is less than one-third of the size of their helper phage (**Figure 3A, B**). To date, four main families have been described: P4-like satellites in *Enterobacteriaceae*, phage-inducible chromosomal islands (PICIs) in Proteobacteria and Firmicutes, phage-inducible chromosomal island-like elements (PLEs) in *Vibrio cholerae*, and phage-inducible chromosomal minimalist islands (PICMIs) in *Vibrionaceae*<sup>86,87</sup>.

The effect a satellite has on the replication of its helper phage can range from a near complete inhibition, as described for PLEs, to a more moderate effect, as it is the case for P4 satellite on P2 helper phage or SaPIs, the prototypical PICI in *S. aureus*<sup>25</sup>. Due to the strong inhibition PLEs exert on ICP1 phages, they were initially described as an anti-phage system<sup>88</sup>. This strong inhibition results from a conserved PLE protein, NixI, which nicks the ICP1 genome at specific sites, thereby preventing its replication<sup>89</sup>.

Phage satellites not only block their helper phages, but also other phages by dedicated defense genes located in diversity hotspots. For example, P4 satellites have a defense hotspot between the conserved genes *int* and *psu* (**Figure 3B**), where more than 80 groups of genetic systems have been identified<sup>19</sup>. In PICIs and PICMIs, defense systems are located at the 5' and 3' ends of the element<sup>25,87</sup>, with PICIs carrying a high diversity of systems between their small terminase gene *terS* and the *attR* attachment site. These conserved locations in phages and satellites might allow their exchange between different phages by homologous or illegitimate recombination<sup>90</sup>. For example, the *gpA-gpQ* hotspot of P2 and the hotspot of P4 contain a *cos* packaging signal, which is common to both elements and could provide opportunities for genetic exchanges between both hotspots during coinfection through recombination of the cohesive ends<sup>19</sup>.

The strategy employed by satellites to hijack their helper phage can affect the amount of accessory DNA they are able to carry. For example, P4 and PICIs shrink the capsid of their helper phage to ensure the preferential packaging of their own smaller genome<sup>91</sup>. This might explain why satellite defense hotspots are typically smaller than those of prophages with P4- hotspots having an average size of 2.6 kb, 0.6 kb less than the hotspot of its helper phage P2<sup>19</sup>. On the other hand, PICMIs do not shrink the capsid of the helper phage but rather form concatemeric DNA to fill the whole capsid<sup>87</sup>. Therefore, they do not suffer from the size constraint of other satellites and have been found to carry large defense systems such as type I RM, Septu, or both, reaching sizes more than twice their average size (6.7 kb)<sup>87</sup>.

### 3.4 Integrative conjugative elements and defense systems

Mobile elements transferred by conjugation such as integrative conjugative elements (ICEs), and integrative mobilizable elements (IMEs), are widespread across bacteria, with more than 37% of bacterial genomes carrying at least one of these elements<sup>92</sup>. They can reach  $\geq 500$  kb in size<sup>93</sup>, being less constrained in size than phages to carry cargo genes (**Figure 3**). Akin to prophages, ICEs/IMEs are inherited concomitantly with the host chromosome until they excise from the chromosome, either spontaneously or triggered by environmental conditions (**Figure 1B**). They then transfer to a recipient cell, either by means of their own conjugation machinery in the case of ICEs, or by hijacking the machinery of co-occurring elements in the case of IMEs<sup>35</sup>.

Overall, it is estimated that 43% and 31% of ICEs and IMEs, respectively, carry at least one defense system<sup>92</sup>. Remarkably, ICEs encoding RM and BREX systems can result in fluctuating-selection dynamics, explaining differences in phage resistance of clinical *V. cholerae* isolates<sup>24</sup>. These defense systems are also typically located in hotspots of genetic variability (**Figure 3C**). As with phage hotspots, it is not clear why defense systems accumulate in certain locations within these MGEs. We can hypothesize that they integrate in-between functional modules without disrupting their integrity (**Figure 3C**) and that some locations are more prone to recombination events, facilitating the exchange or capture of new defense systems.

The detection of these conjugative elements, particularly IMEs, and their specific boundaries remains challenging, likely leading to an underestimation of the number of defense elements they carry. For example, the defense region R2 of *Vibrio crassostreae*, contains a predicted recombinase and a relaxase, suggesting it represents an IME<sup>45</sup>. Another example is defense systems adjacent to mating pair formation proteins in *P. aeruginosa*, which might correspond to a functional ICE<sup>49,94</sup>. Recent bioinformatic approaches and databases<sup>95,96</sup> will shed more light on the precise contribution of chromosomal conjugative elements in bacterial immunity.

### 3.5 Plasmids and defense systems

Plasmids are self-replicating, extrachromosomal MGEs that can exist in multiple copies within a cell. Like ICEs and IMEs, they can be classified as conjugative or mobilizable, but also as non-transferrable<sup>97</sup>. In *E. coli*, various defense systems such as Gabija, tmn, PifA, ppl, and AbiQ are mainly carried by plasmids<sup>53</sup>. The Nhi defense system, a single protein defense which degrades specific phage DNA replication intermediates, was initially identified within chromosomal defense islands, but is frequently found on plasmids<sup>98</sup>. Only a small percentage of plasmids encode RM ( $\sim 3\%$ ) or CRISPR-Cas systems ( $\sim 1.4\%$ )<sup>99,100</sup>, in contrast with their ubiquity in bacterial chromosomes (80% and 40%, respectively). However, when accounting for the size of plasmid and the chromosome, CRISPR-Cas are in fact enriched on plasmids<sup>101</sup>. Besides antimicrobial and virulence genes, defense systems on plasmids

offer an additional mechanism for these elements to be selected<sup>102,103</sup>. For example, the multidrug resistant pEFER plasmid of *E. fergusonii* encodes BREX and a type IV RM system, which together provide complementary protection from unmethylated and modified phages<sup>104</sup>.

Genes in the leading region of plasmids are expressed immediately after transfer to the new host, and typically include genes such as anti-SOS or anti-restriction genes which ensure the successful establishment of the plasmid in a recipient bacterium<sup>105</sup>. Interestingly, Gabija and tmn defense systems tend to co-localize also in this region<sup>53</sup>, suggesting that blocking other MGEs is also an important factor in the establishment of plasmids in new hosts. Given that many plasmid proteins have unknown functions, plasmids are likely to carry uncharacterized defense systems. For example, strains of the marine bacterium *Sulfitobacter pontiacus* carry plasmids which can block the induction of prophages, through a currently unknown mechanism<sup>106</sup>.

### 3.6 Transposons and defense systems

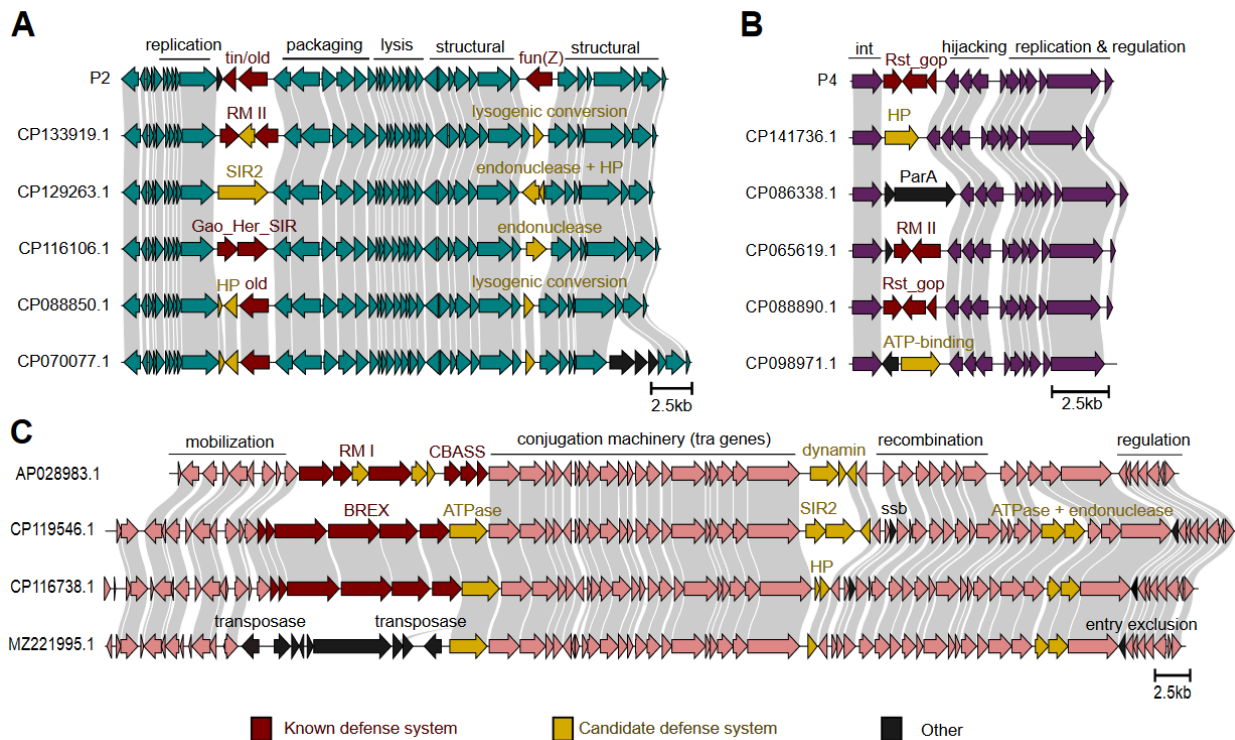
Transposons frequently move within a cell by inserting or excising themselves by means of a “cut and paste” mechanism<sup>107</sup>. They can move between cells through natural transformation, or more often by jumping into and transfer along other MGEs<sup>108</sup> (**Figure 1C**). Some transposons exhibit a high degree of homogeneity in their mobilization mechanisms, while others like Tn-7 employ multiple transposition pathways<sup>109</sup>.

Overall, it is estimated that 26% and 6% of Tn-7 transposons carry known innate and adaptive defense-systems, respectively, with toxin-antitoxin (TA) (16%), RM (14%), Gabija (5%), and type I-F CRISPR-Cas (4%), being the most frequent<sup>21</sup>. CRISPR associated transposons (CASTs) are a special class of Tn-7 like transposons, which use RNA-guided DNA integration for their transposition. CASTs also encode diverse defense systems including RM and Druantia, a particularly large system (~12 kb) with unknown mechanism<sup>110</sup>.

Genomic Island with three Integrases (GInt), a Tn6571-family transposon in *E. coli*, and tychepons in cyanobacteria were also shown to carry defense systems in their cargo<sup>27,111</sup>. Many of the accessory components of transposons and tychepons are associated with carbon metabolism or nutrient acquisition, with defense systems still accounting for a much smaller fraction<sup>21,111</sup>. However, since the majority of transposon cargo genes have unknown function, it is likely that they encode a substantial number of undescribed defense systems.

Altogether, the recognition of this diversity of defense-encoding MGEs heralds an exciting phase of further discovery. For instance, the analysis of the accessory genome of P4-like satellites indicates an open pangenome, with new accessory genes being found as more P4 genomes are analyzed<sup>112</sup>. Thus, it is expected that many of these satellites’ unknown accessory genes, as well as those from other MGEs,

act as defense systems. Analyzing closely related MGEs and their defense hotspots is a good starting point to identify them (Figure 3).



**Figure 3. Defense systems within MGEs are located at diversity hotspots.** Sequence comparison of closely related MGEs are shown for **A)** P2-like prophages **B)** P4-like satellites, and **C)** SXT-ICEs. Homology regions are highlighted in grey<sup>113</sup>. Functional modules of MGEs are also indicated. Known defense systems and domains were detected with defense-finder<sup>40</sup>. HP: hypothetical protein.

## 4. Chromosomal defense hotspots - a snapshot of MGE dynamics?

While many bioinformatically predicted MGEs may be active, a significant proportion could also represent decaying elements that are no longer mobile. Instead, these decaying MGEs may have been domesticated by the host and used for its own benefit<sup>114</sup>. Domestication of MGEs can even result in the repurposing of MGE core components for defensive functions, a well-known case of exaptation<sup>115,116</sup>. For example, the spacer acquisition machinery of CRISPR-Cas systems has evolved from the integrase of casposons, a special class of transposon<sup>117</sup>. Likewise, the Cas9 nuclease of type II CRISPR-Cas systems evolved from IS200/IS605-like transposons<sup>118</sup>.

Whether they originate from proteins involved in the mobility of genetic elements or not, most bacterial defense systems are associated with MGE components or found at MGE integration hotspots. Chromosomal MGEs, such as prophages and transposons, preferentially integrate at certain genomic

locations, such as tRNA loci, due to the site-specificity of their integrase<sup>119</sup>. The preference for tRNA loci might be explained by sequence conservation of tRNA genes, which is on average 6-fold higher than that of protein-coding genes, possibly increasing the host range of such MGEs<sup>119</sup>. This specific integration drives the acquisition of defense systems near the vicinity of tRNA genes or other MGE landing pads, resulting in chromosomal defense hotspots, genomic regions containing a diversity of defense elements among closely-related bacterial genomes (**Figure 4**). Importantly, while a defense hotspot is defined by its genetic context, i.e. its localization between conserved genes, a defense island is defined by its content, i.e. the colocalization of multiple defense systems. Therefore, chromosomal defense hotspots can contain a single defense system or a defense island depending on its size (**Figure 4**).

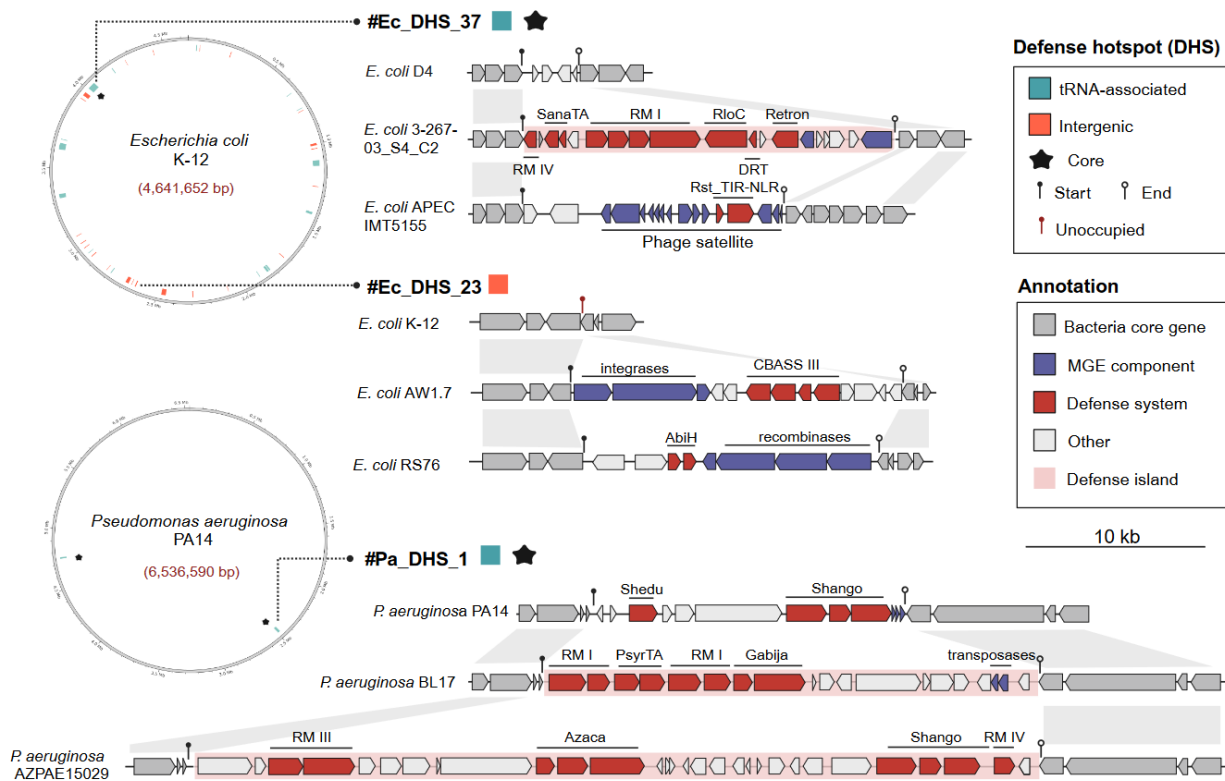
In *E. coli*, Hochhauser et al. identified 41 chromosomal defense hotspots, of which 90% were associated with MGEs and nearly half of them were located downstream of a tRNA<sup>27</sup>. Only a few strains had accessory genes at these positions, suggesting a recent integration of the MGE into the chromosomal hotspot. The exception was hotspot #37, found to carry defense systems in 97.3% of *E. coli* genomes and thus referred to as a “core defense hotspot” (**Figure 4**). Notably, the *E. coli* hotspot between genes *yegR* and *yegQ*, the integration site of P2-like prophages, illustrates how defense systems encoded within MGE hotspots contribute to chromosomal defense hotspots (**Figure 1C**).

In *P. aeruginosa*, Johnson et al., identified two core defense hotspots, cDHS1 and cDHS2, also associated with tRNA genes<sup>94</sup> (**Figure 4**). Unlike the accessory hotspots in *E. coli*, which usually contain one or two defense cassettes, core hotspots in *P. aeruginosa* are often occupied by dozens of defense systems, constituting defense islands that can reach up to 224 kb<sup>94</sup>.

The mechanisms that drive the accumulation of defense systems within large genomic islands remain poorly understood, but MGEs almost certainly play an important role<sup>29</sup>. Defense islands are also found at MGE integration sites and associated with a mixture of MGE elements such as recombinases and integrases<sup>27,94</sup> (**Figure 4**). After a MGE carrying a defense system integrates into the chromosome, MGE core functions can be rapidly lost, resulting in a defective MGE<sup>114</sup>. Then, mutations, genome rearrangements, or the integration of new MGEs can result in the growth of the island<sup>29,111</sup> (**Figure 1C**). Defense systems might be positively selected if they confer a benefit to the host cell, leading to their accumulation into an additive genomic island<sup>90</sup>. In addition to selection, genetic drift might play an important role in the growth or decay of defense islands<sup>120</sup>. As such, defense islands likely represent a snapshot of a continuum of different stages of shuffling MGEs and defense elements<sup>29,115</sup>. Some of these may have retained their ability to self-transfer, while others might rely on the mobility mechanisms of other nearby elements. For instance, lateral transduction can mobilize large sections of the bacterial genome downstream of a prophage<sup>121</sup>.

Overall, the location of defense systems in bacterial chromosomes highlights the important role of MGEs in their acquisition. While some defense systems seem to have been recently acquired, others may have accumulated in defense islands over extended periods. The current observations in *E. coli* and

*P. aeruginosa* provide valuable insights<sup>27,94</sup>, but further research is needed to uncover broader patterns in diverse bacterial species.



**Figure 4. Defense systems localize at chromosomal diversity hotspots linked to MGEs' landing pads.** Examples from *E. coli* and *P. aeruginosa* defense chromosomal hotspots (DHS) are shown<sup>27,94</sup>. tRNA-associated indicates a hotspot downstream of a tRNA or tmRNA loci. Core hotspot indicates genomic regions which are occupied by accessory genes in most of the strains of the species. Note that each work used a different methodology to detect defense hotspots. Therefore, this figure is not intended for direct comparison between the studies, but rather to provide an overview of the identified hotspots in each species.

## 5. The relative contribution of MGEs to the immune arsenal of bacteria

The pervasiveness of defense systems in MGEs is clearly demonstrated by a recent study of marine, soil, and gut metagenomes. In all environments, defense systems were found to be more prevalent in MGEs than in bacterial chromosomes<sup>28,41</sup>. In *E. coli*, this is further supported by experimental data<sup>20</sup>. It is however interesting to note that the contribution of MGEs to the immune arsenal of bacteria might differ between clades. While in many species defense systems appear to be linked to active MGEs<sup>20,22,24,27,45</sup>, in Actinobacteria only 2% of defense systems are encoded in prophages and 3.4% in plasmids<sup>41</sup>. These differences might result from taxa-specific dynamics and defense systems, but also from the fact that besides well-characterized bacteria, detecting MGEs and their boundaries remains challenging. For example, phage satellites are unknown in most hosts, and they are not

considered in most analyses<sup>28,41</sup>. However, they seem to be widespread and recent work has described satellite-like elements in marine bacteria<sup>111,122</sup>. Intriguingly, similar defense islands have been found in distantly-related *Pseudomonas* strains, but they lacked association with any known MGE<sup>94</sup>. Their mobility might result from an undescribed mode of transfer, or they may represent unknown MGEs themselves. For example, defense islands carrying integrases, but no other known mobility gene have been proposed to represent unidentified types of transposon or satellite-like elements<sup>27,111</sup>.

Despite these limitations, the prevalence of defense systems in all main MGE families (See section 3) raises the question of their relative contribution to the bacterial defense arsenal. In metagenomes, ICEs/IMEs contribute the most to the total number of defense systems compared to plasmids and prophages. However, the defensive repertoire of plasmids is far more diverse, carrying more distinct defense systems compared to ICEs/IMEs<sup>28</sup>. On the other hand, defense hotspots in *E. coli* are mostly occupied by prophages and phage satellites, followed by ICEs/IMEs (12%), and transposons (1%)<sup>20,27</sup>. It is worth noting that the dynamics of different MGEs can be varied. For instance, the high diversity of defense systems in plasmids can be attributed to their increased plasticity and promiscuity compared to other MGEs<sup>28,103</sup>. Distinguishing between MGE types can also be challenging as MGEs can exist on a continuum rather than as discrete categories. For example, smaller MGEs such as transposons are often nested within larger MGEs such as plasmids and ICEs<sup>123</sup> (**Figure 1C**). Conjugative defense islands can exist in either integrated or episomal forms<sup>49</sup>. The recent characterization of phage-plasmids, a hybrid MGE that combines features of both phages and plasmids, further illustrates the blurred lines between MGE types<sup>124</sup>.

The systematic detection of defense systems across metagenomes highlights the diversity of associations between defense systems and different types of MGEs<sup>28</sup>. However, some defense systems are more frequently found in specific MGEs, which suggests that some MGEs have coevolved with specific defense systems. For example, defense systems such as BstA, a single-protein system spanning less than 1 kb<sup>84</sup>, and RexAB, a compact two-protein system initially characterized in phage lambda<sup>125</sup>, are predominantly observed in phages<sup>40</sup>. Such association could partly be driven by the physical constraints on the amount of DNA that different MGEs can carry, with phages being enriched in smaller defense systems. In contrast, larger defense systems like BREX, a system that often comprises 6 genes<sup>10</sup>, are enriched in ICEs/IMEs<sup>27</sup>. In addition, the high prevalence for some MGE-defense associations likely result from the specific targets of these defense systems, and the relationship they have with the host. We describe examples of such associations in the following section.

## 6. The role of defense systems in a complex network of MGEs

The presence of defense systems within MGEs can be interpreted as a consequence of their selfish nature, aiming to ensure their own survival and propagation<sup>29,115</sup>. Defense systems carried by

MGEs affect not only the survival of the host cell and the focal MGE, but also the success of other interacting elements, ultimately shaping both the MGE and defensive repertoires of the bacterium. MGEs rarely occur alone within their host but continuously interact with other MGEs. For example, *Klebsiella pneumoniae* strains harbor a mean of 4.5 prophages and 3.4 plasmids<sup>126</sup>. Interactions between MGEs can be mutualistic, enhancing each other's replication and/or transmission. For example, transposable elements can jump from chromosome to plasmid, benefiting from plasmid-provided mobility while in turn providing the plasmid with adaptive traits, thereby securing plasmid persistence<sup>108</sup>. Similarly, mobilizable plasmids and integrative elements rely on other conjugative elements, and in some cases on phages<sup>127</sup>, for their motility. However, as this final section will illustrate, interactions among MGEs are often antagonistic, with the defensive arsenal they carry playing a major role.

## 6.1 Defense systems in the interactions between phages

After a temperate phage integrates in the host genome and enters the lysogeny state, subsequent phage infections pose an immediate threat, due to the risk of lysis and within-host competition for resources<sup>128</sup>. Therefore, prophages block closely related phages, in a phenomenon known as superinfection exclusion (Sie), or distantly related phages by encoding diverse anti-phage mechanisms<sup>62,129</sup>.

The mechanisms used by prophages to block other phages are varied<sup>130</sup>. First, prophages can modify the cell envelope, hindering the recognition of cell receptors by other phages. For example, *Pseudomonas* prophages block other phages by modifying the type IV pilus, while *E. coli* prophages block K1 phages by K1 capsule O-acetylation<sup>71,131</sup> (**Figure 2**). After adsorption, residing prophages can prevent translocation of the viral genome across the inner membrane, like *Escherichia* prophage HK97 blocking other HK97 or related HK75 phages, or *Mycobacterium* prophages blocking DNA injection of distant phages<sup>81</sup> (**Figure 2**). Additionally, the very mechanism that controls the lysogeny decision, and ensures that genes involved in the lytic cycle are silenced, will also silence the lytic genes of related phages<sup>81</sup>. This phenomenon called homotypic exclusion can be viewed as a form of phage defense resulting from the regulation of the prophage lysogenic cycle.

Next, prophages are protected from other phages by encoding dedicated DNA interference mechanisms. For example, RM-like systems are frequently encoded by *Escherichia* P2-like prophages<sup>19</sup> (**Figures 2 & 3**). Complete CRISPR-Cas systems are rare in prophages, but they can encode mini-arrays which can be functional after hijacking the required Cas machinery from the host cell<sup>17,132,133</sup>. The analysis of prophage-encoded CRISPR-arrays reveals other closely- and distantly-related prophages as the preferred target<sup>132</sup>.

Finally, systems that lead to cell death are also found in prophages, preventing competing phages from spreading to other cells of the host population at the cost of killing their own host cell<sup>46,74,84,129</sup>. This outcome can be mediated by defenses targeting host processes essential for phage replication. For example, the CapRel system carried by *Enterobacteria* prophages detects viral capsids and leads to the modification of the host tRNAs, ultimately blocking viral translation<sup>46</sup>(**Figure 2**). Interestingly, the same outcome can result from the action of systems targeting phage assembly, such as the recently discovered Tab system from *P. aeruginosa* prophages. Tab inhibits tail assembly after recognizing the tape measure protein of other phages, which ultimately results in cell death through the action of the phage lysis genes<sup>14</sup>. It is not clear whether prophage-encoded Abi systems preferentially target closely or distantly related phages. An example of the first is the BstA system carried by enterobacteria phages<sup>84</sup>. To avoid self-immunity, BstA encoding prophages carry an anti-BstA (aba) element, which guarantees immunity to the replicating prophage while preventing infection of other BstA-encoding phages<sup>84</sup>.

From an evolutionary perspective, defense-encoding prophages likely have a competitive advantage over other invading viruses. For example, a phage with superinfection exclusion is predicted to outcompete a superinfecting phage, even if the latter has a higher growth rate<sup>134</sup>. Additionally, phage cooperation can also play a role in defense-encoding prophages, particularly in cases where the prophage can block adsorption of other phages of the same kin, helping them to find more permissive phage-free hosts<sup>135</sup>. Abi systems encoded by prophages can also be seen as “public goods”. When activated, they can be extremely costly to the prophage, but they can provide protection to related phages in the bacterial population. Overall, virus-virus interactions likely determine the defense arsenal of prophages.

It is also interesting to consider the specificities of defense against temperate phages. Most studies have focused on defense against productive infections of the target phage, but some defense systems are known to tolerate lysogeny while still restricting the lytic cycle. This has for instance been shown for type III CRISPR-Cas systems whose activation depends on the transcription of the target gene<sup>136</sup> or for a *Pseudomonas* CBASS system which senses the major capsid gene of target phages<sup>137</sup>. This is also the case for some prophage encoded defense systems including BstA<sup>84</sup>. We can further expect this to be true for other anti-phage systems activated by molecular patterns produced during the phage lytic cycle<sup>85</sup>. We can hypothesize that in some circumstances, letting other prophages in, together with the potentially beneficial moron genes they carry, could be beneficial both for the host and the existing lysogens. Some anti-phage systems might even favor lysogeny, as was recently described for the MazEF TA system of *B. subtilis* which promotes lysogeny of phage  $\phi$ 3T at high MOI<sup>138</sup>. More work is needed to understand the mechanisms deployed by phages to control each other's life cycles, and how these interactions explain the distribution of prophages in bacteria, with some species being mostly poly-lysogenic<sup>139</sup>, while other species only harbor a single prophage on average<sup>79</sup>.

## 6.2 Defense systems in the interactions between satellites and phages

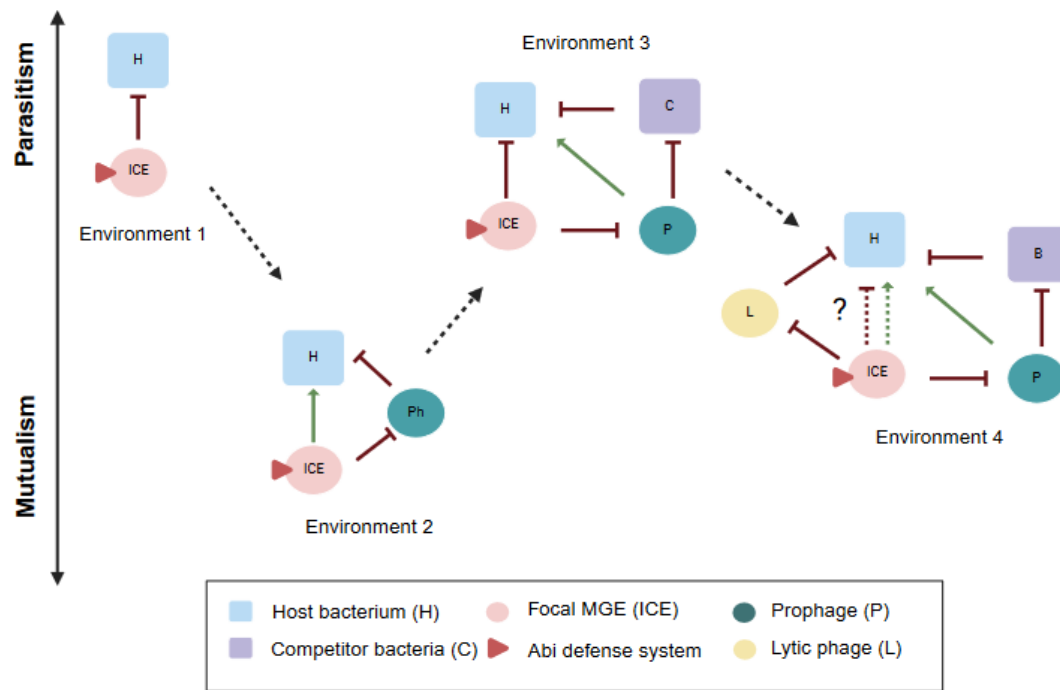
The interest of phage satellites, often referred to as hyperparasites, is also not always aligned with that of the host cell, as the satellite can benefit from the death of its host during infection by a helper phage. Satellites move between hosts along with their helper phage and can sometimes integrate into the same host chromosome. Interestingly, the accessory defense systems found in PICIs or P4 satellites rarely inhibit their helper phage<sup>19,25</sup> (**Figure 1D**). Satellites of temperate phages could thus have a mutualistic relationship with their helper phage rather than a parasitic one: the helper phage provides mobility to the satellite, while the satellite expands the bacterial defensive arsenal that benefits the helper phage by protecting the common host. Such a symbiotic relationship would break down if a virulent helper phage is unable to lysogenize. This is the case for the ICP1 phage of *Vibrio*, which shows a strongly antagonistic relationship with its PLE satellite and thus carries counter measures such as a CRISPR-Cas targeting PLEs<sup>140</sup>. As opposed to other satellites, PLEs completely block replication of their helper phage and have not been found to carry accessory defense genes against other viruses. We hypothesize that the lifestyle of the helper phage, its relationship with its satellite, and the resulting selection pressure can determine whether satellites carry accessory defenses or not.

Defense systems carried by satellites have also been found to block other satellites, as seen for PICI-encoded defense protecting against other PICIs<sup>25</sup>. P4-like and PICI satellites were shown to co-occur less than expected within *E. coli* hosts<sup>86</sup>, which suggests an antagonistic relationship. However, the complex network of interactions between the different satellites, their respective helper phages and the host makes it extremely challenging to ascertain the evolutionary forces driving such negative association.

## 6.3 Defense systems in the interactions between plasmids

The presence of anti-phage systems in plasmids may suggest that their primary function is to protect their host from phages. However, plasmids also interact with other plasmids, and similarly to homotypic exclusion in prophages, plasmids from the same incompatibility group cannot stably co-exist within the same cell<sup>141</sup>. In addition, plasmids encode many DNA targeting systems (RM, BREX, and CRISPR-Cas)<sup>101,104</sup>. A clear example of this is type IV CRISPR-Cas, which is almost exclusively encoded on plasmids and primarily targets other plasmids<sup>142,143</sup>. This raises the question of whether plasmid-defense associations have evolved primarily to protect their hosts from phages, or as a result of plasmid competition. The answer likely lies somewhere in between: plasmids may compete with other plasmids for host resources, act selfishly to ensure their maintenance, but also face a selective pressure to increase host survival by protecting from phage infection. Plasmids therefore benefit from DNA targeting systems able to block other MGEs regardless of their nature. Plasmid-encoded TA modules

might also kill two birds with one stone: they ensure plasmid maintenance by post-segregational killing of plasmid-free progeny, but some TAs have also been found to act as anti-phage systems<sup>144–147</sup>. Such multifaceted blocking of MGEs, however, comes at the cost of decreased gene flow and thus bacterial adaptation, representing a double-edged sword for the host.



**Figure 5. The parasitism-mutualism continuum of MGEs encoding defenses depends on the environment and resulting MGE interactions.** To illustrate this concept, we used the coexistence of two antagonist elements from *B. subtilis*: an ICE carrying the Abi system *spbK* active against a residing  $\text{SP}\beta$  prophage<sup>148</sup>. In Environment 1, the ICE imposes a cost on the host cell (H) due to the metabolic burden of the conjugation machinery and the leaky action of the Abi system. Therefore, the ICE can be seen as a bacterial parasite. However, in the presence of the prophage  $\text{SP}\beta$  (Environment 2), the ICE-encoding *spbK* system blocks the lytic induction of the prophage and its spread to the surrounding host population<sup>148</sup>. In this case, the interaction between the host and the ICE becomes mutualistic. In Environment 3, the host bacterium competes with another bacterium (B) and has a competitive advantage, conferred by the bacteriocin encoded by the  $\text{SP}\beta$  prophage<sup>149</sup>. Consequently, the ICE has a negative effect on the host cell due to *spbK* targeting  $\text{SP}\beta$  prophage. Finally, if a lytic phage (L) infects the host cell (Environment 4), the ICE *spbK* gene might provide protection. In this context, whether the ICE benefits the host or not depends on the balances of selective forces between all these elements. Note that some of the interactions depicted here have not been demonstrated experimentally and are shown for illustrative purposes.

## Concluding remarks

MGEs are bacterial symbionts with their own evolutionary interest. They can act as parasites but also confer beneficial traits to their hosts, including defense systems against other MGEs (**Figure 1**). Transition from parasitism to mutualism can occur as a result of environmental changes, which includes

a complex network of interacting MGEs<sup>29,115,150</sup> (**Figure 5**). For instance, the ICEBs1 element co-exists with the SP $\beta$  prophage in most strains of *B. subtilis*, despite ICEBs1 carrying an Abi system triggered by that phage lytic cycle<sup>148</sup>. SP $\beta$  carries a bacteriocin and therefore its presence can be advantageous to the host bacteria<sup>149</sup>. The Abi system itself might provide defense against other phages as well, leading to a complex landscape in which the balance of selective forces results in the stable coexistence of seemingly antagonistic elements (**Figure 5**). The composition of the bacterial immune repertoire is thus largely determined by the outcome of the conflicts between MGEs that use their bacterial hosts as a battleground.

On the other hand, bacteria can benefit from the defenses provided by MGEs, and their high mobility within bacterial populations offers opportunities for rapid adaptation to new or fluctuating threats. A remarkable example is that of *Vibrio* strains harboring SXT-ICEs with phage defense systems. Phage pressure increases the rate at which defense-encoding ICEs are transferred to other strains, ultimately providing phage resistance to initially susceptible bacteria<sup>24</sup>. The acquisition of a MGE, however, can also result in the loss of defenses. This was recently described in coral associated microbes, where the co-transfer of both, an IME and ICE from competitor *Pseudoalteromonas* spp. resulted in the loss of a phage defense island in *Vibrio alginolyticus*, rendering it susceptible to phage infection<sup>151</sup>.

Depending on the point of view, we can either see MGEs as tools manipulated by bacteria or as agents that use bacteria as their playground. MGEs have likely co-evolved with bacteria since their very beginning, resulting in a complex network of interactions in which defense systems play a crucial role. Understanding these interactions is essential for comprehending bacterial immunity, with many questions still awaiting answers (**Box 1**). This has significant implications for both fundamental biology and the advancement of biotechnology and medicine. Phages and conjugative elements are being repurposed as therapeutic tools in the fight against antibiotic resistance<sup>91,152,153</sup>. However, the ubiquity and diversity of defense systems presents a limitation to their application<sup>50,51,154</sup>. Thus, determining the network of interactions between defenses and MGEs, as well as identifying the corresponding anti-defense genes, is crucial for the success of these therapeutic strategies.

### **Box 1. Outstanding questions about MGEs and defense systems**

- What drives the localization of defense systems within MGEs and defense islands? Is this linked to their mode of acquisition, regulation or exchange? The organization of defense systems, their location, the size of the hotspots and their diversity differs between genetic elements, as well as between bacterial species. More work is needed to describe this organization in diverse species and the factors involved.

- What are the targets of defense systems beyond lytic phages? Most of the work performed on defense systems against MGEs investigated the inhibition of lytic phages, but little is known about the full spectrum of elements targeted.
- Which factors drive the specific association of certain MGEs and defense elements? Little is known about how the ecological and evolutionary interactions between different types of MGEs and the host might favor different defense mechanisms, and how these interactions are modulated by the environment. Do defense systems completely exclude targeted MGEs, or can they provide a more fine-grained control, enabling the presence of target MGEs while limiting their cost?
- Conversely, what can defense systems teach us about the ecological and evolutionary interactions between different MGEs? Studying defense systems will likely help us to better understand the selective pressures faced by different genetic elements, their network of interactions and host range.
- How much of the unknown “cargo” of MGEs still represents novel defense systems? More than 150 systems have been described to date. Will newly discovered systems simply provide small variations on known systems, such as new combinations of known sensor and effector domains or demonstrate entirely novel molecular complexes and mechanisms of action? The study of systems encoded by understudied MGEs might provide unique insights.
- How much of the defensible, including defense islands, are indeed mobile? What is their mechanism of mobility and how is this mobility regulated? We still lack evidence for the mobility of many elements carrying defense systems. Their study might reveal unique modes of horizontal gene transfer.

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## Declaration of Interests

David Bikard is a co-founder and shareholder of Eligo Bioscience.

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