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Mobile Genetic Element Flexibility as an Underlying Principle to Bacterial Evolution

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Abstract

Mobile genetic elements are key to the evolution of bacteria and traits that affect host and ecosystem health. Here, we use a framework of a hierarchical and modular system that scales from genes to populations to synthesize recent findings on mobile genetic elements (MGEs) of bacteria. Doing so highlights the role that emergent properties of flexibility, robustness, and genetic capacitance of MGEs have on the evolution of bacteria. Some of their traits can be stored, shared, and diversified across different MGEs, taxa of bacteria, and time. Collectively, these properties contribute to maintaining functionality against perturbations while allowing changes to accumulate in order to diversify and give rise to new traits. These properties of MGEs have long challenged our abilities to study them. Implementation of new technologies and strategies allows for MGEs to be analyzed in new and powerful ways.

Contents

1. INTRODUCTION

Plasmid: an extrachromosomal replicon, circular or linear, that is often replicated independent of the chromosome; can be conjugative, mobilizable, or nonmobilizable

Integrative and conjugative element (ICE): DNA element that encodes the capacity to integrate into and excise out of a chromosome and has the ability to transfer

Integrative and mobilizable element (IME): DNA element that encodes the capacity to integrate into and excise out of a chromosome and has the ability to be transferred

Integron: DNA elements composed of an integrase, a promoter, and rearrangeable modules of genes flanked by repeats

Mobile genetic elements (MGEs) can move within and between genomes to profoundly affect the evolution of bacteria. MGEs scramble genomes, alter genome composition, shift lifestyles, and trigger the emergence of new lineages (44, 104, 118, 124). Conversely, these elements limit genomic changes by introducing immunity that protects against invasion by other MGEs (14). MGEs also affect the health of eukaryotes and ecosystems that host bacteria by vectoring traits such as metabolism, virulence, symbiosis, and host specificity (37, 113). One of the more pressing concerns is antimicrobial resistance (AMR), such as in the clinically important ESKAPE pathogens (composed of the nosocomial pathogens *Enterococcus faecium*, *Staphylococcus aureus*, *Klebsiella pneumoniae*, *Acinetobacter baumannii*, *Pseudomonas aeruginosa*, and *Enterobacter* spp., which have multidrug resistance) (19, 36, 45). The success of MGEs in rapidly promoting evolution stems from their capacity to mobilize broadly. Horizontal gene transfer is hypothesized to be 50 times more frequent than gene duplication and can significantly affect members of an environment (90, 128).

Further underscoring their importance is the prevalence of MGEs across bacteria (1, 112). MGEs are diverse and include plasmids, integrative and conjugative elements (ICEs), integrative and mobilizable elements (IMEs), integrons, transposons, insertion sequence (IS) elements, and phages (46). ICEs are present in most bacterial lineages and are the most abundant large MGEs; as recently reported, greater than half of all sequenced bacterial genomes have at least one predicted ICE-like element (54, 76). In another study, more than 84% of the analyzed genomes of *P. aeruginosa* carry one or more ICEs, with roughly half of these ICEs having AMR genes (20). Within taxonomic groups, the estimated amount that MGEs contribute to genomes can be substantial (65, 138). This review does not discuss phages and we direct the reader to other excellent reviews (24, 30, 126).

Understanding processes and properties that shape MGEs is central to understanding the evolution of bacteria and the impacts MGEs have. First, we use the perspective of a system to describe recent work and highlight emergent properties of MGEs (96, 132). Robustness is one such emergent property (77) (see the sidebar titled Importance of Contextualizing Robustness). Robustness maintains functionality (phenotypes or traits) in the face of perturbations such as genetic variation and environmental changes. Modularity, particularly one that is hierarchical, is another emergent property and an organizing principle that promotes flexibility to complex biological systems (133). Together, modularity and flexibility underpin robustness. Phenotypes robust against genetic variation have capacitance (92). Under conditions in which a phenotype is robust against a perturbation, the phenotype is maintained but also allows for nonlethal mutations or new combinations of alleles to be generated. When exposed to a perturbation that breaks down robustness, capacitance provides the variation that enables a new phenotype to emerge. Second, we describe barriers to

IMPORTANCE OF CONTEXTUALIZING ROBUSTNESS

Robustness needs to be properly contextualized (77–80). It is essential to explicitly define the phenotype(s) of interest, the components of the system and their interactions, and the perturbation(s) that the phenotype is robust against. It is crucial to understand that robustness pertains to the phenotype of interest, not to the components that give rise to the phenotypes (79). In fact, robustness requires flexibility and components must vary and interact in diverse ways to yield a similar phenotype.

MGE acquisition and how emergent properties of MGEs overcome these barriers. Finally, we highlight approaches for studying MGEs. This is an exciting and fast-paced time for studying MGEs, with many new and insightful findings constantly being reported.

2. MGEs ARE PART OF MODULAR SYSTEMS

The lens we choose to look through influences how we see MGEs. When we examine MGEs in an individual strain, MGEs and the traits they vector may be considered unstable. For example, the genome of *Borrelia burgdorferi* strain B31 MI comprises a chromosome and up to 21 circular and linear plasmids, with some frequently lost when the strain is cultured in vitro (27, 52). Likewise, in agricultural systems, the mobility of symbiosis MGEs may be seen as a negative, as it often results in less effective native rhizobia acquiring an MGE and outcompeting inoculant strains (121). However, if we examine mobility from the perspective of natural ecosystems, transfer and acquisition may allow rhizobia to change and benefit diverse legume species (137). When we examine a single trait encoded by an MGE present in a single lineage, we may see a pattern of clonal expansion and global spread of superfit pathogens (6). When we examine traits detrimental to health, we may consider the mobility of MGEs as problematic, but when we examine beneficial traits, their mobility may be advantageous.

An alternative lens is to view MGEs as components of a hierarchical and modular system, and doing so focuses on their emergent properties to provide generalizable concepts. Bacteria that interact, or have the potential to interact, over an evolutionary timescale are the highest level of such a system (12). The mobilome, or ecosystem of MGEs, is the next hierarchical level. Individual strains can potentially host multiple MGEs, and two strains with identical sets of core genes can conceivably have different MGEs and lifestyles (56, 135). The potential for high numbers of MGE combinations to be in a population enables members to explore a large gene space for combinations that provide selective advantages under diverse pressures. Members of the agrobacteria/rhizobia complex have diverse *repABC* plasmids and can have up to five different plasmids (136). These plasmids are hypothesized to be involved in catabolism and could make cells more competitive for different nutrients. In addition, *repABC* plasmids exist among a backdrop of other MGEs, which further amplifies the number of possible MGE combinations. This modularity is analogous to drawing from a deck of cards, where each MGE is a card, to yield a combination that could possibly beat the hands of opponents. MGEs also provide an easier and lower-risk path than chromosomes that allows trading in and drawing new cards to improve combinations before going all in.

The next level consists of functional modules (25, 71, 127). A module is a system of highly connected components integrated with a biological process and more loosely connected to others. Genes of MGEs that contribute to a common function or subfunction tend to be clustered and syntenic across different MGEs. The most fundamental modules consist of core MGE genes, which

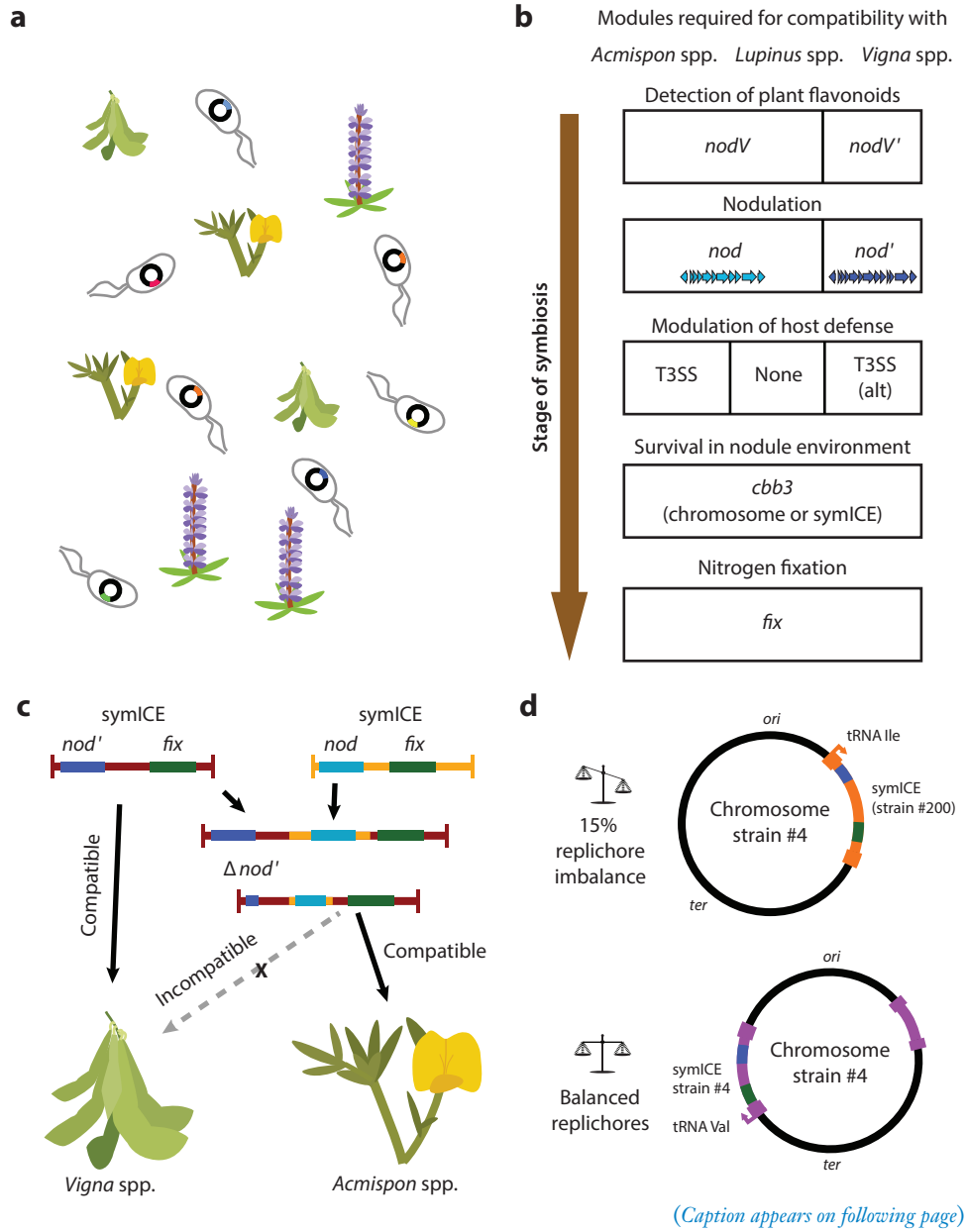
Transposon: DNA element that encodes for the capacity to excise and move between regions of a genome; may carry accessory genes

Insertion sequence (IS) element: DNA element that can excise and transpose from one region of the genome to another

Emergent property: a feature that occurs in a system but not in its components on their own; features emerge when components interact

encode functions necessary for maintenance and transmission. Conjugative elements also include genes that encode for a type IV secretion system and associated functions. Within MGE families, core genes have homologs present in each member and their sequences have been traditionally used for phylogenetic analyses to infer evolutionary histories (100, 102).

Accessory genes can be shuffled extensively within a family of MGEs (32, 99). They can also recombine into diverse MGEs varying in core functions (**Figure 1*a***). This yields a pool of molecules encoding a common trait, which allows diverse strains to quickly adapt under changing



(*Caption appears on following page*)

Figure 1 (*Figure appears on preceding page*)

Modularity diversifies traits and mechanisms of replication and/or transmission. Complex traits, such as symbiotic nitrogen fixation, are encoded by a system of modules distributed within and across MGEs. These modules can be reshuffled to generate new combinations that preserve but diversify the overall function. (*a*) An ecological community composed of diverse legume hosts and *Bradyrhizobium* symbionts. The rhizobia genomes carry diverse symbiosis ICEs that confer symbiotic nitrogen fixation with specific host plants. (*b*) Traits that are collectively necessary for symbiotic nitrogen fixation by *Bradyrhizobium* spp. are encoded in discrete modules. Different host groups require their symbionts to carry a specific combination of modules. (*c*) Exchange between MGEs can result in changes to symbiotic nitrogen fixation. The shuffling of a nodulation module from one symbiosis ICE to another results in a shift in host specificity. (*d*) Shuffling of modules onto different ICE backgrounds increases the number of possible chromosome–ICE combinations to explore compatible interactions. ICEs target different integration sites in the chromosome. Depending on the target location and their size, some ICEs may disrupt chromosome balance and cause fitness defects, while others result in less of a disruption. Abbreviations: ICE, integrative and conjugative element; MGE, mobile genetic element; symICE, symbiosis ICE; T3SS, type III secretion system.

environments. Varied combinations of AMR genes can be found on plasmids or multiple ICEs in multidrug-resistant *Haemophilus influenzae* (62). Similarly, regions, some of which include AMR genes and are identical in sequence, have been identified on diverse plasmids and ICEs in hospital-associated strains of different genera (43). In another example, the dissemination of carbapenemase genes among *K. pneumoniae* strains was modeled to have occurred via three different trajectories: on one plasmid in multiple lineages, on multiple plasmids in multiple lineages, and on multiple plasmids in one lineage (34).

The potential impact that different MGE and chromosomal backbones have on trait spread was exemplified by characterizing in vitro chimeras of the ICEs Tn*916* and ICE*Bs1* (13). Derived variants with new combinations of genes conferring integration/replication or conjugation differed in rates of transfer and transformation efficiency, dependent on the genetic background of the host bacteria. Moreover, the variants had host ranges that differed from either parental ICE, and some variants are able to spread to more diverse microbial hosts. In fact, structures of MGEs may promote the diversification of core functions. Models have suggested that the mechanism by which some symbiosis ICEs excise leads to a reorganization of genes, such that symbiosis gene modules are physically separated from core genes (137). This simplifies the process of swapping core (or accessory) genes to associate the symbiotic nitrogen-fixing trait to diverse ICEs (31, 51, 60, 105, 137).

Overall, we can think of MGEs as amalgamations of mobilizable functional modules, and understanding how and why they came together is one of the major goals. Modules themselves are organized in a hierarchy. On the one hand, modules can interact to yield a higher-level function and new combinations give rise to genetic variation. For example, members of *Agrobacterium* require an oncogenic Ti or Ri plasmid to genetically transform and cause disease to plants. These plasmids carry at least one transferred region (T-DNA) necessary for causing disease symptoms and a *vir* superoperon necessary for transferring the T-DNA (68). One of the Vir proteins recognizes sequences that border the T-DNAs to process them for transfer. These two modules are interchangeable presumably because border sequences of T-DNAs are highly conserved across all those that have been described. Consequently, functionality is retained among a diversity of T-DNAs and *vir* module combinations, including the occurrence of multiple T-DNAs per single *vir* locus. On the other hand, modules may be composed of submodules that can be shuffled into new combinations. In some *Agrobacterium* strains, the *vir* genes originate from different sources and are fragmented into multiple loci within an oncogenic plasmid or across plasmids (59, 136). These cases exemplify the impact of modularity and flexibility on robustness, which in this case is the capacity to withstand genetic perturbations while maintaining virulence.

A similar hierarchy of modules occurs in ICEs. In symbiosis ICEs, a set of *fix* genes confer nitrogen fixation, which in combination with nodulation (*nod*) genes gives rise to symbiotic nitrogen fixation (93) (**Figure 1*a*,*b***). While loss of either *fix* or *nod* genes renders a cell incapable of symbiotic nitrogen fixation, from the perspective of a system, the function is still robust because traits can be (re)acquired from the population (78) (**Figure 1*a***). This has been observed in *Mesorhizobium* spp. isolated from fields where following the introduction of a beneficial strain, new beneficial lineages with the same symbiotic ICE were detected (121). Additionally, exchange can generate new combinations and alter host range (**Figure 1*b***). This has been detected in *Bradyrhizobium* spp., where a swap of *nod* genes was identified and hypothesized to cause a change in host specificity (137) (**Figure 1*c***). Many symbiotic ICEs also have a locus that encodes for a type III secretion system and secreted effectors. This secretion system is not necessary for symbiosis, but its presence/absence and variations in its collection of secreted proteins demonstrably influence host range (123) (**Figure 1*b***). Therefore, like plasmids, the hierarchy and modularity of ICEs provide flexibility and, in *Bradyrhizobium* spp., alter host specificity while maintaining the overall function of symbiotic nitrogen fixation.

Recombination is not restricted to within classes of MGEs, and traits can be swapped between plasmids and ICEs (137). ICEs are predicted to have broader host ranges than conjugative plasmids, and the latter are predicted to have greater levels of plasticity with more variable collections of genes (33). Plasmids are more likely to carry AMR genes, and ICEs are more likely to carry metabolism genes. Among plasmids, the conjugative plasmids have a higher proportion of accessory genes than do mobilizable or nonmobilizable plasmids (95). Transfer of traits from ICEs to plasmids can overcome potential conflict caused by ICEs integrating into the chromosome, the latter of which is described in Section 5. Shuffling can also occur between MGEs and chromosomes (**Figure 1*b***). Rhizobia must be capable of surviving in microoxic root nodules, where they fix nitrogen. Survival in such an environment requires a particular set of *fix* genes, which in *Bradyrhizobium* spp. are typically located within chromosomes. Evidence suggested that these *fix* genes were acquired by diverse symbiosis ICEs and such variants can be found in strains that lack homologs in their chromosomes (137). This extended the symbiotic nitrogen fixation to strains that are not normally capable of supporting the trait (137). A similar example for the virulence trait of a plant pathogenic bacterium has been reported (103). Plasmids in *Chlamydiaceae* also are frequent vectors of gene flow among chromosomes (81).

3. RECOMBINATIONAL HOT SPOTS OF MGES

Core genes and sequences of MGEs can promote shuffling. In fact, because core genes are conserved, they are sites for homologous recombination and can promote the acquisition of linked accessory genes (109, 134). One possible mechanism of recombination is through cointegration of MGEs (**Figure 2*a***). Evidence supports such a mechanism among agrobacterial oncogenic plasmids, which have formed cointegrates with each other and with non-oncogenic *repABC* plasmids (134, 136). Another mechanism is the formation of exchangeable cassettes by flanking accessory loci with homologous core sequences. Several families of multidrug-resistance (MDR) plasmids found in nosocomial *A. baumannii* have few core regions yet have extensive variation in modular accessory loci (16, 86, 99). However, in these plasmids, genes are bordered by inversely oriented plasmid-*dif* (p*dif*) sequences (7, 16) (**Figure 2*c***). These sequences are conserved and typically function with XerC/XerD recombinases to resolve circular molecules linked following replication (99). In members of the GR34 plasmids of *A. baumannii*, so-called *dif* modules can exhibit extreme variation in composition and arrangement (86). Moreover, several *dif* modules in the GR13 plasmid pDETAB5 are highly similar to those in the GR34 plasmid pDETAB2, found in another strain

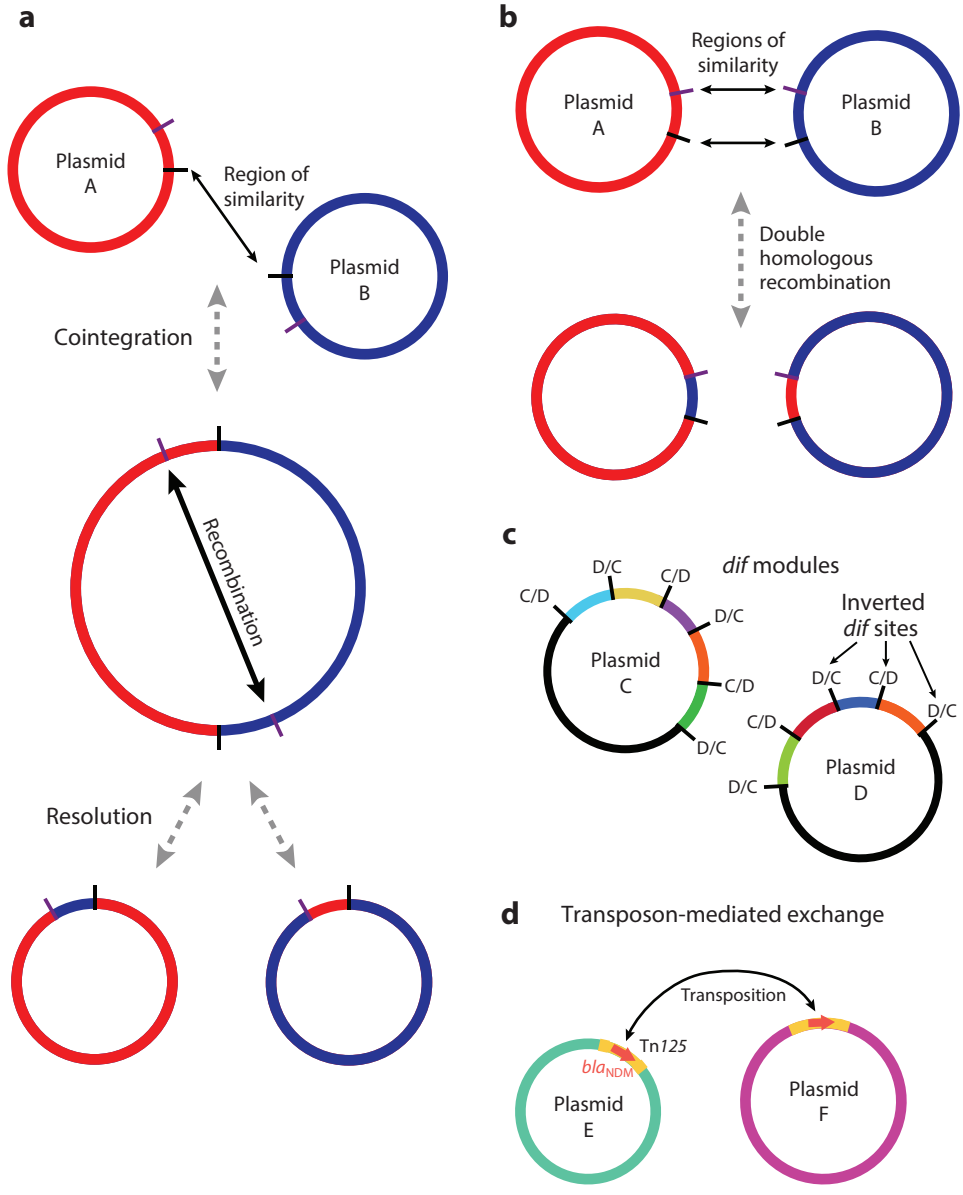


Figure 2

Possible mechanisms for diversifying MGEs. Cointegration and homologous recombination can result in exchange of modules among MGEs. (*a*) Recombination at homologous regions between two plasmids can result in their cointegration. Subsequent homologous recombination at another site can separate elements but cause exchange of regions between those sites. (*b*) Homologous flanking sequences can result in the exchange of intervening sequences. (*c*) Conserved sequences (e.g., p*dif* sites) can promote modularity. Plasmids with similar backbones can carry different accessory gene modules. Recombination mediated by XerC/XerD recombinases at *dif* sites can result in the gain, loss, or exchange of modules between plasmids. (*d*) Transposons carrying accessory genes, or composite transposons flanking an accessory region, can mediate exchange between MGEs. AMR genes, such as bla_{NDM}, can be mobilized from one plasmid to another on transposons. Abbreviations: AMR, antimicrobial resistance; MGE, mobile genetic element; p*dif*, plasmid-*dif*.

isolated from the same hospital a month earlier, suggesting that modules were transferred by recombination of *dif* sequences between different plasmid families (99). An important consequence of recombination among core genes is that it challenges the appropriateness of using them to infer evolutionary relationships.

In other MGEs, hot spots are associated with accessory sequences and promote recombination among just a subset of plasmids or within specific regions of plasmids. In several of the *repABC* plasmid families of rhizobia, accessory regions are at specific sites and are bordered by homologous sequences (136). Recombination is predicted to have swapped modules between plasmids of different families (**Figure 2*b***). Similarly, hot spots have also been identified or predicted to be present in ICEs. The SXT/R391 ICEs of *Vibrio cholerae* have a conserved backbone and five predicted hot spot regions. The proximity of hot spots to accessory genes has led to hypotheses that recombination has diversified SXT/R391 ICEs and mediated a coevolutionary arms race with phages (84).

Small MGEs located within larger MGEs can be hot spots for recombination. IS elements and transposons are major drivers because they can move between locations in a genome and proliferate (118). Transposons, unlike IS elements, can directly mobilize accessory loci. For example, the carbapenem-resistant gene bla_{NDM} is hypothesized to have originated on a Tn*125* transposon and recombined into more than 1,000 plasmid backbones and other transposons in various bacterial species (2). Importantly, the proliferation of either element can yield homology for recombination among different genetic molecules. Although neither can transfer between cells autonomously, both tend to be enriched in larger MGEs and can mobilize as hitchhikers (15). They can also amplify by replication of larger MGEs if transposed into one. IS elements have been implicated in mobilizing AMR genes to diverse conjugative plasmid backgrounds and host lineages (29, 95). IS elements have also been implicated in dramatic variation among members of a multidrug-resistant megaplasmid family in clinical isolates of *Pseudomonas* spp. These megaplasmids have a syntenic core backbone that represents only ~50% of the plasmid gene content, with the other half consisting of large arrays of AMR genes that differ extensively among members (28). Variable regions are enriched for IS elements and transposases.

IS elements and transposons have been associated with more complex evolution of larger MGEs. The FII-33 family of MDR plasmids has undergone extensive recombination with other MGEs (64). These *Enterobacterales* plasmids acquired a primary resistance region (PRR) that has varying numbers of AMR genes. The PRR is a remnant of a Tn*2670* transposon predicted to have been acquired via homologous recombination. A second event was IS*26*-mediated cointegration with another plasmid that provided a $bla_{\text{KPC-2}}$ carbapenemase gene, but it disrupted genes in the FII-33 plasmid backbone necessary for plasmid conjugation. The members of the IS*26* family were also predicted to be responsible for further recombination events that resulted in deletions in portions of the backbone and further alterations to the PRR (64). Many of the FII-33 family plasmids have additional plasmid replication loci of various plasmid backgrounds, suggesting cointegration is common and may extend the effective host range of the PRR. These replication genes are always flanked by IS*26* elements.

IS elements and transposases can also cooperate with conserved sequences to generate a hierarchy of modules. A Tn*125* transposon containing the ble_{MBL} bleomycin-resistant and $bla_{\text{NDM-1}}$ carbapenemase genes is inserted into one *dif* module of a GR34 plasmid and can conceivably be transferred by either transposition or p*dif*-mediated recombination (86). Different IS elements can also target different p*dif* sites and integrate near modules, providing more opportunities for mobilizing modules and mediating deletions that result in the chimerization of adjacent *dif* modules (16, 99). Similarly, in a study of one hospital, the carbapenemase gene bla_{KPC} is hypothesized to have been mobilized in strains between patients, on plasmids between strains, and between

plasmids via transposons (117). In all strains, *bla*$_{KPC}$ is present on a Tn*4401* transposon, which likely transposed from plasmid to plasmid. In several cases, the transposon is hypothesized to have repeatedly mobilized and nested into Tn*2*-like transposon elements, which could serve as templates for homologous recombination between otherwise unrelated plasmids (117). Therefore, even if plasmids carry diverse sets of genes, the presence of homologous IS elements and transposases can mediate recombination to exchange modules broadly (16, 99, 100, 117).

Accessory genome: the set of genes that vary in their presence among related members of a group

Following recombination, regions that are duplicated or are causes of genetic conflict may be lost over time. Selection acts to clean up chimeric molecules, but the original composition of genes does not need to be maintained. As described in Section 2, modularity allows for mixing and matching of subfunctional modules, such that genes with different evolutionary histories can come together and preserve a function (136, 137). Moreover, cleanup can disrupt operons or even alter the function and regulation of the original module. It is unclear whether the structure of a mosaic MGE will be eventually restored to one of its original states or whether the mosaic MGEs are fated to go down a different path (104, 118). Nonetheless, recombination of IS elements could play a role in streamlining this process by restoring original operons or generating new ones (75).

4. CONSEQUENCES OF EMERGENT PROPERTIES ON BACTERIAL FITNESS AND EVOLUTION

As described in Section 2, MGEs (or modules) are analogous to cards in a deck. This resource is immediately accessible by members of a microbial community. Moreover, drawn cards can be passed directly or indirectly over evolutionary time. Diverse MGEs conferring AMR have been transferred many times among human gut commensals and enteropathogens, sometimes across phyla (45). The beauty of MGEs is that they can be shuffled, and a new hand can be dealt. This ensures that variation can be rapidly generated. Many taxa of plant pathogenic bacteria rely on a type III secretion system to deliver effectors to interfere with one form of plant immunity (50). However, the deployment of effectors risks revealing the pathogen because certain plant genotypes can have resistance genes that perceive a cognate effector and elicit a second form of immunity (72). *Pseudomonas syringae* pv. *maculicola* strain ES4326 has tandem ICEs that carry genes encoding effectors delivered by the type III secretion system (9). Their presence on ICEs gives strains a mechanism to rapidly diversify effector gene collections in the population. Communities of nearly clonal *Vibrio lentus* isolates carry diverse MGEs that encode for phage defense systems (67). This provides a standing pool of variation that reduces the number of members susceptible to any given phage and enables populations to respond quickly to changes in phage populations (67). A similar mechanism in *Vibrio crassostreae* has been observed (106). Most of its phage types can attack only specific lineages. This specificity is partially due to phage defense genes present within diverse MGEs that can be exchanged or gained/lost. Remarkably, their MGEs represent only ~20% of the accessory genome but have >92% of the antiphage defense genes. Another example is found in photosynthetic nitrogen-fixing *Bradyrhizobium* spp. Many strains characterized thus far and capable of fixing nitrogen lack *nod* genes and have highly restricted host ranges. However, some members independently acquired an ICE with *nod* genes and have expanded host ranges (55).

Coupling MGE mobilization to changes in environmental pressures can accelerate and broaden the spread of modules. The Ti plasmids of *Agrobacterium* are maintained as a single copy. Upon detection of certain nutrients, whose production is initiated for the benefit of the infecting bacteria, the copy number of the Ti plasmid can be amplified concurrent with induction of interbacterial conjugation (41, 101). In *Vibrio* spp., induction of interbacterial competition, which is associated with the release of DNA, is coinduced with natural competence to promote uptake of DNA (18, 70). Another related example is the reshuffling of integron cassettes during the

SOS response to change the order and influence the expression of cassettes to help members of the population emerge from extreme pressures (8, 53, 119).

SOS response: an inducible DNA repair program

Heteroresistance: a phenotype in which a subpopulation of cells has higher levels of resistance to an antibiotic

A cell can maintain a collection of plasmids under permissive conditions and can optimize and adjust the collection in response to selective pressures (26). Plasmids may impose minimal fitness costs when selection is relaxed, but when plasmids coreside with other plasmids that encode the same function, positive selection may result in loss of the less beneficial plasmid. In another example, the *Shigella* AMR plasmids pKSR100 and pAPR100 provide resistance to antibiotics but differ in their epidemic trajectories (89). The more successful pKSR100 plasmid has a reduced conjugation rate and a lower fitness cost, and the host bacteria shows a weaker SOS response in the presence of antibiotics relative to strains with the pAPR100 plasmid.

The concept that plasmid collections can be refined in response to selection also extends to bacterial communities. Members may differ in their ability to host any particular MGE, but as long as some members can act as reservoirs, the MGE can spread more broadly if favorable conditions arise. In a soil microcosm experiment, members of a community differed in their ability to stably host a conjugative mercury-resistant plasmid (pQBR103) under relaxed conditions (82). However, the plasmid eventually reached higher levels of community abundance under high-mercury conditions if it was first introduced into a proficient host species rather than a less proficient one (82).

Because MGEs can experience genetic changes relatively easily, they are more apt to accumulate changes and potentially increase capacitance. Experimental evolution experiments found that under antibiotic selection, plasmids can accumulate mutations that increased copy number and rates of conjugation (39). The accumulated mutations protected against higher concentrations of antibiotics and dispersed AMR to more members of a community. Consistent with this finding, a temporal study of within-patient isolates revealed the emergence of increased-copy-number variants of pOXA-48 in response to antibiotic treatments (38). While AMR variants of pOXA-48 have a higher fitness cost relative to variants not conferring carbapenem resistance under normal conditions, they become advantageous in the presence of that antibiotic and spread quickly (38). Likewise, plasmids with transposons conferring AMR may emerge under strong antibiotic selection, as multicopy plasmids provide higher numbers of AMR genes than a chromosome could (139). Treatment with antibiotics can also lead to AMR plasmids or host chromosomal genes acquiring compensatory mutations that alleviate the fitness costs of carrying that MGE. The plasmid is then able to persist in populations even after cessation of treatment (73). Another example that is like capacitance in clinical isolates of *A. baumannii* was reported. Isolates with a single copy of *aphA1* are resistant to kanamycin and neomycin but are susceptible to tobramycin. In resistant strains isolated from clinics, treatment with the antibiotic tobramycin was associated with the amplification of this cassette, in some cases up to 65 tandem copies, and heteroresistance to tobramycin (58). Cassettes are bordered by IS*26* elements, suggesting that this MGE mediated amplification of *aphA1* and gained resistance to tobramycin. Adaptation is generally faster if there is preexisting genetic variation than if new mutations are required to arise (11).

5. BARRIERS TO MGE TRANSMISSION OR ACQUISITION

The flexibility of MGEs is not unencumbered. Acquisition of MGEs can be influenced directly by surface exclusion and genome defense mechanisms. Surface exclusion occurs when a barrier erected by recipients carrying related plasmids reduces the frequencies of transfer (47). Surface-associated barriers need not be driven by related plasmids. Chromosomally encoded outer membrane proteins of recipient cells interact with the plasmid conjugation-locus-encoded TraN to confer mating pair stabilization (88). Central to host specificity is that different outer

membrane proteins interact with different variants of TraN (88). Some ICEs also encode for exclusion systems that prevent the host cell from receiving a second copy of the ICE (35). These exclusion systems have specificity based on interactions between exclusion proteins and proteins encoded in the conjugation locus (35). Presumably, ICEs with similar conjugation loci and exclusion systems but different accessory genes may be excluded by this system. However, even if an MGE is immune to the effects of these outer barriers, other barriers, such as plasmid incompatibility loci, exist and can prevent multiple plasmids with similar loci from persisting in a cell (21). But such barriers still allow temporary coexistence and opportunities for cointegration and recombination among MGEs. Not all MGEs are effectively precluded by these types of barriers, as some can have a broad host range and can be found across phyla (15, 19). In addition, as described in Section 2, transfer of traits to MGEs with different core functions can overcome such barriers.

Bacteria have a diversity of defense systems to protect against invasion by MGEs (115). As described in Section 4, MGEs can also encode for defense systems (15, 19, 69, 107, 115). Plasmid-encoded CRISPRs typically target other plasmids, while ICE-/IME-encoded CRISPRs preferentially target other ICEs (19, 107). However, an ICE in *Campylobacter* spp. has a CRISPR-Cas locus predicted to protect against other *Campylobacter* plasmids (130). Recently, a novel defense system in *V. cholerae* strains was unearthed and answered the long-standing question about why plasmids are rare in this group of bacteria. Strains carry two plasmid defense systems, DdmABC and DdmDE, that target small multicopy plasmids for degradation and reduce the fitness of strains carrying large conjugative plasmids (69). These types of defenses typically detect a sequence, not a trait, to discriminate between self and nonself. Thus, defense mechanisms, while protecting genomes against detrimental changes, can potentially limit the acquisition of beneficial genes (84).

MGEs may impose fitness costs. Findings have shown that plasmids impose a short-term acquisition cost on recipient cells immediately following conjugation (108). MGEs may carry genes that are costly to express, which has long been thought to be a major limitation to the spread of plasmids (22). This costliness of MGEs has led to the theory of a plasmid paradox: that fitness cost, insufficient rates of conjugation, and transfer of beneficial genes to the chromosome should lead to the eventual loss of a plasmid (22, 63). But the generalizability of this to all bacteria–MGE combinations is unclear. Either many plasmids do not impose a fitness cost or costs are conditional. Alternatively, costs can be minimal or relatively easily mitigated by mutation or other factors. In many cases, fitness costs result from specific genetic conflicts rather than a general cost of plasmid carriage and can be ameliorated by single compensatory mutations (57). Importantly, as described in Section 4, as long as permissive strains exist, the MGE has the potential to persist and increase in frequency if conditions favor it. In experimental conjugation assays, *Escherichia coli* strains representative of different lineages differed in their ability to acquire the plasmid pLL35 from *K. pneumoniae* (40). Once acquired, the plasmid was stably maintained in all lineages, but fitness costs varied from mildly costly to beneficial across strains (40). In another example, acquisition of the plasmid pOXA-48_K8 by *E. coli* or *K. pneumoniae* resulted in deleterious to slightly beneficial effects that varied across examined strains (4).

Integrative MGEs have the burden of integrating into chromosomes where disruptions to overall architecture risk lethal effects (42, 61, 114, 138). For bacteriophage, it has been suggested that natural selection has shaped the location of integration sites to avoid conflict (17). The case may also be the same for ICEs. These MGEs have integrases that typically mediate site-specific integration at cognate attachment (*att*) sites in the chromosome. A simulation was used to shuffle symbiosis ICEs into cognate sites that vary in locations in genomes of bacteria that represent different clades. Consistent with predictions, ICE–genome pairs not identified from natural settings had an increased probability of experiencing a disruption to genome architecture (138). In addition, a subset of symbiosis ICEs have a polypartite structure that introduces large-scale

rearrangements to the genome upon integration/excision (31, 60, 137). The order by which elements excise can have dramatic effects on genome organization and bacterial fitness (61). Simulations showed that integration of polypartite ICEs into cognate sites of genomes that normally have monopartite ICEs results in large deletions and loss of predicted essential genes. Therefore, a pool of symbiosis ICEs that vary in where and how they integrate provides flexibility to recombine into the best-fitting chromosomes to limit conflict and spread the trait broadly across the genus (137).

***k*-mer:** sequences of length *k*

Jaccard index (JI): a measure to determine similarity within and differences between two sets

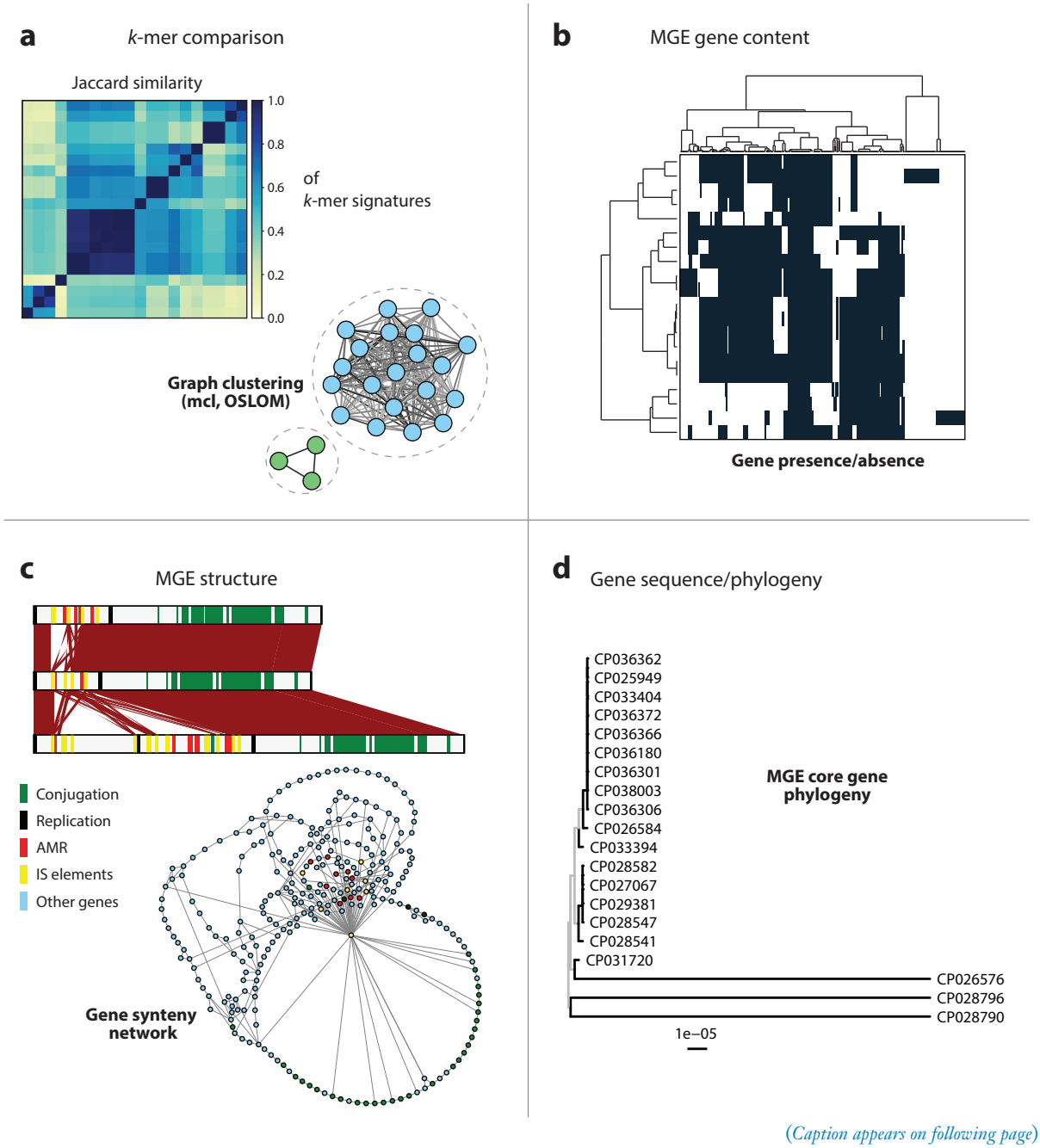
6. IMPACT OF MGE FLEXIBILITY ON ANALYSES OF ITS EVOLUTION

The study of MGEs is challenging because their emergent properties may mask the evolutionary histories of the elements and the genes they carry. As components of nonessential accessory genomes, MGEs can experience mutations, gains/losses, and shuffling to generate new combinations more easily than chromosomes can (122). Gene homologs can be present across diverse MGEs with different modes of transmission, locations in genomes as extrachromosomal or integrated elements, and copy numbers. Therefore, selective pressures and other fundamental factors, such as mutation rate, may not be constant over time, even within a class of MGEs (63). Though MGEs are mobile and can be acquired horizontally, they are more frequently vertically inherited. Consequently, analyses need to differentiate between inheritance patterns. Altogether, MGEs violate the assumptions of conventional evolutionary analyses and make tasks such as classification difficult.

Innovations in sequencing technologies have transformed how we study MGEs. We can leverage deep sampling to sequence large populations of related MGEs to uncover trends within families. Sampling can be environment centric (e.g., in clinics) as opposed to taxon centric (e.g., *P. aeruginosa*) to study traits vectored by MGEs and the taxonomic breadth to which they are disseminated (28, 38, 85, 90, 117, 120). The use of such an approach uncovered evidence for within-patient transmission of a plasmid as another mechanism that contributes to AMR spread (85). In this study, because plasmids were so similar in sequence, researchers examined only rare single nucleotide polymorphism variants and discovered four examples of such variants present in nonclonal bacteria. A similar approach was also used to uncover instances of virulence plasmids being mobilized in agricultural settings (134). However, because of the sampling design, data could not be used to infer whether transfer was direct or indirect.

Several strategies to infer the evolution of MGEs have been developed (**Figure 3**). They generally follow a similar workflow. One of the first steps is to classify MGEs into groups of related molecules. One straightforward method uses *k*-mers to capture genetic signatures and their similarities to evaluate relationships among MGEs. These *k*-mers account for sequence and content of an MGE and therefore represent total genetic variation. The elegance of using *k*-mers is that they are easily and rapidly identifiable. The relationship between pairs of MGEs can be approximated by calculating the Jaccard index (JI) of *k*-mer signatures (23). MGEs can be clustered by generating a graph of Jaccard similarities and using graph clustering algorithms to identify relationships (1, 94, 112, 134) (**Figure 3*a***). This approach was often concordant with other measures of genetic similarity (1, 94, 95, 112, 134, 136, 137). However, few criteria for choosing a JI threshold have been established, and the choice of threshold can influence clustering. In the example data set in **Figure 3**, a minimum JI threshold of 0.1 was used to build the network. This threshold was sufficient to group the 20 plasmids separate from the 3 outgroup plasmids (**Figure 3*a***). But an examination of the JI heatmap shows subclustering within the network, and the use of higher thresholds would further subtype the plasmids. The support for classifying at the subgroup level is provided by clustering based on gene presence/absence patterns (**Figure 3*b***).

A more comprehensive classification strategy couples results from *k*-mer analysis with results from analyses of gene presence/absence patterns, phylogenies of conserved genes, and MGE structure (**Figure 3**). Heatmaps of gene presence/absence can be used for visualization and are a first step to identify genes with similar inheritance patterns and relate changes to MGE structure (**Figure 3*b***). Alternatively, a pangenome graph can be used to visualize relationships between



(*Caption appears on following page*)

Figure 3 (*Figure appears on preceding page*)

Strategies for classifying and analyzing MGEs. (*a*) Classification of MGEs based on overall signatures, such as *k*-mers. Jaccard similarities can be quantified and visualized in a heatmap (*left*) or graph (*right*). (*b*) Comparison of MGEs based on patterns of gene presence/absence. In the heatmap, rows represent plasmids and columns represent genes. Black lines indicate the presence of homologs of a gene; white space indicates its absence. Plasmids and genes were clustered by ward.D2 clustering of binary distances. (*c*) Comparison of MGE structure and gene synteny. Synteny can be visualized in alignments (*top*) or in a gene synteny network (*bottom*). In the alignment, horizontal lines represent plasmids, and red bars/blocks indicate regions of similarity. In the gene synteny network, nodes represent individual genes, and lines connect genes that are adjacent in at least one plasmid. Genes with specific functional annotations were color coded. Highly connected nodes are IS elements. Bubbles are alternative paths and reveal regions of variation between plasmids. (*d*) Phylogeny of core or conserved MGE genes can be used to infer relationships among MGEs, and comparisons with gene trees can be used to identify genes that exhibit different evolutionary histories. The 20 AMR plasmids from Hu et al. (64) and 3 unrelated plasmids (panel *a*) were analyzed for this figure. Abbreviations: AMR, antimicrobial resistance; IS, insertion sequence; mcl, Markov clustering algorithm; MGE, mobile genetic element; OSLOM, order statistics local optimization method.

gene presence/absence and MGE structure. Graphs based on different scales of information, such as gene presence/absence and synteny, and de Bruijn graphs based on component *k*-mers can be generated (97, 125, 134). Pangenome graphs of multiple related MGEs can be visualized with network software (116) (**Figure 3*c***).

The phylogeny of core and/or accessory genes can help provide context for inferring MGE classification and evolution; however, for reasons described in Sections 2 and 3, care must be taken when interpreting results. In **Figure 3*d***, a core gene phylogeny generated on the basis of sequences from 13 genes reveals little variation. Eleven of the gene sequences have no variation across the 20 plasmids, and 17 plasmids cluster in a monophyletic clade with short branches. Yet 3 plasmids have long branch lengths relative to the others because there are polymorphic sites in two other genes included in the analysis. In fact, all 20 plasmids clustered in the *k*-mer network, suggesting that they are related but that they vary in presence/absence patterns of accessory genes (**Figure 3*b***). Broader analyses of all types of genetic variation provide the necessary context to determine similarities and differences between MGEs and their potential causes.

The next steps in the analysis of MGEs vary according to whether interests lie in the evolution of the MGEs or of a trait vectored by MGEs. If one is interested in the former, the next steps are to identify conserved backbones for elements and to determine which regions have recombined into backbones and diversified the backbone. Characterizing the genetic variation also helps to identify the regions driving these changes and to reflect on modularity and flexibility. It is important to examine modularity at each of the different levels. This examination requires developing a phylogenetic framework of the strains that host the MGEs to provide context for their distribution patterns. Classifying strains into species or even lineages and sublineages can clarify whether strains are members of different groups and how MGEs are exchanged among groups.

If one is interested in the trait, it is crucial to address the potential for gene homologs present on closely related MGEs to have different evolutionary histories or for those on more distantly related MGEs to share a similar evolutionary history that differs from those of other gene groups. It is important to infer the relationships of gene homologs independent of the class of MGEs (or chromosomal replicons) that vectors the trait. In addition, some genes may be transferred horizontally in some contexts and vertically inherited in others. Once phylogenies of individual genes are generated, the evolutionary histories of different genes can be compared to infer similarities or differences. Informatic tools can be used to group genes into sets on the basis of similar tree topologies (i.e., those having similar evolutionary histories) (49, 110). Methods designed for virus evolution that account for recombination may also be useful for analyzing MGEs (10, 91, 111). Phylogenetic network analysis, based on gene sequences or a set of phylogenetic trees, can also be used to indicate and visualize the extent of recombination, though these analyses cannot discriminate between recombination and lack of phylogenetic signal (66).

7. CONCLUSIONS

MGEs have properties ideally suited for promoting tremendous phenotypic diversity and adaptability. Their modularity and flexibility generate natural variation, a prerequisite of evolution, and promote robustness, which allows traits to persist and adapt to changing environments. These properties are challenging to deal with in environments with strong selective pressures, such as in clinics and agricultural settings, but they also provide opportunities for biotechnology and ways to innovate practices to leverage traits with beneficial uses. Although this review focused on bacteria, concepts apply broadly (3, 48, 74, 129, 131). Archaea can carry large and diverse plasmids encoding for metabolic pathways (3). Transposable elements have been implicated in mobilizing resistance to metals, influencing meiotic drive, and diversifying virulence-associated genes in fungi (48, 129, 131). Plant pathogenic fungi can have supernumerary minichromosomes that behave like MGEs of bacteria (5, 83, 87, 98). A remarkable example in *Magnaporthe oryzae* was reported, in which gene-containing regions are shed from its chromosomes (74). The resulting circular DNA elements that formed have several important features similar to those of plasmids and ICEs, such as the capacity to increase gene copy numbers, to be diverse, to be integrated into hot spots in the chromosome, and to carry virulence-associated genes (74).

This is an exciting and fast-paced time for studying MGEs, with many new and insightful findings constantly being reported. New innovations and new perspectives will continue to change how we look at MGEs and their role in the evolution of diverse microbial systems. The future is bright for illuminating more about the marvelous biology of MGEs.

SUMMARY POINTS

1. Mobile genetic elements (MGEs) have many important roles in the evolution of bacteria.
2. MGEs can change the genetic content of a strain quickly and are a genetic playground for strains to play with different combinations of genes.
3. MGEs are highly modular and can exchange genes quickly.
4. MGEs enable microbial populations to store genes in a population that may be selected for when environmental change occurs.
5. MGEs can preserve the functionality of traits while also allowing variation to accumulate and lead to the emergence of new traits.
6. The properties of MGEs challenge our abilities to study them, but new technologies and strategies enable their characterization and evolutionary analysis.

FUTURE ISSUES

1. The genetic factors and mechanisms that restrict MGE host range or maintain compatibility with a particular strain or lineage remain to be determined.
2. Researchers still need to resolve if and/or how MGEs are streamlined over evolutionary time.
3. Advance use of long-read sequencing technologies and new approaches to associate MGEs to chromosomes in metagenome sequencing projects need to be developed.

4. The perspective of a trait or natural ecosystem should be used to further advance studies of MGEs.
5. The applications of long-read sequencing should continue to be improved to advance our understanding of the role of MGEs in Eukarya and Archaea.

DISCLOSURE STATEMENT

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