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Fitness Costs of Plasmids: a Limit to Plasmid Transmission

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ABSTRACT Plasmids mediate the horizontal transmission of genetic information between bacteria, facilitating their adaptation to multiple environmental conditions. An especially important example of the ability of plasmids to catalyze bacterial adaptation and evolution is their instrumental role in the global spread of antibiotic resistance, which constitutes a major threat to public health. Plasmids provide bacteria with new adaptive tools, but they also entail a metabolic burden that, in the absence of selection for plasmid-encoded traits, reduces the competitiveness of the plasmid-carrying clone. Although this fitness reduction can be alleviated over time through compensatory evolution, the initial cost associated with plasmid carriage is the main constraint on the vertical and horizontal replication of these genetic elements. The fitness effects of plasmids therefore have a crucial influence on their ability to associate with new bacterial hosts and consequently on the evolution of plasmid-mediated antibiotic resistance. However, the molecular mechanisms underlying plasmid fitness cost remain poorly understood. Here, we analyze the literature in the field and examine the potential fitness effects produced by plasmids throughout their life cycle in the host bacterium. We also explore the various mechanisms evolved by plasmids and bacteria to minimize the cost entailed by these mobile genetic elements. Finally, we discuss potential future research directions in the field.

INTRODUCTION

Horizontal gene transfer (HGT) is a key source of genetic diversity in bacteria (1), and plasmids are one of the main vehicles driving this process (2). Plasmids are widely distributed across prokaryotes, and help bacteria adapt to a myriad of different environments, conditions, and stresses (3, 4), playing a key role in bacterial ecology and evolution (5–7). The most vivid testimony to the power of plasmids as catalysts for bacterial adaptation is their role in the spread of antibiotic resistance among clinical pathogens (8), which has emerged as a major health problem over the past decades (9).

Despite the potential benefits conferred by plasmids, they also produce a burden (fitness cost) in the host, manifesting as a reduced growth rate and weakened competitiveness of plasmid-bearing strains under conditions that do not select for plasmid-encoded genes (10, 11). This fitness cost imposed by plasmids, coupled with the potential plasmid loss during bacterial cell division, hinders the survival of plasmids in bacterial communities. Moreover, any beneficial gene carried by the plasmid could eventually move to the chromosome (12), making it difficult to understand why plasmids persist (the "plasmid paradox"). Several early studies investigated the consequences of the fitness costs of plasmids on their ability to survive in bacterial populations (13–18). These studies established the "existence conditions" for plasmids, which in general involved a relatively high rate of horizontal spread by conjugation, especially when plasmids acted as pure genetic parasites (in the absence of selection for plasmid-encoded traits). Most recently, Harrison and Brockhurst proposed that the plasmid paradox could be explained by compensatory evolution (19). The idea underlying this hypothesis is that even if

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plasmids produce a cost when they first arrive in a new bacterial host, this cost could be alleviated over time through compensatory mutations in the plasmid and/or the host chromosome (20). In recent years, several studies have analyzed the molecular basis of the cost and compensation of plasmids in bacterial populations (21–28), generating new models of the existence conditions of plasmids (22, 24). These studies evaluate a number of selection, transfer, and compensation regimes that could explain plasmid survival in bacterial populations, and have revealed new evidence about the molecular mechanisms underlying the cost of and adaptation to plasmids.

This review expands on previous reviews on the origins of HGT-related costs in light of new data available in the plasmid literature (10). Specifically, we analyze the potential fitness effects produced by plasmids during each step of their life cycle in the host bacterium, as well as the mechanisms that help bacteria and plasmids control these effects. Here, we focus on the effects of plasmids on the bacterial host, so, unless otherwise specified, when we talk about fitness (or fitness costs) we refer to the bacterium, which is not necessarily linked to plasmid fitness due to the potential for HGT of these genetic elements. Finally, we explore some of the main challenges and questions in the field of plasmid evolution.

DISSECTING THE FITNESS COSTS PRODUCED BY PLASMIDS

Infection by a plasmid usually entails a fitness cost that reduces the reproductive rate of the host bacterium (11). This fitness cost plays a key role in the population biology of plasmids by generating selection against plasmid-carrying strains. The origins of these costs are manifold (10), and we are only beginning to understand them. Deciphering the molecular mechanisms underlying plasmid fitness costs is essential for efforts to predict future plasmid-bacteria associations driving the evolution of antibiotic resistance. In this section, we dissect the costs produced by plasmids during the different phases of their biology in the host bacterium (Fig. 1). The actual cost of a plasmid likely emerges from the combination of and interactions among these effects; however, each of these factors is likely to make a different relative contribution to the overall fitness cost.

Plasmid Reception

The first step in plasmid acquisition by a new host is the physical arrival of the plasmid in the cell. Plasmids can be transmitted by any of the three HGT mechanisms: transformation (29, 30), phage-mediated transduction (31), and conjugation (3). Conjugation is considered the most important mechanism of plasmid transmission, and the conjugative process has been extensively studied (discussed below). During conjugation, the plasmid enters the new cell as single-stranded DNA (32), producing a transient activation of the SOS response (33). The SOS response is a bacterial stress response triggered by an increase in single-stranded DNA in the cell, which leads to a rise in mutation and recombination rates (34). Activation of the SOS regulon also results in inhibition of cell division, which by definition translates into a decrease in bacterial fitness in the short term (35). The potential impact of conjugation-mediated SOS response activation on recipient cell fitness is indicated by the presence of anti-SOS genes, such as *psiB*, in conjugative plasmids (36). These factors are transferred as part of the plasmid-leading region and are expressed upon arrival of the plasmid in the new cell (37, 38). PsiB binds to the SOS regulon activator RecA, impeding the formation of RecA nucleoproteins that trigger the SOS response (33, 36). The SOS response can also be triggered by plasmid transformation and transduction (39, 40), so plasmid reception through these pathways will also produce a potential decrease in fitness.

Plasmid reception also entails a transient transcriptional overshooting of plasmid genes regulated by negative feedback loops (41, 42). Until the plasmid-encoded transcriptional repressors are expressed in the recipient cell, expensive plasmid functions such as conjugation will be derepressed, producing a transient elevation in the fitness cost produced by the plasmid. The effect of this cost is to reduce vertical plasmid transmission; however, the transient overexpression of conjugative genes helps the plasmid to rapidly spread horizontally in a bacterial population with available recipient cells (41).

Plasmid Integration

Following successful transfer to a recipient cell, certain plasmids can integrate into the chromosome of the host (43). Although the integration of a plasmid carrying adaptive genes on the chromosome may be potentially advantageous for the host, this process can also produce deleterious effects associated with the disruption of host genes (44). This is the case with integrative and conjugative elements (ICEs), which integrate into and excise from the host cell chromosome thanks to an ICE-encoded recombinase (43). Some ICE families target a single specific attachment site in the bacterial chromosome, usually in a tRNA gene (45), suggesting that these

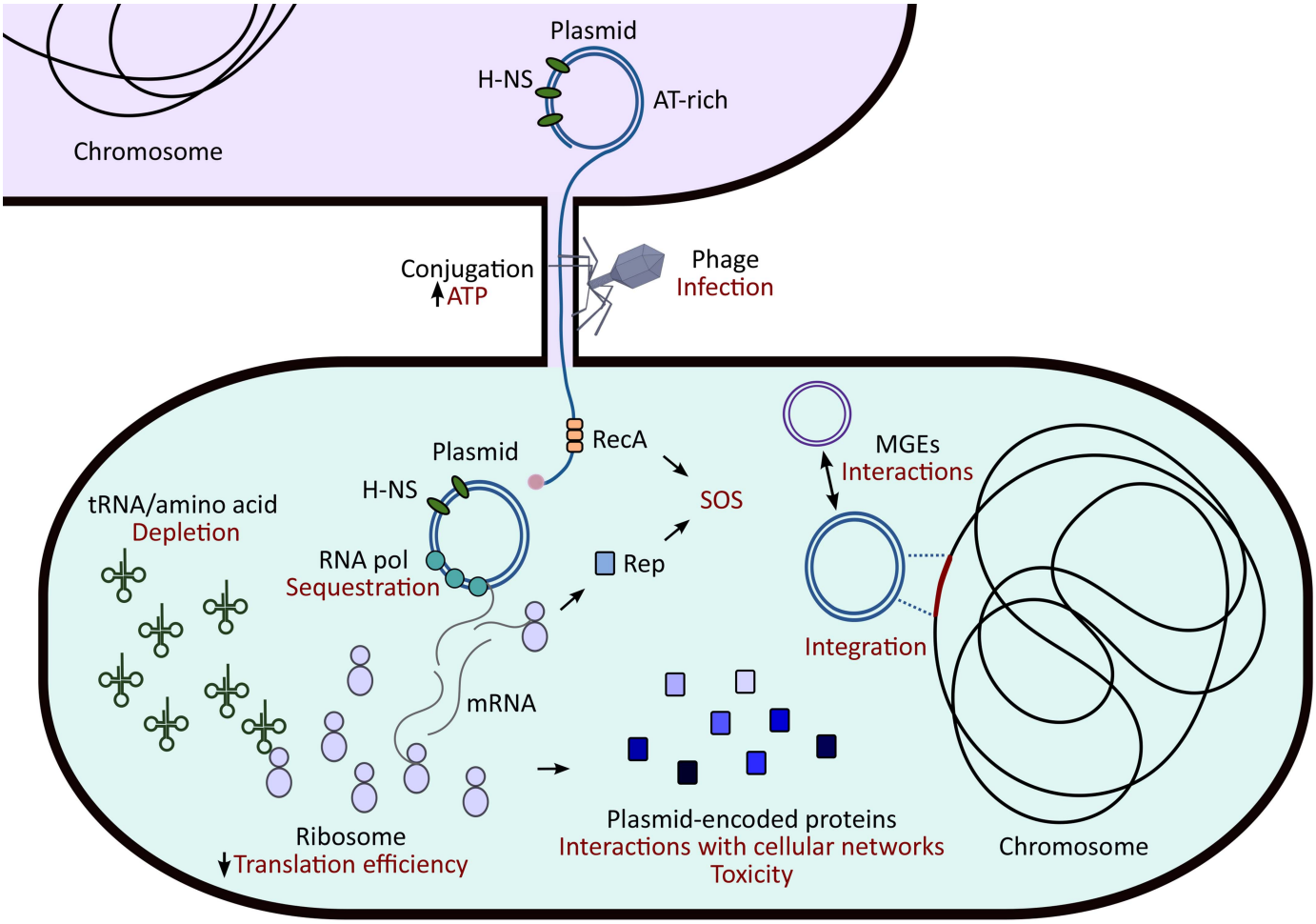

FIGURE 1 Fitness costs produced by plasmids. Potential fitness effects produced by plasmids during their life cycle in the bacterial host.

plasmids have evolved a strategy to avoid arbitrary disruption of the host genome during integration. However, other ICE families target multiple attachment sites (46), and some families, like Tn*916*, integrate almost randomly in the host chromosome (47). This nonspecific integration can disrupt protein coding or regulatory regions or can interfere in the expression of genes flanking the integration site, entailing fitness costs in the recipient bacterium.

Plasmid Replication

Plasmids usually produce only a small increase in the total amount of DNA in the bacterium. It is therefore widely accepted that the main fitness cost associated with plasmids comes not from replication but from downstream events such as expression of plasmid genes (10, 11). There is, nevertheless, evidence indicating that plasmid replication does incur a fitness cost for the bacterial host. A particularly interesting observation regarding replication-related fitness costs is that plasmid DNA is significantly richer in AT than the host chromosome (48, 49). This difference is also observed for other "intracellular parasites" such as endosymbionts or phages (48, 50). Rocha and Danchin proposed that this bias might reflect the higher energy cost of G and C and the lower availability of these nucleotides in the host cell compared with A and T/U (48). This hypothesis predicts that replication of plasmids with a higher GC content would produce a higher cost; however, testing this experimentally is difficult because AT-rich transcripts tend to produce a higher cost than GC-rich transcripts in bacteria (51), and this would produce a confounding effect. Indeed, this selective advantage of GC-rich genes has been proposed as the cause of the wide variation in GC content in prokaryote genomes (51), despite mutation in bacteria being biased toward AT (52, 53). The AT bias in plasmids therefore remains intriguing, and an alternative explanation could be "xenogeneic silencing,"

discussed below under "Mechanisms for Minimizing Plasmid Costs" (54).

An important side effect of plasmid replication is the pleiotropic effects of plasmid-encoded replication initiation (Rep) proteins (23, 55). Plasmids usually encode their own Rep proteins, enabling auto control of plasmid copy number (56). These proteins subsequently recruit several other DNA polymerases and helicases from the bacterial host to proceed with plasmid replication (55). Overexpression of plasmid replication proteins can therefore lead to sequestration of the cellular DNA replication machinery, stalling chromosomal replication, inducing the SOS response, and inhibiting cell division (23, 55). The control of plasmid Rep protein expression can be altered when the plasmid arrives in a new bacterial host due to interactions with chromosomal-encoded genes (23). This deleterious effect has been observed in bacteria carrying small multicopy plasmids, such as pNUK73 in *Pseudomonas aeruginosa* or pSC101 in *Escherichia coli* (23, 55). Multicopy plasmids probably require a high level of Rep protein expression to maintain their high copy number, increasing the potential for this deleterious effect.

Conjugation

Plasmids can transfer directly between bacteria in a process known as conjugation (57). During the classical conjugation process in Gram-negative bacteria, plasmid DNA travels through a type IV secretion system (T4SS) between donor and recipient bacteria (58, 59). Conjugative plasmids carry all the genetic information required to synthetize the T4SS and the proteins needed to escort plasmid DNA to the new host (3). Conjugation provides plasmids with the opportunity to replicate horizontally, and, all else being equal, this should increase the relative frequency of the plasmid in the population. However, conjugation is energetically expensive (it requires three plasmid-encoded ATPases [58]) and entails a reduction in bacterial host fitness, thus reducing the rate of vertical plasmid transmission (60). The fitness cost associated with conjugation comes primarily from the high ATP demand for mating-channel formation and plasmid DNA translocation (58). To minimize this cost, plasmids tightly control the expression of conjugative systems (41, 61–63). The result is a general repression of conjugation genes, with only a few cells in the population expressing the conjugative machinery (63). Conjugation can, however, be derepressed, either through chemical signaling (61, 62) or through transcriptional overshooting of conjugative genes in new recipient cells (41, 42), enabling a wave of plasmid transfer when there are recipient bacteria available in the population.

Several reports pinpoint conjugation as a source of plasmid cost for the bacterial host (28, 64, 65). For example, the fitness cost associated with plasmids R1 and RP4 in *E. coli* is reduced after the conjugation rate is decreased or abolished by natural selection in an experimental setting (64). Also, the expression of plasmid R1 conjugation genes has been shown to activate stress responses in *E. coli* (65), although it is not clear if this is cause or consequence of the cost of plasmid carriage. Finally, in a recent analysis of the IncN antibiotic resistance plasmid pKP33, Porse and colleagues showed that the loss of the conjugation region reduced the burden associated with plasmid carriage in *E. coli* (28).

Another potentially deadly consequence of conjugation is bacteriophage infection. Certain phages use the conjugative T4SS as an attachment site for invading the bacterial cell (66, 67). T4SS-specific phages can therefore select against plasmid-carrying bacteria (68) and have been proposed as an alternative approach to counteracting the plasmid-mediated spread of antibiotic resistance (69).

Expression of Plasmid-Encoded Genes

The main biosynthetic burden associated with plasmid carriage is likely the expression of plasmid-encoded genes. For example, it is well established that highly expressed genes are less likely to be transferred horizontally (70, 71), demonstrating that the cost of expressing newly acquired genes can have profound evolutionary consequences. The costs associated with gene expression can arise from gene transcription, translation, or subsequent interactions between plasmid-encoded proteins and cellular networks (explored in the next section). Transcription is not considered a major cost (51), and the cost of plasmid gene expression appears to be predominantly derived from the translation of protein-encoding plasmid genes (71). Nevertheless, a recent report showed that acquired AT-rich genes can produce toxic effects due to the sequestration of RNA polymerases (72).

The cost of translation is determined by the discrepancy between plasmid mRNA abundance and the availability of cellular tRNAs, amino acids, and ribosomes. In general, for highly expressed genes the principal source of translation-associated cost is thought to be the imbalance between codon usage by the foreign genes and the available tRNA pool in the recipient bacterium (73). Several reports show that HGT is favored when there is codon usage compatibility between

foreign genes and the bacterial host (translational selection) (74, 75). Due to the general AT bias of plasmids, plasmid gene codon usage is very likely to differ from the optimal codon usage by chromosomal genes (73). This difference will affect the translation efficiency (initiation, speed, and accuracy) of plasmid genes (73), leading to inefficient ribosome allocation and ribosome pausing. These alterations will produce costly effects such as increased mRNA degradation, ribosome sequestration, protein mistranslation, and protein misfolding (76–79; reviewed in 73 and 80). The deleterious effects of low translation efficiency will increase with gene expression level. Since some plasmid genes are tightly repressed (e.g., conjugation genes) and others are highly expressed (e.g., integron cassettes), one might expect highly expressed plasmid genes to incur a heavier fitness cost than less expressed genes due to codon usage differences compared to chromosomal genes. Another possible deleterious effect of plasmid gene translation is the depletion of the host cell amino acid pool (81). Amino acid starvation reduces bacterial growth rate and activates the bacterial stringent response (82), leading to increased abundance of ribosomes and charged tRNAs (83, 84). This response serves to alleviate the amino acid depletion; however, the starvation-induced tRNA pools will alter the cellular balance between mRNA and tRNA, impacting host bacterium physiology and fitness.

The centrality of translation as the main plasmid fitness cost is exemplified by a recent study of pQBR103 (25). This mega plasmid from *Pseudomonas fluorescens* produces a major burden for the host cell due to the increased transcriptional demand imposed by the plasmid-encoded genes. This demand induces a marked increase in the expression of genes involved in protein production (25). Interestingly, bacteria are able to compensate this cost during experimental evolution by reducing cellular translational requirements. This reduction is achieved by mutations in the bacterial regulatory system *gacA*/*gacS*, which controls the biosynthesis of a wide range of secondary metabolites (25).

Effects of Plasmid-Encoded Proteins on Bacterial Physiology

Plasmids bring new proteins to the host bacteria, and the potential effects of these proteins on bacterial physiology are impossible to predict. The most prominent examples of plasmid-encoded proteins having a deleterious effect on the host cell are postsegregational killing systems (PSKSs) (85). PSKSs are plasmid addiction mechanisms, usually encoding a stable toxin and a labile cognate antitoxin. If the plasmid is lost during cell division, the plasmid-free cell will succumb to the vertically inherited toxin protein because there will be no antitoxin to counteract its effect. PSKSs thus ensure the stability of the plasmid in the host bacteria, and they are able to expand the plasmid host range (26). However, if the partitioning systems are not fine-tuned, PSKSs will kill many plasmid-free segregant cells. Similarly to PSKSs, plasmid-encoded bacteriocins that are secreted outside the cell will also promote plasmid maintenance, since immunity genes are carried by the plasmids as well (86).

Plasmid-encoded proteins can also cause fitness costs due to unwanted interactions with cellular networks or cytotoxic effects. At the end of last century, Jain and colleagues observed that genes encoding proteins involved in complex interactions with other proteins are horizontally transferred less frequently than those encoding proteins that form part of simpler systems with fewer interactions (87), and these authors developed the "complexity hypothesis" to explain this bias. More recently, Cohen et al. confirmed that protein connectivity (the number of protein-protein interactions) correlated negatively with the transferability of the coding genes (88). On the other hand, transferability is decreased by a lack of recipient cell proteins that physiologically couple with the acquired proteins, because the acquired protein is not in a hospitable metabolic context (89). Interactions between plasmid-encoded proteins and cellular networks thus appear to have the potential to produce a variety of deleterious effects that can influence plasmid transferability.

There are a handful of reports in the literature demonstrating major fitness costs as a consequence of cellular network alterations by specific plasmid-encoded proteins (23, 26, 55, 90). In most cases, the deleterious interactions involve the plasmid Rep protein. Rep proteins connect extensively with host protein networks because they need to recruit many cellular enzymes, such as DNA polymerases and helicases, in order for plasmid replication to proceed (91). These interactions can result in the sequestration of the cellular replication machinery, altering the replication network and activating stress responses (23, 55). Interestingly, host cell helicases appear to play a key role in Rep-induced costs (23, 26, 90). In *P. aeruginosa*, costly overexpression of the Rep protein encoded by the small plasmid pNUK73 is dependent on an accessory helicase, inactivation of which fully compensates the cost of the plasmid (23). Moreover, the cost of an IncP-1b minireplicon is compensated by mutations in chromosomal helicases in *Pseudomonas moraviensis* (26) and by mutations in the

helicase-binding domain of the plasmid Rep protein in *Shewanella oneidensis* (90).

Fitness Effects Due to Interactions between Mobile Genetic Elements

Bacteria often carry multiple mobile genetic elements (MGEs), and interactions among MGEs can affect host fitness. These interactions are common; for example, plasmids can inhibit the conjugative transfer of other coresident plasmids in a process known as fertility inhibition (132). The coexistence of several plasmids in the same host can either potentiate or alleviate plasmid-mediated costs, as recently reported for *P. aeruginosa*, *E. coli*, and *Agrobacterium tumefaciens* (21, 92, 93). In addition to direct MGE interactions, MGEs can influence each other indirectly through their effects on host elements. For example, plasmid-mediated activation of the SOS response can trigger phage induction or mobilize genomic islands (94). MGE interactions may have originated through a shared coevolutionary history (95, 96) or simply be the result of accidental interactions (23). Accidental interactions are a more likely explanation for costly interactions, because HGT brings together genes with different evolutionary histories, and interactions are unlikely to be mutually beneficial through chance alone.

Many types of MGE interactions have been reported, and most of them are likely to affect bacterial fitness. One of the most interesting cases is the cross talk between phages and pathogenicity islands in *Staphylococcus aureus*, in which phage genome and chromosomal island compete for packaging in the phage capsid in an ongoing arms race (95). Another example is the destabilizing interaction between partitioning proteins from a genomic island and an Inc-P7 plasmid in *Pseudomonas putida* (96). There are also specific examples of MGE interactions producing deleterious effects in the host. In *Enterococcus faecium*, the coexistence of Tn*5386* and Tn*916* ICEs produces genomic deletions in the host genome. In *P. aeruginosa*, interactions between proteins encoded by a phage, a small genomic island, and the plasmid pNUK73 produce a major reduction in bacterial fitness (23).

MECHANISMS FOR MINIMIZING PLASMID COSTS

In this section, we discuss the mechanisms aimed at reducing plasmid costs on the host once the plasmid has been acquired. However, for a detailed explanation on host and plasmid barriers to plasmid acquisition see (132). The fitness costs of plasmids limit their existence in bacterial populations, and plasmids are therefore under strong selection pressure to control these costs in order to maximize their vertical spread. The host bacterium also needs to adopt strategies to cope with the presence of plasmids once they are successfully established. The previous section outlines some of the mechanisms controlling the fitness costs of plasmids, such as the action of anti-SOS genes on conjugative plasmids or the tight control of the expression of conjugative systems. Most plasmid-associated costs arise upon the expression of plasmid-carried genes, and therefore transcriptional regulation is a key route to minimizing plasmids costs. This section briefly outlines some of the mechanisms that plasmids and host bacteria have evolved to control the expression of plasmid genes.

Plasmid gene expression can be controlled by a range of different nucleoid-associated proteins (97), such as H-NS (histone-like nucleoid structuring protein) from enterobacteria. These proteins act as transcriptional repressors that silence the expression of acquired genes, a phenomenon called xenogeneic silencing (98–100). Silencing of foreign DNA by H-NS is based on its ability to target sequences with a higher AT content than the host genome (99, 101), which it achieves by binding not to a specific target sequence but to a consensus curved DNA structure commonly associated with promoters (102, 103). H-NS thus enables plasmid acquisition by reducing both the potential deleterious effects of plasmid-encoded proteins (99) and the potential sequestration of RNA polymerases by AT-rich genes (72). H-NS-mediated repression is modulated by environmental conditions and multiple countersilencing mechanisms (54), so that plasmid genes are still expressed to a certain extent, providing potentially adaptive benefits to the host bacteria. Interestingly, conjugative plasmids can also carry H-NS-like genes that repress the expression of foreign genes, including their own (104–106). Plasmid-encoded H-NS-like genes have been proposed to help plasmid conjugation by reducing the cost of plasmid acquisition (105). This hypothesis is supported by the high prevalence of these genes in conjugative plasmids (104). Taken together, these pieces of evidence suggest that xenogeneic silencing could be beneficial both for plasmid, enabling plasmid propagation, and bacterium, providing new genes at low cost. Therefore, we argue that selection for xenogeneic silencing could be responsible for maintaining the general AT bias in plasmids and other MGEs compared to their host chromosomes.

Plasmids also use specific regulators to control the transcription of their own genes, especially those related

to plasmid housekeeping processes. Genes involved in the energetically expensive conjugative process are tightly self-regulated (41), as is the expression of partition and replication genes (91, 107, 108). In contrast, the transcription of plasmid-encoded accessory genes (mediating bacterial adaptation to the environment) is not always fine-tuned, and represents an important potential source of fitness cost. These accessory genes are frequently encoded in genetic elements that generate high expression levels, such as integrons. Integrons are genetic platforms that acquire open reading frames, called cassettes, through the action of the integron integrase (109). These cassettes are expressed from a single strong promoter, creating a gradient of cassette transcription (110, 133). Integrons are very prevalent in plasmids and are generally related to antibiotic resistance (111). Transcriptional analysis has shown that integron cassettes are among the highest-expressed plasmid genes, representing an important potential source of fitness cost to host bacteria (112–114).

CHALLENGES IN THE FIELD

Antibiotic resistance in bacteria is a major health problem, and plasmids are essential vectors of the dissemination of antibiotic resistance to clinically relevant pathogens (8). Plasmids can promote bacterial survival in the presence of antibiotics, but, as we have seen, they can also impose a fitness cost when they enter a new bacterial host. The past few years have witnessed growing interest in the fitness effects of plasmids (21–28, 115), and some of the general principles underlying these effects are now beginning to be identified. However, we still are a long way from understanding the specific molecular basis of these costs or being able to predict plasmid fitness effects in a bacterial host. This final section discusses future research directions that may help to answer some of the outstanding questions about the fitness effects of plasmids and how they dictate the evolution of plasmid-mediated antibiotic resistance: What makes a bacterial-antibiotic resistance plasmid association successful? What is the molecular basis of the fitness effects of antibiotic resistance plasmids in clinical strains? Can we predict which plasmid-bacteria associations are likely to arise in the future?

Investigating Clinically Relevant Plasmid-Bacterium Combinations

One of the main challenges in the field is to develop experimental models of clinically relevant plasmid-bacterium combinations. Most studies of plasmid fitness costs have involved laboratory-adapted bacterial strains and plasmids of low clinical relevance. If we want to understand the evolution of plasmid-mediated antibiotic resistance, we will need to investigate the fitness effects of antibiotic resistance plasmids in high-risk pathogenic bacteria. Gram-negative pathogens, particularly enterobacteria such as *E. coli* and *Klebsiella pneumoni*ae, are currently the most concerning cause of multiresistant infections in hospitals (116–118), and these bacterial strains acquire resistance to front-line antibiotics such as β-lactams primarily by plasmid transmission (8). Interestingly, associations between plasmid types and enterobacteria clones are not random. Rather, certain bacterial clones carry highly specific types of resistance plasmids (e.g., *E. coli* ST131 and IncF plasmids are often associated [119]) but not others, even when clone and plasmid coincide in time and space (e.g., *E. coli* ST131 and pOXA-48 are seldom associated [119, 120]). Studying the molecular basis of the effect of epidemic plasmids on clinically relevant enterobacteria strains may help to reveal why some plasmid-bacterium associations are especially successful. These approaches could also improve our capacity to predict the evolution of plasmid-mediated antibiotic resistance in clinically relevant scenarios.

Expanding the Experimental Conditions

Another limitation of studies of the fitness effects of plasmids in bacteria is that they are usually carried out *in vitro*, using conditions very different from the natural environments of the bacterial hosts. Several experimental models can be used to measure fitness under conditions that more closely reproduce the natural habitat of the host bacteria. These range from *in vitro* systems, such as Biolog plates, that provide a variety of carbon sources (121, 122), through more-complex synthetic systems or *ex vivo* animal models (123, 124), all the way to *in vivo* analysis in nematodes (e.g., *Caenorhabditis elegans* [125]), insects (e.g., *Galleria mellonella* [126]), and mice or pigs (127, 128). Nevertheless, despite the evident differences between laboratory culture medium and the natural bacterial habitat, the overall fitness effects of plasmids measured *in vitro* correlate quite well with their effects measured in mouse models (11).

Exploring Interactions between MGEs

Previous studies revealed that interactions between MGEs play an important part in the fitness effects produced by these elements (21, 23, 92, 95). Moreover, interactions between plasmids and other MGEs can also alter the evolutionary trajectories of plasmid-carrying

bacteria, as recently shown for a plasmid-phage combination in *P. fluorescens* (129). In natural microbial communities, bacteria are exposed to a wide range of MGEs, and understanding how interactions between these elements shape the fitness effects and evolution of MGE-bacteria associations is an exciting challenge. Understanding interactions between different plasmids and between plasmids and other MGEs may help to explain plasmid distribution in bacteria (21, 130).

High-Throughput Approaches

Predicting the fitness effects of specific plasmid types in specific bacterial clones is of paramount importance if we are to predict which plasmid-bacteria associations are likely to arise in the future and anticipate the evolution and epidemiology of plasmid-mediated antibiotic resistance. In a recent meta-analysis, Vogwill and MacLean analyzed available data on the fitness effects of 49 plasmids examined in 16 studies (11). Remarkably, these authors found that the same plasmid generally has a very different fitness cost in different hosts, and that the variation coefficient for fitness effects of a single plasmid in different hosts does not differ significantly from that for the fitness effects of different plasmids in the same host (11). These results highlight the impossibility of using the fitness cost of a plasmid measured in one bacterial host to predict the effects of the same plasmid in other bacterial clones. This question will therefore need to be addressed through high-throughput studies using factorial designs combining multiple plasmids and multiple bacterial clones. Such studies could provide insight into the general trends of the fitness effects of specific plasmid types on specific bacterial groups (genus, species, and high-risk clonal complexes). It will also be necessary to test not only initial plasmid costs but also the ability of compensatory evolution to reduce the cost of specific combinations. Understanding these general trends in fitness effects and the capacity for compensation may assist predictions of which plasmid-bacteria associations we are likely to encounter in real-life scenarios.

Integrative Analysis of Plasmid Costs

A full understanding of the origin of the costs produced by plasmids requires detailed knowledge of the molecular basis underlying them. To date, only a handful of studies have investigated the genetic basis of plasmid fitness costs (23, 25–28) or the transcriptional effects of plasmids on the bacterial host (23, 25, 113, 131). In our experience, the best way to investigate the molecular mechanisms underlying the fitness costs of plasmids is to first obtain a compensated clone from the parental plasmid-carrying strain (22, 23). One can then analyze and compare the plasmid-carrying and plasmid-free naive and compensated strains, obtaining a clearer picture of the basis of cost and compensation (23, 25). Most studies using this approach include the sequencing of the whole genome of the plasmid-carrying strains before and after compensatory adaptation, allowing the genetic basis of cost and compensation to be determined (23, 25, 26, 28). In two studies, the origins of the cost were further investigated using transcriptomic analysis of naive and compensated plasmid-carrying strains (23, 25). Because gene expression is such a key determinant of the cost of plasmid carriage, transcriptomic approaches are an essential tool in the investigation of the mechanisms underlying the cost of plasmid carriage. However, a major challenge in the field is the adoption of a more integrated approach to the analysis of plasmid fitness effects. Genomics and transcriptomics should therefore be complemented with metabolomic and proteomic analyses to yield a full picture of the origins of the costs of plasmids.

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