Beyond horizontal gene transfer: the role of plasmids in bacterial evolution

Jeronimo Rodriguez-Beltran[1†], Javier DelaFuente[1], Ricardo Leon-Sampedro[1], R. Craig MacLean[2] and Alvaro San Millan[1,3†]

[1]Department of Microbiology, Hospital Universitario Ramon y Cajal, Instituto Ramón y Cajal de Investigación Sanitaria (IRYCIS), Madrid, Spain.

[2]Department of Zoology, University of Oxford, Oxford, UK.

[3]Centro Nacional de Biotecnología–CSIC, Madrid, Spain.

[†]e-mail: jeronimo.rodriguez.beltran@gmail.com; asanmillan@cnb.csic.es

Abstract

Plasmids have a key role in bacterial ecology and evolution because they mobilize accessory genes by horizontal gene transfer. However, recent studies have revealed that the evolutionary impact of plasmids goes above and beyond being mere gene delivery platforms. Plasmids are usually kept at multiple copies per cell, producing islands of polyploidy in the bacterial genome. As a consequence, the evolution of plasmid-encoded genes is governed by a different set of rules than those affecting chromosomal genes, and these rules are shaped by unusual concepts in bacterial genetics such as genetic dominance, heteroplasmy or segregational drift. In this Review, we discuss recent advances that underscore the importance of plasmids in bacterial ecology and evolution beyond horizontal gene transfer. We focus on new evidence that suggests that plasmids might accelerate bacterial evolution, mainly by promoting the evolution of plasmid-encoded genes, but also by enhancing the adaptation of their host chromosome. Finally, we integrate the most relevant

theoretical and empirical studies providing a global understanding of the forces that govern plasmid-mediated evolution in bacteria.

[H1] Introduction

Ever since small genetic molecules competed for self-replication in the early origins of life, the evolution of every organism has been shaped by the coexistence of diverse DNA molecules within the cell. Among these, plasmids — autonomously-replicating DNA molecules that stably coexist with chromosomes — stand out as drivers of horizontal gene transfer **[G]** (HGT) in prokaryotic evolution. The word plasmid was first coined by Joshua Lederberg in 1952 as a "generic term for any extra-chromosomal hereditary determinant"[1], but it was not until the characterization of the first conjugative plasmids in the early 1960s that the term rose in popularity[2]. Since then, the study of plasmid biology has produced a remarkable wealth of knowledge, which has nourished the fields of bacterial genetics and molecular biology, among others.

From an evolutionary perspective, plasmids may be considered selfish (or parasitic) genetic elements, as they do not necessarily share evolutionary interests with the chromosome[3,4]. Plasmids encode genes to ensure their maintenance and transmission that are often disadvantageous to the host, such as post-segregational killing systems **[G]** [5]. However, plasmids have an important role in bacterial evolution by transferring beneficial traits within and between species of bacteria, positively contributing to host fitness. Therefore, there is a clear trade-off between plasmid parasitic and mutualistic lifestyles, challenging our understanding of their existence conditions (BOX 1).

Recent work has shown that plasmid-carried genes evolve in fundamentally different ways from chromosomal genes, suggesting that plasmids are more than just vehicles for gene transfer. Deciphering the evolutionary opportunities and constraints associated with plasmids is key to understanding and predicting the evolution of plasmid traits, such as antibiotic resistance and bacterial virulence. In this Review, we discuss recent advances that highlight plasmids as catalysts of bacterial evolution beyond HGT, with a special focus on the evolution of antibiotic resistance.

[H1] Anatomy of plasmids

[H2] Plasmid prevalence and types

Plasmids are remarkably diverse. Plasmids vary in size, copy number, GC content, replication mechanism, transmission mode, DNA topology (that is, circular or linear), genetic cargo and host range, among other features. From the small parasitic 2μm plasmid of *Saccharomyces cerevisiae* to the worrisome enterobacterial antibiotic resistance plasmids, the wide variety of plasmid types mirrors the extreme diversity of their hosts, which span all the domains of life[6]. Bacterial plasmids have received particular attention because of their prevalence (FIG. 1a) and are the main subject of this Review. In spite of their diversity, bacterial plasmids can largely be classified into two groups (FIG. 1b): low-copy number plasmids (LCPs) and high-copy number plasmids (HCPs). LCPs are typically large (from tens to hundreds of kilobases), they present a low copy number, and are frequently conjugative. On the other hand, HCPs are in general small, kept at high copy numbers and typically lack a functional conjugative system, but some of them can be mobilised through the conjugative apparatus of other coexisting plasmids[7]. Plasmids can be further classified based on replication[8] and mobility proteins[9], or based on general similarity in genetic content and nucleotide homology[10–12]. However, for the purposes of this Review, we focus on the broad scale distinction between LCPs and HCPs.

[H2] Plasmid-encoded traits

Plasmids encode a vast catalogue of traits that promote their own survival and increase the range of environmental conditions in which their host can thrive. Plasmids carry a backbone or 'core' genes that are instrumental to their vertical and horizontal transmission such as those coding replicative and conjugative proteins. Additionally, plasmids can encode specialized systems to improve their transmission and stability. Some examples include anti-restriction proteins that contribute to the evasion of host defences[13,14], post-segregational killing systems that ensure plasmid persistence[5], and type IV CRISPR–Cas systems that mediate inter-plasmid competition[15].

Although some plasmids are cryptic and do not encode any known function beyond those devoted to their own survival[16,17], most plasmids encode 'accessory' genes that can expand the ecological niche of their hosts by degrading toxic compounds or providing new metabolic capabilities. For instance, plasmids carrying virulence and antibiotic resistance genes are key contributors to the uncontrollable spread of bacterial pathogens, particularly in clinical settings[18,19]. Beyond human health, plasmid-associated traits are responsible for major transitions in the evolution of several bacterial clades. Some prominent examples include the ability to undergo anoxygenic photosynthesis in marine bacteria of the *Rhodobacteriaceae* family[20], and the metabolic plasmids of the aphid endosymbiont *Buchnera aphidicola,* which are responsible for synthesizing essential amino acids and vitamins required for the bacterium–aphid symbiotic relationship, and have had a key role in the transition of *B. aphidicola* from free-living to obligate endosymbiont[21]. These examples, and several others[22,23], highlight the crucial importance of plasmid traits for the diversity of bacterial lifestyles.

[H2] Plasmids as vectors of horizontal gene transfer

HGT is a major force fuelling prokaryotic evolution[24]. HGT is pervasive and allows taxonomically different organisms to share a common genetic pool which blurs the boundaries between distinct phylogenetic clades and explains why closely related prokaryotes greatly differ in gene content[25]. Plasmids are key drivers of HGT and can be transferred at seemingly high rates through several mechanisms, mainly conjugation (including plasmid mobilization and conduction), but also transduction, transformation and vesiduction (BOX 2). Several factors are known to affect the transferability of plasmids in natural settings[26,27], yet direct measurements of transfer in the environment are still scarce (reviewed in REF.[28]). Recent studies have focused on quantifying the extent of plasmid-mediated gene transfer in the human gut. Longitudinal whole genome sequencing of bacterial samples from human donors demonstrated that plasmid transfer is pervasive, even in the absence of antibiotic treament[29–31]. This view has been further confirmed using tailored CRISPR spacer acquisition systems to record the history and diversity of HGT among human microbiota[32]. Although plasmid transfer is one of the major drivers of prokaryotic evolution, this Review focuses on the less explored mechanisms of plasmid-mediated evolution that go beyond HGT, so we direct the reader to excellent Reviews on plasmids and HGT for further information[24,28].

[H2] Multi-copy nature of plasmids

Arguably, one of the most far reaching properties of plasmids is that they are kept at several copies per cell. Plasmid copy number (PCN) ranges from only slightly higher than chromosomal copy number in LCPs, to tens of copies per cell in HCPs[33]. This fundamental difference in PCN is associated with the presence or absence of active partitioning systems. HCPs generally lack these systems, and therefore they rely on a high PCN to be stably inherited, avoiding loss during plasmid segregation **[G]** at

cell division. Despite these differences, all plasmids (LCPs and HCPs) present on average more than one copy per bacterial cell.

Plasmids dynamically adjust their copy number during bacterial growth through negative feed-back loops[34]. These systems ensure that mean copy number is constant at the population level, yet there is substantial stochastic heterogeneity at the cellular level[35–37], with cells showing up to a 10-fold higher copy number than others[38]. Additionally, although PCN is an evolutionary robust trait[39], certain mutations in replicative genes can stably increase copy number and are routinely isolated in laboratory and clinical settings[39–43]. This variability in plasmid copy number can be further compounded in cases where environmental cues modulate PCN[44–48]. This is the case for the virulence plasmids of two phylogenetically distant bacterial species: *Yersinia* spp.[45] and *Agrobacterium tumefaciens*[44,48]. These pathogens carry plasmids that are essential for their infectious lifestyle, the copy number of which is up-regulated in response to signals emitted by their target cells[44,45]. The key insight from these examples is that PCN is a plastic trait, and PCN varies widely between cells, even if they belong to the same clonal population.

It is becoming clear that most bacterial chromosomes are effectively polyploid at least in some growth conditions[49]. For instance, increased ploidy levels are typically associated with fast growth in *Escherichia coli*, but not with slow growth or stationary phase[49]. By contrast, plasmids are stable polyploid molecules that remain multi-copy throughout the whole bacterial life cycle[50,51]. In the following sections we focus on the evolutionary consequences of the multi-copy nature of plasmids, although some of the principles addressed below could also be extrapolated to the chromosomes of polyploid bacteria.

[H1] Plasmid copy number and evolution

[H2] Increased gene expression

Owing to their copy number, plasmid genes usually have higher gene dosage effects **[G]** than chromosomal genes and, as a result, plasmid genes tend to be highly expressed[52]. Consequently, plasmids have traditionally been harnessed for large-scale protein production. From an evolutionary perspective, high gene expression is a major barrier to HGT[53], yet it can be beneficial under several circumstances. For instance, some members of the genus *Aureimonas* lack a chromosomal copy of the rRNA (*rrn*) operon. Instead, these bacteria carry their sole *rrn* operon on a small HCP held at 18 to 34 copies per cell[22]. High gene dosage of *rrn* operons is known to promote rapid adaptation to changing environmental conditions by facilitating a sudden increase in rRNA expression[54]. Thus, the *Aureimonas* spp. plasmid-encoded *rrn* might help to cope with the common fluctuating conditions that these bacteria face in their natural habitat[22,55]. Other paradigmatic examples are provided by antimicrobial resistance enzymes that are typically plasmid-encoded. For instance, β-lactamases — enzymes that are able to degrade β-lactam antibiotics — are found both on plasmids and chromosomes, but resistance levels are higher (up to 128-fold) when these enzymes are plasmid-encoded[40,42,56]. Therefore, plasmid-driven gene dosage effects might help to predict which antibiotic resistance genes are more likely to associate with plasmids (that is, those that provide more resistance with increased gene dosage).

As stated in the previous section, subpopulations of cells with a relatively high PCN randomly appear as a by-product of plasmid segregation and replication dynamics. Thus, in any plasmid-carrying bacterial population of reasonable size, subpopulations

showing elevated PCN are likely to exist. This PCN intrapopulation variability might lead to potentially beneficial phenotypic plasticity, by which only a fraction of cells show increased expression of plasmid-encoded genes. An illustrative example is provided by bacterial heteroresistance, a phenomenon by which clonal subpopulations of bacteria show lower susceptibility to an antibiotic insult than the main population[57]. Several genetic mechanisms underlie heteroresistance (reviewed in REF.[58]), although alteration of gene dosage is the most common among Gram-negative bacteria[59]. By amplifying gene dosage of genes that confer low level resistance, heteroresistant bacteria can acquire transient high-level antimicrobial resistance[59]. A recent study found that tandem genetic duplications **[G]** were involved in most of heteroresistance instances[59]. Crucially, when antibiotic resistance genes were plasmid-encoded, the effect of tandem duplications was further enhanced by increases in PCN[59], suggesting that both mechanisms act synergistically to drastically increase gene dosage in a fraction of the bacterial population. These resistant subpopulations might lead to antibiotic treatment failure, as they are likely to be undetected by conventional antimicrobial susceptibility testing[58].

Colicin production offers another paradigmatic example of the effects of PCN in microbial lifestyles. Colicins are plasmid-encoded toxins produced by enteric bacteria that shape microbial interactions by killing competing bacteria[60]. Colicin production leads to producer cell death as a side-effect of colicin release, and thus it is extremely costly to the population[61]. As a consequence, colicin production is stochastically triggered only in a small fraction of cells[61,62]. Among the genetic factors that control colicin synthesis, PCN variability has a crucial role, as cells with high PCN are more likely to undergo colicin release[62].

These studies suggest that population heterogeneity in PCN might work as a bet-hedging **[G]** strategy: a fraction of the population is best suited to survive an

environmental perturbation, but do so at the cost of overexpressing plasmid-encoded genes, which produces a competitive disadvantage when selection for high expression is absent[63].

[H2] Increased genetic variability

Mutation and recombination **[G]** provide the evolutionary 'raw material' in which selection acts in classic models of evolution. Mutation rates per gene linearly increase with gene copy number, and recombination is more frequent in plasmids than chromosomes[64,65], suggesting that plasmid-encoded genes should evolve faster than chromosomal genes. This is indeed the case in the evolution of β-lactamases. Certain mutations in the bla_{TEM-1} gene expand its range of activity, conferring resistance to a wide variety of β-lactam antibiotics. One study evolved wild-type and mutator populations of *E. coli* carrying bla_{TEM-1} to increasing concentrations of cefotaxime, a third-generation cephalosporin. Interestingly, despite the high mutation supply provided by mutators **[G]**, no bla_{TEM-1} resistance mutations were found when the gene was located in the chromosome. By contrast, double and triple mutants readily emerged when the bla_{TEM-1} gene was located on a plasmid[66]. Similarly, experimental evolution of resistance to a different β-lactam antibiotic (ceftazidime) showed that emergence of high-level resistance is contingent on the presence of the bla_{TEM-1} allele on a plasmid and it is not accessible when the gene is located on the chromosome[40]. These results can be explained by the interplay of two direct consequences of the multi-copy nature of plasmids. First, plasmid genes offer a large mutational target due to their high copy number, and thus beneficial mutations will occur at higher frequencies (that is, more mutations per gene) than in chromosomal genes. This effect is simple to reconcile with classical evolutionary theory: increasing the rate at which beneficial mutations are introduced into populations increases the rate of adaptation[67]. Second, PCN amplifies the gene dosage of new mutations, and

their expression levels are rapidly maximised at the population level. As stated before, resistance levels conferred by β-lactamases drastically depend on their gene dosage[40,56], and thus mutations improving bla_{TEM-1} activity confer higher resistance levels when they are encoded on plasmids rather than on chromosomes[40]. Similarly, directed evolution experiments, in which plasmids containing a gene of interest are mutagenized, have shown that mutations that increase PCN are positively selected alongside mutations that directly improve the catalytic activity of the gene of interest[68]. Together, these results demonstrate that PCN not only contributes to optimizing existing traits but also facilitates the evolution of new biochemical activities, and that gene amplification and mutation — two of the dominant mechanisms of bacterial evolution — are tightly linked due to the multi-copy nature of plasmids.

Bacterial plasmids are prone to recombination. First, the high density of repeated sequences on plasmids, such as those associated with transposable elements **[G]**, creates recombinational hot-spots that lead to a high rate of *en bloc* deletions and capture of new genetic elements from distinct sources. In fact, more than 40% of plasmids deposited in the NCBI database are mosaic plasmids that carry substantial fragments of other plasmids[69]. A particularly revealing example comes from the plasmids of the phylum Firmicutes, which are characterised by their recombination-driven modularity. These plasmids show a high degree of mosaicism, often including multiple origins of replication within the same plasmid backbone[70]. Second, host defence systems cause an increase in plasmid recombination as a side-effect. Host defences, such as CRISPR–Cas and restriction-modification systems, typically prevent plasmid establishment by producing double-strand breaks in foreign DNA[71]. Double-strand breaks, in turn, promote plasmid recombination, as they are the preferred substrate of most bacterial recombinases. Some plasmids are equipped

with dedicated genes to repair double-strand breaks through recombination[72], suggesting that recombinational evasion of host defences might a be a common strategy among plasmids.

Recombination has had a major role in the evolution of plasmid-associated antibiotic resistance genes such as β-lactamases[73,74] and fluoroquinolone resistance *qnr* genes (which protect DNA gyrase and topoisomerase IV from fluoroquinolone binding[75]). The evolution of these gene families is characterized by frequent recombination between different alleles, combining mutations that had emerged separately[73,75]. At a broader scale, recombination can create chimeric plasmids that contain antibiotic resistance cassettes from different plasmids, resulting in multi-resistance replicons that pose a great clinical concern[76–79]. For instance, non-conjugative plasmids carrying the colistin resistance gene *mcr-1* have been shown to recombine with conjugative plasmids carrying additional resistance determinants such as the bla_{NDM} and $bla_{CTX\text{-}M}$ β-lactamases[76,77]. Importantly, these multi-resistance plasmid hybrids are stably maintained, and spread through conjugation among bacterial populations[76,77]. Plasmid recombination occurs more frequently in pathogenic *E. coli* strains than in their commensal counterparts[64], suggesting that chimeric plasmids might be more common among the former. Additionally, several plasmid families are known to engage in plasmid-mediated gene capture, also known as retrotransfer, by which plasmids are able to transfer to a new host, incorporate genomic fragments of the recipient, and be transferred back to the original donor[13].

Plasmid recombination has driven the evolution of IncP-1, IncW and IncF plasmid families by increasing plasmid fitness through capture and combination of different genes, facilitating their adaptation to different hosts[80–83]. Other paradigmatic examples come from the agrobacterial (tumour-inducing) Ti and (root-inducing) Ri plasmids. The presence of Ti and Ri plasmids in *Agrobacterium* species drives their

transition from non-pathogenic bacteria to tumour-inducing plant pathogens[84]. These plasmids descend from only a handful of parental lineages and are remarkably conserved[85]. However, extensive recombination has reshuffled functional modules between distinct lineages, promoting their diversification and facilitating their massive spread in agricultural settings[85].

Recombination also affects plasmid evolution on shorter time scales. For instance, IncQ plasmids replicate through a strand displacement mechanism that renders them particularly prone to recombination[86]. Once an IncQ plasmid arrives in a new host, recombination rapidly creates variants with smaller sizes that can lack both accessory and essential plasmid genes. Interestingly, these streamlined variants compete for intracellular replication with the full-length version of the plasmid, reducing its PCN and the fitness burden associated with it[87]. This is potentially beneficial for the host, as it can maintain burdensome, but potentially beneficial plasmid genes at a low physiological cost[87]. This example, and others[88,89], highlight the role of recombination in streamlining plasmid genomes to reduce the cost of plasmid maintenance. Together, these studies suggest that plasmids, owing to their copy number, gene content, and replication modes are particularly prone to engaging in recombination, which in turn promotes plasmid evolution.

[H2] Complex population dynamics of plasmid-encoded alleles

One important consequence of the multi-copy nature of plasmids is that once a novel allele arises on a plasmid-encoded locus, it will be initially present in only one of the plasmid copies. Therefore, plasmids carrying novel alleles will coexist with plasmid copies carrying the ancestral allele until they are eventually segregated into distinct cell lineages. The coexistence of distinct variants of the same plasmid is termed heteroplasmy **[G]**[90]. In other words, heteroplasmid cells carry heterozygous plasmid alleles whereas homoplasmid cells carry homozygous plasmid alleles[90].

Heteroplasmy poses consequences for plasmid evolution that are reminiscent of classical population genetics (FIG. 2).

First, because segregation of mutant and non-mutant plasmids copies is random, daughter cells often inherit a different allelic composition than that of the parental cell. Plasmid evolution is thus subjected to an extra layer of genetic drift **[G]**, known as segregational drift, by which intracellular frequencies of plasmid-encoded alleles change due to random inheritance[91]. Segregational drift reduces the establishment probability of plasmid neutral alleles and increases the fixation time of plasmid beneficial mutations compared to chromosomal mutations[91] (FIG. 2). In fact, experimental evolution under selective conditions for plasmid maintenance during 800 generations showed that HCPs accumulate less mutations than expected by chance due to segregational drift[91]. Segregational drift might help explain the striking conservation in the core genomes of some plasmid families that show worldwide distribution, such as the broad-host range IncP-1 family[92].

If a beneficial mutation emerges in a plasmid copy before a former mutation in a different copy reaches fixation, a second effect termed plasmid interference takes place. Plasmid interference produces an analogous effect to clonal interference **[G]**, but at the cellular level instead of at the population level. During plasmid interference, two (or more) beneficial mutations coexist and compete for establishment in the plasmid pool of a single cell lineage under heteroplasmy[88]. One study evolved populations of *E. coli* carrying costly versions of the chloramphenicol resistance *cat* gene in a HCP. Sequencing of evolved clonal populations showed two to three coexisting beneficial mutations in the *cat* gene competing for fixation at the cellular level. This plasmid interference translated into population-level polymorphisms that were maintained over large time-scales[88]. Therefore, as in clonal interference, plasmid interference delays the fixation of beneficial mutations in plasmid populations

and can even lead to the loss of beneficial mutations[88]. However, recombination between plasmid variants is expected to frequently assort competing mutations in the same plasmid backbone[73], modulating the impact of plasmid interference on the evolution of plasmid encoded traits.

Due to the factors mentioned above, plasmid mutations experience considerably longer fixation times than chromosomal mutations[91,93,94]. This negatively affects the establishment probability of novel plasmid alleles, yet it also implies that plasmid loci remain polymorphic for dozens of bacterial generations. Therefore, by shielding mutations from selection, plasmids provide a source of standing genetic variation **[G]** [96]. Upon environmental change, standing genetic variation allows bacterial populations to adapt through pre-existing mutations, and occurs faster than adaptation through *de novo* mutations[95]. Growing populations of bacteria accumulate mutations randomly, but selection and drift rapidly reduce the frequency of chromosomal mutations in the population. In plasmids, these polymorphisms are maintained over larger timescales, which increases the chances of bacterial populations escaping extinction caused by sudden environmental changes such as antibiotic treatment[94].

From a different perspective, when a gene evolves to gain a new function, it often does so at the expense of its original function. These negative correlations between ancestral and novel activities are called trade-offs **[G]** and are a pervasive constraint in protein evolution[96]. Trade-offs occur because beneficial mutations in one environment are often deleterious if the context changes. For instance, evolution of antibiotic resistance is dominated by trade-offs in which a mutation confers resistance to a new antibiotic, but at the expense of a reduced activity against the antibiotic to which it originally conferred resistance[97]. For example, the $bla_{\text{TEM-1}}$ β-lactamase confers high level resistance to penicillin-derived antibiotics such as

ampicillin, but has negligible activity against third-generation cephalosporins such as ceftazidime. Evolution of *bla*$_{TEM-1}$ towards ceftazidime resistance has been shown to occur predominantly through the acquisition of a single mutation that gives rise to the *bla*$_{TEM-12}$ allele[97]. The evolved allele confers high-level resistance to ceftazidime, but does so at the expense of reducing its activity against ampicillin. As a result, when both antibiotics are present either simultaneously or alternatively during fluctuating selective pressures, trade-offs limit the evolution of antibiotic resistance[97]. However, when those alleles are plasmid-encoded, they co-exist within the cell for hundreds of bacterial generations under heteroplasmy, relieving the constraints imposed by trade-offs in the evolution of antibiotic resistance[98]. Plasmids therefore represent excellent scaffolds for the evolution of novel functions because they can maintain genetic diversity both intracellularly and at the population level[98]. Interestingly, the plasmid-like genomes of bacterium-derived eukaryotic organelles also overcame evolutionary trade-offs by accumulating genetic variation in the form of long-lasting heteroplasmy. For instance, the mitochondrial genome of pill-bugs (*Armadillidium vulgare*) shows a conserved polymorphism in their alanine and valine alloaceptor tRNA that has persisted for more than thirty million years[99].

[H2] The role of genetic dominance

A given mutation can have profoundly different phenotypic effects depending on whether it occurs on a chromosomal or plasmid gene. Besides the effects on gene expression levels discussed above, the phenotypic effect of plasmid mutations will depend on their degree of genetic dominance. Genetic dominance is the relationship between alleles of the same gene, in which the phenotypic contribution of one allele (termed recessive) is masked by the presence of a second allele (dominant). However, it can occur that one of the alleles is not completely dominant over the other. In these cases, both alleles are manifested either simultaneously (termed co-

dominance) or as a blend of the phenotype of both alleles (incomplete dominance). The notion of genetic dominance dates back to Gregor Mendel and is considered to drive the evolution of polyploid and diploid organisms. This is because recessive mutations are less likely to contribute to the phenotype than dominant mutations, and as a consequence there is a bias against the establishment of recessive beneficial mutations in polyploid organisms, an effect known as Haldane's sieve[100]. As most bacteria have been assumed to carry a unique copy of their chromosomes[49], the effect of genetic dominance has traditionally been neglected in bacterial evolution (but see REF.[101] where the effect of genetic dominance on the fate of chromosomal mutations is explored). However, genetic dominance has far reaching consequences for plasmid evolution[94,102].

Recessive plasmid alleles will be unable to contribute to phenotypes as long as they co-exist with wild-type alleles within the same cell (FIG. 3). As explained above, plasmids alleles can stably co-exist for dozens of generations under heteroplasmy, and thus genetic dominance should have a key role on determining the establishment probability of novel plasmid mutations[94,102]. Genetic dominance has two major implications for plasmid evolution. First, plasmids should evolve mainly through dominant mutations and therefore have fewer mutations available than bacterial chromosomes (which evolve through both dominant and recessive mutations)[94,102]. In fact, the rate of mutations conferring antibiotic resistance was found to be 3 to 10 times lower in plasmids than in chromosomes when the available mutations were recessive, and 4 times higher when the selected mutations were dominant[94,102]. Second, genetic dominance appears to dictate the fate of horizontally transferred alleles if the recipient bacterium already carries an allele of the transferred gene on its chromosome[94,102]. This was demonstrated experimentally by showing that conjugative transfer of a recessive, quinolone resistant allele of the *gyrA*

gene provided no resistance in the recipient cell[102], as a wild-type version of this housekeeping gene is naturally present on most bacterial chromosomes. Genetic redundancy between plasmids and chromosomes is likely to be common, considering that plasmids and chromosomes frequently share genetic content[26,82,83,103,104], and that plasmid transfer typically occurs between close relatives[105]. Although the pervasiveness of the effects of genetic dominance in bacterial evolution are yet to be established, the interplay between the aforementioned two effects should shape the genetic repertoire of plasmids. In fact, bioinformatic evidence suggests that recessive alleles of antibiotic resistance genes tend to be under-represented on plasmids and other mobile genetic elements in comparison to chromosomes[102]. This might have important implications for bacterial evolution, but also for human health, as it offers a framework to predict which antibiotic resistance genes are likely to be mobilised by plasmids and other mobile genetic elements (BOX 3).

[H1] Further plasmid effects on evolution

Beyond the effects associated with the multi-copy nature of plasmids, other plasmid properties may also contribute to accelerate bacterial evolution. During conjugation, plasmids are transferred as single stranded DNA, which in turn activates the bacterial SOS stress response **[G]** [106]. The SOS response coordinates the expression of dozens of bacterial genes involved in DNA repair and cell cycle control, and it is known to fuel bacterial evolvability through an increase in recombination and mutagenesis[107]. For instance, conjugation of antibiotic resistance plasmids from *E. coli* to *Vibrio cholerae* strongly induces the SOS response, leading to genomic rearrangements mediated by the site-specific integron **[G]** integrase[106].

A large proportion of conjugative plasmids encode SOS-regulated genes that directly enhance mutagenesis[108,109]. Most of these plasmids encode functional homologues of chromosomal UmuDC proteins. The *umuDC* operon encodes an error-prone DNA polymerase, which is capable of replicating heavily damaged DNA templates, but does so at a reduced fidelity. Expression of plasmid *umuDC* genes is tightly controlled as part of the bacterial SOS response and leads to transient mutagenesis upon plasmid transfer[110]. For instance, rizhobial symbiotic plasmids encode complex traits that mediate the relationship between legume plants and bacteria[23]. These plasmids frequently encode UmuDC homologues that boost the mutation rate of the bacterial host upon entry[111]. Experimental replay of rizhobium evolution has shown that co-transfer of UmuDC with symbiotic genes on a plasmid accelerates the transition of plant pathogens into legume symbionts by generating a burst of genome diversity after transiently increasing the chromosomal mutation rate[111]. Thus, SOS-mediated mutagenesis is not restricted to plasmid DNA and also affects chromosomal DNA, promoting the evolution of the bacterial host[111]. Additionally, it has been hypothesized that plasmid-mediated SOS induction might also serve to accelerate the adaptation of the bacterial host to incoming plasmids, and thus might be particularly exploited by broad-host range plasmids[106]. In agreement with this hypothesis, narrow-host range plasmids often carry dedicated genes to prevent SOS induction[106].

Plasmids also facilitate the evolution of their bacterial host through mechanisms independent of SOS induction. For instance, non-growing populations of *E. coli* have been shown to experience increased rates of mutagenesis. This mutagenesis, which seems to be restricted to plasmid genomes, occurs through an increase in PCN in a subpopulation of cells. Conjugative transfer of these plasmids between clone mates

further amplifies plasmid genomes, which are then subject to extensive mutagenesis mediated by the plasmid-encoded, error-prone polymerase DinB[47,112].

Additionally, plasmids are often associated with other mobile genetic elements, such as transposable elements and integrons. Once a plasmid arrives in a new host, these elements commonly transfer to the chromosome, where they foster bacterial adaptation[26,104]. For instance, transposons encoding mercury resistance were shown to transfer between distinct *Pseudomonas* species using conjugative plasmids as vehicles. The transposons were able to mobilize from chromosomes to plasmids, and back to chromosomes, disseminating mercury resistance among soil communities and allowing their hosts to colonize mercury polluted environments[26].

Finally, epistatic interactions **[G]** have been shown to occur between chromosomal and plasmid genes[113,114]. As a consequence, plasmids can alter host evolution by changing the selective effects of chromosomal mutations. For example, epistatic interactions between conjugative plasmids and chromosomal mutations alter the evolutionary paths that *E. coli* follows to evolve antibiotic resistance[113]. Antibiotic resistance chromosomal mutations are usually costly to the bacterial host. However, epistasis between plasmids and chromosomal mutations has been shown to reduce the cost of resistance, and therefore an initially deleterious mutation may become beneficial upon plasmid acquisition[113]. Therefore, these epistatic interactions might pave the way for the emergence of multidrug resistant bacteria at very low fitness costs. Additionally, epistasis often occurs between genes that have similar functional roles[115], and we speculate that chromosome–plasmid epistasis should be especially pronounced for traits like antibiotic resistance and metabolism that are prevalent on both plasmids and chromosomes.

[H1] Conclusions and future perspectives

Despite 80 years of extensive study of plasmid biology, several outstanding questions remain to be answered (BOX 4). The recent advances reviewed here highlight that plasmids are likely to evolve differently than bacterial chromosomes, and that plasmids create a scenario in which opposing forces determine the evolution of plasmid-encoded traits. On the one hand, plasmids generate genetic variability at higher rates than bacterial chromosomes, show dedicated mechanisms to increase their evolvability, and can store genetic variation for hundreds of bacterial generations. On the other hand, genetic dominance, plasmid interference and segregational drift may limit the evolutionary potential of plasmids. This leads to the question of whether plasmids evolve faster than bacterial chromosomes. Several lines of evidence suggest that this is indeed the case. First, experimental evolution has shown that plasmids accelerate evolution when selective pressure is high[40,66]. Second, mathematical models predict that plasmids will increase the rate of beneficial mutations as long as these mutations are dominant or have strong gene dosage effects[40,94,102]. Third, plasmids are enriched in dispensable genes for the bacterial host. This dispensability should reduce purifying selection **[G]**, allowing plasmids to accumulate genetic variability during evolutionary relevant time scales[116]. Fourth, recombination is frequent among plasmid genomes. The genetic plasticity provided by recombination is further enhanced because plasmids frequently engage in HGT, and thus they cohabit with a wide variety of bacterial genetic backgrounds. This increases the repertoire of beneficial variants that can recombine with plasmid genomes, speeding up the evolution of plasmid-encoded traits[117]. Altogether, these evidences suggest that plasmids might act as evolutionary catalysts, promoting their own evolution and that of their bacterial host through a plethora of distinct mechanisms that go above and beyond HGT. It is our view that gaining a better

understanding of plasmid evolution will shed light on the mechanisms that fuel bacterial diversity and help explain the extreme ecological success of prokaryotes.

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Author contributions

All authors contributed substantially to the conceptualization and writing of the manuscript, which was originally conceived and drafted by J.R.-B. and A.S.M.

Competing interests

The authors declare no competing interests.

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Figure 1. Plasmid prevalence and types. a| Phylogenetic tree highlighting the widespread distribution of known plasmids in the most relevant prokaryotic phyla. The outer track surrounding the tree is colour-coded according to the percentage of prokaryotic classes within each phylum that contain plasmids (see the plasmid prevalence key). The inner track delineates phyla depicted in the tree. Note that only four major prokaryotic phyla lack plasmids. The tree was built using 1,688 sequences obtained from a previously published concatenated ribosomal protein alignment[118] and includes every prokaryotic phylum with at least 50 genomes deposited in the NCBI database. **b|** Plot showing the distribution of plasmid sizes for representative bacterial families and uncultured bacteria, showing the common bimodal (or multimodal) distribution of plasmid sizes. Red dashed line represents the local minor density point between the highest peaks for each bacterial family. Data was retrieved from the PLSDB plasmid database[119].

Figure 2. Evolutionary forces that drive the evolution of plasmid-encoded genes. a| Scheme summarising the evolutionary consequences of the multi-copy nature of plasmids. After the emergence of allele A (orange plasmid) in the plasmid pool of the top cell, plasmids are randomly segregated to daughter cells at each cell division. This simple process leads to the generation of heteroplasmid cells (green shading), carrying an allelic mixture. In some cells, mutation A is lost to segregational drift (blue shading). If a second independent mutation appears (mutation B; green plasmids) plasmid interference between mutations A and can B occur. Eventually, mutation A reaches fixation, leading to homoplasmid mutant cells (red shading). **b|** Frequency of a plasmidic beneficial allele under positive selection over time in a bacterial population. A higher plasmid copy number increases the fixation time of the allele, but also the time in which both novel and ancestral alleles coexist under heteroplasmy (depicted as a dotted line). **c|** Only heteroplasmid cells carrying

ancestral and evolved alleles can survive under periodic or simultaneous selection for both alleles.

Figure 3. The role of genetic dominance in plasmid evolution. a| Dominance relationship among three alleles of the same gene. The allele A (green) is dominant over alleles B (blue) and C (orange). Allele B is dominant over allele C. **b|** The phenotypic effect of new alleles will depend on their degree of genetic dominance. If a new plasmid allele emerges by mutation (left) or is acquired through horizontal gene transfer (HGT; right) it will only produce a phenotype if it is dominant over the resident alleles. **c|** Fitness effect of mutations depending on the number of plasmids carrying the mutant allele for dominant mutations (blue line), and recessive mutations (red line). **d|** Effect of genetic dominance on phenotypic mutation rates for plasmid-encoded genes. The plot shows the mutation rates for a plasmid-carried gene at different copy numbers compared with a chromosomal copy of the same gene (blue line). The frequency of mutants able to produce a phenotype increases with plasmid copy number for mutations of high dominance, but decreases for mutations of low dominance. Part d is adapted with permission from REF.[102].

BOX 1. The plasmid paradox

Plasmid carriage imposes a burden on bacterial physiology, which often translates into a reduction in bacterial fitness[52,120,121]. Plasmid costs can originate both as a consequence of plasmid life cycle (for example, plasmid conjugation, replication or gene expression), as well as from the interactions of the plasmid or plasmid-encoded proteins with the host bacterium[120]. Fitness costs associated with plasmids are arguably the main limit to plasmid persistence in bacterial populations. Despite a number of effective mechanisms designed to ensure a correct plasmid segregation (such as active partitioning systems[122] and post-segregational killing systems[5]), the probability of plasmid loss during cell division is higher than zero. In the absence of selection for plasmid-encoded traits, the plasmid-free cells that emerge due to imperfect plasmid segregation will eventually outcompete plasmid-carrying bacteria,

and only those plasmids with a particularly high conjugation rate can persist as genetic parasites. In the presence of constant selection for plasmid-encoded traits, beneficial genes may eventually integrate into the bacterial chromosome, rendering the plasmid dispensable[26,104]. Consequently, it is difficult to explain the widespread prevalence of plasmids in bacterial populations, a puzzle known as 'the plasmid paradox'[123].

Since the pioneering work of Stewart and Levin[124], multiple studies have explored the population biology principles that explain the stable maintenance of plasmids in bacterial populations. Some key discoveries from those studies are helping to solve the plasmid paradox. First, the costs of plasmids can be alleviated over time through mutations in either plasmids or chromosomes (compensatory evolution **[G]**), or through the acquisition of further plasmids[125]. Compensatory evolution leads to the formation of stable bacteria–plasmid associations[126–129], and in some cases can lead to co-dependence[130]. Second, the presence of 'cost-free' hosts promotes the maintenance of plasmids in complex communities[131–133], for example by recurrent transfer from cost-free hosts to species where plasmids are unstable[134]. Third, certain bacterial lifestyles, such as growth within biofilms, can promote plasmid persistence[135,136]. Fourth, many plasmids may indeed conjugate at high enough rates to be maintained in the absence of selection[137]. Fifth, the multi-copy nature of plasmids allows the formation of transient plasmid forms, such as satellite plasmids[87], which reduce the costly expression of accessory genes. Finally, some of the plasmid-mediated evolution mechanisms described in this Review may contribute to plasmid persistence in bacterial populations through second order selection **[G]**, when plasmids are maintained by virtue of their association to high fitness bacterial clones that they generate, for example when mutations caused by plasmid-carried DNA polymerases generate beneficial mutations in the bacterial chromosome[138].

BOX 2. Mechanisms for plasmid mobility

There are several mechanisms for plasmid mobility (see the figure):

- Conjugation is the principal mechanism of plasmid mobility and requires cell-to-cell contact involving pilus formation[9]. Conjugation can occur between phylogenetically distant organisms and several studies highlight this mechanism as a key factor in the spread of antibiotic resistance[19,29,139,140].

- Conjugative mobilization allows mobilizable plasmids, which lack part of the conjugation machinery, to be transferred by the conjugative apparatus of other conjugative elements in the cell. Recent reports suggest plasmid mobilization as an important mechanism of plasmid spread[6,141–143]. Non mobilizable plasmids can be additionally transferred through plasmid conduction[144]. Conduction occurs through the co-integration via recombination of non-mobilizable and mobilizable or conjugative plasmids. The co-integrate plasmid is then transferred to a new recipient where it can be resolved.

- Plasmid transduction occurs when a bacteriophage (phage) packages plasmid DNA in viral particles and infects another bacterium. Once inside the new host, the plasmid can excise and re-circularize again. Phages are the most abundant gene-transfer particles, and new findings strongly suggest that they are likely to have an important role in horizontal gene transfer (HGT) between closely related bacteria[145], but the extent to which transduction contributes to long distance HGT remains unclear[146].

- Plasmid transformation is the uptake of free plasmid DNA by organisms. This mechanism is expected to occur in low rates constrained by the presence of free plasmids in the environment and by the physiology of the host[9].
- Recent reports show the presence of other non-canonical mechanisms of plasmid spread. Some plasmids use specialized vesicles to disseminate in a process known as vesiduction[147], which is hypothesized to occur at high rates in biofilms[148]. Plasmid transmission via intracellular connections mediated by nanotubes has also been suggested[149].

BOX 3. Genetic dominance shapes plasmid-mediated antibiotic resistance

Upon the massive introduction of antibiotics in clinical practice, some chromosomal resistance genes have been mobilised to plasmids, where they uncontrollably spread among pathogenic bacteria. But, why do some antibiotic resistance genes remain associated to chromosomes whereas others are successfully transferred? Among the multiple factors that may limit transferability of antibiotic resistance genes[150], genetic dominance stands out as one of the major forces explaining the commonly observed divergence between chromosomal and plasmid resistance alleles[102].

Several examples highlight that dominant versions of housekeeping antibiotic resistance genes (that is, those able to confer resistance regardless of the genetic context) have been successfully transferred from chromosomes to plasmids. Perhaps the clearest case is sulfonamide resistance *sul1* and *sul2* genes, which originated from a mobilization event of housekeeping *folP* genes present in Rhodobiaceae and Leptospiraceae families[151]. Other examples include the fosfomycin resistance *fosA* gene, which originated from a chromosomal glutathione-S-transferase of *Klebsiella*

pneumoniae[152], or class A β-lactamases that likely derived from penicilling-binding proteins of actinomycetes[153].

On the contrary, other resistance determinants are completely absent from bacterial plasmids, such as the antibiotic resistance alleles of *rpsL*, *rpoB* or *gyrA*, which confer resistance to streptomycin, rifampicin and quinolones, respectively[154]. These alleles are recessive to the wild-type sensitive variant, which is naturally present in most bacterial chromosomes. Consequently, if these alleles were transferred by plasmids, they would not confer any resistance to the recipient bacteria[94,101,102], a fact that likely explains why they are exclusively found on chromosomes.

Obtaining knowledge of the degree of dominance of a given resistance allele is thus crucial to predict which antibiotic resistance genes are likely to spread horizontally and to rationally assess the risk of spread for resistance alleles to novel antibiotics.

Box 4. Outstanding questions

- Why are plasmids extremely prevalent in most bacterial taxa but absent from others? Why are microbial eukaryotes relatively depleted of plasmids?
- Do the evolutionary benefits associated with plasmids offset the costs imposed by their parasitic lifestyles?
- How are antibiotic resistance genes mobilised to plasmids? Once a resistance gene becomes mobile, what are the forces that shape its evolution?
- Can we selectively eliminate resistance plasmids from complex bacterial communities?
- The function of most plasmid genes is not known[11□]. Does this plasmid 'dark matter' contain genes that are beneficial, or is it simply parasitic DNA that spreads by virtue of association with successful plasmid replicons? How does plasmid 'dark matter' alter evolvability?

Glossary

Horizontal gene transfer: Transfer of genetic material between cells that do not share an ancestor–descendant relationship.

Post-segregational killing systems: Genetic systems that ensure plasmid maintenance. They typically rely on the production of a long-lasting toxin and a short-lived antitoxin. If the plasmid is lost in a daughter cell, the antitoxin is rapidly degraded and the stable toxin kills the plasmid-free cell.

Type IV CRISPR–Cas systems: A recently characterized type of CRISPR–Cas system that are found predominantly on plasmids and primarily target other plasmids. Type IV CRISPR–Cas systems are thus believed to have a role in mediating inter-plasmid competition.

Plasmid segregation: Physical separation of plasmid molecules to be inherited by daughter cells during cell division.

Tandem genetic duplications: Duplication of a region of DNA adjacent to the original one.

Bet-hedging: Stochastic process by which some individuals in a community are better suited to tackle environmental perturbations, usually at the price of a reduced growth rate in the short term.

Recombination: Exchange of genetic information between two distinct DNA molecules.

Mutator strains: Strains that permanently show unusually high mutation rates due to a malfunction of a DNA repair mechanism.

Transposable elements: DNA sequences that can move within genomes by a cut-and-paste mechanism.

Heteroplasmy: Co-existence of two different plasmids sharing the same nucleotide sequences for all regions involved in replication and maintenance system within the same cell. Cells carrying plasmids under heteroplasmy are dubbed heteroplasmid cells, whereas cells carrying a unique version of a plasmid are termed homoplasmid cells.

Genetic drift: Change in allele frequency in a population due to random sampling.

Clonal interference: Competition between cellular lineages in a population arising from different beneficial mutations in asexually reproducing organisms.

Standing genetic variation: The presence of more than one allele at a locus in a population before environmental change.

Trade-off: In evolution, trade-offs are negative correlations between ancestral and novel traits.

Evolutionary innovation: Evolutionary changes that allow a species to expand its ecological niche and thrive in new environments.

SOS stress response: Coordinated cellular response to genotoxic stress which involves the expression of more than 40 genes whose main function is to repair damaged DNA.

Gene dosage effects: Effect by which the phenotype of a given mutation is proportional to the cumulative number of mutant alleles present in the cell.

Integron: Genetic element composed by an integrase gene and a recombination site in which gene cassettes can be directionally integrated or excised by integrase-mediated site-specific recombination.

Purifying selection: Selective pressure that eliminates deleterious alleles from populations.

Compensatory evolution: Process by which the fitness cost produced by the acquisition of a plasmid is ameliorated through mutations in the chromosome and/or the plasmid.

Epistatic interactions: Phenomenon by which the phenotypic contribution of a gene varies depending on the presence or absence of another gene. The phenotypic effect of both genes in combination is thus different than the expected according to the phenotypes they conferred separately.

Second order selection. Process by which evolution, while directly selecting for adaptive genetic variability, indirectly selects for the system that created that variability.

Table of contents blurb

Recent studies have revealed that the evolutionary impact of plasmids goes above and beyond being mere gene delivery platforms. In this Review, Rodríguez-Beltrán, San Millán and colleagues discuss the advances that underscore the importance of plasmids in bacterial ecology and evolution beyond horizontal gene transfer.