Received: 8 July 2024 | Revised: 4 October 2024 | Accepted: 28 October 2024

DOI: 10.1002/bies.202400164

BioEssays

PROBLEMS & PARADIGMS

Prospects & Overviews

The metabolic burden associated with plasmid acquisition: An assessment of the unrecognized benefits to host cells

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Funding information
NIGMS, Grant/Award Number: 1R35GM150871-01 (AJL)

Abstract

Bacterial conjugation, wherein DNA is transferred between cells through direct contact, is highly prevalent in complex microbial communities and is responsible for spreading myriad genes related to human and environmental health. Despite their importance, much remains unknown regarding the mechanisms driving the spread and persistence of these plasmids in situ. Studies have demonstrated that transferring, acquiring, and maintaining a plasmid imposes a significant metabolic burden on the host. Simultaneously, emerging evidence suggests that the presence of a conjugative plasmid can also provide both obvious and unexpected benefits to their host and local community. Combined, this highlights a continuous cost-benefit tradeoff at the population level, likely contributing to overall plasmid abundance and long-term persistence. Yet, while the metabolic burdens of plasmid conjugation, and their causes, are widely studied, their attendant potential advantages are less clear. Here, we summarize current perspectives on conjugative plasmids' metabolic burden and then highlight the lesser-appreciated yet critical benefits that plasmid-mediated metabolic burdens may provide. We argue that this largely unexplored tradeoff is critical to both a fundamental theory of microbial populations and engineering applications and therefore warrants further detailed study.

KEYWORDS
conjugation, fitness cost, plasmid

INTRODUCTION

The importance of understanding the ecology and evolution of infectious diseases has never been as painfully apparent as in the past four years following the COVID-19 pandemic. Like viruses, the emergence and spread of bacterial pathogens is of paramount clinical and agricultural concern. While this proliferation is driven in part by clonal expansion of pathogenic strains, horizontal gene transfer (HGT) also plays a major role in establishing new pathogenic lineages with diverse genetic and phenotypic traits.[1]

Plasmid conjugation—a type of HGT that describes the transfer of plasmids between two cells via direct contact—is considered a main driver of pathogen evolution, as plasmids often have broad host ranges and commonly encode multiple antibiotic resistance and/or virulence genes. Population genomic studies of infectious microbes around the world have revealed that global pathogens are often characterized by one/few successful clonal lineages, known as epidemic clones;[1–5] these epidemic clones can often be associated with specific HGT events, most commonly driven by conjugative plasmids carrying antibiotic resistance genes.[6–8] These conjugation events are thought to be particularly abundant in microbial communities[9] (e.g., soil, water, and gut microbiomes), which can harbor thousands of plasmids.[10] Indeed, although some phylogenetic barriers to conjugation exist, microbiomes provide both commensal microbes and opportunistic

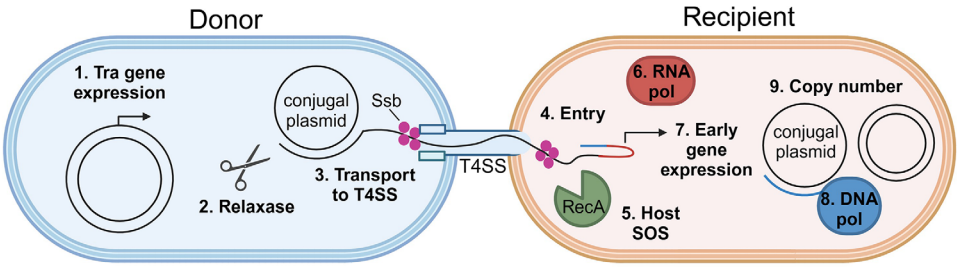

FIGURE 1 Conjugative F-like plasmid lifecycle. For conjugation to begin, first the TrA genes are expressed from the conjugative plasmid (step 1). Following expression, plasmid-encoded relaxase nicks the single strand plasmid at the origin of transfer (oriT) (step 2). The oriT is bound by the relaxase proteins and the complex is transported to the type IV secretion system and through the pilus into the recipient cell (step 3 and 4). Upon entry into the recipient cell, host proteins respond to the single-stranded DNA and RecA proteins remove the single-strand binding protein (Ssb) (step 5), removal of which facilitates the RNA polymerase primer generation (step 6) and early gene expression can begin right away (step 7). Complementary strand synthesis occurs shortly thereafter in the recipient cell (step 8). (We note complementary strand synthesis also occurs in the donor cell concurrently). Lastly, the conjugative plasmid copy number is reached (step 9). Figure created with BioRender.com.

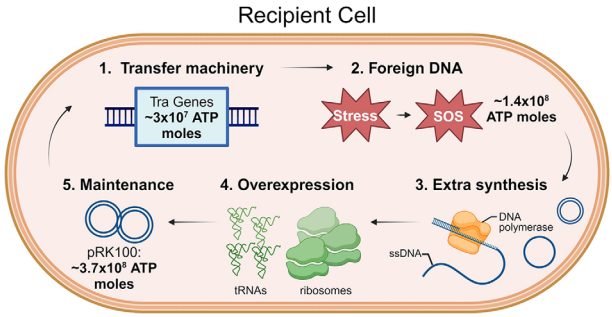

FIGURE 2 The role of metabolism in the F-like conjugative plasmid lifecycle. Metabolic impact begins with the expression of the transfer machinery, which is estimated to cost approximately 3×10^7 ATP moles from the total E. coli cell's ATP reservoir (step 1). The introduction of foreign DNA can induce the SOS response, thereby requiring approximately 1.42×10^8 ATP moles (step 2). The additional DNA synthesis required represents an additional metabolic burden during conjugative plasmid transfer (step 3). The overexpression of plasmid-required genes is a direct demand on the cells' tRNAs and ribosomes (step 4). The average maintenance cost of the conjugative plasmid pRK100 accounts for about 3.7×10^8 ATP moles (step 5). All ATP estimates are based on our in-house calculations using our data combined with the work of Lynch and Marinov, 2015[11]. Figure created with BioRender.com.

pathogens immediate access to a diverse gene pool. Therefore, understanding the molecular factors driving plasmid dynamics in situ is critical for accurately predicting the emergence of new pathogens and developing novel strategies to control its occurrence (Figures 1–3).

As with all biological phenomena, conjugation's ecological and evolutionary consequences are dependent on many underlying cellular processes and interactions. Chief among them is cellular metabolism: plasmid conjugation depends on, and therefore significantly impacts, energy availability, allocation, and usage, which will be thoroughly dissected throughout the following sections; here, we refer to "metabolism" generally as the collective set of all cellular pathways and processes that generate energy for biomass and non-biomass functions.[11] Briefly, conjugation occurs between a donor cell carrying a plasmid, and a recipient cell that receives the plasmid, via direct cell-cell contact. On the donor side, significant amounts of ATP and other macromolecular resources are needed to initiate, coordinate, and physically mobilize the transfer of conjugative plasmids. On the recipient side, new DNA introduces an immediate metabolic disturbance that results in a cascade of stress-related and adaptive responses whose mitigation likewise requires a major energetic investment. Moreover, maintaining a recently accepted plasmid requires adapting to a new intracellular homeostasis, which involves reallocating intracellular resources, likely for optimal growth. Altogether, metabolism is crucial to every step of conjugation.

Given conjugation's impact on metabolism, it is unsurprising that bacteria carrying conjugative plasmids often grow at a lower rate than their plasmid-free counterparts.[12–16] This growth reduction, traditionally referred to as the "fitness cost" of carrying the plasmid, is generally attributed to the additional demand driven by protein expression of plasmid-encoded genes.[12,14,17,18] That a plasmid imposes a fitness cost makes intuitive sense; it is not difficult to find examples of naturally occurring costly plasmids, in both environmental and clinical contexts.[17,19] These costs can readily explain many natural phenomena, ranging from plasmid strain specificity due to coevolution[20] to the prevalence of low-cost plasmids.[21] Yet, despite their intuitive relevance, plasmid fitness costs are not nearly as simple as they initially appear: not all plasmids are costly, and in many cases, the lack of cost cannot be readily explained.[16,22,23] Moreover, even costly plasmids are not always outcompeted in nature and can persist in environments without any obvious selection benefits.[24] Finally, plasmid cost is often context-dependent; host strain,[9,25–27] plasmid type,[28] and environmental conditions[29,30] can all modulate fitness costs. Thus, as it stands, fitness costs alone are insufficient to thoroughly explain plasmid persistence.[16]

Clearly, the underlying assumption that plasmids are inherently burdensome is not universally applicable. Instead, studies are beginning to reveal that plasmids may confer underappreciated yet important benefits to the cell, even under conditions where accompanying growth defects are observed. A more holistic, systems-level perspective is essential to fully understand the metabolic implications of plasmids on

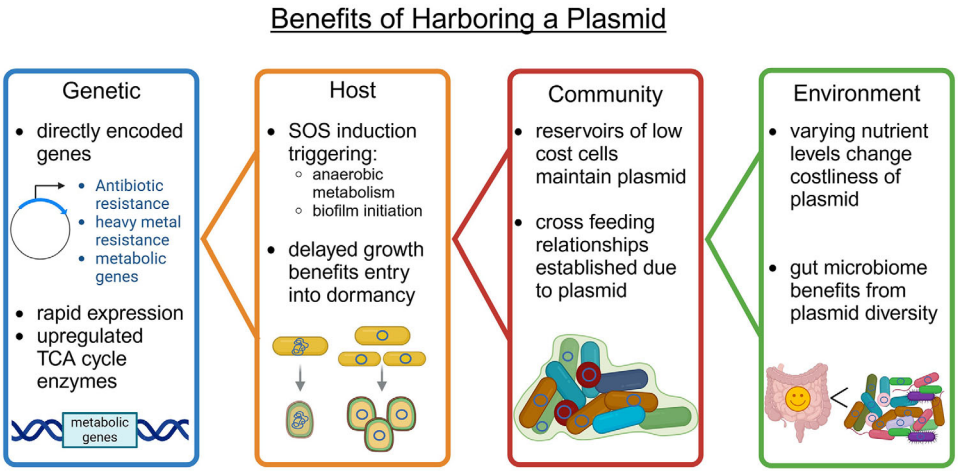

FIGURE 3 The benefits provided to host cells that harbor a plasmid are depicted above in the four categories discussed in this review. On the genetic level (left), a plasmid provides new genes that can enable changes in metabolic profiles despite being initially burdensome. Next, at the host level (middle, left) acquiring a plasmid can induce the SOS response, biofilm formation, or even delayed dormancy which can overall be beneficial for cell survival. At the community level (middle, right), plasmids being carried by a few cells ultimately contribute to the community's success. Lastly, at the environmental level (right), plasmid benefits may outweigh the costs under different environmental conditions such as nutrient levels. Created with BioRender.com.

downstream ecological effects. Indeed, "metabolism" involves highly diverse, interdependent, and often redundant pathways and processes that are often not directly related to a single phenotype. Unraveling the multifaceted ways that bacterial metabolism and plasmid presence impact and interact with one another is key to understanding fitness' overall role in plasmid dynamics.

Here we highlight the current understanding of metabolism's role in the plasmid "lifecycle," specifically focusing on the transfer, acquisition, and maintenance of conjugative plasmids. We first give a brief overview of the general steps of plasmid transfer. Then, we describe the currently known metabolic demands imposed by plasmid conjugation. Finally, we critically examine how burdens may also provide benefits to the cell. Understanding these tradeoffs is a key aspect to explaining, and ultimately predicting, plasmid dynamics in natural communities, which has great relevance to fields as diverse as biomedical therapeutics and agricultural health.

A BRIEF OVERVIEW OF THE BIOCHEMICAL STEPS INVOLVED IN PLASMID TRANSFER

A plasmid is considered conjugative (i.e., self-transmissible) if it encodes both a **mobility (MOB)** module, which consists of an origin of transfer (oriT), a relaxase complex, and a type IV coupling protein (T4CP), along with a **mating pair formation (MPF)** module, which consists of the type IV secretion system (T4SS). These components are often modular, giving rise to mobile plasmids that do not themselves encode all MOB and MPF components but can be mobilized by conjugation machinery in trans[24] (i.e., housed on a separate plasmid). Current estimates suggest that approximately 50% of all plasmids are transferrable (mobile or conjugative), with half of these being fully self-transmissible.[31–33] This distribution likely varies across different contexts (e.g., environment, species); for example, a recent paper that examined ~2000 plasmids from *Escherichia coli* actually found that ~75% of them were transferrable.[34]

Plasmid transfer can only occur once both the MPF and MOB proteins are synthesized in the donor. In some cases, transfer genes are constitutively expressed, such as in the well-studied F plasmid.[35] In other cases, transfer machinery is inducible via external or internal stimuli, including quorum-sensing hormones,[36] environmentally-responsive cAMP levels,[37] and specific antibiotics.[38–42] Once expressed, transfer is initiated when the relaxase protein nicks a plasmid's oriT sequence (see Figure 1, step 2). Then, with the help of coupling proteins (reviewed in Llosa[43]), linearized single-strand plasmid DNA is transported to the T4SS, which extends from the inner membrane to the extracellular environment[44] and probes for a suitable recipient (see Figure 1, step 3).The resulting mating pair formation has long been thought to enable stability for highly efficient DNA transfer,[45] although the precise mechanisms involved remain an open area of study.[45,46] Indeed, only recently, microscopy experiments confirmed that single-stranded DNA can be transported through F plasmid pili even when donor and recipient remain physically distant.[47] Interestingly, the mating pair formation is not necessarily critical in all cases, and instead, the conjugative pilus alone may be sufficient to support plasmid transfer, based on recent cryoelectron microscopy images.[48]

Upon entry into the recipient cell (see Figure 1, step 4), the single-stranded DNA is both immediately recircularized via plasmid-bound recognition proteins (e.g., TraI in F),[49] and coated with host single-strand binding protein (Ssb) (see Figure 1, step 5).[49,50] While Ssb is a known trigger of the SOS-induced protein RecA,[51] recent work showed that additional host proteins, specifically the UvrD helicase, acts to remove RecA from the single-strand;[52] removal of RecA likely facilitates continued replication (e.g., allowing DNA polymerase to initiate synthesis of the complementary strand[49] and/or RNA polymerase

to initiate expression from promoters on the single-strand[50]) (see Figure 1, steps 6 and 8). Additionally, early gene expression (see Figure 1, step 7) from the incoming single-strand includes plasmid-derived Ssb,[49,50,53,54] which can also aid in dampening the host SOS response.[55]

Once all genes on the plasmid are expressed, any encoded regulatory networks begin to stabilize, including those controlling plasmid replication, maintenance, and subsequent conjugation. At this point, and only a few minutes since conjugation began, the recipient has become a transconjugant and can act as a plasmid donor once more.

METABOLIC DEMANDS OF DNA ACQUISITION

Clearly, conjugation is a complex, multi-step process that requires the coordinated expression and regulation of many proteins (see Figure 1). To achieve this exquisite level of cell-wide coordination, DNA synthesis, and physical transfer, both donor and recipient cells must make significant energy investments at the expense of other processes. These metabolic demands imposed on recipient cells are described below and illustrated in Figure 2.

When the cost begins: Plasmid transfer

Conjugation machinery expression involves coordinating, on average, a ~40-gene network accounting for roughly > ~1150 kDa of protein mass (for F-like plasmids). When fully induced, conjugation-related proteins account for a significant portion of a typical bacteria's proteome, equivalent to ~1% in *E. coli*, which represents a massive metabolic undertaking.[11] The T4SS alone consists of ~12–30 proteins, making up a multi-mega-Dalton assembly embedded into the cell's membrane, is powered by three ATPases (VirB4, VirB11, and VirD4[48]). The expression of this complex machinery can account for ~1%–5% of the cell's energy budget.[56,57] Moreover, these conjugation-powering ATPases have been identified at several points in the cell membrane, highlighting that more than one conjugation apparatus can be present during a mating event.[58]

Despite the clear energetic investment of expressing the conjugation machinery, determining the donor's overall cost of undergoing conjugation is difficult, as the process is quite rapid and isolating actively conjugating cells remains a technical challenge. However, precise estimates of plasmid transfer rates may provide some insights into biophysical limits. For example, conjugation kinetics strongly depend on available intracellular energy pools[24,59] largely due to *tra* expression requirements, which vary depending on the physiological growth stage, ATP production rates,[34,37] and nutrient availability;[37] specific mechanisms underlying these relationships remain to be determined. Thus, there is a clear energetic dependence for undergoing conjugation; quantifying the resulting donor "conjugation cost" would be particularly valuable for determining the theoretical limits of conjugation.

Adjusting to a new cost: Plasmid acquisition

Separate from the donor, physically accepting new DNA also imposes a unique energetic demand on the recipient cell through various metabolic pathways.[60,61] Disruption to recipient cell homeostasis may occur directly, due to the plasmid's presence, and/or through secondary and/or transient expression effects. As an example of the former case, the discrepancy of codon usage compatibility (plasmids tend to be AT-biased, while chromosomal genes tend to be GC-biased) is known to influence successful plasmid transfer frequencies.[12,62,63] Differences in codon usage lead to costly effects, including low translation efficiency and ribosome sequestration, resulting in increasing mRNA degradation, protein mistranslation, and misfolding.[64–67] In the latter indirect case, dysregulated and/or induced gene networks can impose a transient metabolic burden. For example, immediately following the transfer of RP4, R6Kdrd, and R388 conjugative plasmids, newly formed transconjugants exhibit transient expression of the SOS genes *sfiA* and *recN* in *E. coli* cells.[24,60] Notably, the SOS response accounts for a considerable proportion of the recipient's pool of metabolic resources.[55] Consistent with this, conjugative plasmids often encode one or more anti-SOS factors (e.g., the *psiB* gene of R64*drd* and R100-1,[60,68,69] and the *ssb*[70] and *ardA*[61] genes of ColIb-P9), located close to the oriT, which are expressed early in the transfer process and serve to minimize the recipient's stress response. Similarly, overexpression of plasmid-encoded genes occurs in recently generated transconjugants,[71–73] adding additional protein burden. This unnecessary protein cost is ultimately alleviated once the plasmid achieves its steady-state regulatory control.

Measuring the metabolic burden of this initial recipient-to-transconjugant adaptation period is challenging due to its short duration, the diversity of underlying cellular processes, and the fact that newly formed transconjugant cells are typically present at relatively low frequencies in a population. However, we recently showed that the costs of acquiring a new plasmid can be measured by quantifying the growth defects exhibited by newly formed transconjugants compared to their adapted counterparts.[68,74] Interestingly, single-colony resolution revealed that acquisition costs manifest in prolonged lag times, rather than reduced growth rates, consistent with the metabolic delay that occurs during cellular adaptation to a new environment.[74] Moreover, various plasmids were shown to impose a range of acquisition costs across various host strains and environmental conditions. A more detailed, mechanistic understanding of the molecular factors and processes that give rise to this delay will undoubtedly provide better insights into potential cellular advantages, mitigations, and downstream engineering applications.

When the cost remains: Plasmid maintenance

Self-transmissible plasmids are typically large (> 60 kb), multi-copy (~2–15), and encode a multitude of genes (including many directly involved in metabolic processes) that can introduce long-term growth

effects on their hosts.[14,75,76] Once established, steady-state expression of plasmid-encoded genes and overall plasmid maintenance requires additional metabolic adaptation. To get a sense of the magnitude of this protein burden relative to the rest of the coding material in the cell, we used in-house transcriptomics data and recent calculations on the number of ATP molecules required to synthesize proteins[11] to estimate the steady-state maintenance cost of the conjugative F-like plasmid pRK100 (110 kb) carried by *E. coli* BW25113. We found this plasmid accounts for up to ~5% of the coding material in an *E. coli* cell and consumes > ~2% of the total ATP budget, an overall staggering amount.

As described above, the effects of this increased plasmid burden are typically quantified as a fitness cost, which describes the reproductive advantage of plasmid-free cells to plasmid-carrying ones. Protein production contributes a major portion of this cost[14] – a single plasmid-expressed copy of a penicillin hydrolase can sequester enough ATP to impact growth.[77–79] These effects are exacerbated with increasing copy number, which is inversely correlated with growth rate;[80] the slope of this relationship depends on the nutrient type and availability, highlighting the distinct role of metabolism in cost phenotypes.

Despite its clear importance, protein production alone cannot fully explain the breadth of fitness costs observed. Indeed, studies have illustrated that plasmids can impose a wide range of fitness costs, often without clear relationships to plasmid characteristics (i.e., size, identity, proteome size, etc.). For example, contrary to intuition, increased genetic material does not necessarily result in higher fitness costs; when the conjugative *Pseudomonas* syringae megaplasmid (~976 Kbp[81]) pMPPla107 is transferred into a naïve host, it imparts a fitness cost equivalent to, or even less than, that of much smaller plasmids.[14,82] Similarly, some plasmids have no detectable fitness cost at all, despite inevitable increases in protein synthesis.[19] Moreover, fitness costs are highly context-dependent, and can change over time[20] and across strains.[30,83]

Many cell- and population-level mechanisms exist to counteract metabolic burdens, including compensatory mutations that ameliorate fitness costs, community reservoirs that retain costly plasmids within the population, and sufficiently high transfer rates that disseminate plasmids faster than they can be outcompeted. Indeed, ameliorating mutations that reduce the metabolic burden of plasmid-bearing cells (and thus fitness cost) have been widely documented[16,23,84] in clinically[19,26] and environmentally[85] relevant scenarios. However, the reasons for the persistence of un-evolved costly plasmids remain unclear; intuitively, we expect some ecological advantage to outweigh further evolution to lower costs, but further elucidation requires precise quantification of cost magnitudes, underlying genetic drivers, and perturbations.

BENEFITS OF A PLASMID-INDUCED METABOLIC BURDEN

Clearly, acquiring and maintaining a conjugative plasmid is often metabolically taxing. In many cases, these costs are worthwhile, as plasmids provide obvious benefits (e.g., access to diverse genes that augment functionality and/or accelerate evolution). Moreover, recent work is highlighting that in many cases, metabolic burdens are context-specific and can even confer highly nuanced secondary benefits. Such benefits likely play a critical role in supporting the continued persistence of conjugative plasmids. The benefits discussed below are summarized in Figure 3.

Directly beneficial plasmid-encoded genes

Undoubtedly, plasmids encode genes that directly help their host survive in a given environment. This accessory content varies greatly, ranging from nutrient catabolism to virulence factors to xenobiotic resistance. Some of these genes provide clear survival benefits in lethal environments, for example antibiotic resistance genes that protect cells under selective pressure. Similarly, many plasmids resident in soil microbiomes exposed to heavy metal[86] and herbicide[87] stressors have been found to harbor corresponding resistance markers. In these cases, so long as the corresponding selection agent is present, plasmid-bearing cells have a clear advantage over non-plasmid-bearing kin, even if the plasmid itself is costly to maintain.

Costly plasmids can persist in environments even without obvious selection,[88,89] and several recent works have shown the diversity of plasmid content extends far beyond protection against environmental stress. For example, we recently found that plasmids more often carry a prevalence of genes implicated in general cell metabolism, such as nucleotide or lipid biosynthesis, rather than specific functional advantages, for example, antibiotic resistance.[34] Interestingly, beyond their primary function, several of these metabolic genes also protected against carbenicillin treatment, highlighting the challenges inherent in connecting plasmid genetics to environment-specific benefits. Similarly, a recent study of plasmid content during human microbiome development also found high levels of plasmid-borne metabolic genes.[90] In this case, diverse metabolic capabilities were thought to contribute to bacterial survival during rapidly changing environments. Consistent with this, a separate group isolated a multi-drug resistant conjugative IncFII plasmid that conferred a growth advantage over plasmid-free cells in both rich and minimal media.[29] Further analysis revealed genes related to iron transport were responsible for this growth phenotype, highlighting nutrient catabolism as the plasmid-derived benefit.

Non-specific plasmid benefits

Separate from, but complementary to, the direct role of plasmid-encoded gene function on host fitness, plasmids themselves may indirectly potentiate the activity of genes they carry. Plasmid-encoded genes can be expressed at higher rates and levels than chromosomally encoded counterparts due to high plasmid copy numbers.[91] For example, some bacterial genera, including *Aureimonas* and *Oecophyllibacter*, house additional copies of ribosomal RNA (rRNA)

operon on a small high-copy number plasmid;[92] this supplementation resulted in a quicker response when adapting to a change in environmental conditions, for example, shifting from nutrient-starved to nutrient-rich conditions, that require increased ribosome synthesis.[93]

Independent of its genetic content, a plasmid's presence alone has been shown to rewire intracellular metabolic processes, which can inadvertently provide conditional benefits.[94] For example, intermediate copy number plasmids can alter the relative levels of common metabolic proteins, such as tricarboxylic acid (TCA) cycle enzymes,[95] which may be beneficial for cells under conditions that impose translational demand. Likewise, some plasmids, for example, the conjugative incF plasmid pLL35 in *E. coli*,[96] have been shown to induce metabolic changes that promote anaerobic metabolism[97] useful in environments such as the human gut and waste-water treatment plants.

These indirect plasmid benefits likely depend on their hosts, and even other plasmids, in non-intuitive ways. For example, a recent study found that cells carrying one plasmid did not incur additional fitness costs upon acquiring a second, even when the second plasmid was independently costly; remarkably, cells carrying both plasmids exhibited consistent expression levels and copy numbers across all plasmid-encoded genes compared to their single-plasmid counterparts.[98] That plasmid burden is not necessarily additive highlights that overall fitness costs likely mask underlying benefits that are difficult to identify from a limited context. For example, acquiring one large plasmid can increase the probability of acquiring a second one,[25,99] yielding more potentially beneficial genes for the host. Similarly, in competition assays, harboring multiple plasmids can increase cell adaptability, thereby prolonging host persistence in fluctuating environments.[98]

While the indirect benefits of plasmids are clear in some cases, other plasmids' roles remain a mystery, despite their natural abundance. For example, the cryptic non-conjugative plasmid pBI143, encoding only a single mobilization and replication gene, was found to be prevalent in the gut of ~90% of tested individuals.[22] Although some variants of pBI143 encoding genes related to host metabolism (e.g., galacturonosidase, pentapeptide transferase, phosphatase, and histidine kinase) were observed, these were relatively sparse, and could not account for the plasmid's overall prevalence. Interestingly, the authors found an association between pBI143 copy number and the incidence of irritable bowel syndrome (IBS), though a mechanistic explanation for this remains to be confirmed.

Host-specific metabolic benefits for plasmid-bearing cells

As discussed above, the acquisition of DNA during conjugation induces multiple defense mechanisms in the recipient. In some cases, such as the bacterial SOS system,[60] these responses are generic to any foreign DNA. In other cases, host immunity is sequence-specific, for example, CRISPR-CAS and restriction-modification systems[100] that only function if the corresponding target DNA is present. In all scenarios, these systems require energetic investment by the recipient cell; as we detail below, these investments often pay off.

The SOS response can activate other systems in the cell that, in turn, facilitate survival.[101] In the context of conjugation, SOS activation can directly regulate processes encoded on the newly acquired plasmid itself. For example, LexA, a key regulator of the SOS response, was recently shown to regulate the expression of multiple colicin-resistance genes[102] commonly found on IncFIII conjugative plasmids.[103] Moreover, many examples of SOS-mediated benefits have been found in non-plasmid-specific contexts as well but may ultimately facilitate plasmid persistence indirectly. For example, under antibiotic treatment, the bacterial SOS system can induce biofilm formation and persister states.[104] Both these phenotypes are beneficial to host cell survival[105,106] against stressors such as antibiotic treatment, mammalian host immune response, and nutrient limitation.[104,107] These results suggest that plasmid-mediated SOS induction may indirectly facilitate survival under antibiotic exposure, though this intuition has not been directly validated.

There are many examples, as in the case of the SOS response, where the presence of a mobile element unexpectedly leads to evolutionary benefits. For example, cells harboring the integrative and costly conjugative element ICEBs1 delayed their transition into dormancy,[108] allowing them to reach a higher proportion of the population.[108] Similarly, the compensatory response to plasmid burden can actually lead to elevated fitness (i.e., growth rate) compared to the burden-free comparator. As an example, one group observed that the fitness cost of a plasmid carrying the NDM variant of beta-lactamase (bla_{NDM}) was ameliorated by mutations in host genes related to oxidative stress, nucleotide and short-chain fatty acid metabolism, and cell membranes.[20] This amelioration not only increased cell growth rate, thus favoring transconjugants, but also occurred with and without antibiotic selection, indicating an ultimately successful relationship between plasmid and host.

Community benefit is greater than the individual burden

Community dynamics are an integral part of bacterial survival and success, and their role in plasmid persistence and abundance is no exception. Intuitively, the diversity of species and strains in bacterial communities' results in plasmid-carrying sub-populations with varying fitness costs. This diversity is essential for sustaining plasmids, since they may stably persist in a low-cost host while still transferring amongst more costly members.[30,109] Moreover, costly plasmids have been shown to promote community success. Indeed, recent characterization of the gut plasmidome revealed the beneficial role of conjugative plasmids in microbiome community dynamics. For example, a recent study found the highest abundance and diversity of plasmids in the infant gut, with 67% of the 328 plasmid replicon groups represented; this distribution then decreased and shifted over time to resemble the mother's plasmidome diversity by 12 months of age. The

authors speculated that high initial plasmid diversity was instrumental in expanding the gene repertoire of gut bacteria,[90] allowing the community to adapt faster to the rapid environmental shifts occurring during infant gut development.[90]

Clearly, costly plasmids can ultimately be advantageous at the ecological level when present in more complex population structures.[21,23,30,84,86,109,110] For example, recent work showed that *L. reuteri*, which produces the plasmid-encoded toxin reuterin, and *E. faecalis*, which carries plasmid-encoded reuterin resistance, each exhibited increased fitness when they shared metabolites.[111] Thus, by harboring each plasmid individually, a stable interaction between these two species that was overall beneficial for each was established. Interestingly, these plasmid-mediated ecological benefits have been demonstrated even when the plasmid is directly costly. For example, plasmid acquisition-mediated growth delays can likely aid the successful establishment of new plasmid/strain pairs. While these acquisition costs correlate with lag times, suggesting a clear metabolic adaptation period, Ahmad et al recently showed that for two plasmids that both provide a selection advantage compete against one another in mixed populations, that with an intermediate acquisition cost could outcompete those with a higher or lower cost.[74] This intriguing finding suggests that an intermediate cost may be evolutionarily optimal. Thus, having some degree of plasmid cost may allow for more complex and/or longer-term community stability to emerge.

Environmental influence on metabolic plasmid benefits

The local environment fundamentally alters bacterial physiology, which can impact key features of a population undergoing HGT, including growth rate, conjugation dynamics, and expression of specific metabolic genes and pathways.[112–114] In the lab, these environmental factors include easily manipulated variables such as temperature, nutrient composition, and the presence/abundance of other bacteria. In situ, these factors are not nearly as controllable, often changing simultaneously and in unpredictable ways, and are compounded by additional complexities such as fluctuating signals, spatial segregation, and ecological disruptions (e.g., bottlenecking). Considering that environmental perturbations are being actively investigated as interventional strategies to control plasmid/host outcomes,[114,115] understanding *how* metabolism-driven plasmid advantages are environment-dependent could directly translate into strategies that allow for plasmid control, which has both human health and environmental applications.

The burdens associated with maintaining (fitness cost) and acquiring (acquisition cost) plasmids are relative, each utilizing a point of comparison (e.g., a strain without a plasmid, or one that has recently acquired the plasmid, respectively), to quantify their magnitudes. Unsurprisingly, then, certain environments alleviate or exacerbate each type.[68,84,116] For example, Basra et al., observed a weakly correlated tradeoff between antibiotic resistance and growth rate in clinical *E. coli* isolates; however, the strength of the correlation depended on the media used.[117,118] This trend likely emerged from the relative utilization of energy towards biomass production and other non-growth-related processes, and it highlights the importance of metabolic efficiency: that is, the amount of energy that is used per unit of biomass formed. Indeed, bacteria can become more metabolically efficient with sufficient adaptation time. For example, antibiotics were recently shown to select for enhanced metabolic efficiency to offset this tradeoff.[119] Interestingly, plasmid acquisition costs were also shown to depend on metabolic efficiency, with higher costs being observed under less efficient environments.[68] Whether this dynamic exists in in situ populations remains to be seen.

PERSPECTIVES

Understanding and predicting the spread of plasmids is critical to both further fundamental biological understanding and develop practical plasmid-dependent applications, whether therapeutic or surveillance-based. To that end, fitness costs have, in general, been the primary metric for characterizing a plasmid's impact on a given strain. This framing is intuitive, as both conjugation and subsequent plasmid maintenance are costly processes that rely on cellular energy. However, accumulating evidence now suggests that 'cost', as it is currently defined via fitness, is highly context-dependent, likely overly simplistic, and certainly incomplete. As such, predicting the full spectrum of a plasmid's metabolic dependencies and consequences across diverse potential host strains, community members, and environmental conditions is not currently possible.

Work over the past several years has made exciting progress towards more holistically defining and interpreting plasmid costs. The increased accessibility of higher throughput experiments and long-read sequencing capabilities have greatly accelerated both the breadth and depth of plasmid discovery and characterization, revealing multiple instances where costliness alone leads to unexpected outcomes For example, recent work highlighted that plasmid copy number segregation variability[120] can lead to phenotypic heterogeneity within a genetically homogeneous population; this variability enabled the population to withstand higher doses of antibiotics without incurring a detrimental cost in drug-free conditions, by selecting for sub-populations with higher plasmid copy numbers and therefore sufficient levels of resistance gene expression.

While inspecting plasmid sequences can shed some light onto such potential emergent benefits (e.g., positive selection), predicting genotype-phenotype relationships is often challenging; not only do complex phenotypes typically arise from multiple interrelated genetic interactions, but incorrect or unknown functional annotations often complicate an already complex picture.[121] Nonetheless, expanding current experimental work to include predicted metabolism-implemented genes is an important next step for characterizing potentially beneficial plasmid traits conferred to hosts.

As the field continues in this direction, we believe that more emphasis should be placed on characterizing both the growth *and* the metabolic properties of plasmid-carrying cells whenever possible.

Indeed, we anticipate that an increasing number of independent findings to yield a more holistic picture of the general principles underlying plasmid costs and their cell-, population-, and community-level implications. While deep profiling using 'omics profiles is likely overkill in most cases to separate growth and metabolic effects, even simple readouts such as GFP/luminescence dual reporters and/or individual metabolites would add an additional dimension to current practices.

The integration of molecular biology with ecology has been hugely beneficial for plasmid research, leading to a more complete elucidation of molecular mechanisms that lead to community dynamics and more common application of computational tools and techniques to study and predict dynamic systems. Nonetheless, we note that beyond classic kinetic or evolutionary approaches, whole genome network modeling is currently not widely utilized in this field yet could be particularly beneficial for understanding the metabolic implications of plasmids in a strain-specific manner. Such systems-level modeling, when constrained with dynamic population-level outcome information, will likely yield the best of all worlds, allowing us to combine multi-scale observations into elegant predictive frameworks.

Overall, the critical importance of metabolic processes in acquiring and maintaining plasmids is clear; while specific mechanisms are still being uncovered, progress to date has demonstrated tantalizing potential for future clinical, agricultural, and biosynthetic applications. Moreover, recent fundamental research is beginning to clearly highlight the positive effects of carrying a plasmid, ranging from direct plasmid-encoded genes for withstanding environmental stressors to metabolic mutations in the host cell that improve cell growth overall. Carrying plasmids should thus not necessarily be assumed to be inherently costly (i.e., detrimental); instead, the plasmid-host combination should be viewed as an independent state rather than one relative to a plasmid-free counterpart. By examining these plasmid-host pairs in this light, new mutually beneficial properties can be identified that begin to fully capture plasmid dynamics.

AUTHOR CONTRIBUTIONS

Heather D. Curtsinger, Sofía Martínez-Absalón, Yuchang Liu, and Allison J. Lopatkin all wrote and edited the manuscript. Heather D. Curtsinger and Allison J. Lopatkin made the figures. Sofía Martínez-Absalón and Allison J. Lopatkin calculated the ATP cost of plasmid pRK100.

ACKNOWLEDGMENTS

This work was supported by NIH NIGMS 1R35GM150871-01 (AJL).

CONFLICT OF INTEREST STATEMENT

The authors declare no conflicts of interest.

DATA AVAILABILITY STATEMENT

All data generated in this study are available upon request.

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How to cite this article: Curtsinger, H. D., Martínez-Absalón, S., Liu, Y., & Lopatkin, A. J. (2024). The metabolic burden associated with plasmid acquisition: An assessment of the unrecognized benefits to host cells. *BioEssays*, e202400164. https://doi.org/10.1002/bies.202400164