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Review

Impact of plasmid interactions with the chromosome and other plasmids on the spread of antibiotic resistance☆

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ABSTRACT

Naturally occurring plasmids have medical importance given that they frequently code for virulence or antibiotic resistance. In many cases, plasmids impose a fitness cost to their hosts, meaning that the growth rate of plasmid-bearing cells is lower than that of plasmid-free cells. However, this does not fit with the fact that plasmids are ubiquitous in nature nor that plasmids and their hosts adapt to each other very fast – as has been shown in laboratory evolutionary assays. Even when plasmids are costly, they seem to largely interact in such a way that the cost of two plasmids is lower than the cost of one of them alone. Moreover, it has been argued that transfer rates are too low to compensate for plasmid costs and segregation. Several mechanisms involving interactions between plasmids and other replicons could overcome this limitation, hence contributing to the maintenance of plasmids in bacterial populations. We examine the importance of these mechanisms from a clinical point of view, particularly the spread of antibiotic resistance genes.

1. Introduction: plasmid maintenance

Plasmids are accessory genetic elements and understanding their maintenance among bacterial populations is of utmost importance given that they are the major contributors to the spread of antibiotic resistance (Carattoli, 2013). In this review, we discuss the effect of plasmid interactions with the chromosome and with other plasmids on plasmid maintenance and its implications to antibiotic resistance dissemination using an evolutionary perspective.

Among plasmid-bearing bacterial cells, plasmid-free cells sometimes arise stochastically upon cell division. If plasmid-free cells are counter-selected (e.g. if antibiotics, against which the plasmid encodes resistance, exist in the environment), plasmids have their maintenance almost ensured. However, if there is no negative selection for plasmid-free cells or no positive selection for plasmid-borne genes, plasmids' maintenance in bacterial communities may be compromised. Indeed, it has been shown that, in an environment not selecting for plasmids, plasmid-free cells usually grow faster than plasmid-bearing cells (see discussion in the next sections and references therein).

To prevent the emergence of plasmid-free cells, plasmids also require correct distribution to daughter cells during cell division. Mechanisms for plasmid segregation are reviewed in (Ghosh et al., 2006). Plasmid copy number plays an important role in this process, for the higher the number of plasmid copies, the higher is the probability that both cells receive at least one plasmid copy. Thus, high copy number plasmids can just segregate randomly. However, to guarantee correct inheritance, low copy number plasmids require specific mechanisms, such as partition systems. These ensure that plasmid copies migrate to opposite cell poles prior to cell division. Nevertheless, partition systems also determine plasmid incompatibility (reviewed in (Novick, 1987)). Consequently, two plasmids that are recognized by the same system should not be maintained together.

High copy number plasmids, may form oligomers due to recombination of plasmid copies which complicates random segregation (Summers et al., 1993). In this oligomeric form, random segregation of copies becomes more difficult. Multimer resolution systems solve the problem through the recognition of *res* sequences by a resolvase enzyme that returns the oligomers to monomeric form, allowing their later random segregation (Austin et al., 1981; Sharpe et al., 1999; Summers and Sherratt, 1984; Tolmasky et al., 2000).

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Other mechanisms act after cell division to eliminate plasmid-free cells. As a whole, strategies for plasmid maintenance have been reviewed in (Bahl et al., 2009; Zielenkiewicz and Ceglowski, 2001). Post-segregational killing systems represent one of the strategies (reviewed in (Yamaguchi et al., 2011)). These systems consist on two products, a stable toxin and an unstable antitoxin. If the plasmid encodes one of these post-segregational killing systems and is lost in a cell (for example if one of the daughter cells does not receive a plasmid copy), there is no antitoxin synthesis. Given that the antitoxin is unstable, only the toxin remains in the cell, causing cell death. Thus, only cells that retain the plasmid can counter-act the toxin and survive.

Generally, conjugative plasmids can transfer to the newly arising plasmid-free cells. This is possible because conjugative plasmids encode all the machinery required for self-transfer. These plasmids can be maintained in bacterial populations if their transfer rate is high enough to compensate for plasmid loss and growth disadvantage. Using a mass action mathematical model of a chemostat, Stewart and Levin (1977) estimated cell densities and plasmid transfer rates required to compensate for plasmid cost and segregation (as well as for chemostat flow rate). Later, using a collection of bacteria (Ochman and Selander, 1984) isolated from man, domestic and zoo animals, Gordon (1992) found that conjugation efficiency between ten *Escherichia coli* strains (in all directions) was lower than the threshold value calculated by Stewart and Levin (1977). In other words, transfer rates were too low to compensate for costs and segregation (Gordon, 1992; Simonsen et al., 1990; Stewart and Levin, 1977).

A possible explanation for this conundrum is that conjugation efficiency is in fact very low in most bacterial strains, but in a few strains, it drastically improves. These outperforming donor strains could then easily transfer the plasmid to other strains. Dionisio et al. (2002) provided the first experimental evidence favouring this hypothesis (Dionisio et al., 2002). In a set of 13 strains belonging to different Enterobacteriaceae species (including several *E. coli* strains isolated from healthy humans), 11 strains exhibited low transfer rates, but one of the *E. coli* strains used in that study and an *Erwinia chrysanthemi* (now reassigned as *Dickeya dadantii*) strain exhibited exceptionally high transfer rates. If high-efficient donor cells are mixed with plasmid-free low-efficient donor cells, many of the low-efficient donor cells end up receiving a plasmid (Dionisio et al., 2002). Additional support for the "plasmid-amplification hypothesis" was recently provided in a study using *Pseudomonas fluorescens* and *Pseudomonas putida* and the plasmid pQBR57 that confers resistance to mercury. In sterile soil and in the absence of mercury, the frequency of cells harbouring plasmid pQBR57 decreased rapidly in populations of *P. putida* (went extinct in all replicates) but not of *P. fluorescens*. However, in cocultures of the two species, the plasmid was maintained among *P. putida* cells. A mathematical model suggested that these results can be explained by the high transfer rate among *P. fluorescens* cells, and from them to *P. putida*, thus behaving as plasmid-amplifiers (Hall et al., 2016). This amplification hypothesis could explain the ubiquity of plasmids among bacterial populations. However, there are other non-exclusive explanations, mostly involving interactions with the chromosome or other mobile genetic elements.

Only about 25% of the plasmids are conjugative: they are self-transmissible by conjugation because they encode all the genes required for this process. Another 25% are mobilizable because they only encode part of the conjugative machinery, consequently depending on other conjugative elements to be transmitted (Shintani et al., 2015; Smillie et al., 2010).

Interestingly, approximately 50% of all sequenced plasmids may be non-transmissible, meaning that they do not encode conjugation genes (Shintani et al., 2015; Smillie et al., 2010). Therefore, explaining their maintenance in bacterial populations is certainly challenging.

2. Plasmid-host interactions

2.1. Costly interactions and their proximate causes

The fitness effect of a plasmid is defined in this review as the growth rate of plasmid-bearing cells relative to plasmid-free cells. The effect may be positive (i.e., conferring fitness advantage) or negative (fitness cost). For example, fitness cost means that the duplication rate of plasmid-carrying cells is lower than that of plasmid-free cells. Most plasmids have been shown to be costly, but fitness effects may depend on the host strain (Humphrey et al., 2012). Indeed, effects may be different even in hosts differing only in a single nucleotide point mutation (discussed in section 2.4, below) (Silva et al., 2011).

Plasmid costs can have different origins: they may result from the replication of the extra DNA, as well as from the expression of plasmid genes that collectively disrupt the cellular fine tuning (Baltrus, 2013; San Millan and MacLean, 2017). Extra DNA molecules present in the bacterial cell may negatively impact cell's replication rate and therefore, the evolutionary success of plasmids present in the cell. This was shown by studying the effect of plasmid sizes (between 2.7 kb and 8.7 kb) on bacterial density at stationary phase: as plasmid size increased, maximum cell density decreased (Cheah et al., 1987). Moreover, with the largest plasmid (8.7 kb), cell death was accelerated after the stationary phase. This was mostly due to the amount of plasmid DNA and not to gene transcription or translation (Cheah et al., 1987). Nevertheless, the effect of plasmid size has been controversial (Vogwill and MacLean, 2015; Zund and Lebek, 1980). In fact, the total amount of plasmid DNA in the host cell is due to both plasmid size and the number of plasmid copies. For instance, it was shown that in yeast, each extra copy of a plasmid imposed an additional fitness cost of 0.17% (Harrison et al., 2012).

Expression of plasmid genes also has a negative impact on the cell's replication rate. For example, silencing antibiotic resistance genes of IncP-1 plasmid RP1 was beneficial for host fitness (Humphrey et al., 2012). Accordingly, there is a negative relationship between bacterial fitness and the number of plasmid-encoded antibiotic resistance determinants (Vogwill and MacLean, 2015). Additionally, plasmids also affect the expression of chromosomal genes including those involved in amino acid metabolism and energy production (Harr and Schlotterer, 2006; Lang and Johnson, 2015; Shintani et al., 2010; Buckner et al., 2018; San Millan et al., 2018). Therefore, the fitness cost associated with plasmid acquisition may result from the consumption of resources for plasmid gene expression but also from disruption of the fine-tuning of cellular pathways (reviewed in (Baltrus, 2013; San Millan and MacLean, 2017)). Interestingly, during co-evolution, a mutation in host regulatory systems seems to mitigate the fitness costs conferred by the plasmids. This was observed with chromosomal mutations in the *gacA* or *gacS* genes encoding a two-component regulatory system which alleviated the translational demand imposed in the presence of the plasmid (Harrison et al., 2015). Moreover, increased persistence of plasmid pBP136 in *Shewanella oneidensis* was also due to mutations in the gene encoding the global transcription regulator Fur (Stalder et al., 2017) and, increased persistence of plasmid pMS0506 in *Pseudomonas moraviensis* was due to mutations in the gene *cheY*, which encodes part of a two-component system (homolog of GacA) (Loftie-Eaton et al., 2016).

Despite the common trends explaining plasmid costs, it is important to stress that plasmid effects on fitness are variable and just one single mechanism may not be enough to explain why distinct plasmids impose fitness costs (Kottara et al., 2018; San Millan et al., 2018).

2.2. Which plasmids are costly to bacterial cells?

Artificial plasmids are expected to be costly to their hosts because the maintenance systems naturally associated with replicons used to construct vectors were overlooked during their construction (e.g. the

cer-site for multimer resolution of plasmid ColE1 was not included in the pUC series). Moreover, these plasmids never have had to evolve specific mechanisms to ensure their maintenance, i.e. to avoid being overtaken by plasmid-free segregants or other well-adapted cells. Molecular biologists usually help plasmid-bearing cells by using media selective for plasmid-encoded traits, e.g., antibiotic-resistance, to prevent the emergence of segregants. Researchers have measured the fitness effect of several artificial plasmids, e.g. pACYC184, pBR322, pBBR1-based vectors, or pSEVA vectors (Bouma and Lenski, 1988; Gruber et al., 2014; Mcdermott et al., 1993; Modi and Adams, 1991; Silva-Rocha et al., 2013). Not surprisingly, all these plasmids conferred a fitness cost to the host (Bouma and Lenski, 1988; Gruber et al., 2014; Mcdermott et al., 1993; Modi and Adams, 1991).

Nonetheless, many natural plasmids are costly while others are nearly neutral or even beneficial (see references below). Why are there such differences between plasmid effects? What do, for example, beneficial plasmids have in common? Many evolutionary biologists and researchers interested in the spread of antibiotic resistance have addressed these questions, but answers remain elusive. At first glimpse, we have found an interesting relation between the fitness effect of a plasmid and the time-span between plasmid isolation and the measurement of its effect on a host. If the time-span is low, the fitness effect is mostly neutral or positive. For longer time-spans, fitness effects are mostly negative (plasmids become costlier). We illustrate this relation by discussing a few studies.

The best approach to test this hypothesis is measuring the fitness effect of plasmids isolated at very different time points. To our knowledge, there is only one study like that: six plasmids isolated between 1990 and 2002, all of them from *Enterococcus faecium* and encoding vancomycin resistance (*vanA*) (Starikova et al., 2013; Werner et al., 2011). Then, in 2013, Starikova et al. (2013) measured the fitness effect of all these plasmids after transferring them, by conjugation, to plasmid-free otherwise isogenic *E. faecium* cells. One plasmid with a 23 years' time-span decreased host fitness by 25% (that is, fitness effect of −25%) (Starikova et al., 2013). Two plasmids with time-span of 17 years had a fitness effect of −14% and − 16% (Starikova et al., 2013). Finally, three plasmids with time-span of 11 years had a fitness effect of −27%, +0.1%, and + 10% (Starikova et al., 2013). Indeed, this study suggests that, as time-spans decrease (23, 17, and 11 years), plasmid costs decrease (on average, the fitness effects were: −25%, −15% and − 5.6%, respectively).

In two other studies, plasmids were isolated just five years before the measurement of the fitness effect (hence a time-span of five years). In one of the studies, conjugative plasmids that confer apramycin resistance were isolated from commensal *E. coli* of newborn calves that did not take apramycin nor related aminoglycosides for at least 20 months before sampling (Yates et al., 2004). Their fitness effects were − 0.02%, +4.78% and + 7.17%, a positive average of +3.47% (Yates et al., 2006). In the other study, plasmids pHNSHP17, pHNSHP23, pHNSHP24, pHNSHP45, and pHNSHP45–2 were collected from pigs in China and presented the following fitness effects: +2%, +8%, +11%, +16%, and − 15% (a positive average of +5.5%) (Wu et al., 2018).

Plasmids R1 and RP4 represent the opposite case, having been isolated a long time before measurement of fitness effects. These plasmids were isolated, respectively, in 1961 (Datta and Kontomichalou, 1965) and in 1969 (Ingram et al., 1973). Only in 2003 (hence a time-span of 42 and 34 years, respectively) did Dahlberg and Chao measure their fitness effects: −6% and − 21%, respectively (Dahlberg and Chao, 2003).

Finally, we refer to two more studies, now with intermediate time-spans. Plasmids pQBR55, pQBR57 (isolated from *P. putida*) and pQBR103 (from *P. fluorescens*), confer mercury resistance and were isolated between 1992 and 1994 (Lilley and Bailey, 1997; Lilley et al., 1996). The time-span between isolation and fitness effect measurement of these three plasmids varied between 19 and 21 years and their fitness effects were − 14%, −27% and − 46% (average = −29%) (Hall et al., 2015). In another study (Enne et al., 2001), with a time-span of about 13 years, the fitness effects of three plasmids encoding sulfonamide resistance were −4.7%, −9.0% and + 4.1%, an average of −3.2% (Enne et al., 2004).

What happens to plasmids while they are maintained in laboratories for several years? Apparently, plasmids are accumulating deleterious mutations. Every time a few cells are taken from freezers and isolated in a Petri dish, bacterial chromosomes and plasmids replicate and, possibly, mutate. When a colony of about 10^8 cells grows from a single cell, about $\text{Log}_2(10^8/1) \approx 26$ generations were completed. This is done periodically to renovate frozen cultures and similar procedures are made when cultures must be dispatched (Harrigan and McCance, 1966). Most likely, after several years there is an accumulation of deleterious mutations in plasmids (Andersson and Hughes, 1996; Funchain et al., 2000). A series of single-cell bottlenecks likely increases the genetic drift, which is not counteracted by natural selection. The accumulation of deleterious mutations is irreversible, a phenomenon denominated Muller's ratchet (Felsenstein, 1974). Hence cost-avoiding mechanisms genetically encoded in plasmids may suffer loss-of-function mutations.

2.3. Costly interactions – manipulating ultimate causes through evolution

Natural Selection is another important force of Evolutionary Biology and should be able to eliminate or compensate for plasmid cost. This was shown in several works where costly plasmids evolved in the laboratory for hundreds of generations (reviewed by (Carroll and Wong, 2018; Dionisio et al., 2012; Lenski, 1998). This was not done with successive single-cell bottlenecks. On the contrary: the evolutionary experiments discussed below (Bouma and Lenski, 1988) (Harrison et al., 2015) (Dahlberg and Chao, 2003; Dionisio et al., 2005)) were performed with populations composed by millions of cells. Each of these cells may gain a compensatory mutation; moreover, each cell contains plasmids, and each of these plasmids also can gain a beneficial mutation. The probability for the appearance of a beneficial mutation is low for each cell or for each plasmid, but it is much higher among millions of plasmid-bearing cells.

Very different plasmids were used in these evolutionary experiments: artificial, natural, non-conjugative, conjugative, different incompatibility groups, isolated long time ago or more recently, etc. With no exception, costs diminished – in most cases costs totally disappeared. Therefore, it is not surprising that the fitness effect of plasmids shortly after their isolation from nature (where they evolved for much more than a few hundred generations) is mostly neutral or positive.

All these studies where plasmids evolved are extremely relevant for the spread of antibiotic resistance. It is worth focusing on some of them. Bouma and Lenski (1988) evolved *E. coli* B harbouring the artificial plasmid pACYC184 (Chang and Cohen, 1978) for 500 generations. After this time, the cost disappeared but, strikingly, evolved cells cured of the plasmid (hence plasmid-free evolved cells) presented a lower growth rate than the complete dyads (evolved cells harbouring the evolved plasmid), as if evolved bacteria became addicted to the plasmid. An important compensatory mutation(s) arose in the chromosome given that there was also no cost if the ancestral plasmid replaced the evolved plasmid. It was later shown that the beneficial effect only appears if the genes responsible for tetracycline resistance are present in the plasmid – this is the case even with pBR322 which encodes the same tetracycline-resistance genes (Lenski et al., 1994). The physiological basis of this effect was not understood then (Lenski et al., 1994), but has been elucidated more recently (Hellweger, 2013). The TetA efflux pump that mediates tetracycline resistance also mediates potassium uptake and does it more efficiently than the endogenous system Trk. Thus, *trk* mutants solely depend on TetA for potassium uptake, which is less costly and therefore confers a fitness advantage.

Harrison et al. (2015) evolved plasmid pQBR103, which confers

mercury resistance. Plasmid-bearing clones evolved in different concentrations of mercury ($HgCl_2$), ranging from 0 μM (no mercury) to 40 μM. In the absence of mercury, the plasmid was costly but, after about 450 generations, the fitness of plasmid-bearing cells increased up to 120% (Harrison et al., 2015). Strikingly, in most replicates across several conditions, compensatory mutations appeared in the same genes, namely those encoding the two-component system *gacA/gacS*. Mutation of these genes downregulated the expression of ~17% of chromosomal and plasmid genes, thus corroborating that gene expression is an important factor for plasmids' fitness cost. Notably, in all treatments, including the one without mercury, there was a transposition of Tn*5042* to the bacterial chromosome accompanied by loss of the plasmid (Harrison et al., 2015). This was not a surprising observation: the cell retained the advantageous genes while getting rid of the plasmid backbone (Bergstrom et al., 2000). However, unexpectedly, these genotypes were mostly transient, as compensatory mutations spoke louder – i.e. apparently, compensatory mutations mitigated plasmid cost (Harrison et al., 2015) rather than the recruitment of putatively important genes followed by plasmid loss.

In two reports, plasmid R1 was used to study plasmid-host adaptation (Dahlberg and Chao, 2003; Dionisio et al., 2005). In both studies, plasmid fitness costs were ameliorated. However, the results of the two studies were very different concerning the fitness effect of the evolved R1 in the ancestral *E. coli*. In one study, evolved plasmids either had no effect or were costly when they found themselves in the ancestral *E. coli* (Dahlberg and Chao, 2003). In the other study, plasmid R1 evolved in *E. coli* in five replicates and, in two of them, evolved plasmids became advantageous to the ancestral *E. coli* cells (Dionisio et al., 2005). Moreover, one of these plasmids provided an advantage even to *Salmonella enterica* hosts (Dionisio et al., 2005). What are the reasons behind the outcomes of these two studies?

Experimental evolution was performed under different conditions in the two experiments. While Dahlberg and Chao (2003) evolved plasmid-bearing cells alone, Dionisio et al. (Dionisio et al., 2005) evolved the plasmid-bearing cells always in competition with a non-evolving strain – this means that, while one of the strains evolved for 420 generations, the other was not evolving because, every ten generations, its population was killed with two antibiotics (to which only the evolving population was resistant due to chromosomal mutations), and a supply of ancestral non-evolving cells (taken from freezer) were added once again to the evolving community. Therefore, only one of the populations was effectively allowed to evolve. The reasoning for this strategy was that, in a competitive environment, less costly plasmids would evolve (Dionisio et al., 2005). In this evolutionary experiment both strains contained plasmid R1 – hence surface exclusion (common to most conjugative plasmids where a resident plasmid blocks the entry of a similar plasmid (Garcillan-Barcia and de la Cruz, 2008)) prevented plasmid transfer between the evolving and the ancestral strains (Achtman et al., 1977; Dionisio et al., 2005).

There was a second difference between the two studies. Dahlberg and Chao (2003) evolved plasmid-bearing cells in an unstructured (liquid) environment, while Dionisio et al. (2005) evolved plasmid-bearing cells partly in a structured habitat (half of the time, during competition with the non-evolving population; the other half was in liquid during selection for the evolving strain). Unfortunately, it is still unknown which factor (the competitiveness or the structure of the environment) was the one that made plasmid R1 evolve with such different outcomes. However, given that *E. coli* cells commonly live in competitive environments and structured habitats (e.g. the mammalian gut), the ability to increase the fitness of other cells (observed with plasmid R1 (Dionisio et al., 2005)) may be occurring with many other plasmids in nature.

Note that the imposition of a competitive environment *per se* may have a different result if competition occurs between plasmids and not between cells. The reason for a different outcome is that, when competition occurs between plasmids within cells, the success of a plasmid may be due to its competitive ability relative to the other plasmids present in the same cell and not by conferring an advantage to the host. Indeed, this rivalry between plasmids may even decrease host fitness (Dionisio and Gordo, 2006; Frank, 1994; Frank, 1996). In this context, it is interesting to analyze the results of another experiment, this time involving a derivative of plasmid R1 – plasmid R1drd19 (Smith, 2011).

R1drd19 is a natural derivative of plasmid R1 that has a 1000-fold higher conjugation rate than R1 (Dionisio et al., 2002; Levin et al., 1979). R1drd19 is more efficient at conjugating because its transfer genes are constitutively expressed (Koraimann et al., 1991). Smith performed evolution experiments of R1drd19-carrying *E. coli* cells for 400 generations under serial transfer regimes (Smith, 2011). This was done under two different conditions: allowing or not allowing plasmid transfer towards plasmid-free cells (but this difference is not relevant for this discussion). In both treatments, the plasmid evolved to become costlier (Smith, 2011), which, according to the author, is consistent with within-host plasmid competition (Smith, 2011).

In principle, two different evolved R1drd19 plasmids should not meet in the same cell due to entry and surface exclusion. For example, it has been shown the transfer rate of plasmid F towards F-bearing cells is about 100–300 fold less frequent than towards a plasmid-free cell, due to entry and surface exclusion (Garcillan-Barcia and de la Cruz, 2008).

Let's assume that plasmids R1, R1drd19, and F have similar values of surface and entry exclusion (because they do not differ in the genes responsible for exclusion). Because the transfer rate of R1drd19 is 1000-fold higher than that of R1, the transfer-rate between two R1drd19-bearing cells is at least 1000/300 = 3.3 to 1000/100 = 10-fold higher than between an R1-bearing plasmid and a plasmid-free cell. Therefore, there are plenty of opportunities for two different R1drd19 mutant plasmid copies (evolved in the same bacterial population but in different cells) to meet in the same cell and, eventually, compete.

In a situation where most or all cells harbour a plasmid, those that are better at transferring plasmids to plasmid-bearing cells (probably displacing the resident plasmids) increase in frequency even if they impose a strong cost on the host (Frank, 1996; Haft et al., 2009). Therefore, in this case, there is selection for plasmids that are competitive but also costly to hosts. As such, evolved plasmids fell victim to the so-called tragedy of the commons (Dionisio and Gordo, 2006; Dionisio and Gordo, 2007; Hardin, 1968; Rankin et al., 2007): competitive plasmids are better at competing with other plasmids within hosts, but, when cells harbouring these plasmids became common, there is a steep reduction (up to 100-fold) of the density of bacterial cells (Smith, 2012).

In conclusion, plasmid-host adaptation is always observed when millions of cells compete for resources. Plasmid fitness costs diminish mostly through compensatory mutations and not by recruiting useful genes followed by discarding the rest of the plasmid. However, plasmid competition within cells may promote their success inside cells, even if that increases plasmid cost, hence diminishing the fitness of plasmid-bearing cells.

2.4. Costly interactions and evolutionary constraints

Unfortunately, more than one drug-resistance determinant can be found in a single bacterial cell. For example, two chromosomal mutations, each one conferring resistance to a different antibiotic, or the presence of one of these mutations and a drug-resistance plasmid, or even two or more plasmids. To understand how interactions between resistance mutations and plasmids impact *E. coli*'s growth, Silva et al. (2011) studied 50 combinations of 15 resistance-determinants (ten different chromosomal mutations and five natural isolated conjugative plasmids) (Silva et al., 2011). All 15 determinants (mutations or plasmids) were costly to the wild-type *E. coli*, but some combinations of a mutation and a plasmid presented striking results.

Let us now point out several noteworthy cases of interactions in this study (Silva et al., 2011). The mutation *rpoB* R529H confers resistance

to rifampicin but decreases the fitness of the cell to 73.8% of the wild-type cell. Now consider plasmid R124, which has opposite effects on *rpoB* R529H mutant cells and wild-type cells: R124 decreases the fitness of the wild-type cells from 100% to 96.1% but increases the fitness of *rpoB* R529H mutant cells from 73.8% to 92.3%. Note that these fitness values imply that this strain (with both resistant determinants) has little chances of reverting to the original wild-type sensitive state (Carneiro and Hartl, 2010; Weinreich et al., 2006). For that to happen, two changes must occur: (i) revert the resistance mutation in the *rpoB* gene and (ii) lose the plasmid. If the cell reverts this *rpoB* R529H mutation into the wild-type allele, its fitness is expected to increase from 92.3% to 96.1% (i.e., 100% - 3.9%), which is a low increase. Moreover, a reversion of a mutation is a very unlikely event: a cell is expected to have just about 0.003 mutations per genome per replication and the *E. coli* genome has 4.6×10^6 base-pairs (Drake, 1991). Plasmid loss by segregation is also not an option because the fitness of the clone would strongly decrease to 73.8%. So, we are stuck with a cell carrying both the mutation and the plasmid with a fitness as high as 93.3% of that of the wild-type.

This interaction between the *rpoB* R529H mutation and plasmid R124 is just an example. In fact, such interactions (where the plasmid increases the growth rate of the mutant) were observed in 16 combinations out of the 50 combinations (32%) studied (Silva et al., 2011). In all 16 cases, cells with both resistance determinants were constrained in their pathways to go back to full susceptibility. This was a frightening observation.

This study also gave more worrying news: in five cases (10%), a mutation increased the fitness of plasmid-bearing cells (Silva et al., 2011). In other words, in these five cases, resistance mutations behaved as compensatory mutations (Andersson and Levin, 1999); however, unlike usual compensatory mutations, these ones confer further resistance to antibiotics. Finally, three of the 50 strains exhibited fitness not significantly different from that of the fully susceptible wild-type strain (two of these strains harboured plasmid R16 and the other strain harboured R831). Unfortunately, and given the ease with which plasmids and bacterial cells adapt to each other, compensatory mutations may soon appear in all the other cases.

Alternatively, drug-resistance genes may be harboured in distinct plasmids, rather than on the chromosome. Bacterial cells, including pathogens, often carry various plasmids (Casjens et al., 2000; San Millan et al., 2009). For example, in a survey of 223 natural bacterial isolates belonging to seven Enterobactereaceae species (Sherley et al., 2003), it was shown that over 60% of them harboured at least one plasmid and that a single strain could simultaneously contain up to seven plasmids. More recently the number of plasmids present in 1866 bacterial genomes was analyzed and revealed an under-representation of strains carrying a single plasmid and an over-representation of strains harbouring simultaneously multiple plasmids (San Millan et al., 2014). Such a trend was observed across different phyla and independently of the antibiotic pressure acting on pathogenic strains.

San Millan et al. (2014) studied the interactions between co-infecting 'large' (30 kb to 90 kb) natural isolated plasmids and a 'small' (5 kb) artificial plasmid. Although not necessarily isolated from Pseudomonadaceae, all six plasmids are able to replicate in *Pseudomonas aeruginosa*. All these plasmids confer resistance to antibiotics and some of them to mercury. The small plasmid imposed a cost of 13.2%, but the cost was not higher than that when co-inhabiting with four of the five big plasmids, even if three of these big plasmids also imposed a strong cost by their own. After a long-term serial culture experiment without antibiotics, neither the level of antibiotic resistance nor plasmid copy-number changed. However, the stability of the small plasmid was significantly higher when in presence of either of the four big plasmids (San Millan et al., 2014). The icing on the cake of these results was a demonstration, through bioinformatic analysis of 1866 bacterial genomes at GenBank, that the frequency of small plasmids in the strains carrying both large and small plasmids was higher than expected by chance for both Enterobacteriaceae and Bacillaceae (as well as for Staphylococcaceae, though not statistically significant).

Multiple (unrelated) costly conjugative plasmids in a single cell are expected to be costlier than each plasmid alone. In fact, it was observed that strains carrying two conjugative plasmids (eight out of nine combinations) had lower fitness than when they carried only individual plasmids (Silva et al., 2011). This could be due to the expression of the conjugative machinery which is presumed to decrease host fitness – experiments of plasmid evolution revealed fitness increases associated with decreased efficiency of conjugation (Dahlberg and Chao, 2003; Turner, 2004; Turner et al., 1998).

Plasmids may have a way to decrease fitness costs associated with conjugation when other conjugative plasmids occupy the same host – by interfering with each other's ability to transfer (Chao et al., 2000). The reason is that the host wastes fewer resources when plasmids decrease transfer rates. Plasmid interactions that decrease the conjugation efficiency of co-resident plasmids have been observed in several works (Olsen and Shipley, 1975; Sagai et al., 1977; Stanisich, 1974; Gama et al., 2017); reviewed in (Getino and de la Cruz, 2018). Indeed, such negative interactions were observed predominantly when plasmids inhabited the same cell (Gama et al., 2017; Sagai et al., 1977). A study with conjugative plasmids of *Pseudomonas aeruginosa* showed inhibition in 11 of 19 combinations of two conjugative plasmids (Sagai et al., 1977), and more recently, the same trend was observed in *E. coli* with inhibition occurring in 20 out of 40 combinations (Gama et al., 2017).

3. Conclusion

We have seen in the introduction that, in a chemostat, the rate of plasmid transfer must be above a certain threshold value to compensate for fitness-cost, segregation rate and the flow of the chemostat (Stewart and Levin, 1977). Taking into consideration common values of segregation rate and plasmid fitness cost, it was later shown that the growth rate of conjugative plasmids was lower than the necessary threshold value (Gordon, 1992). Accordingly, it was suggested that plasmids cannot be exclusively maintained as parasites – somehow, they must confer an advantage to the host (Gordon, 1992). However, some plasmids seem to be devoid of any useful gene for the host (reviewed by (Dionisio et al., 2012)).

In this review we have shown that, after all, most natural isolated plasmids are probably not costly if their fitness effects are measured within a few years of plasmid isolation from nature. Even if, for some reason, a plasmid is costly to its host, a few hundreds of generations seem to be enough for compensatory mutations to appear in the plasmid, in the chromosome, or in both. While compensatory mutations do not arise, antagonistic interactions between plasmid and chromosomal mutations are pervasive, again allowing the maintenance of costly drug determinants among bacterial populations. Moreover, a few bacterial strains are particularly good plasmid donors, being able to transfer plasmids more efficiently to other strains or species. We have also seen that interactions with other plasmids may strongly affect plasmid transfer and fitness costs.

Most of these phenomena point to worrying messages concerning public health. Yet, knowledge of specific genetic interactions between plasmids, should not be neglected and could be used for our own purposes, for instance by performing in vivo competitions with highly competitive plasmids, not coding for antibiotic resistance, increasing their negative effects on bacterial cells. Or, using a different approach, by targeting cells expressing sex pili, for example using male-specific phages, viruses that infect bacterial cells through sex pilus (Anderson, 1968; Dionisio, 2005; Ojala et al., 2013).

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