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Review

Interactions between plasmids and other mobile genetic elements affect their transmission and persistence☆

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ABSTRACT

Plasmids are genetic elements that play a role in bacterial evolution by providing new genes that promote adaptation to diverse conditions. Plasmids are also known to reduce bacterial competitiveness in the absence of selection for plasmid-encoded traits. It is easier to understand plasmid persistence when considering the evidence that plasmid maintenance can improve during co-evolution with the bacterial host, i.e. the chromosome. However, bacteria isolated from nature often harbor diverse mobile elements: phages, transposons, genomic islands and even other plasmids. Recent interest has emerged on the role such elements play on the persistence and evolution of plasmids. Here, we mainly review interactions between different plasmids, but also discuss their interactions with other genetic elements. We focus on interactions that impact fundamental plasmid traits, such as the fitness effect imposed on their hosts and the transfer efficiency into new host cells. We illustrate these phenomena with examples concerning clinically relevant organisms and the spread of plasmids carrying antibiotic resistance genes and virulence factors.

1. Introduction

Plasmids are autonomously replicating DNA molecules, commonly found in bacteria, that are best-known because they often carry genes encoding antibiotic resistance and thus increase the spread of resistance to antibiotics (Carattoli, 2013). These genetic elements are able to replicate autonomously inside their host cells, and some of them can often disseminate between cells through conjugative transfer (reviewed in (Shintani et al., 2015; Smillie et al., 2010)). However, our knowledge concerning the acquisition rates of resistance in clinical settings, including plasmid transfer, is limited (Levin et al., 2014).

In nature, bacteria can harbor multiple plasmids simultaneously. *Borrelia burgdorferi* is a striking example of multiple plasmid carriage since strains of this species typically harbor a mix of circular and linear plasmids that can exceed a total of 20 different extrachromosomal elements per host (Casjens et al., 2000; Chaconas and Kobryn, 2010). That is an extreme case, but other bacteria have been shown to harbor multiple plasmids. For instance, among a collection of 27 Extended Spectrum β-Lactamase (ESBL)-producing *Escherichia coli* strains, all were shown to carry more than one type of plasmid (Garcia et al., 2007). Another survey characterizing > 200 isolates belonging to seven Enterobacterales species, showed that about 40% of plasmid-carrying isolates carried multiple plasmids (Sherley et al., 2003). Beyond this, several cases of pathogenic bacteria harboring multiple plasmids have been reported (Cameranesi et al., 2018; Rodrigues et al., 2016; Wiesner et al., 2013). In fact, carriage of multiple plasmids seems to be a common phenomenon because in silico analysis of bacterial genomes revealed that bacteria harboring multiple plasmids were more represented than expected (San Millan et al., 2014). This tendency was observed for several phyla whether they concerned clinically relevant species or not and is thus independent of the antibiotic pressure imposed on pathogens (San Millan et al., 2014).

With the notable exception of plasmids encoding similar regulatory mechanisms of replication (therefore being incompatible (Novick, 1987)) and conjugation (thus being able to prevent other plasmids to transfer into the same host cell (Garcillan-Barcia and de la Cruz, 2008)), knowledge concerning plasmids is mostly based on studies involving bacteria carrying a single plasmid type. A limited number of studies have been focused on different plasmids to understand how they interact. Such works date from a few decades ago, but the interest in this topic has been renewed in recent years.

There are two predictable ways for a strain to harbor multiple

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plasmids: i) through sequential plasmid acquisition (e.g. a strain already containing a single plasmid can receive another one through conjugation) or ii) by simultaneous transmission of several plasmids (e.g. a donor strain can transfer more than one plasmid). Stable maintenance of several plasmids depends not only on how they affect each other's transfer but also on how they interact with each other intracellularly. In this mini-review we discuss the interactions between different plasmids that impact their dissemination and persistence in bacterial communities and illustrate how they may affect clinically relevant organisms, especially emphasizing their role on the spread of antibiotic resistance genes.

2. Sequential plasmid acquisition

Conjugation is a mechanism of horizontal gene transfer that requires contact between donor and recipient cells (the mating pair) and leads to the formation of transconjugants – recipient cells that acquired the transferred DNA. According to this process plasmids can be categorized as conjugative, mobilizable or non-mobilizable (Smillie et al., 2010). Conjugative plasmids encode all the machinery required for conjugation: a sequence coding for the origin of transfer, *oriT*; a DNA processing machinery which includes the relaxase that nicks and binds to the *oriT*; a type IV secretion system responsible for the mating pair apparatus and DNA transfer; and a coupling protein that provides the connection of the intracellular machinery with the secretion system. Mobilizable plasmids only encode a fraction of this machinery, and require other conjugative elements to provide the remaining components, to be successfully transferred. Non-mobilizable plasmids are not able to transfer by conjugation.

If a strain carrying a single plasmid acquires other plasmids through conjugation, it becomes a multiple plasmid-carrying strain. The dynamics of such transfer is particularly interesting if the plasmid present in the recipient strain is also conjugative, a phenomenon termed facilitation (Gama et al., 2017b; Sagai et al., 1977).

Facilitation is the process by which the rate of transfer of a conjugative plasmid increases when a second conjugative plasmid is present in the same bacterial strain or in another strain of the same community (Sagai et al., 1977). Such transfer increment was initially witnessed when two conjugative plasmids resided in the same cell (Sagai et al., 1977), being observed for approximately 30% of the plasmid combinations (about 10% if we exclude the combinations comprising a plasmid that was later shown to be mobilizable and not conjugative (Haines et al., 2005)). More recently, facilitation was observed in 27,5% of combinations concerning two conjugative plasmids present in the same donor cell but shown to be more frequent (almost 50% of the combinations) when the plasmids were present in different cells (Gama et al., 2017b). Efficiency of plasmid conjugative transfer seems to be higher if both donor and recipient cells carry plasmids encoding different conjugative machineries, as the donor strain of one of the plasmids simultaneously acts as recipient for the other plasmid, and vice-versa, plasmids can be transferred in both directions. Thus, facilitation favors the acquisition of additional plasmids by both strains. Although facilitation has been observed more frequently when plasmids inhabit different cells, and therefore have a stronger impact on the sequential acquisition of plasmids, the studies focusing on the mechanisms of facilitation (described next) were mainly carried with cells already harboring two plasmids.

Several lines of evidence elucidated the mechanism of facilitation. First, the higher transfer efficiency is not due to recombination of the two plasmids because facilitation is still observed when the donor was unable to promote homologous recombination (Gama et al., 2017b). Nonetheless, plasmid co-integration can be mediated by transposons in a RecA-independent mechanism (Clark and Warren, 1979; Kilbane and Malamy, 1980). This alternative mechanism was initially dismissed as plasmids isolated from transconjugants retained the original properties as individual molecules (Morzejko et al., 1990; Sagai et al., 1977).

Table 1
Conjugation rates during facilitation.

Facilitated plasmid[a]			Facilitating plasmid		Study
	Conjugation efficiency			Conjugation efficiency [b]	
	Single donors[b]	Double donors[c]			
Rlb679	−6,70	−2,00	RP4	−1,10	(Sagai et al., 1977)
		−1,52	Rms163	−1,70	
pEM44	−4,43	−3,96	pEM6	−1,92	(Morzejko et al., 1990)
pUO-StVR2	−7,50 to −7,21	−5,25 to −4,24	pStR12	−4,49 to −2,69	(Montero et al., 2013)
R16a	−3,49	−2,42	F	−0,77	(Gama et al., 2017b)
R57b	−5,11	−3,41			
R6K	−7,58	−3,81			
R16a	−3,49	−2,30	R124	−0,75	
R57b	−5,11	−3,41			
R6K	−7,58	−4,10			
RN3	−3,97	−2,10	R1drd19	−1,87	
R57b	−5,11	−4,16	R702	−4,18	
R57b	−5,11	−4,15	RP4	−3,52	

[a]Plasmids facilitated when another plasmid was also present in the donor strain.
[b/c]Log_{10} of conjugation rates, i.e. when the donor strain contains that plasmid alone (and the recipient strain does not harbor any plasmids) / contains two plasmids. Conjugation rates were calculated according to different formulas between (but not within) studies.

Furthermore, transconjugants transferred both plasmids at the same rate as the original donor strain in subsequent matings (Morzejko et al., 1990).

Second, a plasmid increased its conjugative efficiency if the other plasmid co-resided in the same cell, but there was no such increase when its co-resident plasmid was present in both donor and recipient cells. This showed that facilitation seemed to be dependent on the transfer system of the "facilitating" plasmid. More specifically, facilitation was abolished due to surface/entry exclusion (Morzejko et al., 1990; Sagai et al., 1977), which is a mechanism encoded by conjugative plasmids that decreases the probability of plasmids encoding identical conjugative systems to transfer into the same host cell (Garcillan-Barcia and de la Cruz, 2008). Thus, facilitation does not seem to rely on an intracellular diffusible factor but on a physical factor contributing to the mating pair formation (Morzejko et al., 1990; Sagai et al., 1977).

Third, facilitation was mainly observed when the co-resident plasmid exhibited a higher conjugation rate than the "facilitated" plasmid (Gama et al., 2017b; Sagai et al., 1977) (Table 1) and preferentially when the two plasmids resided in different cells (Gama et al., 2017b). This indicates that the stabilization of the mating pair may be a requirement as it was recently shown with the Δ*traG* derivative of plasmid F (Gama et al., 2017b). Contrary to the wild-type plasmid, this mutant does not promote the formation of the mating pair nor its subsequent stabilization (Firth and Skurray, 1992; Manning et al., 1981). Therefore, by comparing the facilitating effect of the Δ*traG* plasmid with that of the wild-type on the transfer rate of another plasmid, it is possible to understand whether promotion/stabilization of mating pair is required for the phenomenon of facilitation. Facilitation was observed when the wild-type plasmid was present either in the donor or in the recipient strain. By replacing it with the Δ*traG* derivative facilitation was abolished, thus corroborating that mating pair promotion/stabilization is essential for facilitation.

Altogether, conjugation is enhanced because the mating pair becomes more stable, as a result of additional cellular contacts mediated by sex pili expressed by other plasmids that reside in the same, or in different, cells. Thus, plasmid DNA is successfully transferred at higher frequencies because the cells do not separate mid-process.

The clinical importance of facilitation for the dissemination of

plasmids was demonstrated with a derivative of the virulence plasmid pSLT of *Salmonella enterica* serovar Typhimurium (Montero et al., 2013). A derivative plasmid, pUO-StVR2, has acquired antibiotic-resistance genes leading to the emergence of multidrug-resistant clones of *S. typhimurium* (Guerra et al., 2002; Herrero et al., 2008). Plasmid pUO-StVR2 exhibits a low transfer efficiency, which may be due to mutations in the *traG* gene reported for other pSLT-like plasmids (Garcia-Quintanilla and Casadesus, 2011). Interestingly, pUO-StVR2 often co-exists with other plasmids in *S. typhimurium* isolates (Herrero et al., 2009). For instance, in the presence of pStR12 the conjugative efficiency of pUO-StVR2 increases, without any evidence of plasmid recombination (Montero et al., 2013). Taken together, these results support the role of facilitation as a mechanism that promotes the spread of plasmids encoding virulence and antibiotic resistance genes among pathogenic isolates.

3. Plasmid co-transfer

3.1. Mobilization

Recipient cells may alternatively receive several plasmids simultaneously from a donor already containing multiple plasmids. Transfer of multiple plasmids may result from diverse mechanisms. Mobilization allows efficient transfer of mobilizable plasmids (Beijersbergen et al., 1992; Buchanan-Wollaston et al., 1987; Meyer et al., 1982; Rawlings and Woods, 1985). Since co-resident mobilizable and conjugative plasmids use the same conjugation machinery, it is likely to assume that they are transferred to the same recipient cell. This assumption is supported by revisiting the work of (Sagai et al., 1977) now keeping in mind that plasmid Rms149 is mobilizable (Haines et al., 2005). That work showed that transconjugant cells selected for the mobilizable plasmid Rms149 had also received the conjugative plasmid, thus showing that both plasmids were co-transferred. In addition, mobilizable plasmids tend to be isolated from hosts carrying simultaneously conjugative plasmids, thus suggesting once more their co-occurrence (Aoki and Takahashi, 1986; Bryan et al., 1972; L'Abee-Lund and Sorum, 2002; Smalla et al., 2000). Mobilizable plasmids have been previously estimated to represent 25% of all plasmids (Smillie et al., 2010). Recent results challenge this view showing that they may represent a much higher proportion since many plasmids thought to be non-transmissible possess *oriT* regions and/or replicative relaxases that allow their mobilization (O'Brien et al., 2015a; O'Brien et al., 2015b) (reviewed in (Ramsay and Firth, 2017)). Over 90% of *Staphylococcus aureus* plasmids therefore seem to be mobilizable instead of non-transmissible. Thus, it is easier to explain the persistence of many non-transmissible plasmids if they are mobilizable because mobilizable plasmids can co-occur and hitchhike the transfer system of a wide variety of conjugative plasmids.

Mobilizable plasmids containing only *oriT* (identical to that of the conjugative element), but no relaxase, have been shown to be mobilized but exhibiting a relatively low frequency of co-transfer with the conjugative element (Daccord et al., 2010; Lee et al., 2012; O'Brien et al., 2015a). However, it was recently reported that a small rolling circle plasmid containing two copies of *oriT*, closely related to the one encoded by the conjugative plasmid, exhibited a high efficiency (70%) of co-transfer (Moran and Hall, 2019). Many plasmids exhibiting such characteristics were in fact detected in silico indicating that a large family of related plasmids encoding two *oriT* seems to have spread globally (Moran and Hall, 2019).

3.2. Co-integration

Formation of co-integrates is also a mechanism that allows simultaneous transmission of plasmids and relies on recombination between co-resident plasmids which may require homologous sequences and the host enzyme RecA. This is also termed conduction and was initially observed in cells that simultaneously harbored a conjugative and a non-transferable plasmid (reviewed in (Clark and Warren, 1979)). Briefly, plasmids recombine in donor cells, and the co-integrate is transferred through conjugation to recipient cells, where subsequent resolution of the co-integrate produces separate plasmids again. This allows the recipient cell to simultaneously acquire both plasmids. However, co-integrates may have some selective advantage that may halt the emergence of resolved plasmids (Chou and Marx, 2012; Xiao et al., 1994).

Plasmid recombination may also be due to replicative transposition of transposable elements (Clark and Warren, 1979; He et al., 2015; Kilbane and Malamy, 1980). Co-integration mediated by transposable elements may be due to their presence in either the conjugative or in the non-transmissible plasmid (Crisona et al., 1980; Elhai et al., 1994; Goto et al., 1984; Kilbane and Malamy, 1980; Lambert et al., 1987). However, resolution of the later type of co-integration produces plasmids which will both then contain a copy of the transposable element (Lambert et al., 1987; Liu and Berg, 1990). In practical terms, transposons not only allow the conduction of non-transmissible plasmids that otherwise would not disseminate through conjugation but also, since they are known to encode antibiotic resistance, favor the spread of antibiotic resistance genes during transposition events. Indeed, several non-conjugative plasmids (determining antibiotic resistance) that co-integrate with conjugative plasmids due to transposable elements, have been described in several pathogens such as *Enterococcus faecalis*, *E. coli*, *Neisseria gonorrhoeae*, *S. enterica*, *S. aureus* and *Staphylococcus epidermidis* (Chavda et al., 2015; Di Sante et al., 2017; Leelaporn et al., 1996; Liu et al., 2013; Scharbaai-Vazquez et al., 2007; Wiesner et al., 2016; Wiesner et al., 2013).

Other mechanisms, besides homologous and transposable element-mediated recombination, have been uncovered as responsible for plasmid co-integration. Conjugative relaxases have been shown to be responsible for site-specific recombination between *oriT* copies of staphylococcal plasmids, leading to the formation of co-integrates of plasmids pE194 and pT181 (Gennaro et al., 1987). Plasmid pBMB0228 is also a co-integrate resulting from the activity of conjugative relaxases in *Bacillus thuringiensis*. Relaxases were also responsible for resolution of the co-integrate in transconjugants. Nonetheless, the plasmids could fuse back again in the tranconjugants in a frequency of 1:600 (Wang et al., 2013). Alternatively, in a strain of *Acinetobacter baumannii* responsible for the dissemination of carbapenem-resistance, plasmid co-integrates were formed and resolved by the XerCD system (Cameranesi et al., 2018). This system is responsible for resolving chromosome dimers formed during DNA replication and, additionally, of plasmid multimers (Castillo et al., 2017). However, the plasmids studied in that work, harbored a far greater number of sites recognized by the XerCD system which favored the formation of co-integrates (Cameranesi et al., 2018). Nonetheless, the same system was observed to be able to resolve plasmid co-integrates (Cameranesi et al., 2018).

Altogether, there seem to be many mechanisms that lead to plasmid co-integration. The clinical importance of co-integration is highlighted by the increased potential to disseminate genes conferring resistance against last resort antibiotics, such as carbapenems (Cameranesi et al., 2018; Chavda et al., 2015; Chen et al., 2014; Desmet et al., 2018; Rodrigues et al., 2016). In one report, 16 of 20 *Klebsiella pneumoniae* carbapenemase-producing isolates carried the gene bla_{KPC-3} in co-integrated plasmids (Rodrigues et al., 2016). In another study, 30% of 263 carbapenem-resistant *K. pneumoniae* isolates and 22 out of 52 non–*K. pneumoniae* carbapenemase-producing Enterobacterales isolates harbored the same gene in conjugative co-integrated plasmids (Chen et al., 2014). Such co-integrates emerged due to homologous recombination between regions of 4 kb exhibiting > 97% nucleotide similarity, while other bla_{KPC-3} encoding conjugative co-integrates have been originated by IS*26*-mediated recombination. Non-conjugative bla_{KPC-3} encoding plasmid pBK30661 commonly forms both types of these co-integrates, thus possibly enhancing its dissemination (Chavda et al., 2015).

Plasmids of the IncHI2 group encoding resistance genes against heavy metals and a wide range of antibiotics, including the production of ESBLs, have been observed to form co-integrates with plasmids of other incompatibility groups. For instance, among 25 multi-drug-resistance carrying IncHI2 plasmids isolated from *E. coli*, 16 were co-integrates with either IncFII or IncN plasmids (Fang et al., 2016). Plasmids of group IncHI2 transfer optimally at low temperatures but the fusion with IncF replicon leads to increased ability to disseminate by enhancing conjugative efficiency at 37 °C (Garcia et al., 2007). As another example, IncA/C plasmids present in *S. typhimurium* confer antibiotic resistance, which also includes the production of ESBL, but their ability to self-transfer has not been detected. These plasmids can, however, fuse with conjugative plasmids of groups IncX and IncF, becoming transmissible. Moreover, although such co-integration seems to occur at low frequency in donor cells, the co-integrate is not resolved in transconjugants and the respective fused structure is maintained after further rounds of conjugation (Wiesner et al., 2016; Wiesner et al., 2013). Interestingly, the transfer rate of co-integrates can be as high as that of the conjugative plasmid (Garcia et al., 2007; Taylor et al., 1981; Wiesner et al., 2013; Xiao et al., 1994).

Alarmingly, a co-integrate derived from the fusion of a virulence and an antibiotic-resistance plasmid (Dong et al., 2018; Fang et al., 2018; Mangat et al., 2017) have resulted in the generation of hypervirulent carbapenemase-resistant *K. pneumoniae* isolates (Dong et al., 2018) exemplifying the major clinical implication of co-integration.

3.3. Synchronized conjugation

Contrary to what happens with co-resident mobilizable and conjugative plasmids, co-resident unrelated conjugative plasmids do not rely on the same transfer machinery. For this reason, co-transfer of unrelated conjugative plasmids is not so easy to understand. Unlike mobilizable plasmids that express mobilization genes constitutively, conjugative plasmids usually repress the expression of conjugation genes. In general, to be able to transfer, conjugative plasmids must de-repress the expression of the genes responsible for conjugation and such de-repression has been shown to occur transiently. Consequently, only a small proportion of the plasmid-containing cells of a population are able to transfer the plasmid (reviewed in (Frost and Koraimann, 2010; Koraimann and Wagner, 2014)).

In 1969, Romero and Meynell proposed that each conjugative plasmid should de-repress its own genes independently of its co-resident plasmids and, consequently, simultaneous de-repression of both plasmids should be a rare event (Romero and Meynell, 1969). If these two probabilities were independent, the rate of co-transfer should be given by the product of the individual transfer rates of each plasmid. Their observations that in two out of three combinations of natural plasmids, the rate of co-transfer was identical to the product of the individual transfer rates of each plasmid seemed to support the hypothesis. They reached the same conclusion when studying combinations of two de-repressed plasmids or of a repressed and a de-repressed plasmid. However, another study of the same year, conducted with a strain harboring three plasmids, reached a different conclusion: the rate of co-transfer of plasmids was higher than the product on individual rates (Bouanchaud and Chabbert, 1969).

This conundrum was solved more recently (Gama et al., 2017a). With 40 combinations of donor cells carrying two plasmids, it was shown that plasmid co-transfer is limited by the plasmid exhibiting the lowest conjugation rate of the two. In other words, transconjugants selected for the least efficient plasmid had also received the co-resident plasmid. Other reports (Mattila et al., 2017; Montero et al., 2013; Morzejko et al., 1990) support this conclusion. Altogether, the two hypotheses seemed to be conflicting because they predict the same rate of co-transfer if at least one of the plasmids has high conjugative efficiency (Gama et al., 2017a) (Fig. 1).

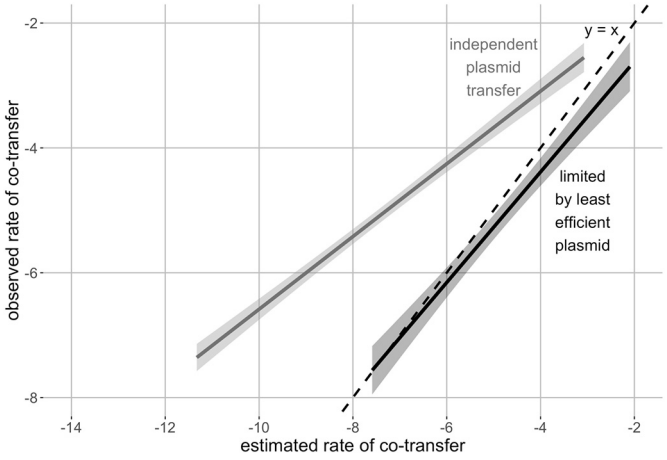

Fig. 1. Models of two-plasmid co-transfer rates. Dashed line represents the identity line y = x, i.e. where the observed rate of co-transfer is identical to the estimated one. The model of co-transfer being limited by the plasmid exhibiting the lowest conjugation rate is closer to y = x. However, the two different models converge (top right) to y = x when the conjugative efficiency of one of the two plasmids is high. Adapted from (Gama et al., 2017a).

The mechanism responsible for synchronized conjugation remains to be elucidated, but it is speculated that physiological and/or environmental signals may simultaneous de-repress co-resident plasmids (Gama et al., 2017a). It remains important to clearly distinguish the processes of facilitation and synchronized conjugation in cells containing multiple plasmids. Synchronized conjugation seems to act at the level of regulation by which co-resident plasmids de-repress conjugative transfer simultaneously, while facilitation is a process that enhances the conjugative efficiency of a single plasmid and is mechanistically due to the stabilization of the mating pair.

4. Co-inhabitation obstacles

4.1. Incompatibility

Although bacteria can acquire multiple plasmids, there is no guarantee that all co-resident plasmids will persist in the host. Some plasmid combinations cannot stably exist in the same host cell due to incompatibility. This happens when plasmids encode related mechanisms of replication or segregation, which interfere with the optimal ability of each plasmid to replicate or transmit to daughter cells upon cell division (reviewed in (Novick, 1987)). For example, a segregation mechanism shared by the two plasmids that acts in such a way that each plasmid is inherited by a different daughter cell would prevent their co-existence in the cell in the absence of selection for both of them (Novick, 1987). However, incompatible plasmids can become compatible (Sykora, 1992).

Recombination between plasmids of different incompatibility groups can lead to the production of co-integrates expressing both incompatibility features (Fang et al., 2016; Froehlich et al., 2005; Nugent and Hedges, 1979; Taylor et al., 1981). Such co-integrates can be stable and co-exist with individually resolved plasmids but can also persist by displacing incompatible plasmids due to the acquisition of additional replication mechanisms (Froehlich et al., 2005; Taylor et al., 1981; Villa et al., 2010). Toxin-Antitoxin (TA) systems provide another alternative to displace incompatible plasmids (Cooper and Heinemann, 2000; Cooper and Heinemann, 2005). These systems consist on a stable toxin and an unstable antitoxin. Host cells die if they lose the plasmid encoding such systems, because they require continuous production of the antitoxin to counteract the effect of the stable toxin. If we consider a cell that carries two incompatible plasmids, and that only one of the plasmids encodes a TA system, after segregation of these incompatible

plasmids, only cells inheriting the plasmid encoding TA systems survive and the cells carrying the other plasmid are eliminated from the population (Cooper and Heinemann, 2000; Cooper and Heinemann, 2005). Overall, co-integration can provide plasmids with additional replication or TA systems that allow them to displace incompatible plasmids.

By acquiring additional replication mechanisms plasmids can also expand their host range, thus being able to replicate in hosts where a parent plasmid would not (Cameranesi et al., 2018; Villa et al., 2010). Indeed, plasmids harboring multiple replicons may experience broader host ranges (reviewed in (del Solar et al., 1996; Jain and Srivastava, 2013)). Plasmids can then suffer mutations in one replicon without becoming inviable because they can rely on a secondary replicon. Such mutations in a replicon can lead to its divergence into a new incompatibility type that becomes compatible with plasmids belonging to the original incompatibility group (Sykora, 1992).

A few studies on the divergence of incompatibility group have focused on the evolution of mobilizable ColE-1-like plasmids, which play an important role in the spread of antibiotic resistance. Single nucleotide polymorphisms in the RNAs controlling plasmid replication can render different ColE-1-like plasmids compatible (Lacatena and Cesareni, 1981; Tomizawa et al., 1977), as shown in vitro (Camps, 2010) and more recently in natural plasmid isolates (Santos-Lopez et al., 2017a). In fact, it was observed that natural distinct ColE-1-like plasmids may stably co-exist. Interestingly, they no longer interact inside the cell and were shown to act independently maintaining their individual copy numbers (Santos-Lopez et al., 2017a; Santos-Lopez et al., 2017b). Maintenance of the different plasmids also led to increased levels of antibiotic resistance. Although such independence allows plasmid co-existence, in the absence of antibiotics cells harboring the two plasmids were not maintained in the population due to their lower growth rate (Santos-Lopez et al., 2017a). However, compensatory evolution leading to host adaptation to the first plasmid allowed the acquisition of further plasmids without slowing down the host's growth rate (Santos-Lopez et al., 2017b). This was possible because the two plasmids were related – thus the cost they imposed may have had the same origin, and therefore host adaptations mitigate the cost imposed by further related plasmids. In silico analysis has further shown that 45% of ColE-1-like plasmids co-exist with at least another plasmid of the same type in the same cell, which is more frequent than what would be expected by chance (Santos-Lopez et al., 2017b).

These examples provide evidence that plasmids may solve incompatibility and may be carried together in natural bacterial isolates.

4.2. Fitness costs

Carriage of plasmids generally creates a fitness cost on the hosts, that is, bacterial cells tend to replicate more slowly, which in turn decreases their competitive ability among bacterial communities. The reasons why plasmids are costly are diverse and have been reviewed elsewhere (Baltrus, 2013; Gama et al., 2018; San Millan and Maclean, 2017). Intuitively, if carriage of a single plasmid is costly, carriage of multiple plasmids should be even more so. Indeed, it was shown with *E. coli* that carriage of combinations of two conjugative plasmids was costlier than harboring each plasmid individually (Silva et al., 2011).

The interactions between a small plasmid (5 kb) and five larger antibiotic resistance plasmids (30 kb to 90 kb), either conjugative or mobilizable, were investigated in *Pseudomonas aeruginosa* (San Millan et al., 2014). The small plasmid imposed a fitness cost of about 13% on plasmid free cells, but this plasmid imposed smaller or undetected additional fitness costs on cells carrying each of the bigger plasmids (observed in four out of five pairs). Consequently, the small plasmid was shown to increase its stability in cells containing the other plasmids. Additionally, it was demonstrated with a bioinformatical approach that cells carrying simultaneously small and big plasmids are more frequent in various bacterial families than expected by chance (San Millan et al., 2014).

It has been proposed that fitness costs could be alleviated due to interference between co-resident conjugative plasmids (Chao et al., 2000). If co-resident conjugative plasmids decrease each other's conjugative efficiency they may prevent the host from wasting resources on their transfer. Interactions between conjugative plasmids that decrease conjugative transfer have been described in the past, but the interest on this subject has been reaffirmed in recent years (Gama et al., 2017b; Olsen and Shipley, 1975; Sagai et al., 1977; Stanisich, 1974). Interactions leading to the inhibition of conjugation of co-resident plasmids have been observed in *P. aeruginosa* (11 out of 19 plasmid pairs) and *E. coli* (20 out of 40 plasmid pairs) (Gama et al., 2017b; Sagai et al., 1977). In the first study (Sagai et al., 1977) all plasmids replicate in *P. aeruginosa* cells and, in the second study all plasmids replicate in *E. coli* cells (Gama et al., 2017b). However, in each case, plasmids might have not resided simultaneously in the same host for long evolutionary periods. We speculate that, if these studies employed a sample of plasmids that have occupied the same host simultaneously for long time periods, an even higher proportion of inhibitory interactions would be observed.

Indeed, interactions between conjugative plasmids leading to inhibition were more frequent than those leading to facilitation (Gama et al., 2017b; Gama et al., 2017c). Moreover, interactions between conjugative plasmids were shown to occur more frequently when the plasmids resided in the same cell than when they resided in distinct cells of the mating pair (Gama et al., 2017b). Interestingly, it was also shown that, in *E. coli*, about half of the pairs of conjugative plasmids tested (four out of nine) were less costly than expected (Silva et al., 2011). Altogether, these observations seem to agree with the hypothesis that interference between conjugative plasmids may reduce fitness costs by decreasing the efficiency of transfer.

The mechanisms responsible for such inhibition of conjugation have been recently reviewed, and in a recent work based on a bioinformatical analysis it was shown that only 129 out of 6878 sequenced plasmids (1.9%) encoded genes that inhibit the conjugative transfer of other plasmids (Getino et al., 2017). Several inhibition systems have been recognized, but only a few have been characterized. Thus, the low proportion of plasmids encoding them could result from the fact that only a few genes with this function are known (Getino and de da Cruz, 2018; Getino et al., 2017). As more genes will be found this percentage is expected to increase to values well above 1.9%.

The inhibitory systems characterized so far seem to comprise at least one of the following strategies: i) prevent the expression of the conjugative machinery, ii) degrade the plasmid's transfer-DNA, iii) block the transport of the plasmid between cells (reviewed in (Getino and de da Cruz, 2018)). A single inhibitory mechanism seems to be efficient against several unrelated plasmids (Getino et al., 2017; Maindola et al., 2014). However, different mechanisms may complement each other. This was found by analyzing the interactions between three conjugative plasmids where it was shown that two plasmids could lead to a stronger inhibitory effect on the other third plasmid (Gama et al., 2017c). Moreover, it was also reported that co-integrated plasmids encoding two different inhibitory systems (each inherited from each parent plasmid) exhibited stronger levels of inhibition (Myandina et al., 1993).

The evolutionary origin of these inhibitors is unknown. One possibility is that they may derive from other plasmid-encoded proteins that acquire additional functions. Indeed, polyvalent proteins – containing different domains performing different activities – are widespread in plasmids (Iyer et al., 2017). A few of the conjugation inhibitors have been shown to be able to have multiple functions in the cell. The PifC protein of plasmid F, for instance, is one of the proteins that inhibits conjugation of unrelated plasmids (Getino et al., 2017; Santini and Stanisich, 1998; Tanimoto and Iino, 1983). However, this protein also regulates the expression of genes involved in plasmid replication (Miller and Malamy, 1983; Miller and Malamy, 1984; Tanimoto and Iino, 1984), and inhibits phage infection (Blumberg et al., 1976; Morrison and Malamy, 1971; Rotman et al., 1983). As another example, the *kilA*

operon confers tellurite resistance (Fong and Stanisich, 1989; Goncharoff et al., 1991) and can be found in transposon Tn*521* (Grewal, 1990). Tellurite-resistance is associated with IncP-1 and IncH plasmids, and more recently an IncP-7 plasmid has been shown to encode the three *kla* genes of the *kilA* operon (Bradley, 1985; Taylor and Summers, 1979; Yano et al., 2007). Additionally, this operon encodes the ability of IncP-1 plasmid to inhibit the conjugative transfer of IncW plasmids (Fong and Stanisich, 1989; Goncharoff et al., 1991; Olsen and Shipley, 1975).

There is another explanation for the origin of these inhibitors exemplified with the *tir* gene. In IncF plasmid R100, this gene is responsible for the inhibition of IncP-1 plasmid RP4 (Tanimoto et al., 1985). However, this gene is also present in several IncL/M plasmids, adjacent to the operon encoding the conjugative machinery, and it was shown to inhibit their own horizontal transfer possibly acting as their own repressor (Mierzejewska et al., 2007; Poirel et al., 2012; Potron et al., 2014; Preston et al., 2014). Therefore, it is possible that plasmids such as R100, acquire the regulatory genes of unrelated plasmids and take advantage of them to inhibit their conjugative transfer. Similarly, some IncI plasmids encode the gene *finQ* responsible for the inhibition of IncF plasmids also adjacently to their conjugation genes (Takahashi et al., 2011). Further supporting this hypothesis, an IncN plasmid was shown to encode the conjugation regulatory system (FinOP) of IncF plasmids (Gasson and Willetts, 1975).

In conclusion, several hypotheses may explain the existence of inhibitory systems, although they need to be tested in the future.

5. Interactions with other mobile genetic elements

Besides interacting with their hosts and other plasmids, plasmids are also subject to interactions with other genetic elements present in the cell. For instance, plasmids have been shown to interact with phages in two different situations: i) to enhance their dissemination as plasmids being transmitted by transduction (for example (Ammann et al., 2008; Arnold et al., 1999; Mann and Slauch, 1997)) or ii) to inhibit phage infection and consequent host cell death (Banniste and Glover, 1968; Marrero et al., 1981; Pecota and Wood, 1996; Riede and Eschbach, 1986; Taylor and Grant, 1976; Taylor and Summers, 1979; Watanabe et al., 1966).

More recently, interactions between plasmids and mobilizable genomic islands have been reported. Salmonella genomic island 1 (SGI1) associated with multidrug-resistance is mobilizable by IncA/C plasmids (Douard et al., 2010; Doublet et al., 2005). SGI1 encodes distant homologs of the plasmid-encoded mating pore subunits which are responsible for efficient mobilization of the genomic island (Carraro et al., 2017). However, replacing the plasmid mating pore by that encoded by SGI1 results in decreased plasmid pVCR94 transfer to recipient cells (Carraro et al., 2017). Moreover, the genomic island and the conjugative plasmid pVCR94 (Carraro et al., 2014) or pRMH760 (Harmer et al., 2016) rarely co-transfer to the same recipient cell. Also, it seems that besides decreasing the conjugative efficiency of pRMH760, SGI1 and SGI2 also destabilize the plasmid. The plasmid was stable when alone in the cells, but when a genomic island was also present in the cells, the plasmid's frequency decreased to ≈50% after one growth cycle and to < 1% after five growth cycles (Harmer et al., 2016); the genomic islands were always stable. SGI2 also destabilizes IncA/C plasmid RA1 which is substantially different from pRMH760 (Harmer et al., 2016). The exact mechanism is unknown, nonetheless an SGI1 variant carrying a 292 bp deletion and two SNPs does not destabilize IncA/C plasmids, suggesting that this region is the one responsible for the destabilization effect (Harmer et al., 2016).

Non-conjugative plasmid pNUK73 imposes a fitness cost in *P. aeruginosa* PAO1 because expression of the plasmid replication protein leads to overexpression of chromosomal genes, such as those involved in SOS response and in transcription/translation pathways. This was due to the interaction with putative helicase and putative protein kinases, as mutations in the genes encoding these enzymes compensated the plasmid-imposed fitness cost and restored the expression of chromosomal genes. The mutated genes exhibited distinct GC content from the *P. aeruginosa* genome and were located in potentially mobile DNA regions (associated with prophages or near transposases), suggesting their recent acquisition through horizontal gene transfer. Moreover, these genes do not play a significant role in *P. aeruginosa* under the studied conditions. Therefore, it seems that the plasmid or the enzymes alone do not affect the host, but when in combination they interact, decreasing host fitness (San Millan et al., 2015). Similarly, evolution of the conjugative plasmid RP4 in *Pseudomonas* sp. *nov.* H2 selected for hosts carrying mutations in accessory helicases (probably acquired by horizontal gene transfer), which turned to exhibit a fitness advantage when carrying the plasmid rather than a cost. Unlike the case of *P. aeruginosa* PAO1 that evolved with plasmid pNUK73, *Pseudomonas* sp. *nov.* H2 with plasmid RP4 revealed general plasmid permissiveness as other plasmids (conjugative or mobilizable) became more stable in the evolved host than in its ancestor (Loftie-Eaton et al., 2017). Together, these two works show that recently acquired helicase genes interact negatively with several types of plasmids.

Overall, interactions with other mobile genetic elements other than plasmids seem also to impact the transmission and selection of plasmids.

6. Conclusion

As discussed recently in (Moran and Hall, 2019), it is generally overlooked that bacterial strains frequently harbor multiple plasmids, and understanding how they interact is of uttermost importance, especially for those relevant in the clinical context. Here, we provided an overview of possible interactions between plasmids, and mechanisms, that affect their transmission and persistence in bacterial communities.

Several mechanisms such as facilitation and co-integration seem to enable more efficient plasmid transfer between bacteria. Although co-integration can be catalyzed by host factors such as RecA and XerCD, it can also be mediated by mobile elements carried on plasmids. Indeed, transposable elements have a relevant role in plasmid evolution as they can, not only rearrange and provide further additional cargo genes (He et al., 2015; Norman et al., 2009; Rowe-Magnus and Mazel, 1999), but also promote rearrangements that alleviate the fitness cost plasmids impose on their hosts (Porse et al., 2016; Turner et al., 2014). Co-integration and mutations further contribute to plasmid stability by expanding host range and compatibility.

Although here we focus mainly on plasmid-plasmid interactions, it is important to keep in mind that plasmid interactions with the host cell and other genetic elements, such as phages that can disseminate plasmids by transduction, should not be disregarded. Indeed, we suggest that the key to understand plasmid ecology and evolution is to go beyond the study of plasmids as individual entities and analyze them in a more integrated view considering how interactions with other genetic elements shape plasmids' life-styles (see, e.g., (Dionisio, 2005)).

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