Plasmid 93 (2017) 6–16

Contents lists available at ScienceDirect

Plasmid

journal homepage: www.elsevier.com/locate/yplas

Conjugation efficiency depends on intra and intercellular interactions between distinct plasmids: Plasmids promote the immigration of other plasmids but repress co-colonizing plasmids

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ARTICLE INFO

Keywords:
Escherichia coli
Conjugative plasmids
Transfer rate
F
R1
R1drd19
R124
R6K
RP4
R702
R477-1
R57b
R16a
R388
RN3

ABSTRACT

Conjugative plasmids encode the genes responsible for the synthesis of conjugative pili and plasmid transfer. Expression of the conjugative machinery (including conjugative pili) may be costly to bacteria, not only due to the energetic/metabolic cost associated with their expression but also because they serve as receptors for certain viruses. Consequently, the presence of two plasmids in the same cell may be disadvantageous to each plasmid, because they may impose a higher fitness cost on the host. Therefore, plasmids may encode mechanisms to cope with co-resident plasmids. Moreover, it is possible that the transfer rate of a plasmid is affected by the presence of a distinct plasmid in the recipient cell. In this work, we measured transfer rates of twelve natural plasmids belonging to seven incompatibility groups in three situations, namely when: (i) donor cells contain a plasmid and recipient cells are plasmid-free; (ii) donor cells contain two unrelated plasmids and recipient cells are plasmid-free; and (iii) half of the cells contain a given plasmid and the other half contain another, unrelated, plasmid. In the third situation, recipient cells of a plasmid are the donor cells of the other plasmid. We show that there are more negative interactions (reduction of a plasmid's conjugative efficiency) between plasmids if they reside in the same cell than if they reside in different cells. However, if plasmids interacted intercellularly, the transfer rate of one of the plasmids was often higher (when the unrelated conjugative plasmid was present in the recipient cell) than if the recipient cell was plasmid-free – a positive effect. Experimental data retrieved from the study of mutant plasmids not expressing conjugative pili on the cell surface suggest that positive effects result from a higher efficiency of mating pair formation. Overall, our results suggest that negative interactions are significantly more frequent when plasmids occupy the same cell. Such interactions may determine how antibiotic resistance disseminates in bacterial populations.

1. Introduction

Conjugative plasmids, commonly present in bacterial cells (Sherley et al., 2003; Shintani et al., 2015), can be transferred to other cells by conjugation. This process often requires sex pili to establish a mating-pair by promoting cell aggregation (Cabezon et al., 2015). Conjugation usually imposes a fitness cost on plasmid hosts as it requires resources, alters cell physiology and determines viral susceptibility because sex pili can serve as receptors for viruses (Baltrus, 2013). These costs may be originated from the extra DNA present in the cell and its replication, as well as from the expression of plasmid genes (Cheah et al., 1987; Harrison et al., 2012; Humphrey et al., 2012; Turner et al., 1998; Vogwill and MacLean, 2015).

Bacterial cells can be colonized by multiple plasmids. To persist in the same cell, the different plasmids cannot belong to the same incompatible group, that is, they cannot encode identical mechanisms of replication or partition (reviewed in Novick, 1987). If that happens, they fail to regulate copy numbers and distribution to daughter cells, originating single-plasmid lines.

Carrying multiple conjugative plasmids may represent an unfortunate situation for bacterial cells for two main reasons. First, more plasmids in the same cell may increase the probability of attack by viruses that use conjugative pili as receptors. Second, when harbouring more than one plasmid, metabolic costs increase and cells replicate slower than when harbouring just a single plasmid (Morton et al., 2014; San Millan et al., 2014; Silva et al., 2011) (though there are exceptions

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http://dx.doi.org/10.1016/j.plasmid.2017.08.003
Received 30 April 2017; Received in revised form 17 August 2017; Accepted 18 August 2017
Available online 24 August 2017
0147-619X/

to this rule (San Millan et al., 2014)).

Plasmids seem to be adapted to deal with other plasmids already present inside recipient cells or transferable plasmids about to arrive into their bacterial host (Cooper et al., 2010; Haft et al., 2009; Dionisio, 2005; Dionisio et al., 2002). This work aims at understanding these adaptations.

Consider two conjugative plasmids, each one in a different cell and having a certain conjugation rate. If the two plasmids move into the same recipient cell, this cell becomes itself a donor of both plasmids. If no interaction occurs between plasmids, the transfer rate of each plasmid towards other cells would not change in the presence of the other plasmid. However, interactions may occur.

The transfer rate of a plasmid may increase in presence of rival plasmids. At least three mechanisms have been identified: conduction, donation (mobilization), and facilitation. Conduction is the process through which a non-mobilizable plasmid is horizontally transmitted by a conjugative or mobilizable plasmid, and requires the physical association of the two plasmids (Clark and Warren, 1979). Donation is the process whereby the cellular contact determined by a conjugative plasmid allows the horizontal transfer of a non-conjugative mobilizable plasmid (Clark and Warren, 1979). Mobilizable plasmids encode only a portion of the genes involved in the horizontal transfer of their DNA. Consequently, they require a conjugative plasmid in the same cell to provide the remaining conjugative machinery, which includes the synthesis of conjugative pili. Facilitation is a process by which the conjugation rate of a plasmid increases when another conjugative plasmid is present in the same host cell (Datta et al., 1971; Sagai et al., 1977).

Alternatively, several works have already described plasmids encoding mechanisms to decrease the conjugation rates of unrelated co-resident plasmids (Datta et al., 1971; Hochmannova et al., 1982; Olsen and Shipley, 1975; Pinney and Smith, 1974; Sagai et al., 1977; Tanimoto and Iino, 1983; Willetts and Skurray, 1980; Winans and Walker, 1985) (see also Chao et al., 2000). For example, at least five different mechanisms encoded by non-F-like plasmids inhibit the conjugation of F-like plasmids. These mechanisms can be rather diverse and some of them can inhibit the whole conjugative system of F-like plasmids, which includes preventing the expression of conjugative pili (Gasson and Willetts, 1975, 1977; Willetts and Skurray, 1980). Plasmid R6K, on the other hand, encodes a mechanism responsible for inhibiting the conjugative transfer of plasmids belonging to three different incompatibility groups: IncN, IncP-1 and IncW (Hochmannova et al., 1985; Hochmannova et al., 1982; Olsen and Shipley, 1975).

The mechanisms of plasmids pKM101 and F that inhibit the conjugation of plasmid RP4 stand among the best characterized. The fertility inhibition (FIN) proteins FipA of plasmid pKM101 and the PifC of plasmid F target the TraG coupling protein of RP4, which is involved in DNA transport (Santini and Stanisich, 1998). However, neither of these two inhibitory mechanisms affect pilus synthesis (Miller et al., 1985; Tanimoto and Iino, 1983; Winans and Walker, 1985). Besides pKM101, another IncN plasmid, RN3, reduces the conjugative transfer of plasmid RP4 and encodes a homologue of FipA (Humphrey et al., 2012; Olsen and Shipley, 1975).

In turn, plasmid RP4 (synonym of R68, RK2 and RP1(Burkardt et al., 1979; Pansegrau et al., 1994; Stokes et al., 1981)), encodes at least two regions involved in the inhibition of plasmid R388: *fiwA* and *fiwB* (Fong and Stanisich, 1989; Yusoff and Stanisich, 1984). The *fiwB* region contains three genes *klaA*, *klaB* and *klaC*, all required to inhibit conjugation of R388 by preventing pilus synthesis (Goncharoff et al., 1991). The product of *fiwA*, the Osa protein of plasmid Sa and the product of gene *p1056.10c* from plasmid ICEhin1056 share some homology (Chen and Kado, 1994; Juhas et al., 2007). Osa (and homologues) seems to interact with the transport machinery of rival plasmids and then degrades transfer-DNA (T-DNA) (Cascales et al., 2005; Maindola et al., 2014).

Active inhibition of co-resident plasmids, however, is not the only successful strategy available to plasmids. When the mobilizable plasmid RSF1010 (nearly identical to R300B and R1162 (Rawlings and Tietze, 2001)) and the conjugative plasmid Ti inhabit the same host cell, the DNA from RSF1010 monopolizes the transport machinery (due to higher copy number), out-competing and inhibiting the transfer of Ti's T-DNA (Cascales et al., 2005; Ward et al., 1991).

The above examples suggest a general rule: if the transfer rate of a plasmid “A” decreases when an unrelated plasmid “B” is present in the same cell, such reduction is caused by genes or mechanisms encoded by plasmid “B”. Thus, it is an antagonistic action of plasmid “B”. For example, plasmid pKM101 interferes with the conjugative machinery of plasmid RP4, by targeting its coupling protein. Given that these plasmids are not genetically related, the selective force for a plasmid to decrease its own transfer rate in the presence of a rival (unrelated) plasmid would be low. The advantage would be shared by both plasmids, “A” and “B”, but only “A” would pay the cost.

Positive interactions (conduction, donation and facilitation) are more difficult to interpret. Suppose that the transfer rate of “A” increases in presence of plasmid “B”. Is plasmid “A” taking advantage of plasmid “B”, or, is plasmid "B", enhancing plasmid “A”? Molecular details may help to answer this question. Cases of positive interactions seem to happen by chance, that is, although the enzymes responsible for the increase of a given plasmid “A” may be encoded by plasmid “B”, those enzymes are involved in fundamental functions of plasmid “B”. For example, conjugative plasmids express the conjugative machinery for their own transfer; however, it can be used by mobilizable plasmids. Moreover, mobilizable plasmids encode a few genes that allow them to interact with the conjugative machinery encoded by rival plasmids.

So far, we have been focused on interactions between plasmids present in the same cell. What happens if the two plasmids reside in different cells, that is, if a plasmid “A” goes into a recipient cell already containing another plasmid, “B”? Should one expect negative, neutral, or positive interactions?

Conjugative plasmids often encode surface and entry-exclusion systems to avoid the incoming of similar plasmids. This saves resident plasmids from competing with similar (probably incompatible) plasmids. To our knowledge, mechanisms blocking unrelated incoming plasmids have never been observed. If no avoidance mechanism exists, one may expect two different scenarios.

First scenario: if stabilization of mating pairs is the limiting factor of plasmid transfer, then it is possible that stabilization of mating pairs by one of the plasmids (plasmid “A”) will increase the probability that another conjugative plasmid (“B”) present in the recipient cell transfers in the opposite direction, and both cells become carriers of both plasmids. In this first scenario, the presence of unrelated conjugative plasmids (“B”) in recipient cells has a positive effect on the transfer rate of plasmid “A” and vice versa.

Second scenario: the case in which the limiting factor of plasmid transfer is the expression of the conjugative machinery. For example, let us assume that only one in a thousand plasmids “A” transfers, since plasmids often repress the conjugative machinery. Let us assume the same for plasmids “B” present in other cells. Only very rarely would a plasmid “A” transfer into a cell with a plasmid “B” also expressing the conjugative machinery and vice versa. In this case, transfer of one of the plasmids (“A”) does not affect the probability that another conjugative plasmid (“B”) present in the recipient cell transfers in the opposite direction. In this second scenario, the presence of unrelated conjugative plasmids (“B”) in recipient cells has neither positive nor negative effects on the transfer rate of plasmid “A”.

According to these two scenarios, unrelated plasmids present in recipient cells either have a positive or null effect on plasmid transfer. However, upon arriving into a cell already harbouring an unrelated plasmid the two plasmids may interact negatively or positively as explained above.

In this work, we measured transfer rates of twelve natural plasmids, belonging to seven incompatibility groups, in three settings and

Table 1
Plasmids used in this study.

Plasmid	Incompatibility Group	Size (kbp)	Resistance markers used[a]	Source[b]
R16a	IncA/C	173.1	AK	S.C.K. – C.E.N.
R57b	IncA/C		AC(K[f])	S.C.K. – C.E.N.
F[c]	IncF I	99.2	T	I. Matic (C.N.R.S.)
R124	IncF IV	125.7	T	S.C.K. – C.E.N.
R1	IncF II	93.9	ACKS	G. Koraimann (Graz Univ.)
R1drd19[d]	IncF II	93.9	ACKS	G. Koraimann (Graz Univ.)
R477-1[e]	IncH I2/S		ST	S.C.K. – C.E.N.
RN3	IncN	54.2	ST	S.C.K. – C.E.N.
R702	IncP-1	69.7	KST	S.C.K. – C.E.N.
RP4	IncP-1	60.1	AKT	S.C.K. – C.E.N.
R388	IncW	33.9	W	S.C.K. – C.E.N.
R6K	IncX	38	AS	DSMZ

[a] A: ampicillin (100 μg/mL); C: chloramphenicol (30 μg/mL); K: kanamycin (100 μg/mL); S: streptomycin (100 μg/mL); T: tetracycline (20 μg/mL); W: trimethoprim (100 μg/mL).
[b] C.N.R.S. – Centre National de la Recherche Scientifique; DSMZ – Leibniz-Institut DSMZ German Collection of Microorganisms and Cell Cultures; S.C.K. - C.E.N. – Belgian Nuclear Research Centre; Graz Univ. – Karl-Franzens-Universität Graz, Institute of Molecular Biosciences.
[c] Constitutive for conjugation (Fernandez-Lopez et al., 2016; Yoshioka et al., 1987).
[d] De-repressed natural mutant of R1 (Koraimann et al., 1991).
[e] Temperature-sensitive for conjugation (Taylor, 2009).
[f] Only low-level of kanamycin resistance, marker not used for selection.

compared their values in order to understand their competitive strategies. The three settings were: (i) donor cells contain a plasmid and recipient cells are plasmid-free; (ii) donor cells contain two unrelated plasmids and recipient cells are plasmid-free; and, (iii) half of the cells contain a given plasmid and the other half contain an unrelated plasmid – in this setting, the recipient cells of a plasmid are the donor cells of the other plasmid.

2. Materials and methods

2.1. Bacterial strains and plasmids

We used the following bacterial strains: *E. coli* K12 MG1655, *E. coli* K12 MG1655 *Δara* (unable to metabolize arabinose) and *E. coli* K12 JW2669 (*Δara* and *ΔrecA* – unable to perform homologous recombination). We used a total of twelve natural conjugative plasmids, summarized in Table 1.

2.2. Generation of plasmid carrying strains

The general method is depicted in Supp. Fig. S1.

We carried out overnight mating experiments, in Lysogeny Broth (LB), between *E. coli* K12 MG1655 *Δara* (recipient) and each of the twelve plasmid-donor *E. coli* strains (either auxotrophic for two amino acids or auxotrophic for one amino acid and unable to metabolize maltose) to produce the twelve single-plasmid donor strains of *E. coli* K12 MG1655 *Δara*. Transconjugants were selected in M9 minimal solid (agar 1.5%) medium supplemented with maltose (0.4%) and the required antibiotics.

The twelve plasmid-carrying *E. coli* K12 MG1655 (ara^+) recipient strains resulted from overnight matings, in LB, between *E. coli* K12 MG1655 (ara^+) and each of the twelve strains of *E. coli* K12 MG1655 *Δara* which carried a single plasmid. Transconjugants were selected in M9 minimal solid medium supplemented with arabinose (0.4%) and the required antibiotics.

We produced a total of 40 strains of *E. coli* K12 MG1655 *Δara* carrying all the possible combinations (due to incompatibility or selective markers) of two plasmids. These strains resulted from overnight matings, in LB, between two strains of *E. coli* K12 MG1655 *Δara*, each carrying a single plasmid. Transconjugants were selected in solid LB (agar 1.5%) medium and the required antibiotics.

We used the same methodology to produce *E. coli* K12 JW2669 (*ΔrecA*) single donors of R57b, F, and R6K plasmids and double-plasmid donors harbouring the three possible combinations of these plasmids.

2.3. Identification of FINs in the plasmids with available sequences

The complete nucleotide sequences are currently available for plasmids F (NC_002483), R16a (KX156773), RN3 (NC_015599), RP4 (BN000925), R388 (BR000038) and R6K (ftp://ftp.sanger.ac.uk/pub/pathogens/Plasmids/R6K.dbs). We used the BLASTP algorithm to search for homology between plasmid proteins and previously described FIN proteins (accession numbers of their amino acid sequences are provided in Supp. Table S1), considering an e-value $< 10^{-4}$. During the course of this work, the sequence of plasmid R6K was not yet annotated. In such case, the BLASTX algorithm (also considering an e-value $< 10^{-4}$) was used instead.

2.4. Deletion of the traG gene of plasmid F

We constructed *ΔtraG* mutants of plasmid F in both *E. coli* MG1655 and *E. coli* MG1655 *Δara* strains. Mutants were constructed using the λ Red recombination method (Baba et al., 2006; Datsenko and Wanner, 2000). We used the λ Red expression vector pKD46, the vector pKD3 as template and the primers traG_fwd_cmpR 5′-CTCAGTCGTTACCAGAACAACTATCACTTCGGAGGGAGCACGCTGTGAAAGTGTAGGCTGGAGCTGCTTC-3′ and traG_rev_cmpR 5′-CTCTCCATACCCTACCCAACATGTTATGATTATTCTTTATGCTGGTAACTCATATGAATATCCTCCTTAGT-3′ designed as described elsewhere (Baba et al., 2006; Datsenko and Wanner, 2000). This way, we replaced the gene *traG* by the FRT-flanked chloramphenicol-resistance cassette of vector pKD3. Subsequently, we transformed the strains with the temperature-sensitive vector pCP20 encoding a flipase to eliminate the chloramphenicol-resistance cassette. Colonies grown at 30 °C in LB solid medium (with ampicillin for pCP20 selection) lost the chloramphenicol-resistance cassette. Subsequently, selected colonies were grown at 42 °C in LB solid medium (without antibiotics) to induce the loss of vectors pCP20 and pKD46. We confirmed gene deletion by colony PCR.

To construct new donor strains harbouring combinations of two plasmids, we mated the *E. coli* MG1655 *Δara* strain carrying the *ΔtraG* plasmid with auxotrophic strains carrying another plasmid, as described before.

2.5. Conjugation assays

Strains were grown at 37 °C in LB overnight with agitation at 170 rpm. *E. coli* K12 ara^+ derived strains served as recipients while plasmid-carrying strains of *E. coli* K12 *Δara* served as donors, in a ratio of 1:1. Approximately 10^8 total bacteria were inoculated into 15 mL tubes containing 5 mL of LB. Tubes were incubated at 37 °C for 90 min without agitation. Next we plated the adequate culture dilutions (in $MgSO_4$ 0.01 M) in Tetrazolium Arabinose (TA) medium to quantify donor and recipient bacteria (which appear respectively as red and white colonies due to differential arabinose metabolism); and in M9 minimal solid medium supplemented with arabinose (0.4%) and suitable antibiotics to quantify transconjugants. Considering D, R and T respectively as the number of donors, recipients and transconjugants per millilitre, the logarithm of conjugation rates (γ) were calculated as: $\gamma = log_{10}\left(\frac{T}{\sqrt{D^*R}}\right)$.

Conjugation rates were measured in three different mating conditions: (i) donor cells containing a single plasmid and plasmid-free recipient cells; (ii) donor cells containing two unrelated plasmids and

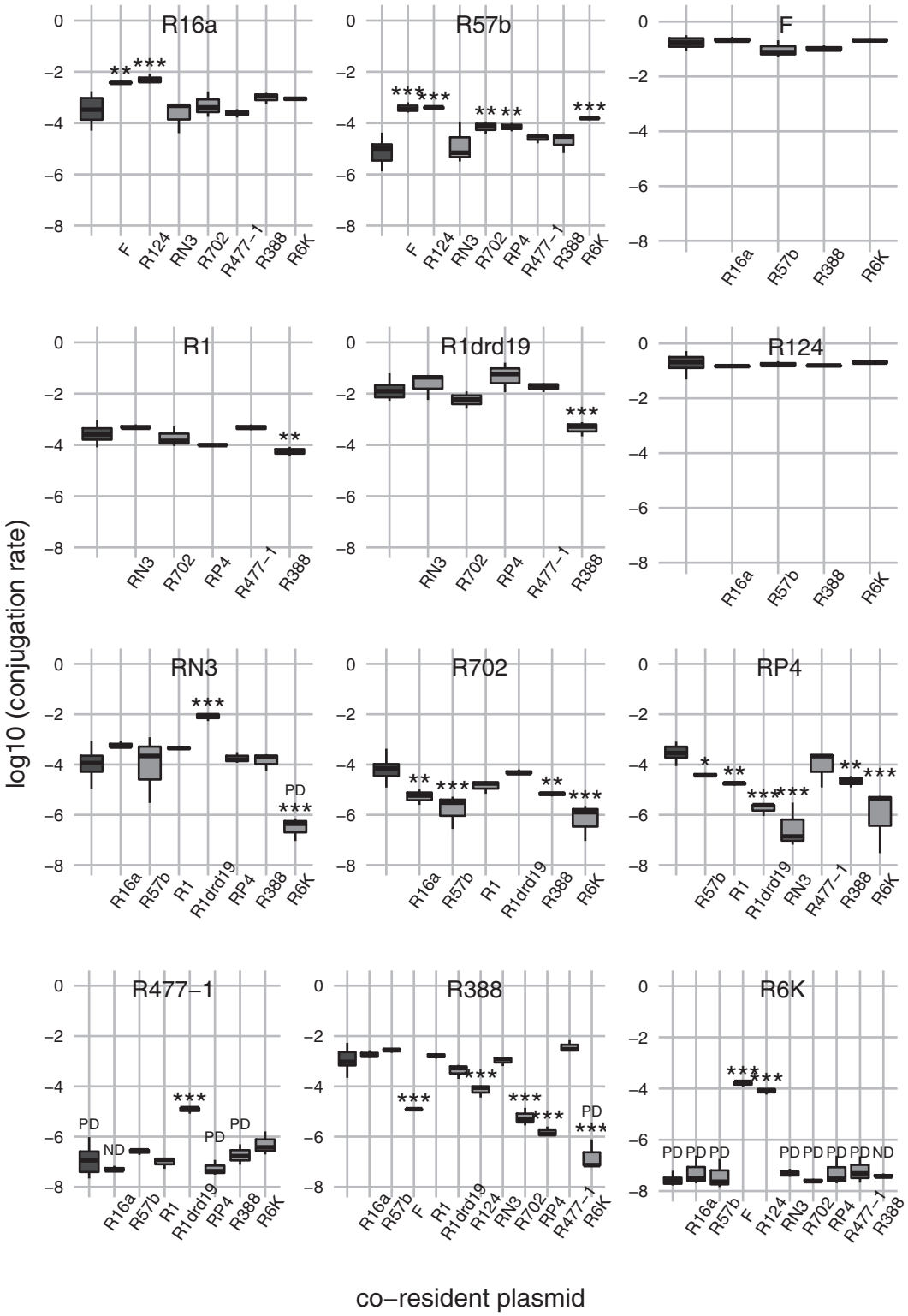

Fig. 1. Effect of a co-resident plasmid. Each boxplot represents the distribution of values: the bottom and top of the box are the first and third quartiles, the horizontal line is the median, the vertical lines are the 1.5 interquartile range. Titles indicate the analysed plasmid and the horizontal axis indicate the co-resident plasmid. The first box of each plot represents the conjugation rate of the analysed plasmid in the absence of co-residents (sample size indicated in Supp. Fig. S3). Conjugation rates in the presence of co-resident plasmids were measured in triplicate. ND – not detected (no transconjugant colonies in any replicates). PD – partially detected (no transconjugant colonies in some replicates). Annotations above the boxes represent the results of Dunnett's multiple comparison test (control group indicated in title): * - p-value $<$ 0.05; ** - p-value $<$ 0.01; *** - p-value $<$ 0.001.

plasmid-free recipient cells; and, (iii) donor cells containing a single plasmid and recipient cells containing a single unrelated plasmid. All variations of this approach are depicted in Supp. Fig. S2. In condition (i) we measured the conjugation rates of each of the twelve natural conjugative plasmids. In condition (ii) we measured conjugation rates of the two plasmids carried in each of 40 double-plasmid carrying strains, thus studying 80 (2×40) interactions. In condition (iii) we only studied 68 interactions (instead of 80) for two reasons. First, we

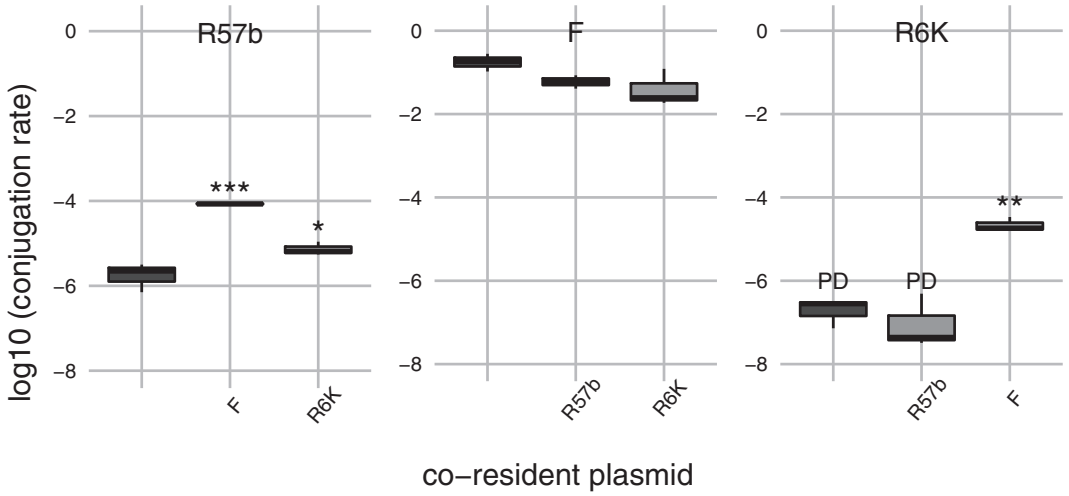

Fig. 2. Effect of a co-resident plasmid in a *ΔrecA* strain. Titles indicate the analysed plasmid and the horizontal axis indicate the co-resident plasmid. The first box of each plot represents the conjugation rate of the analysed plasmid in the absence of co-residents (sample size indicated in Supp. Fig. S3). Conjugation rates in the presence of co-resident plasmids were measured in triplicate. PD – partially detected (no transconjugant colonies in some replicates). Annotations above the boxes represent the results of Dunnett's multiple comparison test (control group indicated in title): * - p-value $<$ 0.05; ** - p-value $<$ 0.01; *** - p-value $<$ 0.001. Each box represents the values as explained in Fig. 1.

had to exclude matings of R6K to recipients carrying RN3 or R702 because the only marker available to select transconjugants of these two matings was ampicillin-resistance (production of β-lactamase). The extracellular presence of β-lactamase in the inoculum (plated) was able to inactivate the antibiotic and for this reason we could not correctly detect transconjugants without changing the methodology (Sambrook, 2001) (see also Domingues et al., 2017 and references therein). Second, due to its temperature-sensitivity for transfer, we tested only intercellular interactions between plasmid R477-1 and either plasmid R1 or R1drd19 (which had a positive intracellular influence on it).

2.6. Statistics

Statistical tests were performed in R version 3.2.0, available at http://www.rstudio.com/ (R Core Team, 2015).

3. Results

3.1. Conjugation rate variability

We measured the conjugation rates of twelve natural plasmids belonging to seven incompatibility groups. Conjugation rates ranged across nearly eight orders of magnitude and centred around -4 (Supp. Fig. S3). Plasmids F (which is de-repressed due to a mutation in the *finO* gene (Yoshioka et al., 1987)) and R124 exhibited the highest conjugation rates (an average of -0.77 and -0.75, respectively). Plasmids R477-1 and R6K exhibited the lowest rates (an average of -6.96 and -7.58) and we only detected transconjugants in some of the replicates.

We tested the conjugation rates of each plasmid for normality by the Shapiro-Wilk test. For all plasmids, the distribution of conjugation rates did not significantly differ from normal distributions (all p-values $>$ 0.05). We then assigned the twelve plasmids to nine clusters, according to their conjugation rates, using the Tukey multiple comparison test (Supp. Fig. S3).

As observed in Supp. Fig. S3, plasmid R1drd19 exhibited an average conjugation rate higher than that of R1, which was expected due to its de-repressed expression of conjugation (Koraimann et al., 1991). The fact that plasmid R477-1 exhibited one of the lowest conjugation rates is also expected since IncH plasmids transfer optimally at temperatures lower than 30 °C (Taylor, 2009).

Additionally, we can observe in Supp. Fig. S3 that, when clustering plasmids according to their conjugation rates, plasmids belonging to the same incompatibility group rarely clustered together. IncF plasmids F and R124 clustered together, but not with R1 nor R1drd19; IncP-1 plasmids R702 and RP4 did not cluster together as well, neither did IncA/C plasmids R16a and R57b. Furthermore, plasmids F and R124 belong to different IncF subgroups (IncF I and IncF IV). Therefore, an association between incompatibility groups and conjugation rates seems unlikely.

3.2. Effect of a co-resident plasmid

We measured the conjugation rates of 40 pairs of plasmids (Fig. 1) (experimental approach depicted in Supp. Fig. S2). Then, we compared the conjugation rates of each plasmid when in the presence of a co-resident plasmid with its own conjugation rate when alone in the host cell. We observed that a plasmid affected its co-resident's transfer rate (Dunnett's multiple comparison test, p-value $<$ 0.05) in 25 (62.5%) of the pairs.

This influence was unidirectional, that is, only the conjugation rate of one of the plasmids was affected in 21 of the pairs. We observed two types of unidirectional effects: i) positive, when the conjugation rate of a plasmid increased, and ii) negative, when the conjugation of a plasmid decreased. Nine pairs of plasmids (22.5%) exhibited positive unidirectional effects while 12 pairs (30%) exhibited negative unidirectional effects.

Conjugation rates of several plasmids (R16a, R57b, RN3, R477-1 and R6K) increased in the presence of plasmids F, R124 or R1drd19, which were the plasmids exhibiting the three highest intrinsic conjugation rates. Plasmid R57b, was particularly striking because its transfer rate increased in the presence of five different co-resident plasmids: F, R124, R702, RP4 and R6K.

Both IncP-1 plasmids, R702 and RP4, along with IncW plasmid R388 served as targets for inhibition of conjugation by most of their co-residents. IncX plasmid R6K displayed an exceptional inhibitory ability, exhibiting the greatest decrease in its co-residents' conjugation rates, being able to do that to plasmids belonging to three different incompatibility groups (IncN, IncP-1 and IncW).

Furthermore, in four pairs of plasmids (10%) the conjugation rates of both plasmids changed. We observed that the conjugation rates of both plasmids decreased in two pairs (5%). These reciprocal negative effects occurred between IncP-1 (R702 and RP4) and IncW (R388) plasmids. We never detected reciprocal positive effects. In another two

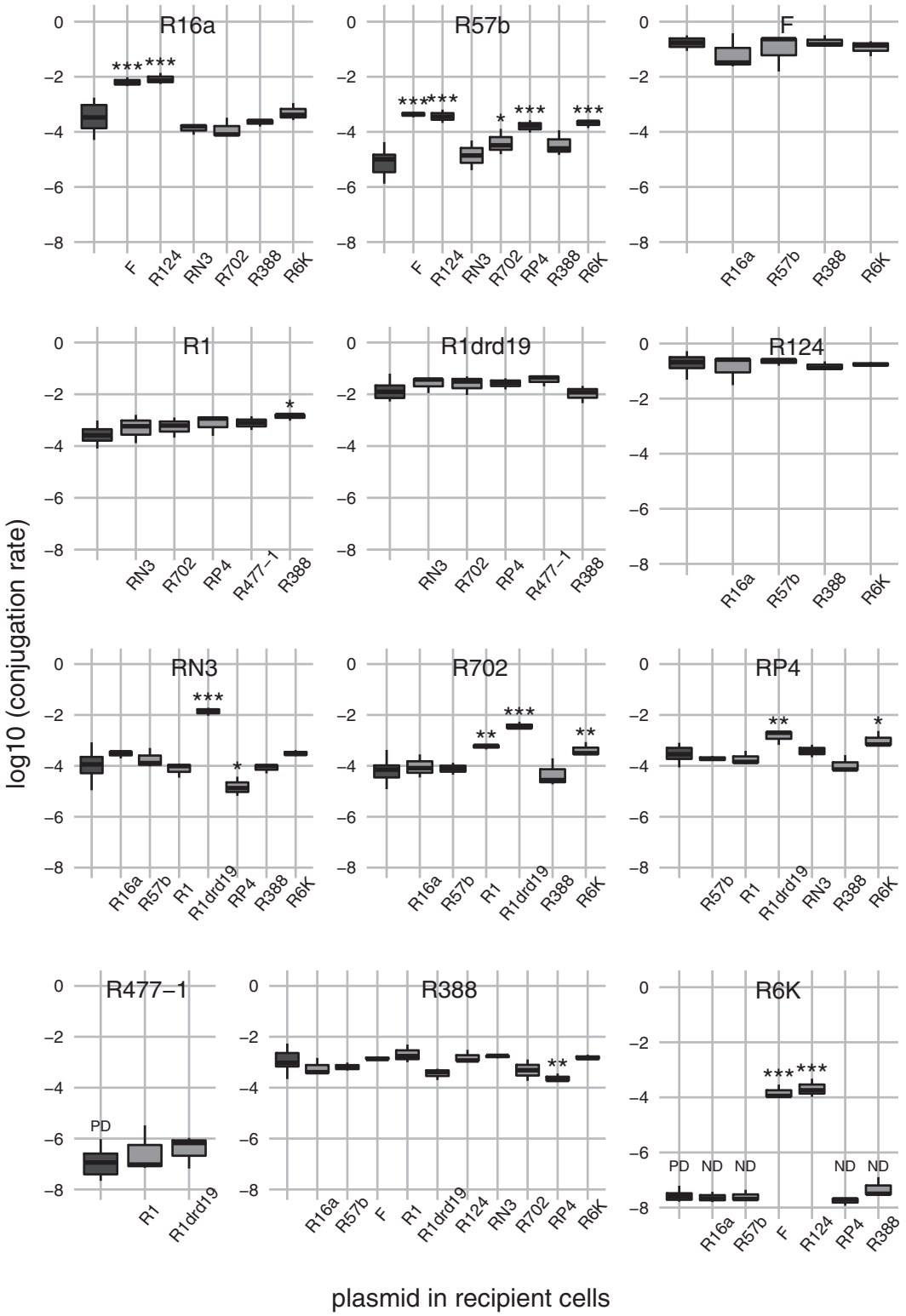

Fig. 3. Effect of a plasmid present in the recipient strain. Titles indicate the analysed plasmid and the horizontal axis indicate the plasmid present in the recipient strain. The first box of each plot represents the conjugation rate of the analysed plasmid to a plasmid free recipient strain (sample size indicated in Supp. Fig. S3). Conjugation rates to plasmid-carrying recipient strains were measured in triplicate. ND – not detected (no transconjugant colonies in any replicates). PD – partially detected (no transconjugant colonies in some replicates). Annotations above the boxes represent the results of Dunnett's multiple comparison test (control group indicated in title): * - p-value $<$ 0.05; ** - p-value $<$ 0.01; *** - p-value $<$ 0.001. Each box represents the values as explained in Fig. 1.

pairs, we observed that conjugation rates of plasmid R57b increased in the presence of both IncP-1 plasmids, R702 and RP4, but conjugation rates of these two plasmids decreased in the presence of plasmid R57b.

It was impossible to determine negative effects on plasmids R477-1 and R6K since their conjugation rates, when alone, were close to the detection limit of transconjugants. Consequently, negative interactions may have been underestimated.

3.3. Identification of FINs in the plasmids studied

Since we observed negative effects between co-resident plasmids, we searched for homologues of known FIN proteins in their sequences. We found a putative FIN homologue in plasmid R16a. This plasmid encodes a protein with homology to FipA of plasmid pKM101. The protein encoded by R16a exhibits 27% of identity with FipA, which means that 27% of the amino acids in the protein of R16a match those in FipA. This alignment covers 44% of the length of the protein of R16a. The region from position 135178 to 135783 (accession AOB42034) in the sequence of R16a encodes this putative FIN protein.

We did not detect any homologue in plasmid R6K. We confirmed that plasmid RN3 codes for a FipA homologue and that plasmid R388 codes for an Osa homologue. FipA of plasmid RN3 shares 99% of identity with that of plasmid pKM101. The Osa homologue of R388 differs from that of plasmid Sa only by being two residues shorter. We also confirmed that plasmid F encodes PifC and that plasmid RP4 encodes FiwA, KlaA, KlaB and KlaC proteins. However, we did not identify any FIN proteins in these plasmids besides those already known.

3.4. Effect of recombination

One of the possible causes for positive effects could be recombination events. Thus, we tested this hypothesis using a strain deficient in homologous recombination, *ΔrecA*, as donor in the mating experiments. We measured the conjugation rates of plasmids F, R57b and R6K, alone and in pairs.

We observed before that the transfer rate of R57b was the main target of positive effects, and now we observed again that the conjugation rate of plasmid R57b increased when in the presence of either F or R6K. On the other hand, plasmid R6K only increased its conjugation rate in the presence of plasmid F, and the conjugation rates of plasmid F remained unchanged in any combination, as observed in the previous experiments (Fig. 2). Thus, at least in these cases, facilitation does not seem to be mediated by plasmid homologous recombination.

3.5. Effect of a plasmid carried in the recipient strain

Plasmids present in recipient cells may express mechanisms, such as entry/surface exclusion, that decrease the conjugation rates of other plasmids. Therefore, we also studied intercellular interactions. To do so, we measured the conjugation rates of each plasmid (alone in the donor cell) in matings to recipient cells carrying a different but compatible plasmid (experimental approach depicted in Supp. Fig. S2). For this, we studied 68 effects (35 pairs, two of which tested only in one direction – see Methods) and found effects in 18 pairs of plasmids (54.6%): 16 pairs (48.5%) exhibited positive effects, while only two pairs (6.1%) exhibited negative effects. All the effects detected were unidirectional.

Conjugation rates of most plasmids increased when plasmids F, R124 or R1drd19 (which had the highest intrinsic conjugation rates) were present in the recipient strain (Fig. 3). Plasmid R57b was again the main plasmid exhibiting such positive effects (five positive effects among seven combinations).

Conjugation rates of plasmids R57b, R702 and RP4 increased when the recipient strain harboured plasmid R6K, although this plasmid exhibited a low transfer rate. Therefore, the conjugation rates of both IncP-1 plasmids decreased if this IncX plasmid was present in the same

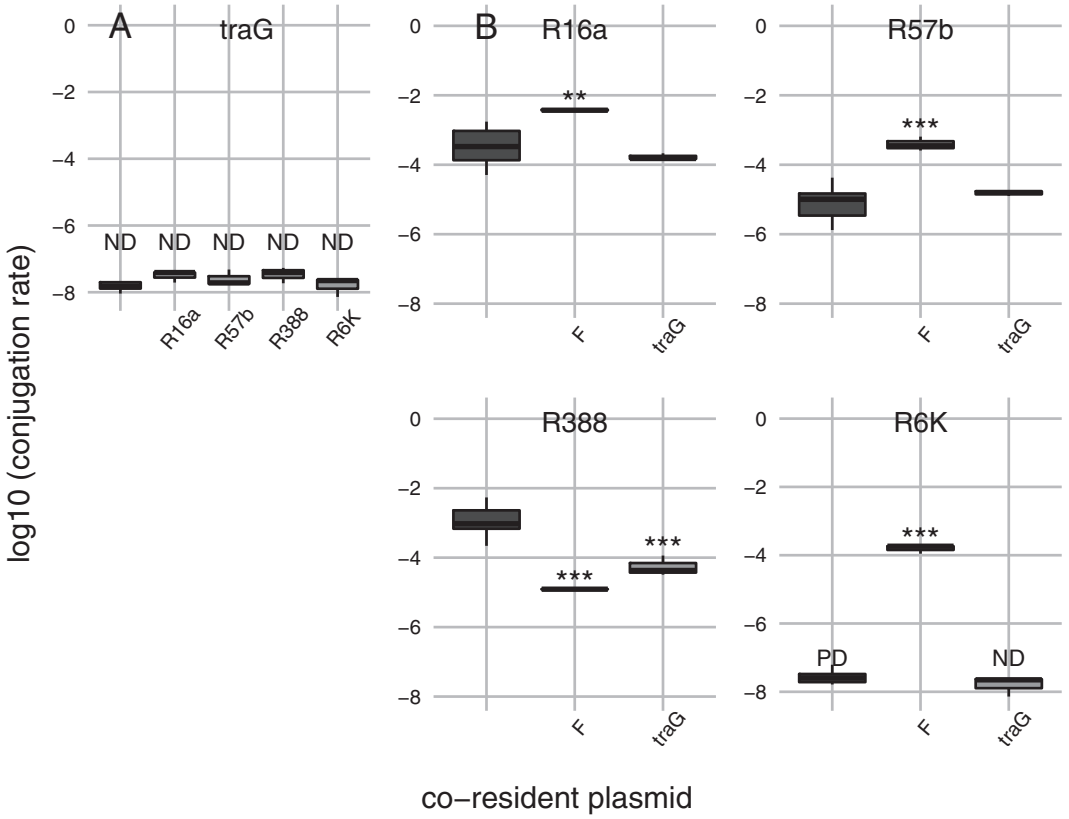

Fig. 4. Effect of a co-resident wild-type F or a mutated F plasmid (*ΔtraG*) impaired for mating pair stabilization. A) Conjugation rates of the mutant *ΔtraG* plasmid; B) Conjugation rates of other plasmids having F or *ΔtraG* as co-resident plasmids. Titles indicate the analysed plasmid and the horizontal axis indicate the co-resident plasmid. The first box of each plot represents the conjugation rate of the analysed plasmid in the absence of co-residents (sample size indicated in Supp. Fig. S3; n = 3 for *ΔtraG*). Conjugation rates in the presence of co-resident plasmids were measured in triplicate. ND – not detected (no transconjugant colonies in any replicates). PD – partially detected (no transconjugant colonies in some replicates). Annotations above the boxes represent the results of Dunnett's multiple comparison test (control group indicated in title): * - p-value < 0.05; ** - p-value < 0.01; *** - p-value < 0.001. Each box represents the values as explained in Fig. 1.

host cell, but increased if it was present in a different cell. Plasmid R388 seems to act similarly towards plasmid R1, such that the conjugation rate of the latter decreased when both plasmids inhabit the same cell, but increased when they were present in different cells. However, R388 had no such positive effect on plasmid R1drd19, presumably because of the latter's higher conjugation rate. On the other hand, RP4 was the only plasmid exhibiting negative interactions, which were directed towards plasmids RN3 and R388. Such effect could result from intracellular inhibition when the recipient cells harbour both plasmids. However, this is unlikely since plasmid RP4 did not inhibit RN3 intracellularly (Fig. 1). Again, it was impossible to determine negative effects on plasmids R477-1 and R6K because their conjugation rates are lower than the limit of detection of our experiments.

3.6. Effect of mating pair stabilization

Given the observation of so many positive effects when plasmids inhabit different host cells, we hypothesised that the increase in conjugation rates is due to formation or stabilization (maintenance) of the mating pair. To test this hypothesis, we constructed a derivative of plasmid F, *ΔtraG*, impaired for pilus production and mating pair stabilization, and checked if this mutant plasmid was able to increase the conjugation rates of other plasmids (Firth and Skurray, 1992; Manning et al., 1981).

Fig. 4 represents the intracellular interactions between the wild-type plasmid F or its non-conjugative mutant, F (*ΔtraG*), and their co-resident plasmids. As expected, the *ΔtraG* mutant was unable to transfer horizontally; indeed, its conjugation rates fell below the limit of detection, even if another plasmid (R16a, R57b, R388 or R6K) co-resided in the same donor cell. Furthermore, conjugation rates of plasmids R16a, R57b and R6K increased in the presence of co-resident wild-type plasmid F, but not of the *ΔtraG* mutant that does not express sex-pili. However, the ability to inhibit the conjugation of plasmid R388 was retained by the *ΔtraG* mutant.

Moreover, the conjugation rate of plasmids R16a, R57b and R6K did not increase if the mutant *ΔtraG* plasmid was present in the recipient cell (Fig. 5) (but there was an increase if recipient cells harboured the wild-type plasmid). Strikingly, plasmid R388 was inhibited intercellularly by the *ΔtraG* mutant but not by the wild-type F plasmid. This suggests that the intracellular negative effect of plasmid F towards R388 (in transconjugant cells now harbouring both plasmids) was cancelled by the reverse intercellular positive effect. Since the *ΔtraG* mutant was unable to provide the intercellular positive effect, we only observed an overall negative intracellular effect towards R388.

Altogether, these results confirm that formation or stabilization of the mating pairs can increase the efficiency of plasmid horizontal transfer. This corroborates the hypothesis that stabilization of the mating pair is responsible for the positive interactions observed.

3.7. Comparison between intracellular and intercellular interactions

Overall, in 51 of the possible interactions, we did not observe intracellular effects (when plasmids were initially in the same cell) and, in 50 of the possible interactions (when plasmids were initially in different cells), we did not detect intercellular effects. However, the amount of positive and negative effects between the two conditions is different. There are fewer positive cases among intracellular interactions than among intercellular interactions (11 cases in 80 possible interactions versus 16 in 68) and more cases of negative effects among intracellular interactions than among intercellular interactions (18 cases in 80 possible interactions versus 2 in 68). These comparisons reveal that the two conditions differ significantly (two-sided Fisher exact test, $p = 0.00097$). Negative effects prevail when both plasmids are present in the same donor cell. Moreover, positive effects are more frequent than negative when plasmids inhabiting different hosts interact.

Comparing the results between intracellular and intercellular

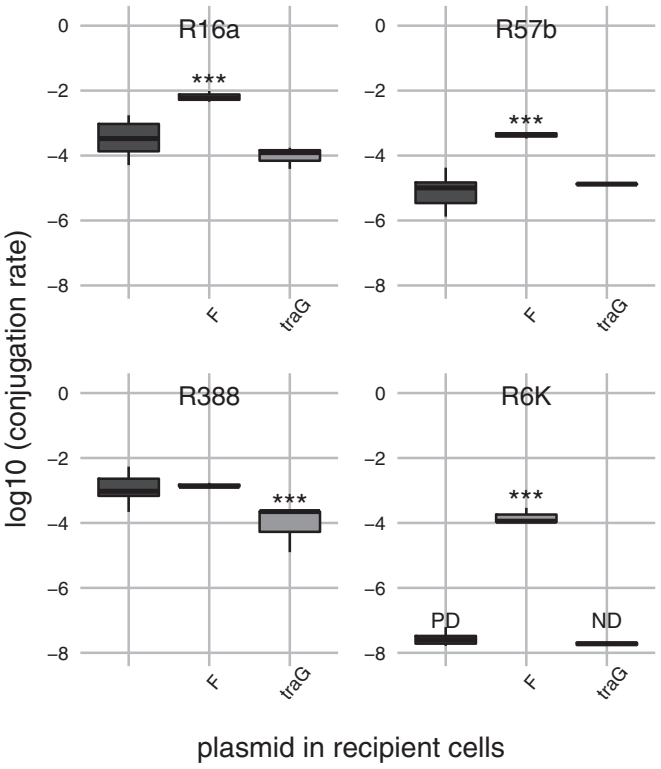

Fig. 5. Effect of a wild-type F plasmid or of a mutated F plasmid (*ΔtraG*) impaired for mating pair stabilization present in the recipient strain. Titles indicate the analysed plasmid and the horizontal axis indicate the plasmid present in the recipient strain. The first box of each plot represents the conjugation rate of the analysed plasmid to a plasmid free recipient strain (sample size indicated in Supp. Fig. S3). Conjugation rates to plasmid-carrying recipient strains were measured in triplicate. ND – not detected (no transconjugant colonies in any replicates). PD – partially detected (no transconjugant colonies in some replicates). Annotations above the boxes represent the results of Dunnett's multiple comparison test (control group indicated in title): * - p-value < 0.05; ** - p-value < 0.01; *** - p-value < 0.001. Each box represents the values as explained in Fig. 1.

conditions, we found additional negative effects in the interactions of plasmids R1 and R1drd19 towards R702. These additional cases may be the result of the following reasoning. In intercellular interactions, the conjugation rate of plasmid R702 increased if recipient cells harboured R1drd19, probably because it further stabilized the mating pair. Such increase most probably also occurs when the two plasmids are in the same cell as well. However, we observed no variation on the transfer rates of plasmid R702 when plasmid R1drd19 inhabited the same donor cell. Therefore, most probably, our observations that R1drd19 has no effect over R702 are a simplification: within the cell, R1drd19 is repressing the transfer of R702 at the same time that it is helping the double donor cell to find a recipient cell and stabilize mating pair. According to this interpretation, there is, indeed, a negative effect of R1drd19 over R702 when present in the same cell. Thus, we did not observe a variation on the transfer rates of plasmid R702 possibly because the opposite intercellular positive and the intracellular negative effects result in a combined net effect. Following the same reasoning, we argue that plasmid R1 acts negatively towards R702. Moreover, results presented in the second accompanying article Gama et al., (2017) suggest that, intracellularly, R6K is negatively affected by plasmids R16a, R57b, and R388. These five cases of intracellular negative effects further strengthen our main conclusion: negative interactions prevail when both plasmids are present in the same donor cell, while they interact only positively, if at all, when inhabiting different host cells.

4. Discussion

In this work, we analysed changes on the transfer rates of conjugative plasmids when other conjugative plasmids resided in the (i) donor cell or (ii) in the recipient cell. We will discuss these two conditions in turn. We observed interactions in 25 out of 40 possible combinations of co-residing plasmids. In total, we detected 11 cases where the conjugation rates increased and 18 cases of decrease.

Increased transfer rates of a plasmid "A", when plasmid "B" is present in the cell, can be interpreted as "A" taking advantage of "B", or as "B" helping "A" (henceforth "help" means that "B" codes for a metabolite specifically to directly increase the transfer rate of plasmid "A"). If the second hypothesis prevailed, cases of mutual help, where both plasmids increased their transfer rates, would have been expected, which we never observed. Actually, we observed the opposite: in two combinations, both plasmids were able to inhibit one another. These two situations of mutual inhibition and the absence of mutual help are consistent with the interpretation that plasmids are not cooperating.

We show that homologous recombination is not responsible for the increase of transfer rates. Homologous recombination is stimulated by SOS response, which in turn is induced by single-stranded DNA (Matic et al., 1995), which may be relevant because plasmids transfer as single-stranded DNA. However, narrow host-range plasmids (such as IncF) prevent SOS response (Baharoglu et al., 2010). The fact that plasmids (F, R124 and R1drd19) that positively influence their co-residents have narrow host range supports the finding that homologous recombination is not responsible for increasing conjugation rates. Alternatively, pilus synthesis and/or stabilization of mating pairs have a role in facilitation processes, either if the two plasmids are present in the same cell (Fig. 4) or in different cells (Fig. 5).

The case of IncX plasmid R6K is thus an intriguing exception, since this plasmid exhibited the lowest transfer rates but was able to increase the transfer rates of plasmids R57b, R702 and RP4. Studies suggest that IncX plasmids frequently carry mobile forms of biofilm-promoting gene cassettes encoding type 3 fimbriae (Burmolle et al., 2012). These fimbriae can affect the efficiency of conjugation positively, which is a plausible explanation for our observation (Burmolle et al., 2008). This effect is however variable for they can also decrease conjugation rates (Ong et al., 2009). Plasmid R477-1 is the other exception because its conjugation rate increased when plasmid R1drd19 was present in the same cell, but not in neighbouring cells. Since plasmid R1 had no observable effect on R477-1, we speculate that some intracellular molecule, produced in excess by R1drd19, is responsible for the enhanced transfer of R477-1. Alternatively, translocation of a transposon from R1drd19 (Crisona and Clark, 1977; Goebel et al., 1977; Guyer, 1978) to R477-1 would lead to conduction of R477-1 due to co-integration.

Broad-host range plasmids RP4 and R702 (IncP-1) and R388 (IncW) were the main targets of inhibition. Therefore, the widespread inhibitory effect against these plasmids could be the result of a strategy preventing them from invading bacterial populations that already harbour resident plasmids. Furthermore, R6K inhibits all three plasmids (Olsen and Shipley, 1975). Although reciprocal inhibition was previously observed between plasmids RP4 and R6K (Gama, 2017), we could not detect negative effects on plasmid R6K (and R477-1) since the intrinsic transfer rate values were close to our limit of detection. Using a different approach, we detected negative effects on plasmid R6K (see next accompanying article Gama et al. 2017).

Some inhibitory mechanisms only prevent the transport of the rival plasmid's DNA instead of preventing the expression of the whole conjugative system, which theoretically would impose a lower cost on the host and confer protection against viruses. By allowing production of additional conjugative pili, plasmids can exploit their rivals to further increase their own transfer.

IncA/C plasmids, despite being broad-host range as IncP-1 and IncW plasmids, were never inhibited. In fact, the conjugation rates of plasmids R57b increased in the presence of many of the plasmids tested and R16a increased in cells harbouring plasmids F or R124. Thus, positive interactions may promote their dispersion and broaden their host ranges.

In the second scenario where the plasmids were present in two distinct cells, positive interactions were detected in 16 combinations, while negative interactions were detected in only two. Given that after plasmid transfer the two rival plasmids will inhabit the same host cell, intracellular effects can either enhance or hide the positive intercellular effects. For example, the conjugation rate of plasmid R1 increased when plasmid R388 was present in (potentially) recipient cells; however, if both plasmids were present in the same host cell, the transfer of plasmid R1 decreased. The two cases of intercellular negative interactions (in which plasmid RP4 decreased the transfer rates of plasmids RN3 and R388) could result from an entry/surface exclusion system, since IncN, IncP-1 and IncW plasmids express somewhat similar sex pili (Bolland et al., 1990; Bradley, 1980; Cabezon et al., 1994; Olsen et al., 1974).

Other intercellular positive interactions may be hidden under negative effects within cells. We can detect such cases by comparing transfer rates when two plasmids are in the same cell versus when they are in different cells. For example, when R388 transfers into cells harbouring F (Fig. 3), it seems that there is no positive effect; however, when both R388 and F occupy the same cell, transfer rate of R388 decreases (Fig. 1). The two effects almost cancel. Experiments involving a *ΔtraG* mutant F plasmid corroborates this hypothesis given that the *ΔtraG* plasmid has a negative effect over R388 both in intracellular and intercellular experiments (Figs. 4 and 5).

An intercellular effect could also mask an intracellular one. We would expect the inhibitory effect of *ΔtraG* on R388 to be stronger than that of F, because *ΔtraG* cannot transfer to the recipient cell and provide the positive effect. Our results (Fig. 4) do not seem to corroborate this hypothesis.

One might consider the existence of retrotransfer, which is the transfer of a plasmid from the recently formed transconjugant back to the donor cell (Ankenbauer, 1997; Mergeay et al., 1987; Szpirer et al., 1999; Top et al., 1992). An important point of retrotransfer is that a plasmid that moves from a donor cell to a recipient cell expresses conjugation genes in the recipient cell. Therefore, one should expect that the conjugation rate of the intracellular case was higher than that of intercellular case because the number of cells harbouring the two plasmids is higher in the first case. However, by comparing conjugation rates of plasmids R16a, R57b, RN3 and R6K in the two conditions (Figs. 2 and 4), one can see that this is not the case.

Overall, our results strongly suggest that stabilization of the mating pairs is crucial for the efficiency of plasmid horizontal transfer. One may envisage at least two major, but non-exclusive, interpretations for the effect of conjugative pili synthesis. First, it is possible that the conjugative pili of a plasmid help stabilizing the transfer of another plasmid. Second, it is possible that the positive effect comes from the increased effective volume of a cell when it expresses a plasmid, hence increasing the probability of interacting with a partner for mating – thus helping other plasmids co-inhabiting the donor cell or present in the recipient cell. This second explanation arises from the following: while an *E. coli* cell has a volume of about 2 μm^3 (Schulz and Jorgensen, 2001), a cell harbouring an F plasmid, hence expressing two or three sex-pili per cell each one about 20 μm long (Sokatch, 1979), has an apparent volume (assuming a sphere) of approximately $4/3 \cdot \pi$ $(20\ \mu m)^3 = 33{,}386\ \mu m^3$, that is, about sixteen thousand times bigger than without sex-pili. With such increased apparent volume, these cells easily interact with a partner for mating, eventually affecting positively other plasmids present in the donor or in the recipient cells.

For bacterial cells, there is a fitness cost associated with conjugation (Turner et al., 1998). In an evolutionary experiment, it was shown that conjugative plasmids evolve towards higher virulence (higher cost to their host), consequently decreasing the density of the bacterial population. Mechanisms that decrease conjugation rate (as observed with the 18 intracellular negative interactions) could be expected to decrease

the fitness cost to their host (Chao et al., 2000) and even prevent the collapse of the bacterial population (Smith, 2012). Future research could test this hypothesis.

Our results are relevant from the clinical point of view, given that plasmids of this study confer antibiotic resistance. In the one hand, positive interactions are worrisome given that they imply an increase of the spread of antibiotic-resistance genes. On the other hand, negative interactions, not only have the opposite effect, but could also be exploited to design strategies to prevent plasmid dissemination. For example, inhibition of conjugation has already been suggested to be used as a prophylactic strategy (reviewed in Williams and Hergenrother, 2008). This approach, once implemented, could also be directed towards plasmids carrying virulence factors.

Some plasmids code for genes that confer resistance to antibiotics by modifying or degrading antibiotic molecules (Wright, 2005). For example, plasmids R1, R1drd19, R16a, R57b, R6K and RP4 encode β-lactamases that hydrolyses β-lactam antibiotics such as ampicillin. In some conditions (Domingues et al., 2017), bacteria harbouring these plasmids can detoxify the environment and prevent sensitive bacteria from dying ((Domingues et al., 2017), see also for non-conjugative plasmids (Nicoloff and Andersson, 2016; Sorg et al., 2016)). Since surviving sensitive cells may harbour plasmids encoding resistance to other antibiotics, these cells and the ones detoxifying the environment may exchange plasmids. Even if sensitive cells do not harbour plasmids, they can latter receive the plasmid coding for the detoxifying determinant.

In conclusion, in this work we show that negative interactions are more frequent when both plasmids occupy the same cell than when plasmids reside in different cells, where positive interactions prevail. Indeed, the transfer rate of each plasmid is higher if other, unrelated, conjugative plasmid is present in the recipient cell than if this cell is plasmid-free. Possibly this may be one of the mechanisms underlying antibiotic resistance dissemination.

Acknowledgements

We thank Doctors Ivan Matic, Günther Koraimann and Max Mergeay for providing the plasmids used in this and accompanying articles. We also thank Doctor Karina Xavier for providing vectors pKD46, pKD3 and pCP20 used to delete the *traG* gene in plasmid F. We also thank two anonymous reviewers and the editor for helpful suggestions which enabled us to increase the quality of the manuscript. Finally, we thank Fundação para a Ciência e Tecnologia for funding through the PhD grant (JAG) (SFRH/BD/86103/2012).

Appendix A. Supplementary data

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.plasmid.2017.08.003.–>

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