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Multiple plasmid interference – Pledging allegiance to my enemy's enemy

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ABSTRACT

As shown in the previous article, two distinct conjugative plasmids sometimes interact within bacterial cells, implicating changes of transfer rates. In most cases of interactions within bacteria, the transfer of one of the plasmids decreases. Less frequently, the transfer rate of one of the plasmids increases. Here we analyse what happens if three distinct conjugative plasmids colonize the same bacterial cell. Our aim is to understand how interactions between two plasmids affect the transfer rate of the third plasmid. After showing that plasmids interact in 59 out of 84 possible interactions we show that, with some exceptions, if the transfer rate of a plasmid decreases in the presence of a second plasmid, a decrease is also observed in the presence of a third plasmid. Moreover, if the conjugation rate of a plasmid increases in the presence of another, an increase is also observed if there is a third plasmid in the cell. Both types of interactions are mostly independent of the third plasmid's identity, even if sometimes the third plasmid quantitatively distorts the interaction of the other two plasmids. There is a bias towards negative intensifying interactions, which provide good news concerning the spread conjugative plasmids encoding antibiotic-resistance genes and virulence factors.

1. Introduction

Interactions between three or more different biological entities often lead to the emergence of new phenomena. Focusing only in the bacterial world, one finds examples in very different contexts such as the ecological effect of colicinogenic bacteria (Kerr et al., 2002), biofilm formation (Mitri et al., 2011; Momeni et al., 2013), pathogenicity in *Salmonella* (Diard et al., 2013) or quorum sensing in *Bacillus subtilis* (Pollak et al., 2016).

Bacterial cells can harbour several plasmids, and they can influence each other employing diverse systems. Exclusion systems prevent host cells from receiving a related plasmid (reviewed in Garcillan-Barcia and de la Cruz, 2008). Incompatibility, due to replication and partition systems, precludes two related plasmids from persisting in the same host cell (reviewed in Novick, 1987). Different plasmids, however, tend to be compatible. Toxin-Antitoxin loci, also known as post-segregational killing (psk) systems consist of 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. During intracellular plasmid competition, psk^+ plasmids displace psk^- plasmids, otherwise the host cell dies (Cooper and Heinemann, 2000). Plasmids may also encode fertility inhibition mechanisms, responsible for repressing their own horizontal transfer. Paradoxically, by inhibiting their own transfer, such plasmids prevail in bacterial populations, while plasmids not repressing their own transfer become too costly to their hosts, which leads to their counter-selection (Haft et al., 2009).

Furthermore, plasmids can employ strategies to affect the horizontal transfer of competitor plasmids ((Cascales et al., 2005; Chen and Kado, 1994; Datta et al., 1971; Fong and Stanisich, 1989; Gasson and Willetts, 1975; Gasson and Willetts, 1977; Goncharoff et al., 1991; Hochmannova et al., 1985; Hochmannova et al., 1982; Juhas et al., 2007; Maindola et al., 2014; Miller et al., 1985; Olsen and Shipley, 1975; Pinney and Smith, 1974; Sagai et al., 1977; Santini and Stanisich, 1998; Tanimoto and Iino, 1983; Ward et al., 1991; Willetts and Skurray, 1980; Winans and Walker, 1985; Yusoff and Stanisich, 1984) and companion article (Gama et al., 2017)).

These previous works, however, are insufficient to predict how two plasmids interact with a third one (in this work we define "interaction" as any influence on the transfer rate of a plasmid). For example, considering three plasmids "A", "B" and "C" where "A" increases the transfer of plasmid "C" and "B" decreases the transfer of "A", what will

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happen when “C” is simultaneously in the presence of those two plasmids? Will the influence of plasmid “B” towards “A” result in an attenuation of the effect of “A” on “C”? Or will “B” be unaffected because “B” targets a function specific for plasmid “A”? As a second example, suppose that both plasmids “A” and “B” decrease the transfer of plasmid “C”. When the three plasmids occupy the same cell simultaneously, will the effect of “A” and “B” complement each other intensifying the inhibition of the transfer of plasmid “C”?

To understand how two plasmids influence the transfer rate of a third one, we compared the conjugation rates of a given plasmid when alone, in the presence of two plasmids and in the presence of either one of them. With these experiments, we will be able to detect distorting interactions in which the values of the conjugation rate of a given plasmid in the presence of a third plasmid differs from those when only a second plasmid is present. Specifically, among negative and positive interactions, we expected two main types of interactions: attenuating distortions, in which the effect of a plasmid on another is alleviated due to the presence of a third plasmid in the cell, or intensifying distortions, in which the effect of a plasmid on another one is heightened when a third plasmid is present in the cell.

2. Materials and methods

2.1. Bacterial strains and plasmids

We used the following bacterial strains: *E. coli* K12 MG1655 and *E. coli* K12 MG1655 Δ*ara* (unable to metabolize arabinose). We used 11 natural conjugative plasmids, whose properties are summarized in Supp. Table S1.

2.2. Generation of plasmid harbouring-strains

We produced a total of 28 strains of *E. coli* K12 MG1655 Δ*ara* carrying all possible combinations of three plasmids (not all combinations were possible due to incompatibility and selective markers). These strains resulted from overnight matings between two strains of *E. coli* K12 MG1655 Δ*ara* (produced in the accompanying article (Gama et al., 2017)), one carrying a single plasmid and the other carrying two plasmids. Transconjugants were selected in Lysogeny Broth (LB) supplemented with agar (1,5%) and the required antibiotics.

2.3. Conjugation assays

After overnight growth at 37 °C with agitation, donor (*E. coli* K12 Δ*ara*) and recipient (*E. coli* K12 *ara*$^+$) strains were inoculated (10^8 total bacteria) in 15 mL tubes containing 5 mL of LB in a ratio of 1:1. Conjugation assays were performed at 37 °C for 90 min without agitation. To quantify donor and recipient bacteria, we plated suitable culture dilutions (in $MgSO_4$ 0.01 M) in Tetrazolium Arabinose (TA) medium, where, due to differences in arabinose metabolism, the donor strain forms red colonies and the recipient strain forms white colonies. To quantify transconjugants, we plated suitable culture dilutions in M9 minimal solid medium supplemented with arabinose (0.4%) and adequate antibiotics. Logarithm of conjugation rates (γ) was calculated as: $\gamma = log_{10}\left(\frac{T}{\sqrt{D*R}}\right)$, considering D, R and T respectively as the number of donors, recipients and transconjugants per millilitre.

2.4. Determination of distorting interactions

Classification of plasmid interactions in triplets (strains carrying three plasmids) follows Supp. Fig. S1 (explained in supplementary information). We use the following definitions:

No-interaction: we considered that the co-resident plasmids did not interact with the analysed plasmid if its conjugation rate was not affected in either the double (strain carrying two plasmids) or in the triplet.

Non-distorting interactions: these interactions occurred if the effect observed in the triplet was identical to the strongest effect observed in the doubles.

Distorting interactions: an interaction is distorting if the effect observed in the triplet differed from the strongest effect observed in the doubles.

The type of interaction could not be determined if the effect observed in the triplet was simultaneously indistinguishable from the strongest effect observed in doubles and from when the analysed plasmid was alone.

2.5. Statistics

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

3. Results and discussion

3.1. To interact or not to interact, that is the question

We measured the conjugation rates of each plasmid present in bacteria harbouring three plasmids simultaneously, using a sample of eleven different naturally-occurring plasmids. For each combination of three plasmids, we studied the transfer rate of each plasmid – this allowed us to check for three putative interactions. Indeed, assuming that a combination comprises plasmids "A", "B" and "C", we have three possible interactions to consider and three questions. First, how does the interaction between "B" and "C" affect the transfer rate of plasmid "A"? Second, how does the interaction between "A" and "C" affects the transfer rate of plasmid "B"? Third, how does the interaction between "A" and "B" affects the transfer rate of plasmid "C"? Since we analysed 28 combinations of three plasmids, we studied a total of 84 possible interactions. We detected plasmid interactions in 59 of the 84 cases (70,2%). Except for combinations R16a/R388/RN3 and R388/R57b/RN3 (Fig. 1), there are interactions in all the other 26 combinations (Supp. Fig. S2).

Through the analysis of the transfer rates in each combination, one can see that, if a co-resident plasmid was inhibitory towards a given plasmid (negative interaction), it remained inhibitory in the presence of a third plasmid. For instance, in the accompanying article (Gama et al., 2017), we observed inhibition of plasmid RP4 in combinations with another plasmid; now we still observe its inhibition when in the presence of two co-resident plasmids (Supp. Fig. S2I). The reverse is also true: a plasmid increased its conjugation rate in the presence of another (positive interaction), independently of the third plasmid's identity, which is illustrated by R16a in any combination involving either plasmid F or R124 (Supp. Fig. S2E). Despite this general trend, there are some exceptions.

We have seen that, in most cases, the presence of a third plasmid did not alter the qualitative effect of the second plasmid, that is, negative interactions continue to be negative even in the presence of a third plasmid, and positive interactions continue to be positive even in the presence of a third plasmid. Quantitatively, however, there may be some distortion, that is, the values of the conjugation rates in the presence of a third plasmid could differ from those when only a second plasmid was present. To test for distortion events, we compared the conjugation rates of a given plasmid when alone, in the presence of both co-resident plasmids and in the presence of each plasmid. Then, we classified the interactions as intensifying or attenuating, based on the groups resulting from the Tukey multiple-comparison test, as outlined in supplementary information (Supp. Fig. S1).

3.2. Distorting interactions: how frequent?

Overall, we observed 59 interactions among the possible 84