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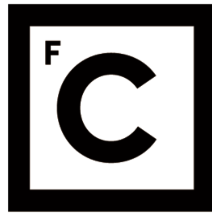

On Plasmids: studies of plasmid-host and plasmid-plasmid interactions

Doutoramento em Biologia
Especialidade em Genética

João Pedro Alves Gama

Tese orientada por:
Professor Doutor Francisco Dionísio
Professora Doutora Rita Zilhão

Documento especialmente elaborado para a obtenção do grau de doutor

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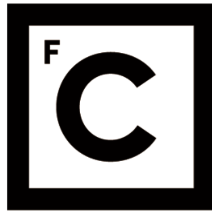

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FCT Fundação para a Ciência e a Tecnologia
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Nota prévia

O autor declara que esta dissertação é resultado do seu próprio trabalho. Uma vez que os trabalhos mencionados foram feitos em colaboração, o autor esclarece a contribuição dos vários autores para os trabalhos efectuados.

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« Great men are forged in fire.

It is the privilege of lesser men to light the flame. »

The War Doctor *in* The Day of the Doctor

Resumo

Os plasmídeos são moléculas de DNA de cadeia dupla frequentemente isoladas a partir de populações bacterianas. Os plasmídeos replicam-se no interior das suas células hospedeiras e dependem dos seus mecanismos replicativos. Os plasmídeos podem também codificar vários factores envolvidos neste processo.

Os plasmídeos podem codificar funções adicionais que asseguram a sua manutenção e estabilidade nos seus hospedeiros. Alguns plasmídeos podem ser transmitidos horizontalmente (entre células). O principal processo envolvido na transmissão horizontal de plasmídeos é designado por conjugação. Esta transmissão requer contacto celular entre as células dadoras e receptoras de plasmídeos. A maquinaria necessária para a conjugação é codificada no genoma dos plasmídeos.

De um modo geral, estas funções definem a amplitude de hospedeiros de um plasmídeo, ou seja, determinam para que células os plasmídeos se podem transferir horizontalmente e em que células se conseguem replicar e manter estavelmente. Um dos objectivos desta tese é avaliar o impacto que cada uma destas funções (replicação, estabilidade e mobilidade) tem na amplitude de hospedeiros dos plasmídeos. Concluímos que a replicação é a função que mais contribui para a amplitude de hospedeiros dos plasmídeos.

Os plasmídeos não interagem apenas com os seus hospedeiros, mas também com outros plasmídeos presentes na mesma população bacteriana. Nesta tese foram analisadas interacções entre plasmídeos conjugativos. Demonstrou-se que a eficiência de conjugação dos plasmídeos depende das suas interações com outros plasmídeos. Mais especificamente, a eficiência de conjugação de um plasmídeo tende a aumentar se outro plasmídeo estiver presente na mesma população bacteriana mas habitando em células diferentes. Contrariamente, quando os dois plasmídeos se encontram simultaneamente na mesma célula hospedeira, a eficiência de conjugação de um deles tende a diminuir. Esta redução da eficiência de conjugação é causada por proteínas de inibição de fertilidade (FIN). Demonstramos também que mecanismos de inibição distintos se podem complementar e consequentemente determinar um maior efeito de inibição.

A expressão da maquinaria conjugativa impõe custos metabólicos, energéticos e/ou fisiológicos, na célula hospedeira. Devido a estes efeitos negativos, os plasmídeos reprimem geralmente a expressão da sua própria maquinaria conjugativa, sendo a sua desrepressão apenas transitória. Devido a esta transitoriedade, pensa-se que a transferência simultânea de vários plasmídeos que co-habitam na mesma célula hospedeira constitua um evento raro, pois requer desrepressão simultânea de ambos os plasmídeos. Resultados obtidos nesta tese opõem-se a esta hipótese, sugerindo que a desrepressão de plasmídeos co-infectantes seja activada pelos mesmo estímulos.

Nesta tese foi medido o *fitness* (determinado como a taxa de crescimento) de células bacterianas que contenham plasmídeos. Podendo assim determinar-se o custo de *fitness* que os plasmídeos impõem à célula hospedeira. Demonstra-se que os custos de *fitness* estão positivamente co-relacionados com a eficiência de conjugação dos plasmídeos. Assim, plasmídeos que exibam maior eficiência de conjugação conferem também maiores custos de *fitness* às células hospedeiras (reduzindo a sua taxa de crescimento). Estes resultados

demonstram um *trade-off* entre conjugação e *fitness*. Além disso, combinações de dois plasmídeos tendem a exibir epistasia negativa, isto é, impõem um custo ao hospedeiro que é superior à soma dos seus custos individuais.

Nem todos os efeitos causados pelos plasmídeos aos seus hospedeiros são negativos. Por exemplo, vários plasmídeos contêm genes que conferem benefícios aos seus hospedeiros. Além disso, os plasmídeos podem aumentar a habilidade das suas células hospedeiras produzirem biofilmes. Nesta tese demonstra-se que as interacções entre plasmídeos contribuem para a formação de biofilmes. Contudo, o resultado dessas interacções varia consoante a combinação de plasmídeos analisada.

Keywords: plasmídeo, *host range*, conjugação, biofilme, custo de *fitness*

Summary

Plasmids are double-stranded DNA molecules commonly isolated from bacterial populations. They replicate inside their host cells and depend on the replication mechanisms of their hosts. However, plasmids also encode several factors involved in their own replication. Indeed, plasmids can code for various functions that affect their stability in host cells and in bacterial populations. Moreover, some plasmids can be transmitted horizontally (between cells). Conjugation is the main process through which plasmids transfer horizontally. This process requires the contact between plasmid-donor and recipient cells and, is encoded in the plasmid genome. Altogether, these functions determine to which hosts plasmids can be transferred and be stably maintained, that is, the plasmid's host range. One of the objectives of this thesis is to evaluate the impact of each of these functions (replication, stability and mobility) on host range. We show in this thesis that replication is a major function affecting the variation of the host range of plasmids.

Plasmids interact with their hosts but also with other plasmids that are present in the same bacterial population or in the same host cell. Here we show that the conjugative efficiency of plasmids depends on such interactions. More specifically, we demonstrate that plasmids tend to increase their conjugative efficiency if a distinct plasmid is present in different cells of the same host population. However, if plasmids are present in the same host cell, the conjugative efficiency of one of the plasmids tends to decrease. This reduction results from the actions of fertility inhibition (FIN) proteins. We also show that distinct FIN mechanisms can complement each other, thus providing stronger inhibition.

Expression of the conjugative machinery imposes a metabolic/energetic burden on host bacteria. Moreover, it also alters cell physiology due to the expression of conjugative pili (appendages that promote cellular contact between donor and receptor cells) on the host's surface. Thus, plasmids often repress the expression of their conjugation machinery. Its de-repression occurs only transiently. For this reason, it is hypothesized that simultaneous transfer of multiple plasmids present in the same host is a rare event, because it requires simultaneous de-repression of both plasmids. Here we show that co-transfer of plasmids is more frequent than anticipated by this hypothesis, suggesting that de-repression of both plasmids obey to common stimuli.

Furthermore, we measured the fitness (as growth rates) of plasmid-carrying bacteria. Thus, we assessed the fitness cost associated with plasmid carriage. We show that fitness costs are positively co-related with the conjugative efficiency of plasmids. Plasmids exhibiting greater conjugative efficiency impose greater fitness costs on their hosts, that is, reduce their fitness (growth rates). This reveals a trade-off between conjugation and fitness. Moreover, we observed that approximately half (16 out of 33) of plasmid pairs (two co-infecting plasmids) impose greater fitness costs than the sum of the individual cost of each plasmid alone, but only about 1/5 of the plasmid pairs (7/33) impose lower fitness costs than the sum of the individual cost of each plasmid alone.

Nevertheless, not all the effects of plasmids on their hosts are negative. For instance, plasmids can carry genes that code for beneficial traits, such as antibiotic-resistance. Moreover, plasmids can also enhance the ability of hosts to form biofilms. We studied this subject and showed that two plasmids affect their

hosts ability to form biofilms in a way that is specific for each two-plasmid combination.

Keywords: plasmid, host range, conjugation, biofilm, fitness cost

Resumo Alargado

Biologicamente, os plasmídeos são moléculas de DNA de cadeia dupla frequentemente isoladas a partir de populações bacterianas. No entanto, os plasmídeos podem ser encontrados quer em células procariotas, quer em células eucariotas. Os plasmídeos replicam-se no interior das suas células hospedeiras e dependem da maquinaria celular para a sua replicação. Os plasmídeos podem também codificar vários factores envolvidos neste processo, deste modo controlando a sua própria replicação. Plasmídeos distintos exibem diferentes níveis de dependência dos seus hospedeiros. Tal dependência especifica em que hospedeiros cada plasmídeo se pode replicar.

Os plasmídeos podem codificar funções adicionais. Entre estas destacam-se mecanismos que actuam de modo a assegurar uma correcta segregação dos plasmídeos para células filhas durante a divisão celular. Deste modo, os plasmídeos codificam diversas estratégias para prevenir a emergência de células que não herdem plasmídeos, como por exemplo: i) expressão de sistemas de partição, ii) manutenção de um elevado número de cópias, iii) expressão de sistema toxina-antitoxina. Os sistemas de partição são sistemas que asseguram uma correcta distribuição dos plasmídeos pelas células filhas. Alternativamente, se a distribuição dos plasmídeos pelas células filhas for aleatória, um maior número de cópias do plasmídeo maximiza a probabilidade de pelo menos uma cópia ser herdada em cada célula filha. Os sistemas toxina-antitoxina actuam através da eliminação de células resultantes da divisão bacteriana que não tenham recebido o plasmídeo. Tal acontece porque a antitoxina é uma molécula instável e requer uma constante expressão. Uma vez perdido o plasmídeo, não há síntese de antitoxina e, a toxina (que é mais estável) elimina esta célula que já não possui o plasmídeo. De um modo geral, estes mecanismos asseguram que os plasmídeos sejam mantidos estavelmente nos seus hospedeiros.

Além de herdados verticalmente durante a divisão celular, alguns plasmídeos podem também ser transmitidos entre células (transmissão horizontal). O principal processo envolvido na transmissão horizontal de plasmídeos é a conjugação. Este processo requer contacto celular entre a célula dadora e receptora do plasmídeo. A maquinaria necessária para a conjugação é codificada no genoma dos plasmídeos. Em termos de mobilidade, os plasmídeos podem ser classificados como: i) conjugativos, ii) mobilizáveis ou iii) não-mobilizáveis. Plasmídeos conjugativos são aqueles que codificam a totalidade da maquinaria de conjugação. Os plasmídeos mobilizáveis codificam apenas uma porção destes genes. Consequentemente, para serem transferidos horizontalmente, os plasmídeos mobilizáveis dependem da existência de plasmídeos conjugativos na mesma célula para fornecerem a maquinaria conjugativa em falta. Os plasmídeos não-mobilizáveis não codificam nenhum dos genes envolvidos no processo de conjugação.

De um modo geral, estas funções definem a amplitude de hospedeiros de um plasmídeo, ou seja, determinam para que células os plasmídeos se podem transferir horizontalmente e em que células se conseguem replicar e manter estavelmente. Um dos objectivos desta tese foi avaliar o impacto que cada uma destas funções (replicação, estabilidade e mobilidade) tem na amplitude de hospedeiros dos plasmídeos. Concluiu-se que a replicação é a função que mais contribui para a amplitude de hospedeiros dos plasmídeos. Os mecanismos de

estabilidade parecem contribuir em menor grau. Trabalhos futuros deverão ser desenvolvidos de modo a avançar o conhecimento relacionado com a manutenção de plasmídeos em populações bacterianas.

Os plasmídeos não interagem apenas com os seus hospedeiros, mas também com outros plasmídeos presentes na mesma população bacteriana, ou até presentes na mesma célula hospedeira. Por exemplo, os plasmídeos mobilizáveis necessitam de interagir com plasmídeos conjugativos para poderem ser transmitidos horizontalmente. Nesta tese foram analisadas apenas interacções entre plasmídeos conjugativos. Demonstrou-se que a eficiência de conjugação dos plasmídeos depende das suas interações com outros plasmídeos. Mais especificamente, mostrou-se que a eficiência de conjugação de um plasmídeo tende a aumentar se outro plasmídeo estiver presente na mesma população bacteriana, mas habitando células diferentes. Verificou-se que este aumento se deve a uma maior estabilização do contacto entre a célula dadora e a receptora, o qual é essencial para o processo de conjugação. Por oposição, quando os dois plasmídeos se encontram simultaneamente na mesma célula hospedeira, a eficiência de conjugação de um deles tende a diminuir.

A redução da eficiência de conjugação é causada por proteínas de inibição de fertilidade (FIN). Algumas proteínas FIN foram já identificadas e os seus mecanismos de actuação caracterizados. Conhecem-se três mecanismos distintos: i) inibição da expressão de pili conjugativos (apêndices que promovem o contacto celular entre a células dadora e a receptoras), ii) degradação de DNA plasmídico, e iii) interacção com a maquinaria de transporte de DNA. Resultados obtidos nesta tese demonstram que mecanismos de inibição distintos se podem complementar e consequentemente determinar um maior efeito de inibição. Estudos futuros acerca de proteínas FIN poderão contribuir para a elaboração de estratégias que previnam a disseminação da resistência a antibióticos codificada em plasmídeos.

A expressão da maquinaria de conjugação requer recursos celulares e, consequentemente, impõe um custo metabólico/energético na célula hospedeira. Além disso, a exposição de pili conjugativos à superfície da célula hospedeira altera a fisiologia celular. Adicionalmente, os pili conjugativos podem ser utilizados como receptores virais, assim susceptibilizando as células hospedeiras a infecções virais. Devido a estes efeitos nefastos, os plasmídeos reprimem geralmente a expressão da sua própria maquinaria conjugativa, sendo a sua desrepressão apenas transitória. Devido a esta transitoriedade, pensa-se que a transferência simultânea de vários plasmídeos que co-habitam na mesma célula hospedeira constitua um evento raro, uma vez que requer a desrepressão simultânea de ambos os plasmídeos. Resultados obtidos nesta tese contrariam esta hipótese, demonstrando que existe uma tendência para os plasmídeos serem co-transferidos tão eficientemente como o plasmídeo que possui menor eficiência de transmissão. Estes resultados sugerem a possibilidade de a desrepressão de plasmídeos co-infectantes ser activada pelos mesmos estímulos. No contexto da resistência a antibióticos, este fenómeno revela que a aquisição de múltiplos plasmídeos, que conferem tais fenótipos, é mais provável do que esperado antes da realização desta tese.

Nesta tese foi também medido o *fitness* (determinado como a taxa de crescismento) de células bacterianas contendo plasmídeos. Desta forma, foi determinado o custo de *fitness* que os plasmídeos impõem à célula hospedeira. Demonstra-se que os custos de *fitness* estão positivamente co-relacionados com

a eficiência de conjugação dos plasmídeos. Assim, plasmídeos que exibam maior eficiência de conjugação conferem também maiores custos de *fitness* às células hospedeiras (reduzindo a sua taxa de crescimento). Estes resultados demonstram um *trade-off* entre conjugação e *fitness*.

Epistasia pode ser definida como interacção genética. Nesta tese será analisada a epistasia entre plasmídeos co-infectantes, em termos de *fitness*. Neste sentido, observamos epistasia se o custo imposto por dois plasmídeos diferir da soma dos seus custos individuais. Observou-se que a maioria das combinações de dois plasmídeos analisadas nesta tese exibe epistasia negativa. Tal significa que o custo de *fitness* imposto por dois plasmídeos é superior àquele que é esperado se os custos fossem aditivos. No contexto, da resistência a antibióticos, bactérias que contenham múltiplos plasmídeos parecem exibir uma desvantagem competitiva em relação àquelas que não contêm plasmídeos e, consequentemente deveriam ser contra-seleccionadas na ausência de antibióticos.

Nem todos os efeitos causados pelos plasmídeos aos seus hospedeiros são negativos. De facto, vários plasmídeos contêm genes que conferem benefícios aos seus hospedeiros. Por exemplo, os plasmídeos podem codificar genes que conferem resistência a antibióticos ou metais pesados. Podem também ser codificados genes que codificam factores de virulência e que aumentam a capacidade das bactérias produzirem infecções. Além disso, os plasmídeos conjugativos podem aumentar a capacidade das suas células hospedeiras produzirem biofilmes (comunidades bacterianas embebidas numa matriz polimérica, que constituem estruturas resistentes a vários factores prejudiciais). Este efeito envolve a produção de pili conjugativos que aumenta a adesão das células. Uma vez que plasmídeos conjugativos interagem entre si, afectando a eficiência da transferência horizontal por conjugação, foi também avaliado se estas interacções afectam a capacidade para formar biofilmes. Revela-se nesta tese que tais interacções afectam a formação de biofilmes. Contudo, o resultado dessas interacções varia consoante a combinação de plasmídeos analisada.

Keywords: plasmídeo, *host range*, conjugação, biofilme, custo de *fitness*

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Chapter 1

General Background

Plasmids – historical overview

Nowadays a plasmid can be broadly defined as a DNA element that replicates autonomously within a suitable host cell (most often bacteria). Along with bacteriophages and transposons, plasmids belong to the classically defined mobile genetic elements. However this classification is outdated due to the discovery of various chimerical elements (Leplae et al., 2004).

The concept of plasmid took some time to be properly formalized. These historic events have been reviewed in (Kado, 2014; Lederberg, 1998; Summers, 2015). Let us briefly travel in time to understand how it happened.

More than a hundred years ago, (Sutton, 1903) and (Boveri, 1904) proposed Mendelian characters, which we now call genes, to be located in the cell nucleus, more specifically in the chromosomes. Several works, including those by Morgan with fruit flies, improved this hypothesis (Morgan, 1926).

The absence of a physically defined cellular nucleus in prokaryotes raised the doubt of whether these organisms possessed genes. In 1946, the work of Lederberg and Tantum shed some light on this subject by identifying a mating system in *Escherichia coli* (Lederberg & Tatum, 1946). This finding allowed the characterization of the genetic map of *E. coli* by studying frequencies of recombination in such matings (Lederberg, 1947). Thus, providing evidence of bacteria as gene-carrying organisms.

Further studies, showed that only a portion of *E. coli* isolates could mate with the strain (K-12) found by Lederberg (Cavalli & Heslot, 1949; Lederberg, 1951b). Hayes later showed that these matings were asymmetric, requiring a donor and a receptor cell, unlike the process of zygote formation through cell fusion (Hayes, 1952). This finding was however insufficient to explain why only some strains could successfully mate – a problem of sexual compatibility. The answer arrived soon as both the groups led by Lederberg and Hayes independently identified the F (“fertility”) “factor”, which seemed to behave as an “infectious particle” (Hayes, 1953; Lederberg et al., 1952). Other “infectious particles” were soon discovered: bacteriophage λ and the colicin (a toxin produced by some strains of *E. coli*) “factor” (Fredericq, 1963; Lederberg, 1951a).

In 1952, Lederberg invented the term “plasmid”, from cytoplasm and the latin suffix “id”, to denote any extrachromosomal genetic element (Lederberg, 1952). At the time, this was a rather general term that could also be used to mention organelles such as mitochondria and chloroplasts. In 1958, Jacob and Wollman proposed the term “episome” to mention genetic elements that could optionally associate with chromosomes, such as bacteriophage λ, the F and colicin factors (Jacob & Wollman, 1958). For many, the terms were interchangeable and “episome” became the preferred expression, instead of “plasmid”.

Bacteriophages (viruses that infect bacteria), were independently discovered by Frederick W. Twort and Felix d’Hérelle, back in 1910’s (d'Herelle, 1917; Duckworth, 1976; Twort, 1915). Meanwhile, bacteriophages from various *Salmonella* serotypes were shown to transfer genes between bacteria (Zinder & Lederberg, 1952) – a process termed transduction.

The discovery of other “factors” flourished very soon. Japan led the way by recognizing R-factors, which were elements with the ability to transfer antibiotic resistance between bacteria (Nakaya et al., 1960; Watanabe, 1963). The term “episome” was still in use, however as evidence was gathered showing that most R-factors were, often, unable to integrate in chromosomes, the term “plasmid” became more frequent.

Plasmids became fairly known for their ability to transfer genes, nonetheless, bacteriophages were preferentially used to study bacterial genetics (Patrick, 2015). However, in 1974, Chang and Cohen successfully cloned genes of *Staphylococcus aureus* in plasmid pSC101 and (after plasmid transformation) expressed them in *E. coli* (Chang & Cohen, 1974). Bacteriophages, employed until then, had one main disadvantage, as they were only useful when working with closely related bacteria. Since 1974, plasmids became the method of choice employed in genetic studies due to their potential to overcome this species barrier (Cohen, 2013).

Due to their usefulness, nowadays, the term plasmid is often associated with genetic engineering. Natural plasmids are still a relevant subject of study nowadays and many have been discovered and sequenced in the past years. The current state of plasmid biology was subject of numerous meetings, one of which is summarized in (Wegrzyn, 2005). For instance, new challenges emerged, such as their classification. By standard definition, plasmids are small elements replicating autonomously and do not encode essential functions. However, megaplasmids and minichromosomes have been found. Additionally, the essentiality of some genes is debatable. On the other hand, old challenges remain, for plasmids have been extensively studied in terms of their genetic mechanisms, yet, eco-evolutionary knowledge is not enough to explain their persistence in many ecosystems. Thus, plasmid biology still constitutes a relevant field of study, hopefully prompting exciting discoveries to come in a near future.

Plasmids in a nutshell

Plasmids can be rather diverse and have already been found (and are able to transfer) in all three domains of life: Archaea, Bacteria and Eukarya (Heinemann, 1991). They exist in various shapes and sizes, either circular or linear and ranging from few kilobase pairs (kbp) to megabase pairs (Mbp), reviewed in (Shintani et al., 2015). Alternatively, these later megaplasmids can be termed chromids or secondary chromosomes (Harrison et al., 2010).

Moreover, plasmids can be of natural or artificial origin. In the scope of this thesis, the term plasmid will be solely employed to mention elements of natural origin, regardless of their size – thus, including chromids. The term vector will be reserved for artificial constructs.

In the next pages we will dissect plasmids, to understand what makes them what they are. Plasmids are organized in modules determining essential and accessory functions (Thomas, 2000; Toussaint & Merlin, 2002). This overview will focus on functions required for plasmid existence; the most fundamental being the ability to replicate – a characteristic shared by plasmids and vectors. Natural selection affects plasmids and shapes them by acting on characters leading to their persistence. Thus, besides replication, we will highlight characters such as those required for maintenance and transmission. We will mention the importance of accessory regions. Furthermore, we will also overview the role of

these features in interactions between plasmids and their hosts but also between plasmids and other plasmids.

The work produced in this thesis (chapters 2 through 8) addresses such interactions. More specifically, in chapter 2, we aim to understand the contribution of several plasmid features to their host range (set of hosts able to sustain a given plasmid), while subsequent chapters (3-8) will be focused on interactions between plasmids and on their contribution to phenotypes such as plasmid transmission.

Replication

Plasmids have a module devoted to replication – the replicon. Such region comprises the origin of replication and the elements involved in the control of replication (reviewed in (del Solar et al., 1998)). Generally, the sequences in the origin of replication are rich in A and T nucleotides, which melt more easily than GC regions, thus facilitating the access of the replicative machinery (Morgan, 2015). Additionally, many replicons encode a Rep protein, which is required for the initiation of replication (reviewed in (del Solar et al., 1998)). Despite their ability to replicate autonomously, plasmids depend on their host's replicative machinery, requiring for instance the action of factors such as DNA polymerases.

Circular and linear plasmids replicate differently. Circular plasmids have been extensively studied and their replication can be classified in three main mechanisms: theta type, rolling circle and strand displacement (reviewed in (del Solar et al., 1998)). Theta type replication has been associated which plasmids frequently found in Gram-negative bacteria while plasmids from Gram-positive species have been associated with rolling circle replication, yet exceptions exist. This association stems from the fact that theta replication has been extensively studied among plasmids of Gram-negative bacteria and vice-versa. Most examples of strand displacement replication were found in plasmids of the IncQ family. Linear plasmids vary in structure, which in turn determines their replication mechanism (reviewed in (Meinhardt et al., 1997)).

In most cases, initiation of theta type replication starts by opening double-stranded DNA at the origin of replication. Most plasmids using this mode of replication require the plasmid-encoded Rep protein and the host's DnaA protein. Plasmids such as ColE1 require host enzymes because they do not code for a Rep protein. Subsequently, a replication complex of host proteins (including DNA polymerase III) is assembled and an RNA primer is synthesized either by plasmid or host enzymes. Both DNA strands replicate simultaneously. Replication can be either uni- or bi-directional and during this process, DNA molecules acquire a characteristic θ-like shape (hence the attributed name).

Rolling circle replication was discovered in bacteriophages and, since then, has been identified mostly in plasmids of Gram-positive bacteria but also in those of Gram-negative bacteria and *archaea*. Most of the plasmids using this replication mechanism are small (less than 10 kbp), nevertheless, not all small plasmids use this mode of replication. Rolling circle replication is uni-directional and involves two phases. In the first phase the plasmid's Rep protein introduces a nick on the plus strand (the one generally transcribed into RNA) at the double-stranded origin. This action, results in a free end that can be used as primer for DNA synthesis carried out by host enzymes including DNA polymerase III. As elongation proceeds, the parental plus strand is displaced. Termination of

replication is also mediated by the Rep protein. At this stage, there is a complete double-stranded DNA molecule and the single-stranded parental plus strand. The second phase involves only host factors, such as RNA polymerase for the synthesis of an RNA primer (at the single-stranded origin) and DNA polymerase III to catalyse DNA synthesis.

Strand displacement replication is typical of IncQ plasmids and has been mainly studied in plasmid RSF1010. This plasmid encodes three proteins: RepA, RepB and RepC. RepC is the initiator protein that binds the origin of replication and then interacts with the helicase RepA. Their action exposes *ssiA* and *ssiB* sites in single-stranded DNA, which are then recognized by the primase RepB. The sites *ssiA* and *ssiB* are adjacent but located in different strands, which allows replication to proceed in both directions with consequent strand displacement. This mode of replication is therefore independent of host initiation factors such as helicase and primase.

Linear plasmids commonly found in spirochetes have terminal inverted repeats and closed hairpin loops at their ends. Similarly to some virus, replication is possible after nicking of the loops allowing the DNA to circularize. Linear plasmids typically found in Actinomycetes have 5' attached proteins that prime DNA synthesis.

Plasmids replicate autonomously but depend, however, on the host's replicative machinery. Thus, plasmids are only able to replicate in hosts providing them with all the required factors. Logically, not only the quantity of factors but also their affinity to interact with plasmids is important for successful plasmid replication. This dependence on the host machinery determines the set of hosts in which a plasmid can replicate, which, for now, will be synonymous to host range. Some plasmids are more dependent on host factors than others, therefore exhibiting narrower host ranges. For instance, IncQ plasmids have broad host ranges and replicate by strand displacement which is independent of host initiation factors. Replication strategies employed by broad host range plasmids are reviewed in (delSolar et al., 1996; Jain & Srivastava, 2013).

Due to the action of the Rep protein, initiation of rolling circle replication is independent of host primases, which can explain the broad host range of plasmids using this mode of replication. However, associations between the Rep protein and a host helicase may be essential to unwind DNA strands. It was shown however that helicases of Gram-positive bacteria can recognize non-cognate DNA, which can contribute to plasmid broad host range. Moreover, the second phase of replication involves only host factors, implying that efficient interaction between the single-stranded origin (*sso*) and host machinery is essential. Distinct types of *sso* have been identified, and some of those can be very efficient in various hosts. Some plasmids can even carry more than one *sso*. Furthermore, some plasmids without a *sso* encode instead an antisense RNA that serves as primer.

Thus, we can speculate from the evidence provided so far that plasmids replicating either by strand displacement or rolling circle mechanisms could attain broader host ranges than θ replicating plasmids that depend on several host initiation factors. Nonetheless, some plasmids, such as those belonging to the IncP family, depend on host initiation factors but exhibit broad host ranges. These plasmids display plasticity in their Rep proteins. The TrfA protein of plasmid RK2 is made in two forms (from two in-frame translational starts), a short (TrfA2) and a longer (TrfA1) version, which differently interact with host factors. For instance,

replication in *Pseudomonas putida* and *Escherichia coli* requires only TrfA2, while in *Pseudomonas aeruginosa* both forms are essential.

The presence of multiple replicons (units of replication) could also broaden plasmid host ranges. In this sense, different origins of replication could function in distinct hosts. Broad host range plasmids such pCU1 and pJD4 contain three origins of replication. Nonetheless plasmid R6K and many IncF plasmids also contain multiple origins of replication but exhibit narrow host ranges. The failure of the latter plasmids to attain broader host ranges could be due to the specificity of host factors with which they can efficiently interact.

Plasmid replicons also determine the number of copies in which the plasmid exists. This is achieved by the elements controlling the initiation of replication. It is known, however, that the copy number of a given plasmid is also affected by the host strain and growth conditions. Diverse regulatory mechanisms exist, which are reviewed in (Chattoraj, 2000; del Solar & Espinosa, 2000). Regulatory mechanisms maintain a plasmid in a constant number of copies, and allow each copy to replicate only once (on average), so that upon cell division both cells inherit the adequate number of copies.

Regulation of copy number is important for plasmid maintenance in the host and it has been shown that plasmid copy number is correlated with host fitness burden (Harrison et al., 2012; Patnaik, 2000). Thus, as the number of copies increases the slower the host reproduces due to the additional metabolic load. Consequently, such hosts can be outcompeted by faster reproducing hosts present in the same habitat. This will lead to counter-selection of plasmid-carrying cells and ultimately decrease plasmid persistence.

Regulatory mechanisms can have a negative effect on plasmid stability, a phenomenon termed plasmid incompatibility, which happens when plasmids exhibiting similar replicons inhabit the same host cell (reviewed in (Novick, 1987)). In this case, copies of two different plasmids are recognized by the same regulatory mechanisms and not allowed to replicate as much as they were supposed to. Instead of “counting” the number of copies of each plasmid, regulatory mechanisms are “counting” the total number of copies of both plasmids. Eventually, this results in plasmid segregation: some cells will only harbour one of the plasmids while other cells will only carry the other. Therefore, plasmids encoding identical replicons can hardly persist in the same host cell for long, unless some kind of selective pressure is exerted in that sense. Such plasmids are said to belong to the same incompatibility group due to their inability to co-exist.

The replicon is thus essential for plasmid replication and specifies in which hosts it can replicate. Regulatory mechanisms encoded in the replicon also determine number of copies in which the plasmid exists, which affects the growth of its host and determines plasmid incompatibility.

Stability

Besides replication, plasmids also require correct distribution to daughter cells during cell division. Otherwise, plasmid-free cells arise. Mechanisms for plasmid segregation are reviewed in (Ghosh et al., 2006). Plasmid copy number plays an important role once more, for the higher the number of plasmid copies, the higher is the probability that both cells receive at least one plasmid copy. Thus, high copy number plasmids can just segregate randomly. However, to

guarantee correct inheritance, low copy number plasmids require specific mechanisms, such as partition systems.

Plasmid encoded partition systems, in a generic way, comprise two proteins ParA and ParB and a centromere-like region *parC*. ParB recognizes and binds to the centromere-like region *parC* and recruits ParA. In turn, ParA has ATPase activity and is required for the migration of plasmid copies away from the centre of the cell towards opposite cell poles. Generally, these systems consist in a bi-cistronic operon. However, they can also be encoded together with the Rep protein as a single operon. Such is the case of the repABC systems found solely in the large plasmids of Alphaproteobacteria (reviewed in (Pinto et al., 2012)). RepC, being the Rep protein is required for initiation of replication, while RepA and RepB are required for partition. Host chromosomes also require partition systems but these are distinct from those used by plasmids.

Partition systems can have a negative impact on plasmids in the same sense that replication control systems do, that is, partition systems determine plasmid incompatibility (reviewed in (Novick, 1987)). Thus, copies of two different plasmids are identically recognized by partition system and, as a consequence, different copies will segregate into different cells.

High copy number, however face another problem, as recombination of plasmid copies leads to the formation of plasmid oligomers (Summers et al., 1993). In this oligomeric form, random segregation of copies becomes more difficult. To solve this difficulty, plasmids encode multimer resolution systems, which comprise *res* sequences and a resolvase enzyme. By recombining *res* sites, this enzyme returns the oligomers to the monomeric form, which can later randomly segregate. Some plasmids encode fully functional multimer resolution systems, while others only possess *res* sequences and require host resolvases (Austin et al., 1981; Sharpe et al., 1999; Summers & Sherratt, 1984; Tolmasky et al., 2000).

The strategies just mentioned are useful prior to cell division, however other strategies exist, which work after cell division to eliminate plasmid-free cells. As a whole, strategies for plasmid maintenance have been reviewed in (Bahl, 2009; Zielenkiewicz & Ceglowski, 2001). Post-segregational killing systems represent one of the strategies involved in plasmid persistence acting after cell division (reviewed in (Yamaguchi et al., 2011)). Alternatively, they can be termed addiction-systems or toxin-antitoxins. These systems consist on two products, a stable toxin and an unstable antitoxin (encoded by the plasmid). In the absence of the antitoxin, cells die due to the action of the toxin. Thus, continuous production of the antitoxin is essential for plasmid persistence. Whenever plasmid loss occurs, cells are no longer able to synthesize the antitoxin. However, the toxin, which is more stable, is still present in the cells and leads to their death. Therefore, this mechanism allows plasmids to eliminate plasmid-free cells upon missegregation.

Type II restriction-modification systems can exert a similar function to that of post-segregational killing systems (Kulakauskas et al., 1995). In general, restriction-modification systems contain two components, a methyl-transferase and a restriction enzyme (reviewed in (Roberts et al., 2003; Vasu & Nagaraja, 2013)). The methyl-transferase is responsible for adding methyl groups to specific DNA sequences. The restriction enzyme recognizes the same sequences and cleaves them when they are un-methylated. These systems can be classified in several types according to their mode of action. A particularity of

type II systems is that the two enzymes can act independently. Thus, type II restriction-modification systems can function analogously to toxin-antitoxin systems. In this sense, the restriction enzyme acts as a toxin that degrades the host genome if the respective antitoxin, the methyl-transferase, is not present in the cell.

In addition to their function as stabilizing agents, addiction systems have been shown to play a role in interactions between plasmids (Cooper & Heinemann, 2000a; Cooper & Heinemann, 2005). Considering two incompatible plasmids inhabiting the same host cell, it was shown that addiction systems could be useful to displace competitor plasmids. That is, after missegregation, cells carrying the plasmid encoding the addictive system (*psk*$^+$) survived. However, cells inheriting the plasmid not encoding the addictive system (*psk*$^-$) died due to the action of the toxin. Moreover, it was shown that cells carrying *psk*$^+$ plasmids reproduce more slowly than those carrying the *psk*$^-$ plasmid. Thus, continuous synthesis of toxin-antitoxin products seems to impose a metabolic burden on plasmid hosts.

Bacteria can produce toxins that are active against closely related species (reviewed in (Riley & Wertz, 2002)). This kind of proteic toxins is termed bacteriocin and many of them are encoded in plasmids. After secretion, toxins adsorb to sensitive cells and kill them. Plasmids not only encode production but also immunity against bacteriocins, rendering host cells insensitive to their lethal action. Therefore, bacteriocins can be useful for plasmid persistence because they enhance the displacement of competitor cells that are plasmid free or carry different plasmids (Zielenkiewicz & Ceglowski, 2001).

Horizontal transmission

In general, plasmids require functions for replication and stability to persist in their hosts. Some plasmids possess a third function – horizontal transmission. This ability allows them to transfer between cells, while vertical transmission solely determines their inheritance from mother to daughter cells. Horizontal gene transfer is mainly mediated by three (classical) mechanisms: transformation, transduction and conjugation (reviewed in (Frost et al., 2005)). Transformation is the ability to uptake DNA from the environment. Transduction requires bacteriophages to mistakenly transport non-viral genetic material. Conjugation was first observed in plasmids and, as previously mentioned, was the property that allowed their discovery. Conjugation is the main mechanism of plasmid horizontal transfer and depends on cellular contact between donor and recipient cells. Acquisition of genes through transformation and transduction requires recombination with host genomes and thus the host recombination machinery. In contrast, conjugation is a process encoded in conjugative elements, such as plasmids and integrative conjugative elements (ICEs). In the scope of this thesis we will only consider plasmids.

Only approximately half of all sequenced plasmids can transfer through conjugation (Shintani et al., 2015; Smillie et al., 2010). Among these, only half (that is, approximately a quarter of the sequenced plasmids) is conjugative, which means that they encode all the machinery required for their self-transfer. The other half of plasmids transferred through conjugation is termed mobilizable. Mobilizable plasmids encode only a portion of the genes required for their transmission. Consequently, they depend on conjugative plasmids (or ICEs) to supply additional functions. Since mobilizable plasmids only encode part of the

conjugative machinery, it is not surprising that they tend to be smaller than conjugative plasmids. Plasmids unable to transfer through conjugation are termed non-mobilizable. Plasmid mobility has been reviewed in (Smillie et al., 2010).

DNA preparation for conjugation resembles the process of replication by rolling circle (reviewed in (de la Cruz et al., 2010; Smillie et al., 2010)). It requires an origin sequence, the origin of transfer *oriT*, and a relaxase which is the protein recognizing *oriT* and that nicks it to produce the DNA strand that will be transferred. These components comprise the relaxosome and are the common requirements of both conjugative and mobilizable plasmids. Conjugative plasmids harbour further genes which encode a type IV secretion system commonly known as mating-pair formation (MPF) complex. In plasmids common among Gram-negative bacteria, sex pili constitute the MPF. The MPF, also known as the transferosome, promotes cellular contact and signals the start of DNA processing and transfer. The connection between the transferosome and the relaxosome is mediated by a type IV coupling protein (T4CP) which forms the conjugative pore and energizes the transport of the relaxase-bound DNA to the recipient cell. Once in the recipient cell, the relaxase promotes the re-circularization of the single-stranded plasmid.

Regarding the MPF, plasmids from Gram-negative bacteria encode many genes required for pilus synthesis. For instance, plasmid F requires at least 15 genes just to synthesize sex pili (Arutyunov & Frost, 2013). Plasmids belonging to the IncI incompatibility group even encode an additional type of pilus devoted to maximize conjugative transfer in liquid habitats (Bradley, 1984; Komano et al., 1994). Gram-positive bacteria have less complex cell structures as they lack outer membrane, and in some cases also lack cell wall, thus, the machinery of plasmids recurrent among Gram-positive bacteria seems to be simpler. Indeed, there is no evidence of sex pili among the plasmids commonly associated with Gram-positive bacteria, supporting the idea that their machinery might be simpler (reviewed in (Grohmann et al., 2003)). Furthermore, *Streptomyces* species exhibit a characteristic growth resembling a mycelium and plasmids found in such species do not seem to encode complex transferosomes to produce cellular contact but instead use a sole protein for DNA transport. Pheromone-dependent plasmids are found in enterococci and their conjugation is stimulated by pheromones produced by recipient cells. For these plasmids, only one surface protein is necessary to establish cellular contact. These examples have been reviewed in (Grohmann et al., 2003).

Conjugative transfer is a controlled process (reviewed in (Frost & Koraimann, 2010)). Availability of recipient cells is one of the factors that can stimulate conjugative transfer. This is well exemplified by the aforementioned pheromone-dependent plasmids of *enterococci* (Clewell, 2007; Dunny, 2007). Recipient cells secrete pheromones that induce the expression of genes required for conjugation in plasmids of neighbouring bacteria. In turn, this leads to aggregation and mating. Moreover, conjugation efficiency depends on host cells and there is evidence that some strains have an amplificatory effect on plasmid dissemination (Dionisio et al., 2002). It has also been shown that quorum sensing promotes conjugation in *Yersinia* and *Agrobacterium* (now classified as *Rhizobium* (Young et al., 2001)) (Atkinson et al., 2006; White & Winans, 2007). Environmental conditions, such as temperature, pH, the availability of oxygen and

nutrients and the growth phase experienced by the host can influence conjugative efficiency too (reviewed in (Frost & Koraimann, 2010)).

Plasmid encoded mechanisms controlling conjugation exist too (reviewed in (Frost & Koraimann, 2010)). The process repressing the expression of the genes required for conjugation is termed fertility inhibition and it has been studied mainly in plasmids belonging to the IncF incompatibility group. In a standard state, plasmids do not express these conjugative traits, although some mutants are constitutively de-repressed. Such is the case of plasmid F due to an insertion sequence (IS3) disrupting the *finO* gene. This gene codes for a protein that prevents the degradation of the antisense RNA, FinP, which in turn downregulates the translation of the activator, TraJ. Thus, the low levels of FinP lead to the de-repressed state of plasmid F. In repressed cells, the levels of FinO and FinP prevent the expression of conjugation. This type of regulation has another consequence a phenomenon designated epidemic spread. Until high enough levels of FinOP repressor are reached, recipient cells that have just acquired the plasmid (transconjugants), exhibit very efficient plasmid transmission.

Besides IncF pasmids, some IncP plasmids have also been studied in terms of their regulation of horizontal transfer. Their strategy is quite different as they modulate gene expression through repression instead of employing an activator. Moreover, these plasmids seem to co-regulate replication, partition and horizontal transmission with only a few key plasmid-encoded regulators. Little is known for plasmids other than those belonging to these two incompatibility groups. Nonetheless, it is interesting to note that, in mobilizable plasmids, expression of mobilization genes seems to be constitutive and associated with replication. Additionally, they have higher copy number than conjugative plasmids, which they exploit for horizontal transfer.

It may seem contradictory, at first sight, that plasmids repress their own horizontal transfer, as it would appear to decrease their persistence. However, by repressing the efficiency of conjugation, plasmids can enhance their chances of maintenance. A recent work showed that plasmids encoding fertility inhibition mechanisms require more time to establish themselves in a bacterial population than plasmids not encoding fertility inhibition mechanisms (Haft et al., 2009). It also showed that fertility inhibition imposes a fitness cost on hosts, that is, due to expression of such regulatory machinery bacteria incur a metabolic cost that decreases their growth rate. Nonetheless, the same work showed that plasmids encoding fertility inhibition enjoy a long-term success. Thus, it seems preferable to incur a small cost by repressing horizontal transmission than maximizing horizontal transmission with the consequence of burdening hosts with a greater cost due to the constitutive expression of conjugative machinery.

Fertility inhibition can confer other advantages. A consequence of conjugation is lethal zygosis (Alfoldi et al., 1957; Gross, 1963; Skurray & Reeves, 1973). This phenomenon leads to the death of recipient cells due to high pili-mediated cellular contact that destabilizes their outer membrane. Inhibition of conjugation can also prevent cell death in specific ecological contexts, for instance in the presence male-specific bacteriophages (Dionisio, 2005; Jalasvuori et al., 2011; Ojala et al., 2013). The later are viruses that infect bacteria through conjugative pili. Thus, fertility inhibition represses pili expression and therefore prevents infection by male-specific bacteriophages. By allowing survival of their hosts, plasmids also increase their own survival.

Fertility inhibition might affect plasmids in an additional condition: when more than one plasmid co-inhabit the same host. Considering, those plasmids as unrelated in terms of conjugative machinery, their regulation would be independent and, as a consequence, so should be their horizontal transfer. Thus, co-transfer of multiple plasmid should be lower than the transfer of each plasmid alone because it would be improbable that two plasmids expressed the conjugative machinery simultaneously. Indeed, there is evidence that such co-transfer seems to be independent (Romero & Meynell, 1969). Nonetheless, there is also evidence that co-transfer is not so low (Bouanchaud & Chabbert, 1969). There are also distinct mechanisms that specifically decrease the horizontal transfer of unrelated plasmids but not that of the self (Farrand et al., 1981; Gasson & Willetts, 1975; Olsen & Shipley, 1975; Pinney & Smith, 1974; Sagai et al., 1977; Santini & Stanisich, 1998; Tanimoto & Iino, 1983; Winans & Walker, 1985; Yusoff & Stanisich, 1984). These can act by inhibiting the production of conjugative pili, degrading plasmid transfer DNA or by interacting with coupling proteins (Cascales et al., 2005; Goncharoff et al., 1991; Maindola et al., 2014; Santini & Stanisich, 1998).

There is another function, generally associated with conjugative machinery: exclusion (reviewed in (Garcillan-Barcia & de la Cruz, 2008)). Exclusion systems prevent plasmids from being transferred into a cell already containing a plasmid encoding identical conjugative mechanisms. At least two types of exclusion systems exist (reviewed in (Garcillan-Barcia & de la Cruz, 2008)). Surface exclusion systems act on the phase of mating pair formation, preventing it. Entry exclusion systems act later, preventing DNA from entering the cell. Curiously, plasmid F encodes both surface and entry exclusion systems. Inability of related plasmids to occupy the same host could act as barrier to prevent their recombination. It was hypothesized, however, that instead exclusion systems may act to disrupt the mating pair after plasmid transfer has occurred (reviewed in (Thomas & Nielsen, 2005)).

Although often associated with plasmid horizontal transmission, conjugation is not the only mechanism responsible for plasmid dissemination. Some bacterial species are able to uptake DNA, including plasmids, through natural transformation (reviewed in (Lorenz & Wackernagel, 1994; Thomas & Nielsen, 2005)). These naturally competent bacteria comprise species of both Gram-positive and Gram-negative eubacteria and also archaea. Indeed, several human pathogens acquire genes through transformation. Chromosomal and plasmid DNA can persist in blood for several hours and it was shown that even DNA from the gastrointestinal tract can enter the bloodstream. Such fact might support the evolution of these competent human pathogens. In addition to transformation, transduction was early recognized as a mechanism responsible for the horizontal transfer of plasmids (Smith & Lovell, 1985). For instance, transduction is suggested as the main mechanism of horizontal gene transfer in *Staphylococcus aureus*, as there is little evidence of transformation and conjugation in this species (Lindsay, 2008). Moreover, evidence for plasmid transduction has been gathered from both marine and freshwater environments (Jiang & Paul, 1998; Saye et al., 1987).

Plasmids transfer by transformation or transduction present, however, some limitations. Genetic material transferred by transduction might be better preserved than that transferred by transformation due to its packaging inside a viral capsid, protecting it from degradation (Lorenz & Wackernagel, 1994; Zeph

et al., 1988). Furthermore, bacteriophages, have been generally recognized to have narrow host ranges, although intergeneric transfer is possible, which restricts genetic transfer to highly related bacteria (Hyman & Abedon, 2010; Lorenz & Wackernagel, 1994). In opposition, conjugative transfer seems to extend such host ranges, as there is even evidence for interkingdom plasmid transfer (Heinemann, 1991).

In addition to the three classical ways, alternative mechanisms of horizontal transfer have been discovered. A system of genetic transfer, coined GTA (gene transfer agent), was discovered in *Rhodobacter capsulatus* (reviewed in (Lang & Beatty, 2001; Lang et al., 2012)). This system relies on bacteriophage-like particles that are encoded in host genomes, while GTAs themselves do not carry the genes encoding the ability to produce more GTAs. GTAs mediate generalized transduction at higher rates than bacteriophages but carry smaller DNA elements. GTAs can transfer both chromosomal and plasmidic DNA (Scolnik & Haselkorn, 1984; Yen et al., 1979). GTAs have been proven to exhibit interspecific gene transfer material and to exhibit higher rates of transfer than transformation and transduction in various marine environments (McDaniel et al., 2010). Another way of transferring DNA relies on membrane vesicles produced from the outer membrane of Gram-negative bacterial species (reviewed in (Berleman & Auer, 2013)). These vesicles were shown to transfer plasmids between *E. coli* cells (Yaron et al., 2000). Vesicles were shown to carry DNA up to 370 Kbps (Velimirov & Hagemann, 2011). Additionally, vesicles from *Neisseria Gonorrhoeae* and *Thermus spp.* can contain plasmids too (Blesa & Berenguer, 2015; Kadurugamuwa & Beveridge, 1995). Nanotubes have also been detected in bacteria (Dubey & Ben-Yehuda, 2011). These structures connect cells in solid medium and enable the transport of small molecules, including plasmids, between cells. Nanotube-mediated transfer was observed among *Bacillus subtilis* cells but also between *B. subtilis* and *S. aureus* and *E. coli*, showing it to be a general feature among bacterial species. An alternate Dnase-I resistant (ADR) mechanism for plasmid acquisition was reported in *Helicobacter pylori*, which was found to be distinct from both transformation and conjugation (Rohrer et al., 2012).

Non-mobilizable plasmids were found to be horizontally transferred by transformation in colony-biofilms, although transfer in liquid was also possible in a lesser extent (Maeda et al., 2006). This phenomenon was found to occur not only on common laboratory culture media but also on food extracts (Ando et al., 2009). Artificial and natural media exhibited comparable levels of transformation, however it was shown that nutrient availability affected the efficiency of transfer, showing that active cell growth is essential. Purified plasmids could not be transferred, suggesting that DNA had to be supplied by cells (Etchuuya et al., 2011). Thus, revealing that this mechanism differs from typical transformation. It was shown that the production of a secreted factor, which acts similarly to a pheromone, promoted cell-to-cell transformation (Etchuuya et al., 2011). Furthermore, cell-to-cell transformation was observed among natural isolates of *E. coli* and shown to promote genetic transfer at higher rates than typical transformation (Matsumoto et al., 2016).

Overall, these mechanisms other than conjugation accounting for the horizontal transfer of plasmids, demonstrate that even plasmids classified as non-mobilizable can be transmitted horizontally. Thus, plasmids may be more mobile than previously expected.

Post-transfer establishment

After horizontal transfer plasmids face various host barriers to their establishment. Such barriers can be envisaged as host (prokaryotic) immune systems and have been mostly studied as functions against viral infection (reviewed in (van Houte et al., 2016)). One of the most commonly known barriers is the action of host encoded restriction-modification systems that target plasmid sequences for degradation (reviewed in (Thomas & Nielsen, 2005; Vasu & Nagaraja, 2013)). Thus, plasmids must employ strategies to overcome host barriers (reviewed in (Bahl, 2009)).

One strategy is to avoid the sequences specifically targeted by restriction-modification systems. Such sequences tend to be short and palindromic, that is to say, a sequence is identical to its reverse complementary sequence. Indeed, this kind of sequences has been shown to be underrepresented in several broad host range plasmids (Rawlings & Tietze, 2001; Wilkins et al., 1996).

Alternatively, plasmids may encode enzymes devoted to anti-restriction (reviewed in (Tock & Dryden, 2005; Wilkins, 2002)). These enzymes seem to alleviate restriction after conjugative transfer, possibly because they prevent DNA recognition. Indeed, *ard*-like genes that encode these enzymes, are co-regulated with transfer genes, which is indicative of their role in post-transfer establishment.

Some plasmids and phages use an additional strategy: encoding solitary methyl-transferases (Murphy et al., 2013; Vasu & Nagaraja, 2013). Solitary methyl-transferases can be seen as incomplete restriction-modification systems lacking a restriction enzyme, thus only encoding the modificating function. The role of solitary methyl-transferases has been mostly studied in bacteriophages, showing that their action protects bacteriophage genomes from host encoded restriction enzymes. Some of these enzymes recognize multiple distinct target sequences, providing simultaneously resistance to more than one restriction enzyme. Reasonably, harbouring solitary methyl-transferases may contribute to broader host ranges of bacteriophages. Indeed, solitary methyl-transferases were shown to be abundant in bacteriophages and conjugative plasmids (Oliveira et al., 2014).

Biofilms

Bacteria have the ability to attach to surfaces and to produce extracellular products. Altogether, bacterial biomass associated with such an extracellular polymeric matrix produces complex structures called biofilms (Hall-Stoodley et al., 2004). There is low diffusion inside these structures, which confer protection to bacteria from several damaging agents such as antibiotics. Moreover, high cell density and close proximity between cells inside biofilms can promote horizontal gene transfer (reviewed in (Molin & Tolker-Nielsen, 2003; Sorensen et al., 2005; Stalder & Top, 2016)). As aforementioned, some mechanisms of plasmid transfer are favoured in solid media, such as biofilms. Nonetheless, the inverse is also plausible, in the sense that horizontal transfer may enhance biofilm formation. For instance, extracellular DNA essential for transformation acts as an adhesion factor (Whitchurch et al., 2002).

Plasmids have also been identified as useful for biofilm formation. Specifically, conjugative plasmids express pili required for conjugation, which in turn, act as adhesion factors in early stages of biofilm formation. This was verified

for plasmids expressing conjugative transfer constitutively (Ghigo, 2001). Moreover, plasmids repressed for conjugation can also enhance biofilm formation, given that conjugative transfer is stimulated, for example, if recipient cells are present in the population (Ghigo, 2001; Reisner et al., 2006). This feature seems to be rather general, as it was verified for diverse plasmids instead of being restricted to a specific group of plasmids (Ghigo, 2001).

Plasmid contribution to biofilm formation is however, more complex and gets complicated when more than one plasmid is present. It could be expected that in the presence of multiple plasmids, the overall biofilm formed corresponded to an additive effect of each plasmid. That is to say, that summing up the contribution of each plasmid would translate into the observed biofilm. However, two plasmids may act synergistically, that is, promoting more biofilm formation than the sum of the effects of the two plasmids separated, as observed in (Dudley et al., 2006). In other words, this means that plasmids enhanced each other's effect. Contrastingly, plasmids can behave antagonistically. Surface exclusion systems inhibit conjugation between cells containing related plasmids. Thus, plasmids encoding such systems prevent interactions with cells containing related plasmids. Consequently, this feature limits the enhancing effect of plasmids, as shown in (Reisner et al., 2006). Another parameter that seems to affect plasmid contribution to biofilm formation is the host. It has been shown that plasmids interact differently with distinct hosts, while in some hosts a given plasmid promotes biofilm formation, in other hosts the same plasmid decreases this feature (Roder et al., 2013).

Besides pili, plasmids can encode other factors that promote biofilm formation. For instance, plasmids belonging to the IncX incompatibility group encode frequently type 3 fimbriae, which are known to mediate attachment to surfaces and consequently affect biofilm development positively (Burmolle et al., 2008; Burmolle et al., 2012; Ong et al., 2009). Plasmids can also mediate the synthesis of host encoded adhesion factors, consequently promoting cell aggregation (Barrios et al., 2006; May & Okabe, 2008). It has also been shown that plasmid encoded addiction systems can favour biofilm formation (Wen et al., 2014).

Therefore, plasmids can exhibit a fundamental role as enhancers of biofilm formation, although their contribution depends on various factors.

Accessory genes

Plasmids are organized in modules (Thomas, 2000). Thus, we can consider that the modules described previously (replication, stability and transmission) constitute the plasmid backbone. Consequently, backbone modules can be envisioned as essential. Other modules are however, accessory and encode non-housekeeping functions instead.

Plasmid carried accessory genes are generally associated with other mobile elements. Such genes can have a chromosomal origin, not being themselves mobile at first. They become mobile when captured by transposons. Composite transposons are constructed after random events when these genes become flanked by insertion sequences (IS). These transposons generally carry only one or two genes. Other transposons, however, harbour integrons. Integrons encode an integrase enzyme, which is a site-specific recombinase, and a recombination site *attI*. This allows the insertion of various gene cassettes at the

attI site. Integrons also encode a promoter near the *attI* site, which leads to the expression of the captured genes. Thus, integrons constitute platforms of accessory genes. Transposons, either composite transposons or integron carrying transposons, have the ability to move within genomes through transposition. This allows them to move into mobile elements such as plasmids. Consequently, genes transported in transposons present in mobile plasmids can be transferred between bacterial cells. This matter has been reviewed in (Rowe-Magnus & Mazel, 1999).

The presence of insertion sequences outside the backbone regions provides plasmids with sites where new genes can be introduced without the risk of disrupting any backbone gene. Therefore, plasmid functionality can be preserved without compromising essential genes. Plasmids can thus encode adaptive regions, that is, they harbour genes that enable hosts to adapt to specific environments. Because of their ability to mobilize such accessory genes, plasmids contribute to a communal gene pool, which means that they provide genes to a community of hosts generally sharing the same environmental niche (reviewed in (Norman et al., 2009)). Indeed, horizontal gene transfer frequently occurs between organisms residing in the same environments (Jain et al., 2003).

Initially, many plasmids have been found because they conferred antibiotic resistance (Nakaya et al., 1960; Watanabe, 1963). Indeed, many integrons harbour gene cassettes determining antibiotic resistance (Gillings, 2014). In particular, plasmids shared by *Enterobacteriaceae* species carry antibiotic resistance genes (Carattoli, 2009). Resistance to other compounds such as heavy metals is also commonly determined by plasmids and transposons (Foster, 1983; Silver, 1992; Silver & Misra, 1988).

Integron-carried gene cassettes determine factors involved in diverse additional functions such as virulence and secondary metabolism (Gillings, 2014). Therefore, plasmids carrying accessory genes have a determinant effect on the life styles of their hosts, which can be illustrated by plasmids encoding virulence factors (reviewed in (Gyles & Boerlin, 2014)). For instance, distinct features responsible for the differentiation between commensal and pathogenic strains of *E. coli* are virulence factors encoded in plasmids or other genetic elements. Moreover, the Gram-positive bacteria *Bacillus anthracis* and *Bacillus cereus*, which belong to the same species (*Bacillus cereus sensu lato*) and possess identical genomes, cause different diseases due to the distinct plasmids they carry. Acquisition of virulence traits through plasmids is also a characteristic of phytopathogenic bacteria (reviewed in (Sundin, 2007)).

Plasmids are also relevant for the adaptation of soil bacteria (reviewed in (Heuer & Smalla, 2012)). Broad host range plasmids found in soil bacteria carry diverse accessory genes, in such a way that they seem to sample the soil mobilome (reviewed in (Heuer & Smalla, 2012)). Indeed, catabolic plasmids, which are plasmids encoding metabolic pathways to degrade man-made compounds (xenobiotics), generally exhibit broad host ranges (Top et al., 2003). Such pathways seem to result from the combination of genes with different origins. Degradation of natural compounds on the other hand, is often encoded in plasmids exhibiting narrow host ranges. While narrow host range plasmids inhibit the SOS response of their natural hosts, broad host range plasmids do not (Baharoglu et al., 2010). In turn, induction of SOS response stimulates homologous recombination of gene cassettes (Matic et al., 1995). Consequently, this allows broad host range plasmids to expand the functions they encode and

consequently adapt to other hosts. Narrow host range plasmids not having to frequently adapt to different hosts, prevent recombination, maintaining their integrity. Indeed, virulence traits of *E. coli* are often encoded in narrow host range plasmids (Johnson & Nolan, 2009).

Classification

Classification systems are helpful when studying plasmids because they encode many diverse features. Since the early 1970s, plasmids have been classified into incompatibility groups, which reflects their systems of replication and partition (plasmid classification was extensively reviewed in (Shintani et al., 2015)). Thus, since these are the most important and in some cases the only functions encoded by plasmids, they should provide a means to classify all known plasmids. However, testing incompatibility in the laboratory can be laborious as any newly isolated plasmid must be tested for incompatibility against reference plasmids belonging to each incompatibility group (del Solar et al., 1998). PCR-based methods to type plasmids were also developed but due to plasmid evolution and sequence variation, false negative results can be obtained as primers fail to amplify various replicons (Carattoli et al., 2005; Rosvoll et al., 2010). In recent years, more feasible methods have been developed, which allow plasmid classification *in silico*, based on sequence similarities among Rep proteins (Carattoli et al., 2014; Shintani et al., 2015).

There are some difficulties associated to plasmid typing (Shintani et al., 2015). Firstly, because most plasmid studies concern only plasmids isolated from *Enterobacteriaceae* and thus little information about other plasmids is available. Secondly, plasmids belong to multiple incompatibility groups have already been described.

Classification into incompatibility has been typically restricted to plasmids found in *Enterobacteriaceae*, *Pseudomonas* and *Staphylococcus*. There are approximately 27 groups in *Enterobacteriaceae*, 18 in *Pseudomonas* and 18 in *Staphylococcus*. Moreover, some groups from *Enterobacteriaceae* and *Pseudomonas* are redundant, for example IncP and IncQ from *Enterobacteriaceae* are equivalent to IncP-1 and IncP-4 from *Pseudomonas.* Furthermore, some Rep proteins from distinct groups exhibit high homology, such is the case among the *Enterobacteriaceae* groups IncB, IncI, IncF, IncK, IncZ and pCD-type; and inc4, inc9, inc10 and inc14 from *Staphylococcus*. Plasmids belonging to inc4, inc8, inc9, inc10, inc11, inc13 and inc14 determine rolling-circle replication, while those belonging to inc1, inc7 and inc18 determine θ type replication. Alternatively, plasmids from *Firmicutes* can be classified in 26 Rep families and 10 unique families. Overlap between inc groups and Rep families can occur. Recently, a classification of *Acinetobacter* plasmids based on PCR-typing has been proposed, resulting in 19 (GR1-GR19) groups (Bertini et al., 2010). *

Most of the big plasmids found in *Alphaproteobacteria* encode RepABC proteins, while classification of smaller plasmids is still difficult. Plasmids found in *Spirochaetes* were identified in *Borrelia* species and tend to be linear. Genomic

* To facilitate the comprehension of these groups, in this thesis Inc groups refer to incompatibility groups found in Enterobacteriaceae , IncP- groups to those frequently found among Pseudomonas, inc and Rep to those found in Firmicutes and GR to those found in Acinetobacter.

rearrangements are frequent among these plasmids, however 28 compatibility groups have been proposed by (Casjens et al., 2012). Among plasmids from Actinobacteria more than 40 types of Rep proteins exist. On the other hand, there is a lack of information for plasmids of *Cyanobacteria* and *Archaea* and further characterization is required.

An alternative to incompatibility groups has recently been proposed based on plasmid mobility (Garcillan-Barcia et al., 2009). Plasmids can be assigned to MOB types according to the relaxase protein they encode. However, this classification is restricted to plasmids transferable through conjugation.

Nonetheless, this alternative can complement the classification into incompatibility groups, since there is a lack of characterization of many Rep proteins. Plasmids can be assigned to seven MOB types (Smillie et al., 2010). In addition, conjugative plasmids can be classified according to their T4SS (Smillie et al., 2010). An association between incompatibility groups and transfer systems can exist, as the morphology of conjugative pili varies between plasmids belonging to different incompatibility groups (Bradley et al., 1980).

These classification systems can also be useful to understand the host range of plasmids. Indeed, (Suzuki et al., 2010) showed that related plasmids, which share identical gene content and replicative machinery exhibit similar host ranges. Thus, it is possible to associate incompatibility groups and host range. In that study, it was concluded that plasmids belonging to IncI and IncF exhibited narrow host ranges, those belonging to IncA/C, IncP, IncP-9, IncQ, IncU, PromA and those resembling Ri/Ti plasmids from *Rhizobium* exhibited broad host ranges, while intermediate host ranges were attributed to incompatibility groups IncH, IncN and IncL/M. Moreover, among the plasmids in *Acinetobacter*, most GR groups were confined to this genus, while plasmids belonging to GR03 exhibited broader host ranges (Shintani et al., 2015). MOB types are also differently represented among plasmids (Smillie et al., 2010). MOBF and MOBH predominate among conjugative plasmids, while MOBV is overrepresented among mobilizable plasmids. Moreover, MOBQ and MOBV relaxases are more promiscuous and can interact with diverse T4SS.

Eco-evolutive perspectives of plasmid selection

As has been shown, plasmids are organized in distinct modules. Thus, at first glance, plasmids can be simply regarded as a set of functional genetic building blocks (Toussaint & Merlin, 2002). However, plasmids are subject to natural selection and play a significant role in the ecology and evolution of their hosts. Thus, they can be considered living entities and classified as parasites or mutualists (Eberhard, 1990).

Perceiving plasmids as mutualists is intuitive since, as mentioned earlier, plasmids can carry accessory genes that benefit their hosts. Keeping accessory genes in mobile genetic elements provides bacteria with a shared gene pool that allows them to rapidly adapt to new ecological conditions (Eberhard, 1990; Norman et al., 2009; Summers, 1996b). However, such advantage tends to be transitory (Eberhard, 1990; Summers, 1996b). For instance, antibiotic resistance is a trait that only benefits bacteria in the presence of antibiotics but not in their absence, thus conferring only a temporary selective advantage. As another example, plasmid encoding virulence factors are only useful for facultative pathogens when these bacteria are in an infectious context.

Alternatively, plasmids can be viewed as parasites because they can impose a fitness cost on their hosts, which can be defined as any detrimental effect on cell physiology or energetical waste caused by a plasmid that decreases its hosts ability to persist (reviewed in (Baltrus, 2013)). Such metabolic burdens can result in the lengthening of bacterial lag phases or reducing maximal growth rates. One of the causes of these fitness costs is the sequestration of host resources such as ATP and host machinery. For example, plasmid replication depends on host enzymes, nucleotides and ATP. However, metabolic burdens are mostly associated with transcription and translation. For instance, essential host proteins may not be readily synthesized because ribosomes are occupied in translating products of plasmidic origin. Thus, instead of devoted to host housekeeping processes, these resources are diverted for plasmid functions. Therefore, plasmids not only consume energy but also delay host processes essential for cell maintenance.

Moreover, recent acquisition of plasmids through horizontal gene transfer may result in carriage of a plasmid that did not coevolve with that specific host (reviewed in (Baltrus, 2013)). Such plasmid misadaption has implications on host pathways, as some proteins may not function optimally in the new host's cellular context. For instance, if plasmids encode homologs of host encoded proteins, their expression alter protein dosage and consequently can disrupt the fine-tuning of physiologic pathways. These pathways can also be disturbed if plasmids are able to integrate in the host chromosome, which can result in the disruption of genes. Additionally, their host cell may not possess all protein chaperones to which the plasmid adapted. This leads to misfolding of plasmid proteins, which in turn may cause cytotoxicity, for example due to stress at the membrane level. Furthermore, composition of plasmid sequences may also contribute to the metabolic burden (reviewed in (Baltrus, 2013)). AT-rich codons tend to encode amino acids that require more energy to be synthesized, thus, such codons should be counter selected so that plasmids decrease the cost imposed on hosts.

Although plasmids impose a fitness burden on their hosts, during co-evolution, this cost can decrease and eventually disappear (Bouma & Lenski, 1988; Dahlberg & Chao, 2003; Dionisio et al., 2005). This can be the result of mutations (either plasmidic or chromosomal) that promote the adaptation between hosts and plasmids. It has been shown that plasmids, as well as other mobile genetic elements, tend to acquire nucleotide composition identical to that of their hosts (Campbell et al., 1999; Lawrence & Ochman, 1997; Ochman et al., 2000; Vernikos et al., 2007). This "genome amelioration" was shown to decrease fitness costs and consequently favour plasmid persistence (Dahlberg & Chao, 2003). Genetic interactions, or epistasis, also shape plasmid selection (reviewed in (Carneiro & Hartl, 2010; de Visser et al., 2011; Phillips, 2008)). Selection of plasmids is favoured if they exhibit positive epistastic interactions. This means that certain interactions minimize the cost of harbouring a plasmid. Indeed, positive epistasis has been observed between plasmids and chromosomal mutations, and between different plasmids (Morton et al., 2014; San Millan et al., 2014; Silva et al., 2011). Moreover, limiting the expression of non-essential plasmid traits can also reduce the host's metabolic burden. For instance, plasmids no longer expressing antibiotic resistance are less costly to their hosts (Humphrey et al., 2012; Turner et al., 1998).

Since they decrease the growth rates of hosts, costs associated with plasmid carriage can be defined as plasmid virulence. Plasmids and hosts,

therefore, seem to have opposite interests, as plasmid functions favouring its maintenance affect the hosts negatively, which result in conflicts. These conflicts were demonstrated for features such as plasmid copy number (Harrison et al., 2012; Paulsson, 2002). The higher the copy number of a plasmid, the higher is the probability that both daughter cells inherit the plasmid upon cell division. However, the greater the plasmid copy number, the greater the fitness burden imposed on the host. Considering incompatible plasmids, (Paulsson, 2002) demonstrated that plasmid selection acts on two levels: intracellular and intercellular. As discussed earlier, incompatible plasmids are unable to persist in the same cell for long. Thus, plasmids evolving a strategy to increase their own copy number would be favoured by selection, because they outcompete rival plasmids, since they are inherited by a greater number of cells. Such strategy however increases virulence and inversely decreases the growth rate of hosts. Consequently, hosts harbouring plasmids exhibiting lower copy number (least virulent) growth faster and outcompete hosts that growth slower. Therefore, at the intracellular level, plasmids with higher copy numbers are favoured, while at the intercellular level, hosts harbouring costly plasmids are counter-selected (Paulsson, 2002).

The same reasoning can be applied to other plasmid features, as illustrated by addiction systems (Cooper & Heinemann, 2000a; Cooper & Heinemann, 2005). As discussed before, such systems eliminate host cells after plasmid loss, thereby reducing plasmid loss. Addiction systems can also play a relevant role in the context of plasmid competition. Let us consider, for instance, two incompatible plasmids, of which, only one encodes addiction systems. After segregation of these incompatible plasmids, only cells inheriting the plasmid encoding addiction systems survive. Such systems can therefore comprise a strategy to displace rival plasmids, thus this strategy would be favoured at the level of plasmid competition. On the other hand, expression of addiction systems is costly to hosts, and consequently plasmids encoding them should be counter-selected at the intercellular level.

As mentioned before, plasmid conjugation also entails a burden to bacteria. Expression of the conjugative machinery poses a metabolic cost to hosts. Additionally, expression of conjugative pili allows infection by male-specific bacteriophages. Thus, conjugation is a matter of conflict because it favours plasmids by increasing their horizontal transmission but counter selects cells carrying conjugative plasmids as they growth slower and become susceptible to viral infection.

Indeed, a theoretical hypothesis regarding parasite evolution concerns transmission. According to this trade-off hypothesis virulence results as a side effect of the traits that promote parasite transmission (reviewed in (Alizon et al., 2009)). In this sense, parasites with increased transmission tend to be more virulent and vice-versa. Moreover, parasites only transmitted vertically should be less virulent. In this case, parasite success is tied to host success because plasmid persistence depends solely in host's reproduction. Thus, the more the host reproduces the more cells harbour the plasmid, favouring its persistence. On the other hand, higher virulence could be selected if parasite horizontal transmission compensates the cost imposed on the host. In this case, the number of infected hosts emerging due to horizontal transfer of parasites is greater than the number infected hosts that are counter-selected due to the cost of infection. Thus, this shows a trade-off between vertical and horizontal transmission.

Alternatively, another hypothesis suggests that higher virulence is favoured by parasite competition for host resources (Frank, 1996). In this sense, in the context of multiple infections, virulence should increase because parasites optimize their traits to exploit the host, which leads to overexploitation of host resources. These hypotheses are not mutually exclusive because horizontal transmission increases the probability of co-infection and consequently leads to parasite competition. However, the association between parasite competition and virulence is complex and depends on the context of selection (Chao et al., 2000). As stated before, competition would favour parasites better exploiting their hosts and this could be achieved by enhancing traits promoting host exploitation. Nonetheless, parasites could instead evolve traits to interfere with rivals (Chao et al., 2000). By doing so, they do not overexploit the host but decrease the ability of their rivals to succeed. In turn, among all infecting parasites, the ones that better exploit host resources are the ones interfering with rivals. Since this strategy does not overexploit hosts, plasmids would not evolve to be more virulent.

These hypotheses can be applied to plasmid biology. For instance, mechanisms such as incompatibility and conjugative exclusion diminish the ability of plasmids to establishing long-lasting co-infections. Therefore, preventing the evolution towards greater virulence because intracellular competition is minimized. Additionally, conjugative plasmids tend to repress their horizontal transmission through fertility inhibition. Although at first sight this seems to decrease plasmid maintenance, at long term plasmid persistence increases because hosts incur a less fitness burden. Thus, fertility inhibition may be a trait that coordinates the trade-off between vertical and horizontal transmission. Fertility inhibition mechanisms specifically control the expression of the conjugative machinery of related plasmids. However, other mechanisms seem to decrease the horizontal transfer of unrelated plasmids but not that of the self (Farrand et al., 1981; Gasson & Willetts, 1975; Olsen & Shipley, 1975; Pinney & Smith, 1974; Sagai et al., 1977; Santini & Stanisich, 1998; Tanimoto & Iino, 1983; Winans & Walker, 1985; Yusoff & Stanisich, 1984). Therefore, mechanisms that inhibit conjugation of unrelated plasmids could have evolved as a strategy to interfere with rival plasmids during co-infection. These mechanisms would be implicated in plasmid competition as a means to alleviate host exploitation.

Thesis Overview

In Chapter 2 we will study the host range of plasmids. Host range can have various definitions. In this work, using bioinformatic methodology, we will analyse the evolutionary host range of plasmids. This concept defines the set of hosts to which a plasmid can be transferred and in which it may replicate and be stably maintained. More specifically, we aim to understand the contribution of several plasmid traits, involved in replication, maintenance and horizontal transmission, to their host range.

In subsequent chapters, we will study experimentally the interactions between natural conjugative plasmids. This sample of plasmids is summarized in Table 1.1.

Table 1.1. Plasmids used in Chapters 3-8.

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[3])	S.C.K. – C.E.N.
F	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[1]	IncF II	93.9	ACKS	G. Koraimann (Graz Univ.)
R477-1[2]	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

[1] de-repressed natural mutant of R1
[2] temperature-sensitive for conjugation; only used in chapters 3 and 6.
[3] only low-level of kanamycin resistance, marker not used for selection
[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

In Chapters 3 to 5 we will study how interactions between plasmids shape their horizontal transmission. We will focus on the variation of conjugation rates to understand the strategies employed during plasmid competition. That is, we will assess if, in the presence of rivals, plasmids tend to increase their own conjugation rates or if instead they act by decreasing the transmission of their rivals.

In Chapter 6, we will study how plasmids transfer simultaneously from a common donor host cell. Given that plasmids regulate conjugation independently, we aim to understand if co-transfer of plasmids is also independent. In Chapter 7 we will study how plasmids affect biofilm formation. More specifically, we aim to understand if an association between conjugation rates and biofilm formation exists. Additionally, we investigate how interactions between plasmids contribute to their hosts ability to form biofilms.

In Chapter 8 we will study the fitness cost associated with conjugative plasmids and we will estimate the epistatic effects of plasmids combinations.

Additionally, the author contributed to other works during the period concerning this thesis. Such works fall out of the scope of this thesis but are attached in Appendix A-D.

Chapter 2

Plasmid host range is mainly determined by factors involved in replication and mobility

Abstract

The set of hosts in which plasmids evolve defines their host range. A plasmid's host range thus depends on its ability to replicate, to be stably maintained and to be transferred between hosts. Our aim is to understand which plasmid characters mainly determine host range. In order to do so, we evaluated the impact of several plasmid factors on the host ranges of prokaryotic plasmids. We studied factors involved in: i) replication, namely incompatibility groups, ii) mobility, namely relaxase families and, iii) stability, namely palindrome avoidance, addiction systems, carriage of methyltransferases and presence of integrons. We found that incompatibility factors have a predominant effect on the host range of plasmids, explaining approximately 2/3 of the observed variation of host range. We also observed that relaxase families affected host range; conjugative and mobilizable plasmids exhibited broader host ranges than non-mobilizable plasmids. Narrow host range plasmids carry addiction systems and methyltransferases, while broad host range plasmids show a greater tendency to avoid palindromes. Although statistically significant, the factors related to plasmid stability little contributed to explain the variation in host range. We conclude that host range is mainly affected by plasmid features concerning replication and horizontal transmission, both traits implicated in the level of success at dealing with other entities, namely hosts and other plasmids.

Introduction

Throughout the years, plasmids have been isolated from many different bacterial species, but also from archaea and eukaryotic organisms (Summers, 1996a). Not only can plasmids inhabit a vast diversity of hosts, they can also spread between them. Plasmid evolution is affected by the diversity of hosts encountered by its ancestors. In fact, several studies have already provided evidence that plasmids, and other accessory genetic elements, tend to acquire nucleotide frequencies identical to those exhibited by their hosts (Campbell et al., 1999; Lawrence & Ochman, 1997; Ochman et al., 2000; Vernikos et al., 2007). Coined "genome amelioration", this phenomenon can be simply described as a specific homogenization of the plasmidic nucleotide composition resulting from specific mutational bias of hosts. Consequently, genome amelioration can enhance plasmid persistence as the physiological/metabolic cost imposed on hosts decreases (Dahlberg & Chao, 2003). Additionally, the lifestyle of genetic elements also shapes their sequences (reviewed in (Dutta & Paul, 2012). For instance, due to a parasitic lifestyle that often implies competition for scarce resources, plasmids tend to have higher AT content (which is energetically less expensive) (Rocha & Danchin, 2002).

Nucleotide composition of genetic elements constitutes a "genomic signature" which has often been proven useful to envisage their evolutionary

history by identifying in which hosts they have evolved (Deschavanne et al., 2010; Lamprea-Burgunder et al., 2011; Suzuki et al., 2008; Suzuki et al., 2010; van Passel et al., 2006). Moreover, identification of plasmid hosts facilitates the study of plasmidic host range (Suzuki et al., 2010). Commonly, plasmids are qualitatively classified as narrow or broad host range. Such classification is laborious as several traits, reflecting plasmid replication, transmission and maintenance, need to be accessed experimentally. As shown by (Suzuki et al., 2010), the method (*in silico*) concerning genomic signature allows the study of a plasmid's evolutionary host range. This consists on the set of hosts where a plasmid was able to replicate and persist long enough to acquire traces of its host's genomic signature. In the present work, we take advantage of this approach to gain insight on the parameters affecting the host range of plasmids.

For plasmids, as for any other organism, the ability to replicate should be essential. There are distinct mechanisms of plasmid replication, for instance circular and linear plasmids replicate differently (review in (del Solar et al., 1998)). Among circular plasmids, three main replication mechanisms exist: theta replication, strand displacement and rolling circle. Although not necessarily correct, there is a pervasive idea that plasmids from Gram negative bacteria are more often associated with theta type replication, while plasmids from Gram positive bacteria tend to replicate by rolling circle (del Solar et al., 1998). Such notion originates from the fact that most knowledge concerning theta-replication originated in plasmids of Gram-negative bacteria while the study of those from Gram-positive bacteria improved the knowledge of rolling-circle replication (del Solar et al., 1998). Narrow and broad host range plasmids are expected to use different strategies of replication (reviewed in (delSolar et al., 1996; Jain & Srivastava, 2013)). Autonomous initiation of replication could be associated with broad host range plasmids. On the other hand, plasmids more dependent on host replication initiation factors would exhibit narrower host ranges. Replication by rolling circle as well as by strand displacement are thought to favour broad host range plasmids.

Apart from these differences, another important feature concerning plasmid replication is the incompatibility group. An incompatibility group defines a group of plasmids that share identical replication control mechanisms and replication origin sequences. Replication is essential to plasmids, hence this feature has been widely employed in plasmid classification (Novick, 1987). These mechanisms also determine plasmid copy number. Therefore, plasmids having the same control mechanisms, that is belonging to the same incompatibility group, are unable to persist in the same host cell. Replication mechanisms encoded by a plasmid must also be compatible with its host machinery, otherwise the plasmid is unable to replicate. Thus, replication mechanisms are thought to affect host range. Experimental evidence corroborates this hypothesis and more recently the work by (Suzuki et al., 2010) has shown that plasmids of the same incompatibility group tend to have identical evolutionary host ranges. Moreover, there are several broad host range plasmids harbouring more than one origin of replication (reviewed in (delSolar et al., 1996; Jain & Srivastava, 2013)). Such a feature could be associated with their broader host range, however there are also examples of narrow broad host range plasmids containing multiple replicons.

Host range is subject to other relevant features, such as the plasmid's ability to be horizontally transferred. The main mechanism for the horizontal transfer of plasmids is conjugation, which requires cellular contact (reviewed in

(Smillie et al., 2010)). Two kinds of plasmids are able to be transferred by conjugation: conjugative and mobilizable plasmids. Both kinds of plasmids encode an origin of transfer, and a protein that recognizes it – the relaxase. Exceptions exist, as some mobilizable plasmids harbour an origin of transfer but do not encode a relaxase (Smillie et al., 2010). Conjugative plasmids also encode a type IV secretion system (T4SS) and a coupling protein (T4CP) required for DNA transport between cells. Thus, conjugative plasmids are self-sufficient, being able to transfer autonomously. Since mobilizable plasmids cannot promote their own DNA transport, they require the presence of a conjugative plasmid in the same cell to be transferred. Accordingly, mobilizable and conjugative plasmids can be classified into MOB families depending on the relaxases they encode. Such classification also hints that MOB families affect host range (Francia et al., 2004; Garcillan-Barcia et al., 2009).

Additionally, it is also known that transfer of genetic elements, including plasmids, can be achieved by other mechanisms (Ando et al., 2009; Dubey & Ben-Yehuda, 2011; Etchuuya et al., 2011; Kurono et al., 2012; Madsen et al., 2012; Maeda et al., 2006; Matsuda et al., 2012; Molin & Tolker-Nielsen, 2003), which can also enable the horizontal transfer of non-conjugative plasmids. Mechanisms, such as transduction and transformation, can be favoured in certain habitats such as biofilms. In terms of host range, it is expected that horizontally transmitted plasmids exhibit broader hosts ranges (Jain & Srivastava, 2013).

Plasmids also employ strategies to persist after their transfer (reviewed in (Bahl, 2009)). Mechanisms associated with plasmid maintenance can potentially affect host range. Such mechanisms include, among others, partition systems, addiction systems and the ability to escape the action of cellular restriction-modifications systems. Addiction systems are also known as post-segregational killing (PSK) systems or toxin-antitoxin (TA) *loci* (Van Melderen & De Bast, 2009). Through the action of a stable toxin and an unstable antitoxin, these systems promote death of plasmid-free cells that emerge upon plasmid segregation. The toxin is more stable than the antitoxin, thus, cells no longer carrying the plasmid become vulnerable to the action of the toxin due to their inability to synthesize the antitoxin. Therefore, these systems are thought to ensure plasmid maintenance, although recent works support their role in plasmid competition (Cooper & Heinemann, 2000b). Some restriction-modification systems (RMS) can also be viewed as addiction systems (reviewed in (Roberts et al., 2003; Vasu & Nagaraja, 2013)). In most type II restriction-modification systems, the methyltransferase and the restriction endonuclease can act independently, while in other types of RMS the endonuclease (at least) is only active if its cognate methyltransferase is also available. Thus, type II methyltransferases and endonucleases could function respectively as antitoxins and toxins.

Hosts also possess restriction-modification systems. Thus, to persist, incoming mobile genetic elements must overcome such barrier, which can be accomplished by avoiding restriction sites (generally small palindromic sequences) or by encoding methyltransferases that correctly modify their sequences and therefore prevent cellular systems from targeting them for degradation (reviewed in (Bahl, 2009; Bickle, 2004; Gelfand & Koonin, 1997; Tock & Dryden, 2005)). Moreover, broad host range plasmids would be expected to face a greater selective pressure since they confront a wider diversity of RMS as they inhabit more distantly related hosts. In this sense, broad host range plasmids should avoid palindromes to a greater extent than narrow host range

plasmids. Methyltransferases could also account for differences in plasmid host range. There is evidence of some bacteriophages that encode methyltransferases and exhibit broad host range (Murphy et al., 2013).

Plasmids are widely recognized for their role in spreading antibiotic resistance genes. Plasmids may carry other traits benefitting their hosts and consequently improve their persistence. Such traits include virulence factors, catabolic enzymes and resistance to heavy metals. In turn, plasmids acquire many of these accessory genes from other genetic elements such as integrons (Gillings, 2014; Mazel, 2006).

In this Chapter, we will test the impact of several plasmid factors employed in these three phases (replication, transfer and maintenance/stability), in the host range of plasmids. We selected the following factors: 1) for replication – incompatibility groups; 2) for transfer – plasmid mobility (conjugative, mobilizable or non-mobilizable) and relaxase family; 3) for maintenance/stability – plasmid size, avoidance of palindromes, presence of addiction systems and presence of integrons.

Materials & Methods

Experimental data set

We retrieved a total of 4543 prokaryotic plasmid and 2662 prokaryotic genome complete sequences from GenBank (ftp://ftp.ncbi.nih.gov/genomes). We focused only on plasmids with a minimum size of 4 kb, selecting a total of 2956 plasmid sequences. We selected genomes with a minimum size of 500 kb, resulting in a total 2636 chromosomal sequences. Eight genomes were further excluded because no 16S sequence was available.

Estimation of plasmid host range

First, we followed the methods described in (Suzuki et al., 2008; Suzuki et al., 2010) to identify potential hosts for each plasmid. Briefly, we accessed the mono and trinucleotide frequencies in plasmid sequences (both strands). We estimated the relative trinucleotide frequency (x_{ijk}) as: $x_{ijk} = \frac{f_{ijk}}{f_i \cdot f_j \cdot f_k}$, where f_{ijk} is the frequency of trinucleotides observed and $f_i \cdot f_j \cdot f_k$ is the expected frequency (f_i, f_j and f_k represent the observed frequency of nucleotides). We performed a similar approach for genomes, selecting 100 equally inter-spaced 5 kb chromosomal segments instead of the full sequence. To quantify the difference in trinucleotide composition between plasmids and chromosomes, we calculated the Mahalanobis distance between them as: $(X - \bar{Y})^T \cdot S^{-1} \cdot (X - \bar{Y})$, where X is the vector of trinucleotide relative abundance of a plasmid, $\bar{Y}$ is the mean vector of trinucleotide relative abundance of the chromosomal segments, S is the variance-covariance matrix of the trinucleotide relative abundances of the chromosomal segments and S^{-1} is its inverse matrix, ^{T} is the transposition operator. The Mahalanobis distance corrects the covariance of trinucleotides (due to high correlation between reverse complements) and for variabililty along the chromosome (see (Suzuki et al., 2008)).

The Mahalanobis distance calculated reflects the probability of a plasmid's trinucleotide relative abundance belonging to a set of chromosomal trinucleotide relative abundances. Because these distance values have no upper limiter, they were transformed into p-values. To do so, empirical distributions of distances were estimated for each set of chromosome segments. Plasmid hosts were identified when p-values were greater than 0.6.

Secondly, we aligned 16S sequences of one representative per species with MAFFT v7.220, with informative positions selected by trimAl v1.2, using default parameters (Capella-Gutierrez et al., 2009; Katoh & Standley, 2013). Then, we built a phylogenetic tree of prokaryotic genomes with RaxML v8.1.17 using GTR+GAMMA model (Stamatakis, 2014). We computed the host range of each plasmid by calculating the average phylogenetic distance between all pairs of identified hosts. Note that we considered *Escherichia coli* and *Shigella* species as the same species (Brenner, 1981).

Identification of incompatibility groups

We used two independent methods to identify incompatibility groups (Carattoli et al., 2014; Shintani et al., 2015). The first method, PlasmidFinder, uses the BLASTN (BLAST+ suite 2.2.28) algorithm to search for homology between plasmid sequences and a series of probes (database updated on March 2016) specific for each incompatibility group (Carattoli et al., 2014). Hits were selected when they had a minimum sequence identity of 80% and the alignment covered at least 60% of the probe sequence. Best matches were selected according to the highest length scores (LS), estimated as LS = PL – AL + G, where PL is the length of the probe sequence, AL is the alignment length and G is the number of gaps in the alignment (for more details see (Carattoli et al., 2014; Larsen et al., 2012)). If two or more alleles produced the same length score, the one showing highest identity was chosen.

The second method, henceforth termed RepIdentifier, is described in (Shintani et al., 2015). We used the BLASTP algorithm to search for homology between plasmid proteins and (putative) Rep proteins, with the following parameters: identity > 50%, query coverage > 0.5 and e-value < 10^{-5}. For Rep proteins Rep1B (SCP1) and Rep23, TBLASTN was used (same parameters) instead, with the nucleotide sequence encoding the Rep protein. Best matches were selected by choosing the match with the lowest e-value. To search for ColE1-like plasmids we used BLASTN and RNA sequences as query, selecting hits with e-value < 10^{-5}. In addition to the ones provided by (Shintani et al., 2015), we included the following Rep proteins (accession no): X15669 for inc11; YP_006962107 for LowGC; NP_361015, AAF85627, AAF85628 for PromA; WP_000918302 for pXO1-like.

There were cases in which the two classification methods gave different results for the same plasmid. There were also cases of plasmids classified into multiple incompatibility groups. This could be a consequence of high homology between sequences of different incompatibility groups (as previously observed (Carattoli et al., 2014; Shintani et al., 2015)). To simplify, we used the first method to check for homology between PlasmidFinder probes or between these probes and the DNA sequences of the proteins used in RepIdentifier. We also used RepIdentifier to check for homology between Rep proteins. Save for few exceptions, whenever sequences specific for different groups matched each

other, they were considered to define a combined group (see Supp.Table S2.1). This way, we reduced the number of cases of conflicting classification. In a few cases, conflicts remained for a few incompatibility groups such as IncF, IncH and IncI and in such cases we considered the classification obtained according to the PlasmidFinder. We did so, because PlasmidFinder retrieved hits with greater sequence identity than RepIdentifier. For the cases where one or both methods classified plasmids in more than one incompatibility group, we classified the plasmids in multiple groups. In this work, we considered only incompatibility groups (individual or multiple) for which at least three plasmids were identified.

Classification of plasmids according to their mobility

We used the program MacSyFinder (version 1.0.2) to detect genes involved in horizontal transfer (Abby et al., 2014). We used protein profiles built previously (Smillie et al., 2010). Plasmids encoding a relaxase (MOB) but no type 4 secretion system (T4SS) were classified as mobilizable. Plasmids encoding both MOB and T4SS in addition to a type 4 coupling protein (T4CP), disregarding inter-gene distance, were classified as conjugative. Plasmids lacking relaxase genes were considered non-mobilizable. Because we cannot identify the oriT, mobilizable plasmids that do encode a relaxase were regarded as non-mobilizable (although they may encode an oriT). Additionally, identified relaxase genes were used to classify plasmids into MOB families.

Estimation of palindrome avoidance

We used the RMES program (version 3.1.0), with the Gaussian approximation and maximal Markov order options, to estimate the observed and expected counts of all 4-mer and 6-mer sequences in plasmid and genome sequences (in one strand only) (Schbath, 2011). Then we defined a relative palindrome score, calculated as $(\frac{observed - expected}{expected})$, for every 4-mer and 6-mer sequence, in each plasmid and genome. Such scores were ranked in ascending order and, for each plasmid and genome, we calculated the palindrome index as the ratio between the mean rank position of all palindromes over the total number of sequences of the same size. Palindrome index values close to zero reflect underrepresentation of palindromes. Palindrome avoidance was estimated as the mean of the differences between the palindrome index of a plasmid and each of its hosts. Here we considered as hosts, all the genomes of the species identified as potential hosts. We also considered *Escherichia coli* and *Shigella* species as one species only (Brenner, 1981).

Detection of type II RMS and of TA loci

We used the program MacSyFinder (version 1.0.2) to detect type II RMS and TA *loci* in plasmids sequences (Abby et al., 2014). We used unpublished protein profiles built by our group. We defined a system when both components were no more than four genes apart.

Detection of integrons

We used the program IntegronFinder (version 1.5) to detect integrons in plasmid sequences, using default parameters and protein profiles (Cury et al., 2016). We used the 'local_max' option to increase sensitivity and discarded single *att*C sites. We considered that a plasmid contained an integron when at least one of the following was identified: complete integron, In0 element or CALIN element.

Statistics

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

Results

Distribution of host ranges

We identified all the possible plasmid hosts using a previously described method relying on the genomic signature of plasmids and host chromosomes (Suzuki et al., 2008; Suzuki et al., 2010). Since small plasmids tend to exhibit AT rich sequences, such bias could compromise identification of plasmid hosts, thus, we studied plasmids greater than 4 kb. We searched for potential hosts among 2636 complete genome sequences greater than 500 kb. We were able to identify hosts to a total of 2322 (almost 90 %) plasmids. Next, we built a phylogenetic tree for the host genomes, using one rRNA 16S subunit sequence only per prokaryotic species (eight species were excluded due to a lack of 16S sequence). We defined the host range of each plasmid as the average phylogenetic distance between all its identified hosts.

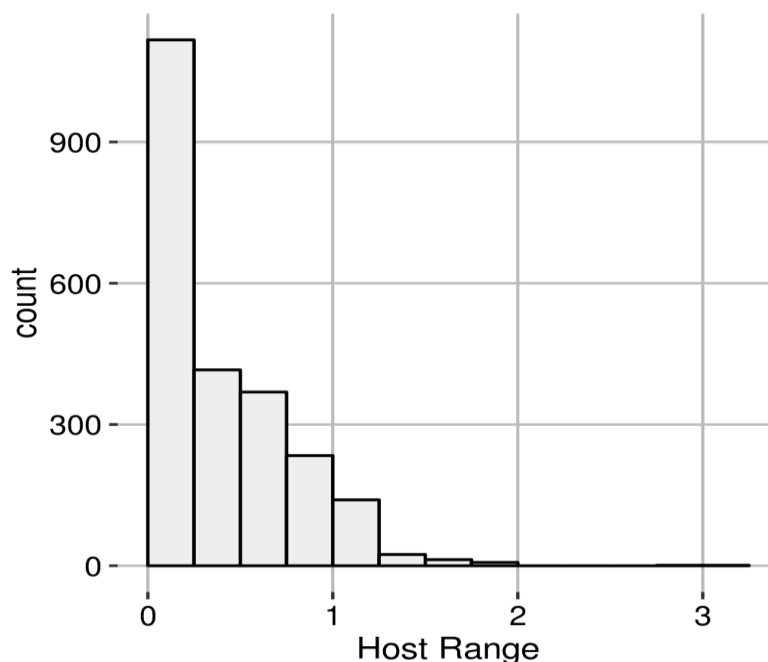

Figure 2.1. Distribution of plasmid host ranges. The x axis represents the host range of plasmids. For each plasmid, host range was measured as the average phylogenetic distance between its hosts.

Host range was measured as the average phylogenetic distance between plasmid hosts, in other words, as the average number of substitutions per site in in 16S sequences. Host ranges spanned continuously between 0 and 3.09 substitutions/site in a non-Gaussian decreasing distribution (Fig. 2.1), with an average of 0.39 and a median of 0.28 substitutions/site. Plasmids for which only one host was found exhibited a host range of 0 substitutions/site, as did plasmids whose hosts all belonged to the same species. Plasmids are commonly just referred as narrow or broad host range. Although we observed a considerable number of plasmids exhibiting very narrow host ranges, we could not identify a clear threshold to split the host range distribution in these two categories. We checked then if plasmid size influenced host range and we found no significant effect (p-value > 0.05, $|\rho| < 0.05$, Spearman correlation test).

Replication mechanisms and host range

Previous experimental observations have classified the host range of some incompatibility groups as broad and narrow. Recently, the association between host range and incompatibility groups has been shown (Suzuki et al., 2010). That work identified potential plasmid hosts by comparing the genomic signature of plasmids to that of various chromosomes, which allow defining host ranges. However, such analysis was restricted to a limited set of incompatibility groups.

Here, we followed the same experimental approach to identify plasmid hosts. Then, we estimated host range as the average phylogenetic distance between all the hosts of a plasmid. Moreover, we attempted to classify plasmids on additional known incompatibility groups, using two previously described methods. We successfully assessed incompatibility groups for 1012 (nearly 45 %) of the 2322 plasmids for which we had already inferred host range values. 860 plasmids belonged to only one incompatibility groups, while 152 belonged to more than one incompatibility group. Testing the host range of plasmids (belonging only to individual incompatibility groups), we observed differences associated with the incompatibility groups they belonged to (p-value < 10^{-15}, Kruskal-Wallis test). For instance, incompatibility groups such as IncI and IncF exhibit narrower host ranges than IncA/C, IncP-1 and IncQ plasmids (Fig. 2.2A). These results are in agreement with those obtained by (Suzuki et al., 2010), thus validating our analysis. We also observed (Fig. 2.2B) differences when comparing plasmids belonging to multiple incompatible groups (p-value < 10^{-12}, Kruskal-Wallis test).

It has been suggested that the presence of multiple replicons could be associated with broad host range (delSolar et al., 1996; Jain & Srivastava, 2013). We tested a different hypothesis by checking for differences between plasmids belonging to only one incompatibility group and those belonging to multiple incompatibility groups. Indeed, we observed (Fig. 2.2C) that plasmids belonging to multiple incompatibility groups exhibited broader host ranges (*p*-value = 0.0003, Wilcoxon test). Next, we compared each of the multiple incompatibility groups against all groups it comprised (data not shown). Such analysis revealed that plasmids belonging to multiple incompatibility groups did not exhibit a significant greater host range than plasmids belonging to at least one of the individual incompatibility groups. These results could be due to an over representation of incompatibility groups associated with broad host ranges

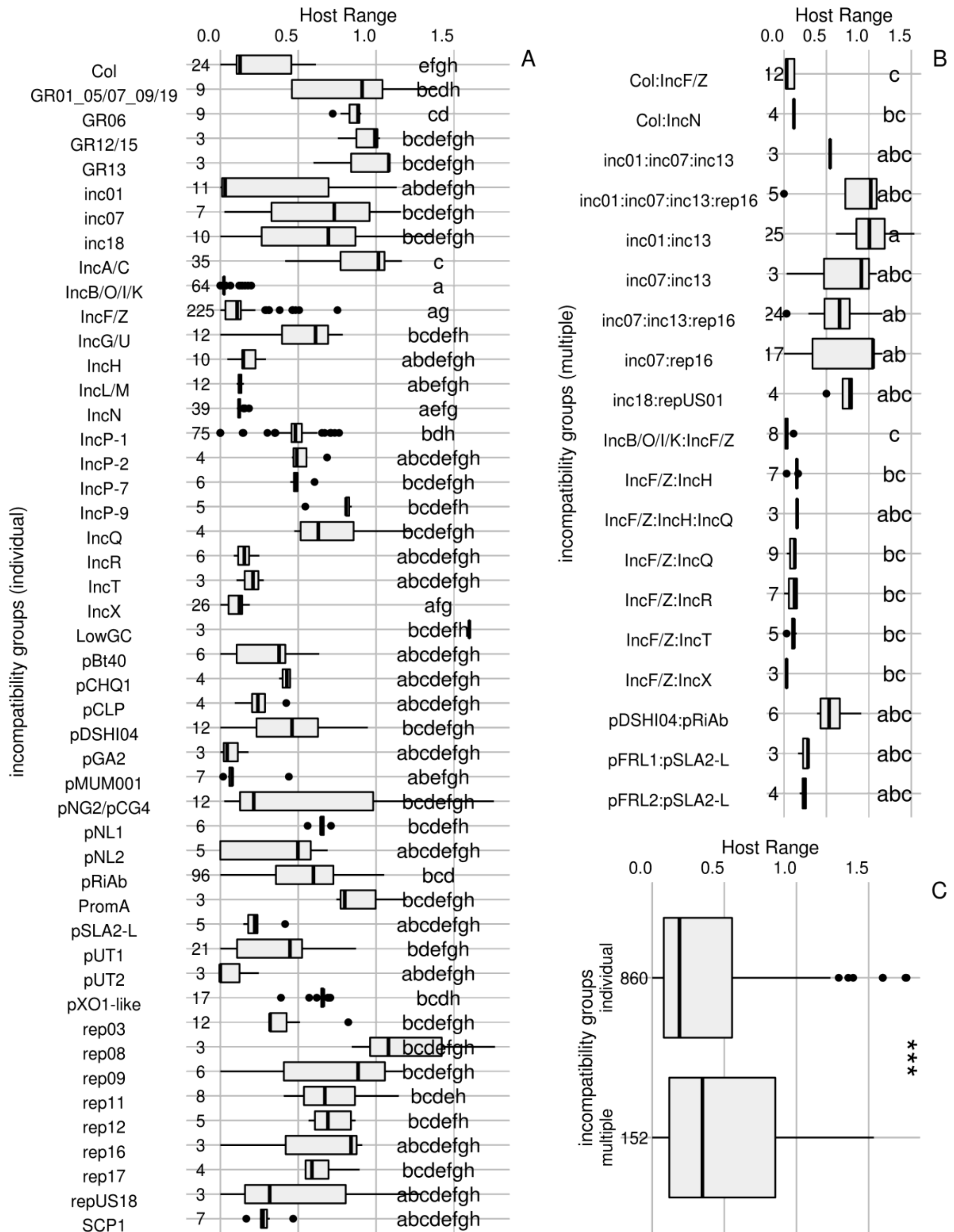

Figure 2.2. Variation of host range determined by incompatibility groups. A – host range variation of plasmids encoding individual incompatibility groups. B – host range variation of plasmids encoding multiple incompatibility groups. C – comparison between plasmids encoding individual and multiple incompatibility groups. For each plasmid, host range was measured as the average phylogenetic distance between its hosts. Annotations at the left of each box represent the number of plasmids. Annotations above at the right of boxes represent the results of post hoc multiple comparison tests; groups with different letters are significantly different (p-value < 0.05). Significant differences in A and B (p-value < 10^{-15} and < 10^{-12} respectively, Kruskal-Wallis test), and C: *** p-value < 0.001, Wilcoxon test.

among multiple incompatibility groups. In fact, of the plasmids belonging to multiple incompatibility groups, approximately 50 % belonged to at least one of the following groups: inc07, inc13, inc18 or rep16, which are characteristic of plasmids associated with Gram-positive bacteria. When considering plasmids assigned to only one incompatibility group, less than 5 % belonged to those four groups. Indeed, among the plasmids belonging to a single incompatibility group, only a minority (less than 10 %) encoded replicons associated with Gram-positive bacteria. Thus, plasmids associated with Gram-positive bacteria, which exhibit broad host range, seem to have a greater tendency to encode multiple replicons.

Plasmid mobility and host range

It is possible that host ranges are affected by plasmid type, namely if it is conjugative, mobilizable or non-mobilizable. Of the 2322 plasmids with defined host ranges, 886 were conjugative and 411 were mobilizable. We observed that the host range varied with plasmid type (p-value < 0.001, Kruskal-Wallis test). Non-mobilizable plasmids exhibited narrower host ranges as expected (Fig. 2.3A), however, no difference was observed between conjugative and mobilizable

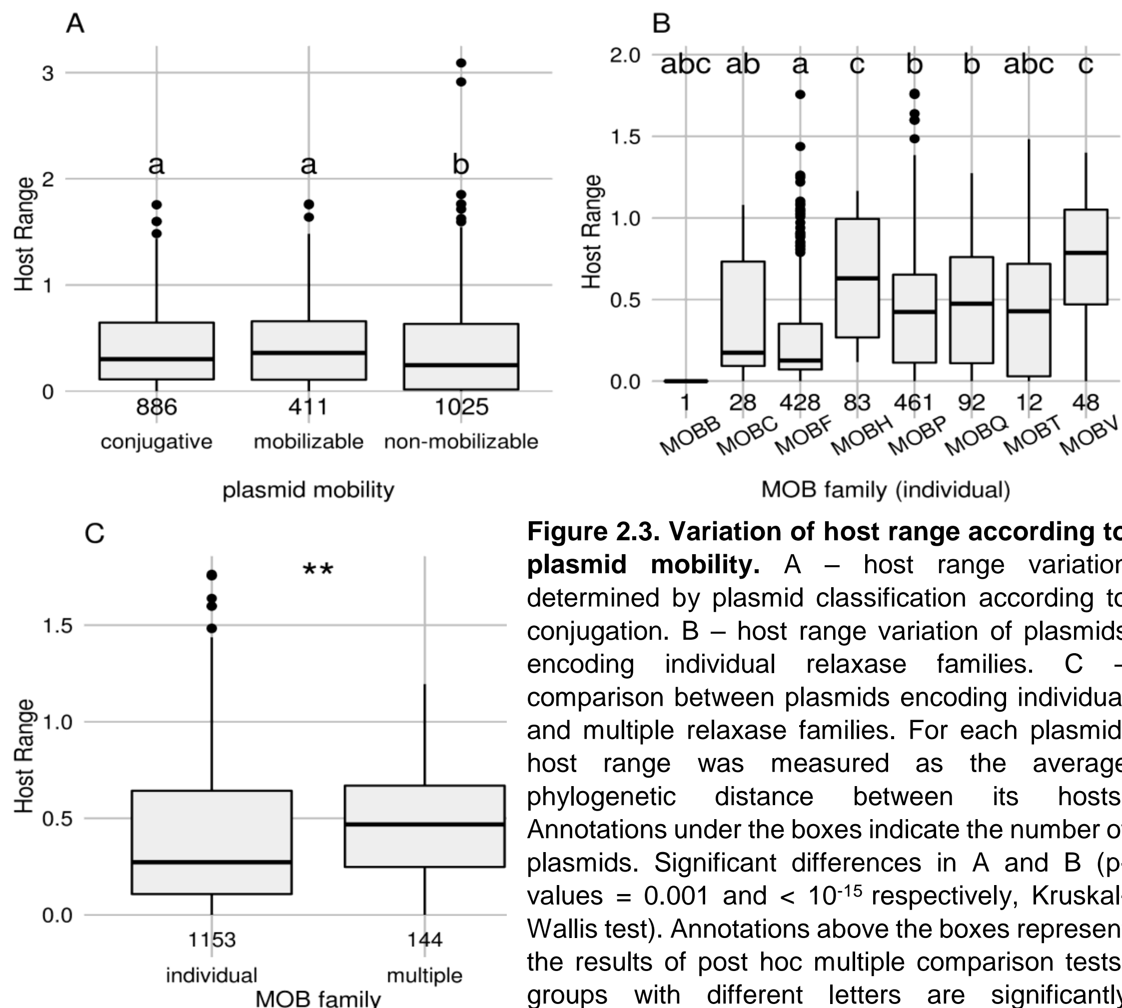

Figure 2.3. Variation of host range according to plasmid mobility. A – host range variation determined by plasmid classification according to conjugation. B – host range variation of plasmids encoding individual relaxase families. C – comparison between plasmids encoding individual and multiple relaxase families. For each plasmid, host range was measured as the average phylogenetic distance between its hosts. Annotations under the boxes indicate the number of plasmids. Significant differences in A and B (p-values = 0.001 and < 10^{-15} respectively, Kruskal-Wallis test). Annotations above the boxes represent the results of post hoc multiple comparison tests; groups with different letters are significantly different (p-value < 0.05). ** p-value < 0.01, Wilcoxon test.

plasmids (post hoc test). Conjugative and mobilizable plasmids were also classified according to their relaxases. Relaxase families displayed different host ranges (p-value < 10^{-15}, Kruskal-Wallis test). MOB_V and MOB_H exhibited the broader host ranges (post hoc test), followed by MOB_P and MOB_Q relaxase families (Fig. 2.3B). Narrower host ranges were attributed to MOB_C and MOB_F relaxases. Due to under-representation, the host range of plasmids encoding MOB_B and MOB_T relaxase families is uncertain.

Approximately 10 % of the plasmids, harboured distinct relaxase families, were classified into multiple types. We then tested for differences between plasmids coding single and multiple relaxase families and found that plasmids carrying more than one relaxase family exhibited broader host ranges (p-value < 0.01, Wilcoxon test) (Fig. 2.3C). We then compared (Kruskal-Wallis tests, Dunnett tests) each of the multiple relaxase families against all the relaxase types it comprised, as done before for incompatibility groups. We found that plasmids encoding multiple relaxase families did not exhibit a significant greater host range than plasmids encoding at least one of those individual relaxase families. In addition, we observed an over representation of relaxase families conferring broad host ranges among plasmids encoding multiple relaxase families. Either MOB_P or MOB_Q were present in approximately 90 % of plasmids encoding distinct relaxases but only in less than 50 % of the plasmids encoding only one type of relaxase. These results are qualitatively similar, either if considering the sample of 2322 plasmids with defined host ranges, or the subsample of 1012 plasmids classified in incompatibility groups.

Ability to overcome host's restriction-modification systems

We tested the association between host range and the ability of plasmids to overcome cellular defence mechanisms, such as restriction-modification systems. Avoidance of small palindromic sequences, which are often recognized

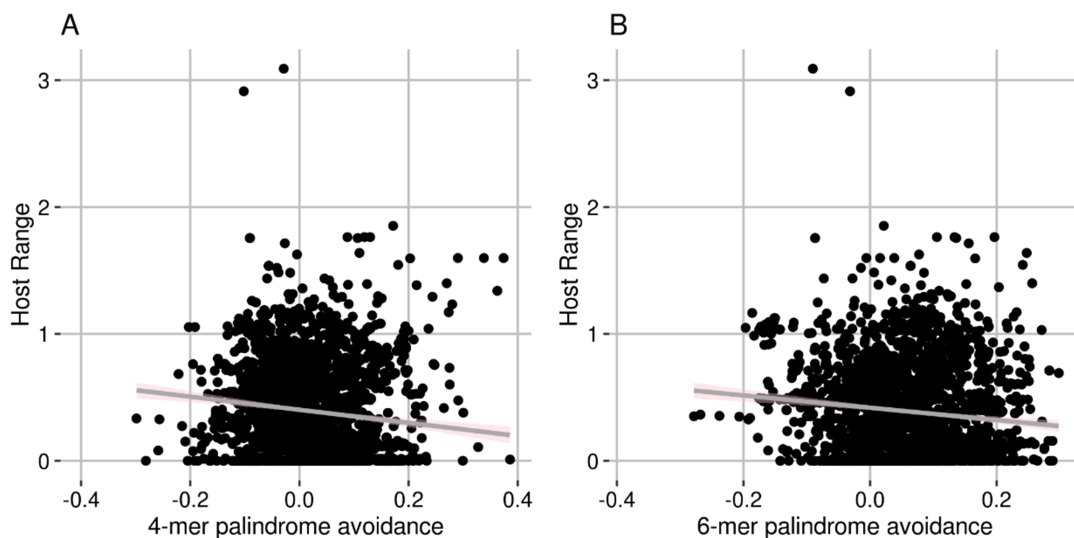

Figure 2.4. Correlation between host range and palindrome avoidance. A – 4-mer palindromes. B – 6-mer palindromes. Palindrome avoidance represents the different frequencies of palindromes in the sequences of plasmids and their respective hosts (see Materials & Methods for details). For each plasmid, host range was measured as the average phylogenetic distance between its hosts. Significant Spearman correlation tests (p-values < 10^{-15} and < 10^{-6}, respectively; ρ = -0.19 and -0.1, respectively).

as restriction sites, constitutes a strategy to overcome the action of restriction-modification systems. We analysed the composition of plasmid sequences in terms of 4- and 6-mer long palindromes. We compared the frequency of palindromes present in each plasmid sequence versus in all its hosts, which resulted in a numeric variable reflecting the mean palindrome avoidance of a plasmid. The more a plasmid avoids palindromes (comparatively to its hosts) the smaller is the value of this variable.

We observed (Fig. 2.4) a significant, but weak, negative correlation between host range and avoidance of size 4 palindromes (p-value $< 10^{-15}$, $\rho = -0.19$, Spearman correlation test) and size 6 palindromes (p-value $< 10^{-6}$, $\rho = -0.1$, Spearman correlation test). That is, plasmids exhibiting wider host ranges are more prone to avoid palindromes. When considering the subsample of plasmids with defined incompatibility groups, correlations became little stronger, however never exceeding $\rho = -0.37$.

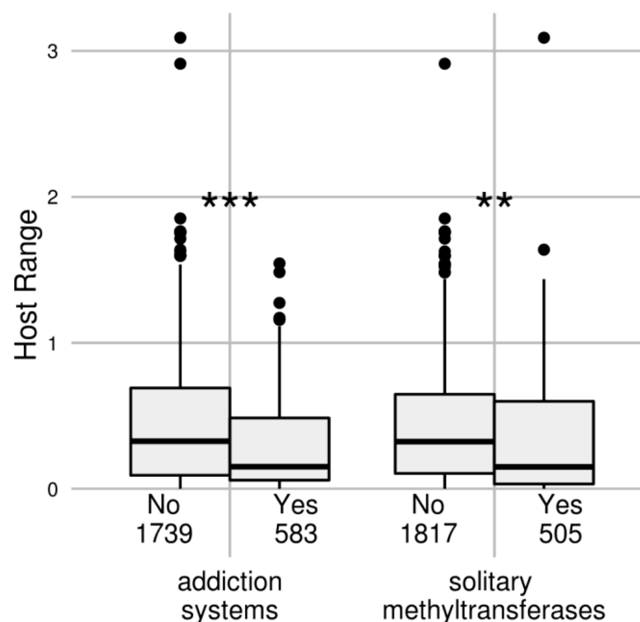

Figure 2.5. Association between host range and addiction systems and methyltransferases. For each plasmid, host range was measured as the average phylogenetic distance between its hosts. Annotations under the boxes indicate the number of plasmids. ** p-value < 0.01. *** p-value < 0.001, Wilcoxon test.

Additionally, we searched for solitary methyltransferases in plasmid sequences and found a total of 505 plasmids encoding methyltransferases among the 2322 plasmids with defined host ranges. Plasmids harbouring them exhibited narrower host ranges (p-value < 0.01, Wilcoxon test; see Fig. 2.5). This result holds if instead we consider the subsample of plasmids with defined incompatibility groups.

Effect of addiction systems

Plasmids can also encode addiction systems, which are thought to contribute to their stability. In this work, we considered type II restriction-modification systems as addictive systems. We found a total of 583 plasmids encoding either TA *loci* (505) or type II RMS (91) among the 2322 plasmids with defined host ranges and they exhibited narrower host ranges than plasmids not encoding such systems (p-value < 10^{-7}, Wilcoxon test) (Fig. 2.5). We obtained identical results whether considering the subsample of plasmids with defined incompatibility groups or the subsample of horizontally transmitted plasmids.

Accessory genes

Plasmids are known to carry accessory genes, which are not required for their replication nor transmission. Accessory genes can encode, for example, antibiotic resistance, pathogenic traits or degradative enzymes. Such genes can be beneficial to bacteria and therefore play a role in plasmid maintenance. An important way for plasmids to acquire non-essential genes is through the acquisition of integrons, which can be defined as platforms that capture and spread genes. We identified integrons in 208 of the 2322 plasmids with defined host ranges. The presence of integrons did not affect host ranges significantly (p-value > 0.05, Wilcoxon test). Testing the subsample of plasmids with defined incompatibility groups yielded qualitatively similar results.

Host range is mainly defined by incompatibility groups

Overall, we observed that several factors affect the host range of plasmids. Thus, we access the relative importance of each factor. We tested the impact of these factors in two phases. First, we made a stepwise regression analysis of whole effects, that is, testing the effect of each variable. Both, forward and backward analysis revealed that incompatibility groups contribute to explain most (67.68 %) of the variance observed in host range. Altogether, incompatibility groups, relaxase family, presence/absence of methyltransferases and avoidance of 6-mer palindromes explained 70% of the observed host range variance (Table 2.1).

Table 2.1. Results of stepwise regression of whole effects

Parameter	R^2 (adjusted)*	AIC criterion	Prob>F
incompatibility groups	0,6768	-305	<,0001
relaxase families	0,6940	-343	<,0001
6-mer palindromes	0,6968	-351	0,0005
methyltransferases	0,6996	-359‡	0,0019

* cumulative effect of the variables on the stepwise regression.
‡ minimum by the AIC criterion.

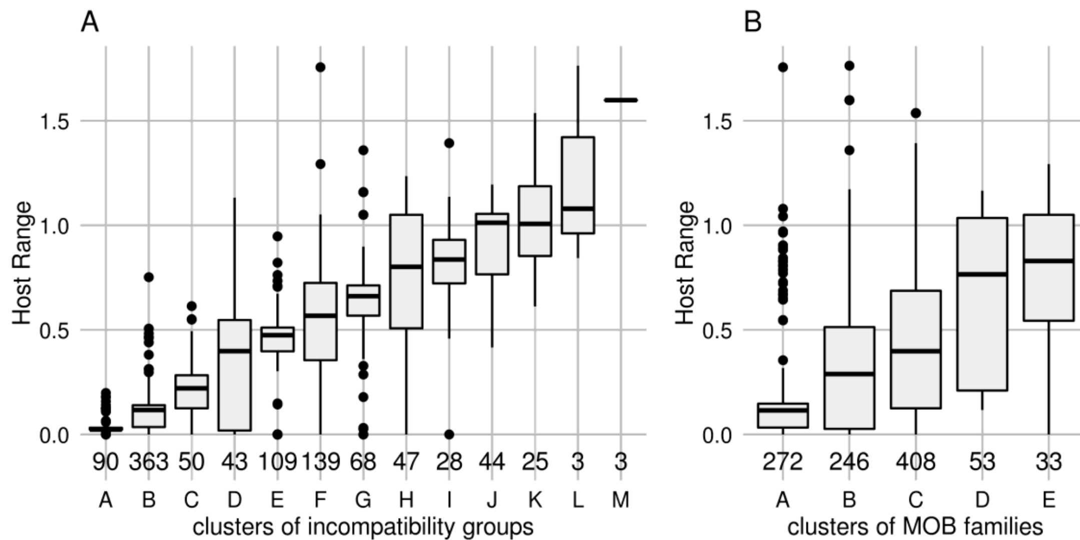

Figure 2.6. Association between host range and clusters of: A – incompatibility groups, B – relaxase families. For each plasmid, host range was measured as the average phylogenetic distance between its hosts. Annotations under each box indicate the number of plasmids. Statistics are shown in Table 2.1 and Table 2.2. The x axis represents clusters of incompatibility groups (A) and of relaxase families (B). Clusters are described in Table 2.3. For example, cluster J of incompatibility groups includes the following: PromA; IncA/C; GR13 and GR12/15.

Next, we made two additional stepwise regression analysis to understand single individual contribution of the hierarchic cluster divisions of incompatibility groups and relaxase families (Fig. 2.6, Tables 2.2 and 2.3). Incompatibility groups span through 12 clusters. The clusters exhibiting narrow host ranges comprised

Table 2.2. Results of stepwise regression of hierarchical clusters

Term		Prob>|t|
Variable	Clusters	
incompatibility groups	[A-D] – [E-M]	<,0001
	[A-B] – [C-D]	<,0001
	A – B	0,0027
	C – D	0,0026
	[E-H] – [I-M]	<,0001
	[E-F] – [G-H]	<,0001
	E – F	<,0001
	G – H	0,0245
	[I-K] – [L-M]	<,0001
	[I-J] – K	0,0003
	I – J	0,0358
	L – M	0,0209
relaxase families	[A-B] – [C-E]	<,0001
	A – B	<,0001
	C – [D-E]	<,0001
	D – E	0,043

The levels of the variable are successively split into groups of levels (clusters) differing in their mean host range. The probability associated with each split is indicated. For example, clusters A through D are separated from clusters E through M (p-value < 0.0001). See Table 2.3 for a description of the groups included in each cluster.

many of the incompatibility groups frequently associated with enterobacterial species, which supports the results shown in Fig. 2.2A. Relaxase families were split in five clusters which also agree with the results shown in Fig. 2.3B as plasmids encoding MOB_C or MOB_F exhibit lower host ranges while plasmids encoding MOB_V and MOB_H exhibit higher host ranges.

Table 2.3. Clusters of incompatibility groups and relaxase families

variable	Cluster	Groups included in each cluster
incompatibility groups	A	IncF/Z:IncX; IncB/O/I/K; IncB/O/I/K:IncF/Z; Col:IncF/Z; pGA2
	B	Put2; IncF/Z:IncR; IncF/Z:IncT; IncF/Z:IncQ; IncF/Z; IncX; pMUM001; Col:IncN; IncN; IncL/M;IncF/Z:IncH; IncF/Z:IncH:IncQ; IncR; IncH
	C	IncT; Col; pFRL2:pSLA2-L; pSLA2-L; pFRL1:pSLA2-L; pCLP; SCP1
	D	pBt40; pNL2; inc01; pUT1
	E	rep03; pDSHI04; pCHQ1; IncP-1; IncP-7
	F	IncG/U; IncP-2; pNG2/pCG4; repUS18; inc01:inc07:inc13; pRiAb; pDSHI04:pRiAb; rep16
	G	inc18; inc07:inc13:rep16; inc07; pXO1-like; pNL1; rep17
	H	inc07:inc13; rep11; rep12; inc18:repUS01; rep09; inc07:rep16; IncQ
	I	IncP-9; inc01:inc07:inc13:rep16; GR01_05/07_09/19; GR06
	J	PromA; IncA/C; GR13; GR12/15
	K	inc01:inc13
	L	rep08
	M	LowGC
relaxase families	A	MOBF:MOBP; MOBC:MOBF; MOBF:MOBH; MOBF; MOBC
	B	MOBP
	C	MOBP:MOBQ; MOBQ; MOBF:MOBH:MOBP; MOBF:MOBQ *
	D	MOBH
	E	MOBF:MOBP:MOBQ; MOBP:MOBV; MOBV

* also includes non-mobilizable plasmids

Discussion

Plasmids evolve in different hosts, which define their host range. Plasmids encode diverse features that can affect their host range. Our aim was to study the host range of prokaryotic plasmids, more specifically, to identify the factors determining it. To do so, we started by identifying their potential hosts. We employed the method developed by (Suzuki et al., 2010), which allowed us to find potential hosts for almost 90% of the studied plasmids. For the remaining plasmids, their hosts might have not been yet sequenced, resulting in our inability to identify them. Furthermore, as discussed in (Suzuki et al., 2010), highly

promiscuous plasmids may spend little time in each host, and consequently are not ameliorated. This fact could also contribute to our inability to identify hosts for some plasmids.

We estimated host range as the average phylogenetic distance of between all the hosts of a plasmid. This approach was successful as our results are in agreement with those of (Suzuki et al., 2010), showing for instance that plasmids belonging to incompatibility groups such as IncI and IncF exhibit narrower host ranges than those encoding IncA/C, IncP-1 or IncQ replicons. Analysing the distribution of host ranges, we did not observe a clear division that could be used to classify plasmids as narrow or broad host range.

In this Chapter, we wanted to understand the association between the host range of plasmids and a set of variables related with plasmid replication, transmission and stability. Among these, we observed that the replication mechanism explained more than 2/3 of the variability in host range. By clustering incompatibility groups, we observed that many of the groups commonly found in enterobacterial species exhibit narrower host ranges than those that are frequent among Gram positive species. Rolling circle and strand-displacement replication mechanisms are associated with broader host ranges (delSolar et al., 1996; Jain & Srivastava, 2013). Due to the historical development of the knowledge of plasmids, replication by rolling circle has also been associated with plasmids of Gram-positive bacteria (del Solar et al., 1998). We did not classify plasmids according to these general mechanisms of replication, but this seems to be relevant for future works. We also observed that plasmids exhibiting multiple incompatibility groups had broader host ranges. However, such broader host range could be the cause for acquiring further replication traits instead of a consequence.

We have been regarding incompatibility groups as a variable associated with replication, thus defining the interactions between plasmids and hosts. However, incompatibility groups may have an additional role in maintenance due to interactions between different plasmids. In this sense, two plasmids encoding incompatible replicons could be maintained if they encoded additional replication mechanisms determining different incompatibility groups. This hypothesis fits with the fact that plasmids exhibiting broad host ranges encode multiple incompatibility groups. This would represent a good strategy to compete with other plasmids sharing one of the incompatibility groups encoded by plasmids exhibiting multiple replicons. Therefore, the dual role of incompatibility groups as a proxy of replication (interactions with hosts) and competition (interactions with other plasmids) would explain why this variable exhibited the strongest association with host range.

The mobility mechanisms of plasmids also helped to explain the observed variability of host range, however to a much lesser extent than incompatibility groups. In general, conjugative and mobilizable plasmids exhibited broader host ranges than non-mobilizable plasmids. We did not observe any significant difference between conjugative and mobilizable plasmids. Additionally, our results show that different relaxase families affect differently plasmid host ranges. As for incompatibility groups, we also noted that plasmids encoding multiple relaxase families exhibited broader host ranges. It is however important to keep in mind that our approach does not allow the detection of origins of transfer. Consequently, we classify all plasmids not encoding a relaxase as non-mobilizable. Likewise, mobilizable plasmids that encode an origin of transfer but

no relaxase exist. Thus, some plasmids classified as non-mobilizable can in fact be mobilizable. Moreover, non-mobilizable plasmids might be transferred by mechanisms other than conjugation, for instance transduction or transformation. Such plasmids can therefore have broader host ranges than expected although they are non-mobilizable.

The set of hosts in which a plasmid can be stably maintained is narrower than that in which it can replicate or be transferred to (De Gelder et al., 2007; Suzuki et al., 2010). Thus, one would expect that traits associated with plasmid stability should strongly affect host range. Yet we failed to observe such effect. We also studied some of the factors thought to affect plasmid stability such as palindrome avoidance, addiction systems, carriage of methyltransferases and integrons. Part of these features exhibited significant effects, but altogether they did not constitute the predominant variable affecting host range. Stability can result from the simultaneous action of several factors and the individual analysis of each factor might be insufficient to explain the variation in host range. Furthermore, distantly related plasmids may employ a wide diversity of mechanisms involved in maintenance and the interactions between distinct mechanism could also be relevant to explain the variation in host range. Thus, it is difficult to choose a common crucial factor simultaneously explaining the stability of a diversity of plasmids. It is also important to state that future works using different in silico analysis, as well as experimental approaches, may shed light on this matter, identifying new mechanisms involved in plasmid maintenance.

Author Contributions

Conceived and designed the experiments: João Alves Gama, Eduardo P. C. Rocha. Performed the experiments: João Alves Gama, Pedro H. Oliveira. Provided tools or data: Marc Garcia-Garcerà. Analysed the data: João Alves Gama Eduardo P. C. Rocha. Wrote the chapter: João Alves Gama, with contributions of Eduardo P. C. Rocha, Francisco Dionísio, Rita Zilhão.

Supplementary Information

Supplemental Table S2.1

Combined group	Groups included
Col	Col; pSC101
GR01_05/07_09/19	GR01; GR02; GR03; GR04; GR05; GR07; GR08; GR09; GR19
GR12/15	GR12; GR15
inc01	inc01; rep20; repUS9; repUS14
inc07	inc07; rep05; rep15; rep19; rep24
inc13	inc13; rep21; rep22; repUS12; repUS13
inc18	inc18; rep01; rep02
IncB/O/I/K *	IncB/O; IncI; IncK
IncF/Z *	IncF; IncZ; pCD1
IncQ	IncQ; pTF-FC2
pKJ50/pNAC2	pKJ50; pNAC2
pSL483	pSL483; pXuzhou21
rep07	inc04; inc09; inc10; inc10; rep07; rep07b
rep10	rep10; rep10b
rep13	rep13; repUS22

Groups on the first column include the groups indicated on the second column.
* Results for PlasmidFinder IncB/O/K/Z probes were not used

Chapter 3

Conjugation efficiency depends on intra and intercellular interactions between plasmids – some neighbours are good Samaritans

Abstract

Natural isolated bacteria harbour conjugative plasmids, extra-chromosomal DNA elements capable of transferring among cells. To transfer, these plasmids are responsible for the synthesis of certain appendages called conjugative pili, whose expression may be costly to bacteria – mainly because conjugative pili serve as receptors for certain viruses but also due to the cost associated to expression of conjugative pili themselves. Therefore, if two different plasmids colonize the same cell, they face a conflict: each plasmid has an individual advantage of expressing their own conjugative pili, but the cost (increasing the risk of viral infection or the burden to the cell) is shared by both plasmids. However, if the two plasmids reside in two different bacterial cells, there is no such conflict. Therefore, plasmids are expected to repress the synthesis of conjugative pili by unrelated plasmids when present in the same cell but not when present in different cells. In this work, we measure transfer rates of twelve natural plasmids, belonging to seven incompatibility groups, in three situations: when donor cells contain a plasmid and recipient cells are plasmid-free; when donor cells contain two unrelated plasmids and recipient cells are plasmid-free; and when half of the cells contain a given plasmid and the other half contain another, unrelated, plasmid – here, the recipient cells of a plasmid are the donor cells of the other plasmid. Our results show that the number of negative interactions (reduction of a plasmid's conjugative efficiency) is significantly higher if interactions occur between plasmids residing in the same cells than between those residing in different cells. Interestingly, however, intercellular interactions were mostly positive, i.e., the transfer rate of each plasmid is higher if another, unrelated, conjugative plasmid is present in the recipient cell than if this cell is plasmid-free. Experimental data retrieved from the study of mutant plasmids not expressing conjugative pili on the cell surface suggest that positive effects come from a higher efficiency of mating pair formation.

Introduction

Plasmids are double-stranded DNA molecules commonly present in bacterial cells. By definition, they do not encode genes essential for bacterial growth under non-stressing conditions. Plasmids are able to replicate independently of the host's chromosome. Nevertheless, plasmids have to use resources of their bacterial host to ensure their own replication and maintenance. Some plasmids, called conjugative plasmids, are able to transfer horizontally to other bacterial cells, through a process termed conjugation. For this, plasmid-encoded appendages, called conjugative pili (or sex-pili), promote cellular contact

between bacterial cells, which is required for plasmid transfer (Cabezon et al., 2015).

Although essential for plasmid transfer, the conjugative process leads to negative effects. Expression of the conjugative machinery imposes a fitness cost on plasmid hosts because it requires resources, alters cell physiology and determines viral susceptibility because conjugative pili serve as receptors for certain viruses (Baltrus, 2013).

Conjugative plasmids transfer among heterogeneous bacterial communities (Dionisio et al., 2002), and so bacteria can be colonized by multiple plasmids. This 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. Some plasmids code for fertility inhibition systems, which reduce their own rate of horizontal transfer thus preventing the infection by those viruses. Second, when harbouring more than one plasmid, metabolic costs increase and cells replicate slower than when harbouring just a plasmid (Morton et al., 2014; San Millan et al., 2014; Silva et al., 2011).

Metabolic costs imposed by plasmids may have different origins: they may originate from the extra DNA present in the cell and its replication, as well as from the expression of plasmid genes. Indeed, it has been shown that extra DNA molecules present in the bacterial cell may negatively impact cell's replication rate and consequently, the evolutionary success of plasmids present in the cell. For example, a study on the impact of plasmid sizes on bacterial growth has shown that, at stationary phase, as plasmid size increased, maximum cell density decreased (Cheah et al., 1987). Moreover, with the largest plasmid, cell death was accelerated after the stationary phase was reached. This effect was mostly due to the amount of plasmidic DNA, not transcription or translation of genes (Cheah et al., 1987). However, plasmid size does not seem to be correlated with fitness cost (Vogwill & MacLean, 2015). This suggests than besides size other plasmidic features affect host's fitness. The total amount of plasmidic DNA in the host cell results from plasmid size, but also from the number of plasmid copies. For instance, in another study, it was shown that, in yeast, each extra copy of a plasmid imposed a higher cost of 0.17% to the cell (Harrison et al., 2012). Moreover, small and big plasmids may exhibit identical fitness costs because small plasmids are present in greater copy numbers inside the host cell (Millan et al., 2010).

Expression of genes also has a negative impact to the cell replication rate. Indeed, it is more costly to the cell if plasmid genes are expressed than if they are not. For example, plasmids expressing antibiotic resistance are more costly to their hosts than plasmids not expressing them (Humphrey et al., 2012; Turner et al., 1998).

Not surprisingly, plasmid behaviour and evolution has evolved to face other plasmids. For example, within-host competition between plasmids can select for toxin-antitoxin systems (Cooper et al., 2010). Toxin-antitoxin systems are those in which an antitoxin counteracts the effect of the toxin unless the system is lost from the cell. Then the unstable antitoxin degrades and the cell becomes susceptible to the toxin. In another study, it was argued that competition between plasmids for limited hosts selects for lower horizontal transfer rates, which helps to explain the persistence of fertility inhibition systems in nature (Rembrandt J F Haft, John E Mittler and Beth Traxler, 2009, ISME J). The reason

is that, for a plasmid, it may be dangerous if another plasmid inside the same cells produces many conjugative pili. Accordingly, it has been argued that plasmids have evolved mechanisms to repress conjugative pili production when alone but not when co-inhabiting with other plasmids – if a second plasmid is present and imposing a cost to the host, there is no advantage for the first plasmid to repress its own transfer (Dionisio, 2005; Dionisio et al., 2002).

According to all the examples mentioned above, plasmids seem to be somehow adapted to deal with other plasmids (conjugative or not) already present inside recipient cells or transferable plasmids about to arrive into their bacterial host. 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 and there are several possibilities.

One of the plasmids (the "focal" plasmid) may have the ability to increase its own transfer rate in presence of rival plasmids. At least four mechanisms have been identified: the so-called processes of "conduction", "donation", facilitation and mobilization. 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 & Warren, 1979). Donation, is the process whereby the cellular contact determined by a conjugative plasmid allows the horizontal transfer of non-conjugative plasmid (Clark & Warren, 1979). 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). Some plasmids (termed 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. This type of interaction constitutes the process of mobilization (Smillie et al., 2010). With any of these four mechanisms, a focal plasmid would be increasing its relative evolutionary success, compensating for any risk associated to the expression of sex-pili by other co-inhabiting plasmids, at least partially.

Alternatively, a likely strategy for a given focal plasmid to decrease the costs associated with the conjugative transfer of rival plasmids co-inhabiting the same cell is by decreasing the transfer rate of rival plasmids. This could be accomplished either by solely averting transfer of DNA or by completely preventing the expression of its transfer, e.g. preventing pilus synthesis, which theoretically is more favourable to the host cell.

In the last strategy (where the focal plasmid decreases the transmission of its rival), if the focal plasmid has no individual cost, the interaction is sometimes referred to as interference (Chao et al., 2000). In fact, 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 & Shipley, 1975; Pinney & Smith, 1974; Sagai et al., 1977; Tanimoto & Iino, 1983; Willetts & Skurray, 1980; Winans & Walker, 1985). For example, there are at least five different mechanisms encoded by non-F-like plasmids that inhibit 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 & Willetts, 1975; Gasson & Willetts, 1977; Willetts & 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 & Shipley, 1975).

The mechanisms of plasmids pKM101 and F that inhibit the conjugation of plasmid RP4 stand among the best characterized. The FipA protein of plasmid pKM101 and the PifC protein of plasmid F target the TraG coupling protein of RP4 which is involved in DNA transport (Santini & Stanisich, 1998). However, neither of these two inhibitory mechanisms affects pilus synthesis (Miller et al., 1985; Tanimoto & Iino, 1983; Winans & Walker, 1985). 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 & Stanisich, 1989; Yusoff & Stanisich, 1984). *fiwB* prevents plasmid R388 from producing conjugative pili. The *fiwB* region contains three genes *klaA*, *klaB* and *klaC*, all required to inhibit conjugation of R388 (Goncharoff et al., 1991). The product of *fiwA* and the Osa protein of plasmid Sa share some homology (Chen & Kado, 1994). Osa interacts with plasmid Ti from *Agrobacterium* species (now included in the genus *Rhizobium* (Young et al., 2001)) (Chen & Kado, 1994; Close & Kado, 1991). Plasmid Ti encodes two conjugative systems: one for its own transmission and another to transfer T-DNA (a portion of the Ti plasmid), which is essential for pathogenicity in plants) (Christie, 2004; Li et al., 1998). 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). Consequently, Osa prevents plasmid Ti from producing oncogenic effects in plants (Chen & Kado, 1996; Close & Kado, 1991; Farrand et al., 1981; Loper & Kado, 1979; Vlasak & Ondrej, 1985).

Active inhibition of co-resident plasmids, however, is not the only successful strategy available to plasmids. RSF1010 (nearly identical to R300B and R1162 (Rawlings & Tietze, 2001)), for example, is a mobilizable plasmid and thus depends on the machinery encoded by conjugative plasmids (such as Ti) to be horizontally transmitted. When both plasmids RSF1010 and Ti inhabit the same host cell, the DNA from RSF1010 accumulates in excess because this plasmid has a higher copy number than plasmid Ti. Consequently, DNA from RSF1010 out-competes the T-DNA of Ti, therefore monopolizing the transport machinery and indirectly inhibiting the horizontal transfer of plasmid's Ti T-DNA (Cascales et al., 2005; Ward et al., 1991).

These examples where molecular details of the interactions between plasmids are known suggest a general rule: if the transfer rate of a given plasmid "A" decreases in the presence of another plasmid "B" within the same cell, such reduction is caused by genes or mechanisms encoded by the plasmid "B". In other words, it is the result of an antagonistic action of the plasmid "B". For example, plasmid pKMH101 encodes the protein FipA that targets the coupling protein of plasmid RP4, thereby interfering with the conjugative machinery of this plasmid.

From the point of view of natural selection, and still following the above example with plasmids "A" and "B", one also should consider these cases as an

antagonistic behaviour of the plasmid “B”. The reason for this is the low selective force for “A” to decrease its own transfer rate in the presence of a rival (unrelated) plasmid “B”. Such behaviour would indeed provide an advantage for both plasmids. However, it would also provide a cost to plasmid “A” because its dissemination would decrease. Consequently, strong selection of this behaviour, contradicts plasmid “A”'s self-preservation, so that by preventing its own horizontal transfer, plasmid “A” could be eliminated from the population

Positive interactions (conduction, donation, facilitation or mobilization), however, are more difficult to interpret. Consider two plasmids, “A” and “B” where the transfer rate of “A” increases in presence of plasmid “B”. Is plasmid “A” taking advantage of plasmid “B”, or, is plasmid B, for some reason, helping plasmid “A”? It can be difficult to answer this question. Once more, molecular details may help. 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 the 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, resulting in their mobilization. Moreover, mobilizable plasmids encode a few genes that allow them to interact with the conjugative machinery encoded by rival plasmids.

Again, from the point of view of natural selection, and still following the example with plasmids “A” and “B”, one also should consider these cases as a selfish behaviour of the plasmid “A” (the mobilizable plasmid of the previous example), not as a “benevolent” behaviour of the unrelated plasmid “B”, because only plasmid “A” benefits from this behaviour.

What happens if the two plasmids are 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 mechanisms (surface and entry-exclusion systems) to avoid the incoming of similar or related plasmids. Being similar, they will probably be incompatible, and so exclusion saves resident plasmids from competing with related plasmids. To our knowledge, mechanisms blocking unrelated incoming plasmids, however, 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 (say, plasmid “A”) will increase the probability that another conjugative plasmid (say, “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 – plasmid “B” in the example above – 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” (due to the expression of a system of fertility inhibition) is expressing 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, stabilization of mating pairs by one of the plasmids (e.g., “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 (plasmid “B”) in recipient cells has no positive nor negative effect 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. One may further foresee other possible scenarios by considering the possibility that after arriving into a cell already harbouring an unrelated plasmid the two plasmids may interact negatively or positively as explained above.

In this work, we measure transfer rates of twelve natural plasmids, belonging to seven incompatibility groups, in three settings and compare their values in order to understand their competitive strategies. The three settings are: (1) donor cells contain a plasmid and recipient cells are plasmid-free; (2) donor cells contain two unrelated plasmids and recipient cells are plasmid-free; and (3), 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.

Materials & Methods

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 1.1.

Generation of plasmid carrying strains

The general method is depicted in Supp.Fig. S3.1.

We carried out overnight mating experiments, in Luria 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*$^+$) receptor 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 combinations 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 LB solid (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.

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 & Wanner, 2000). We used the λ Red expression vector pKD46, the vector pKD3 as template and the primers traG_fwd_cmpR 5'- CTCAGTCGTTACCAGAACAACTATCACTTCGGAGGGAG CACGCTGTGAAAGTGTAGGCTGGAGCTGCTTC-3' and traG_rev_cmpR 5'- CTCTCCATACCCTACCCAACATGTTATGATTATTCTTTATGCTGGTAACTCA TATGAATATCCTCCTTAGT-3' designed as described elsewhere (Baba et al., 2006; Datsenko & 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 30ºC in LB solid medium (with ampicillin for pCP20 selection) lost the chloramphenicol-resistance cassette. Subsequently, selected colonies were grown at 42ºC 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.

Conjugation assays

Strains were grown at 37ºC 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 tube containing 5 mL of LB. Tubes were incubated at 37ºC for 90 minutes without agitation. Next we plated the adequate culture dilutions (in $MgSO_4$ 0.01M) 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, conjugation rates (γ) were calculated as: $\gamma = log_{10}\left(\frac{T}{\sqrt{D*R}}\right)$.

All variations of this approach are depicted in Supp.Fig. S3.2

Statistics

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

Results

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 (Fig. 3.1). Plasmids F 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 (Fig. 3.1).

As observed in Fig. 3.1, 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 30ºC (Taylor, 2009).

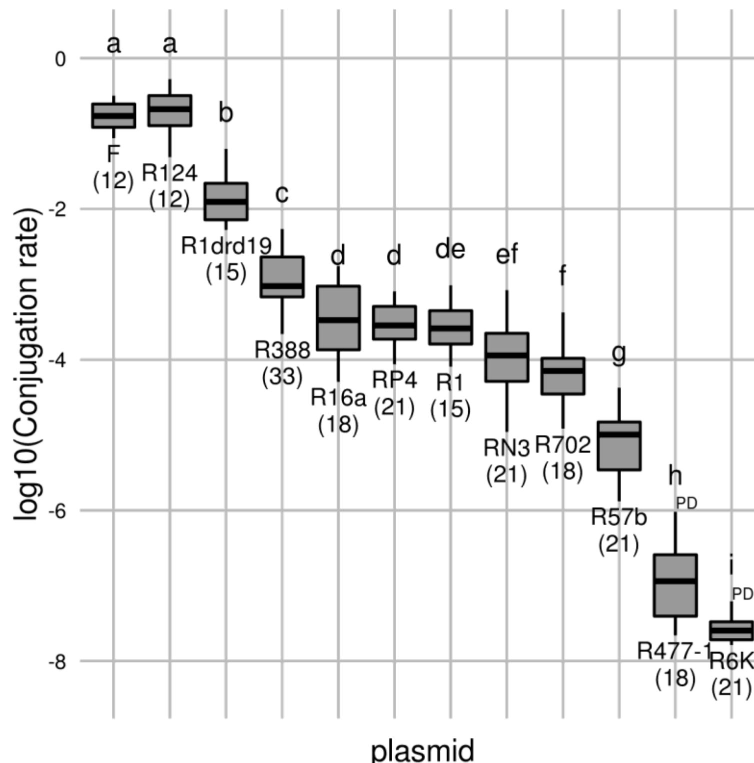

Figure 3.1. Conjugation rates of the plasmids. Annotations under the boxes indicate the identity of the plasmids and, in parenthesis, the sample size. PD – partially detected (no transconjugant colonies in some replicates). Annotations above the boxes represent the results of Tukey's multiple comparison test; plasmids with different letters are significantly different (p-value < 0.05).

Additionally, we can observe in Fig. 3.1 that, when classifying 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.

Effect of a co-resident plasmid

We measured the conjugation rates of 40 pairs of plasmids (Fig. 3.2, Supp.Fig. S3.3) (experimental approach depicted in Supp.Fig. S3.2). We then 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 detected interactions (Dunnett's multiple comparison test, p-value < 0.05) between co-resident plasmids in 25 (62.5%) of the pairs.

Interactions were unidirectional in 21 of the pairs, that is, only the conjugation rate of one of the plasmids was affected. We observed two kinds of unidirectional interactions: i) positive, when a plasmid increased its own conjugation rate, and ii) negative, when a plasmid decreased the conjugation rate of its co-resident. Nine pairs of plasmids (22.5%) exhibited positive unidirectional interactions while 12 pairs (30%) exhibited negative unidirectional interactions.

F	R124	RN3	R477-1	R702	RP4	R388	R6K	
+\0	+\0	0\0	0\0	0\-		0\0	0\0	R16a
+\0	+\0	0\0	0\0	+\-	+\-	0\0	+\0	R57b
		0\0	0\0	0\0	0\-	-\0		R1
		0\+	0\+	0\0	0\-	-\0		R1drd19
		-\0	0\0				-\0	RP4
-\0	-\0	0\0	0\0	-\-	-\-		-\0	R388
+\0	+\0	0\-	0\0	0\-				R6K

Figure 3.2. Effect of a co-resident plasmid. "+", "-" and "0" indicate respectively, positive, negative and no interactions. Effect of plasmids on the row towards plasmids on the columns, are indicated after the backslash and vice-versa.

Several plasmids (R16a, R57b, RN3, R477-1 and R6K) were able to take advantage from 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 it took advantage from 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 decreases 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 both plasmids decreased the conjugation rate of its co-resident in two pairs (5%). These reciprocal negative interactions occurred between IncP-1 (R702 and RP4) and IncW (R388) plasmids. We never

detected reciprocal positive interactions. In another two 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 could have been underestimated.

Effect of recombination

The positive interactions we observed could be explained by recombination events. Thus, we tested this hypothesis using a strain deficient in homologous recombination, Δ*recA*, as donor in the mating experiments. We measured conjugation rates of plasmids F, R57b and R6K, alone and in pairs.

We observed before that R57b was the main target of positive interactions, and now we observed again that the conjugation rate of the R57b plasmid 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. 3.3). Thus, at least in these cases, facilitation does not seem to be mediated by plasmid recombination.

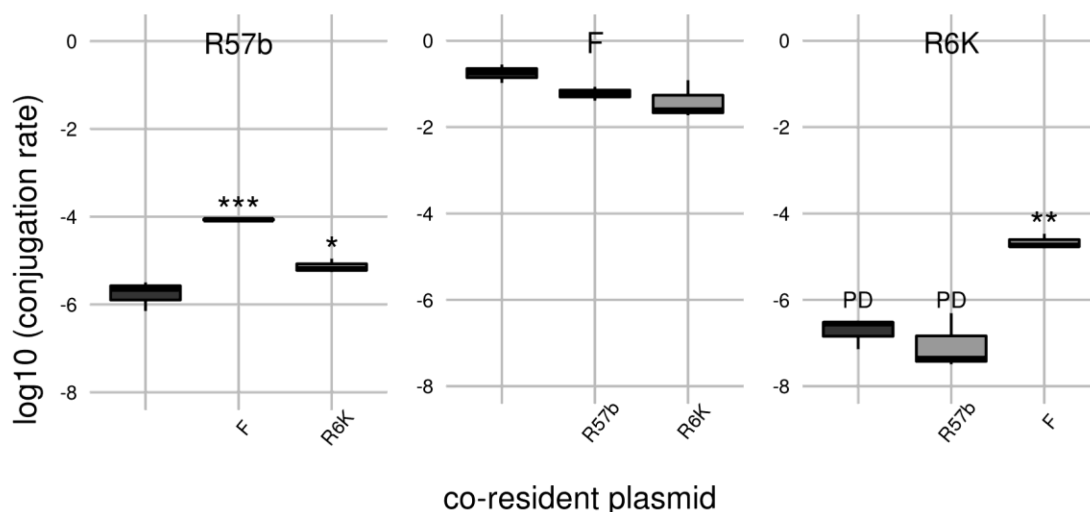

Figure 3.3. Effect of a co-resident plasmid in a *ΔrecA* strain. Titles indicate the focal plasmid and the horizontal axis indicate the co-resident plasmid. The first box of each plot represents the conjugation rate of the focal plasmid in the absence of co-residents (sample size indicated in Fig. 3.1). 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.

Effect of a plasmid carried in the recipient strain

Plasmids can interact not only intracellularly (within cells) but also intercellularly (between cells). 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. S3.2). For this, we studied 68 interactions (35 pairs, two of which tested only in one direction) and found interactions in 18 pairs of plasmids (54.5%): 16 pairs (48.5%) exhibited positive interactions, while only two pairs (6.1%) exhibited negative interactions. All the interactions detected were unidirectional.

Instead of the all the possible 80 interactions between plasmids, we only studied 65 interactions for two reasons. First, we 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 J, 2001) (see also Appendix B). Second, due to its temperature-sensitivity for transfer, we tested only intercellular interactions between plasmid R477-1 and either plasmid R1 or R1drd19 (which exhibited positive intracellular interactions with R477-1).

F	R124	RN3	R477-1	R702	RP4	R388	R6K	
+\0	+\0	0\0		0\0		0\0	0\0	R16a
+\0	+\0	0\0		+\0	+\0	0\0	+\0	R57b
		0\0	0\0	0\+	0\0	+\0		R1
		0\+	0\0	0\+	0\+	0\0		R1drd19
		0\-					+\0	RP4
0\0	0\0	0\0		0\0	-\0		0\0	R388
+\0	+\0	?\0		?\+				R6K

Figure 3.4. Effect of a plasmid present in the recipient strain. "+", "-", "0" and "ND" indicate respectively, positive, negative, no interactions and not determined. Effect of plasmids on the row towards plasmids on the columns, are indicated after the backslash and vice-versa.

Most of the plasmids interacting with plasmids F, R124 or R1drd19 (which had the highest intrinsic conjugation rates) exhibited increased conjugation rates (Fig. 3.4, Supp.Fig. S3.4). Plasmid R57b was again the main plasmid exhibiting such positive interactions (five positive interactions among seven combinations).

Despite its low conjugation rate, plasmid R6K increased the conjugation rates of plasmids R57b, R702 and RP4. Therefore, this IncX plasmid is able to decrease the conjugation rates of both IncP-1 plasmids if present in the same host cell, but increases them if it is carried in a different cell. Plasmid R388 seems to act similarly towards plasmid R1, decreasing the conjugation rate of R1 when both inhabit the same cell, but increasing it when they are present in different cells. However, such positive effect is not observed with plasmid R1drd19, presumably because of its higher conjugation rate. On the other hand, RP4 was the only plasmid exhibiting negative interactions, which were directed towards plasmids RN3 and R388. Again it was impossible to determine negative effects on plasmids R477-1 and R6K because their conjugation rates fall below the limit of detection.

We compared the relative frequency of positive and negative interactions between the two mating conditions: intracellular and intercellular. Both Fisher's exact test and Barnard's two-sided test displayed p-values less than 0.05, therefore revealing a clear difference between the two situations. Positive

interactions prevail when plasmids inhabit different host cells while negative interactions are more frequent when both plasmids are simultaneous present in the same donor cell.

Effect of mating pair stabilization

Since many positive interactions can also be observed 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, which could increase the efficiency of plasmid transfer. To test this hypothesis, we constructed a derivative of plasmid F, *ΔtraG*, impaired for pilus production and mating pair stabilization and checked if this plasmid was able to increase the conjugation rates of other plasmids (Firth & Skurray, 1992; Manning et al., 1981).

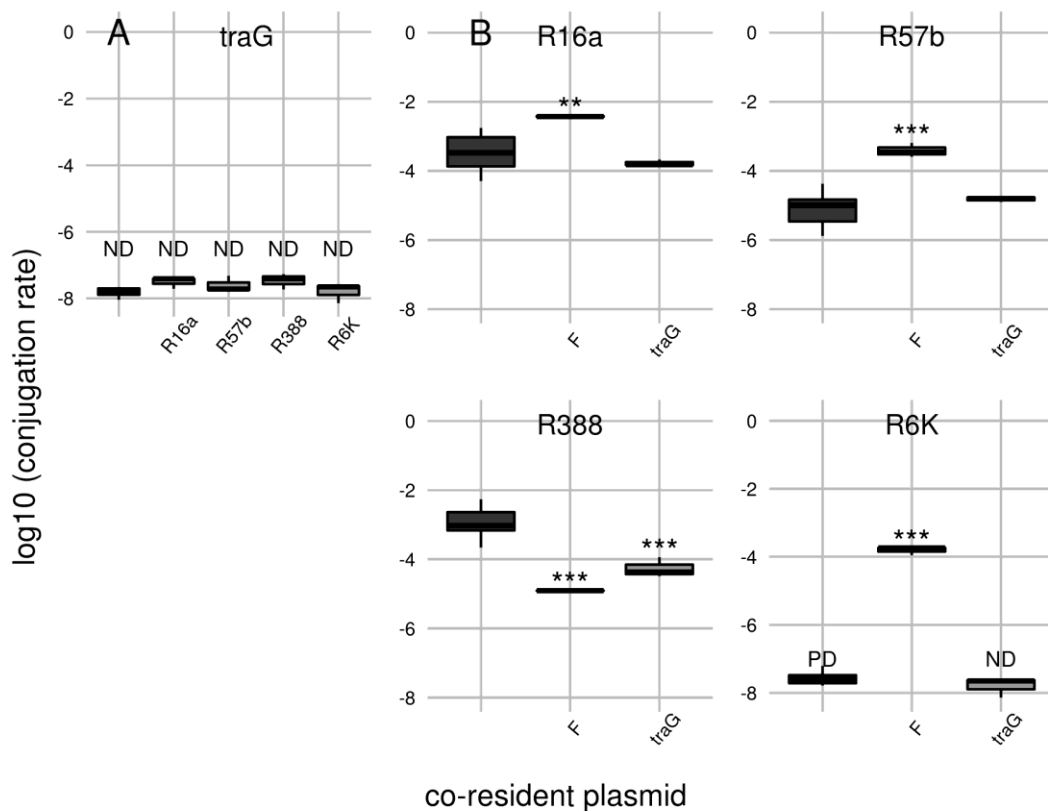

Figure 3.5. Effect of a co-resident mutated F plasmid (*ΔtraG*) impaired for mating pair stabilization: A) Conjugation rates of the mutant *ΔtraG* plasmid; B) Conjugation rates of other plasmids having F or *ΔtraG* as co-resident plasmids. Titles indicate the focal plasmid and the horizontal axis indicate the co-resident plasmid. The first box of each plot represents the conjugation rate of the focal plasmid in the absence of co-residents (sample size indicated in Fig. 3.1; 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.

Fig. 3.5 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, plasmids R16a, R57b and R6K increased their conjugation rates if co-residing with wild-type F plasmid, but not if co-residing with 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.

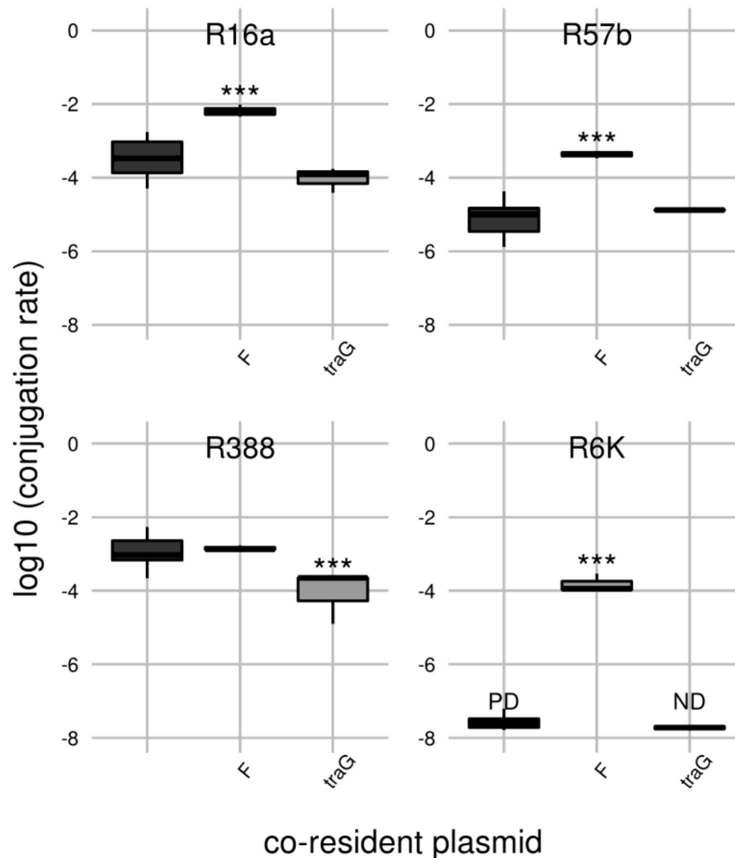

Figure 3.6. Effect of a mutated F plasmid (*ΔtraG*) impaired for mating pair stabilization present in the recipient strain. Titles indicate the focal plasmid and the horizontal axis indicate the plasmid present in the recipient strain. The first box of each plot represents the conjugation rate of the focal plasmid to a plasmid free recipient strain (sample size indicated in Fig. 3.1). 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.

The inability of the mutant plasmid to increase the conjugation rate of plasmids R16a, R57b and R6K could also be observed when either of the other three plasmids inhabited a different host cell (Fig. 3.6). Alternatively, plasmid R388 was only inhibited intercellularly by the *ΔtraG* mutant but not by the wild-type 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.

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

Discussion

In this work we analysed changes of transfer rates of several plasmids when other plasmids reside in the (i) donor cell or (ii) in the recipient cell. We will discuss these two conditions in turn.

Plasmids seem to inhibit other plasmids in the same donor cell or to exploit them

We searched for interactions between plasmids when residing in the same cell. For that, we measured transfer rates of plasmids when alone *versus* when co-inhabiting the same bacterial cell, and compared the values. We observed interactions in 25 out of 40 possible combinations of co-residing plasmids. In total, we detected eleven cases where plasmids increased their own transfer rates and eighteen cases where plasmids decreased the transfer rate of the rival.

As explained before, if the transfer rate of a given plasmid "A" increases when a plasmid "B" is also present in the same cell, this might be interpreted as "A" taking advantage from "B", or as "B" helping "A" (here, the word "help" is used in the sense that plasmid "B" would produce a metabolite that would directly increase the transfer rate of another plasmid, in this case plasmid "A"). Considering the second hypothesis, cases of mutual help, i.e., cases where both plasmids increased their transfer rates, should be expected, something we never observed. However, 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 is consistent with the interpretation that, when a given plasmid "A" increases its transfer rate in presence of a plasmid "B", this should be interpreted as "A" taking advantage from "B", not as "B" helping "A".

Recombination was unlikely to be responsible for the increase of transfer rates since this effect was observed in both conditions where rival plasmids inhabited the same or neighbouring cells. Testing three distinct pairs of plasmids in a strain deficient in homologous recombination, allowed us to confirm that recombination was not associated with the increase of transfer rates (Fig. 3.3). We then tested if this effect was rather due to the cellular contact determined by the rival plasmid. To do so, we constructed a derivative of plasmid F lacking the gene *traG* required for pilus synthesis and stabilization of the mating-pair. Because this mutant F did not synthesize pili nor stabilized the mating pair, it could not contribute to mating-pair formation; in other words, plasmids transferring to cells where this mutant F was present had the same transfer rate as if it was not there. This corroborates the hypothesis that the cellular contact provided by plasmids present in the recipient cell was responsible for the increased transfer rates. It also explained why plasmids increase their transfer rates when their rival has a higher intrinsic transfer rate, such as the cases of plasmids F, R124 or R1drd19. In general, this is in agreement with the works of

(Datta et al., 1971; Sagai et al., 1977), although they did not observe an increase of transfer of RP4 when R1drd19 was present in neighbouring cells.

The case of plasmid R6K is thus an intriguing exception, since in the present work 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, to which plasmid R6K belongs, 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 type 3 fimbriae have also been shown to 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. Furthermore, plasmid R1, which resembles R1drd19 except for being repressed for conjugation, had no observable effect on the conjugation rate of R477-1. We thus speculate that some intracellular protein product, produced in excess by R1drd19, is responsible for this observation.

Plasmid RP4 was inhibited when co-inhabiting with another plasmid in most combinations. This plasmid and the other IncP-1 plasmid of this study, R702, along with IncW plasmids, were the main targets of inhibition. IncP-1 and IncW groups are known to comprise broad-host range plasmids. Therefore, the widespread inhibitory effect against these plasmids could be the result of a strategy that prevented them from invading bacterial populations already harbouring resident plasmids. It is also known that the conjugative systems of IncN, IncP-1 and IncW plasmids share some similarities (delSolar et al., 1996; Jain & Srivastava, 2013). Furthermore, R6K inhibits plasmids belonging to these three incompatibility groups, as already stated by (Olsen & Shipley, 1975). In the work by (Olsen & Shipley, 1975) reciprocal inhibition was observed between plasmids RP4 and R6K, which could not be detected in the present work since the intrinsic transfer rate values of R6K were close to our limit of detection.

IncA/C plasmids are also broad-host range plasmids. Nevertheless, none of the other plasmids used in this work was able to inhibit their transfer. In fact, we observed the opposite scenario, as both IncA/C plasmids R16a and R57b increased their transfer rates when inhabiting host cells also harbouring plasmids F or R124. Furthermore, plasmid R57b could increase its own transfer in the presence of many of the plasmids tested. In this case, the broad-host range of these IncA/C plasmids could be the result from plasmid interactions in a way that these plasmids take advantage from their rivals to increase their dispersal.

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 also confer protection against male specific phages. By allowing production of additional conjugative pili, plasmids can exploit their rivals to further increase their own transfer. The behaviour of plasmid R57b agrees with this hypothesis. Plasmid R57b appears to be the best strategist: for example, in the presence of IncP-1 plasmids, R57b inhibited its rivals and increased its own transfer.

Plasmids seem to take advantage from other plasmids present at the recipient cell

In the second scenario where the two plasmids, instead of being present in the same host cell, were present in two distinct cells – one of the plasmids in one cell and the other plasmid in another cell – positive interactions prevail. We detected increased conjugation rates in 16 combinations and decreased transfer rates in only two combinations.

It is important to note that, after plasmid transfer, the two rival plasmids will inhabit the same host cell. Once that happens, the intracellular inhibitory effect antagonizes the intercellular facilitation effect. For example, plasmid R1 increased its transfer rate when plasmid R388 was present in (potential) 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). Therefore, putting aside these two cases, all observed cases are positive.

Moreover, it is possible that other cases of intercellular positive cases are hidden under negative effects within cells. Indeed, by comparing transfer rates when two plasmids are in the same cell versus when they are in different cells suggests that, at least in some cases, even when one does not see a positive effect in the intercellular case (Supp.Fig. S3.4), perhaps there is a positive effect after all. For example, when plasmid R388 transfers into cells harbouring plasmid F (Supp.Fig. S3.4), it seems that there is no positive effect; however, when both R388 and F occupy the same cell, the plasmid F interacts negatively with R388. Therefore, it seems that in the intercellular case, R388 is indeed taking advantage from the fact that F is forming the mating pair, but then R388 suffers a negative effect from incoming F plasmids once they establish in cells harbouring R388 (as one can see in the intracellular experiments – Supp.Fig. S3.3), and the two effects almost cancel.

The above hypothesis is corroborated by the experiments with the *ΔtraG* mutant F plasmid that does not synthesize conjugative pili: this mutant plasmid has a negative effect over R388 both in intracellular and intercellular experiments (Fig. 3.5 and 3.6). That is, both the wild-type F plasmid and the non-conjugative *ΔtraG* mutant of the F plasmid inhibit R388 intracellularly; however, in intercellular experiments, the wild-type F that forms conjugative pili compensates its intracellular negative effects.

In general, our results involving the *ΔtraG* mutant strongly suggest that formation of the mating pairs is crucial for the efficiency of plasmid horizontal transfer. Thus, it is possible that the conjugative pili of a plasmid (e.g. F) help stabilizing the transfer of another plasmid (for example plasmid R388).

In conclusion, when plasmids interact within cells, the interaction is mostly negative, whereas when plasmids reside in different cells, positive interactions prevail. Indeed, these intercellular interactions were mostly positive, i.e., the transfer rate of each plasmid is higher if another, unrelated, conjugative plasmid is present in the recipient cell than if this cell is plasmid-free. Our experiments with the *ΔtraG* mutant plasmids that do not expressing conjugative pili on the cell surface strongly suggest that positive effects come from a higher efficiency of mating pair formation.

Author Contributions

Conceived and designed the experiments: João Alves Gama, Rita Zilhão, Francisco Dionísio. Performed the experiments: João Alves Gama, with contributions of Rita Zilhão. Analysed the data: João Alves Gama, Rita Zilhão, Francisco Dionísio. Wrote the chapter: João Alves Gama, Francisco Dionísio, with contributions of Rita Zilhão

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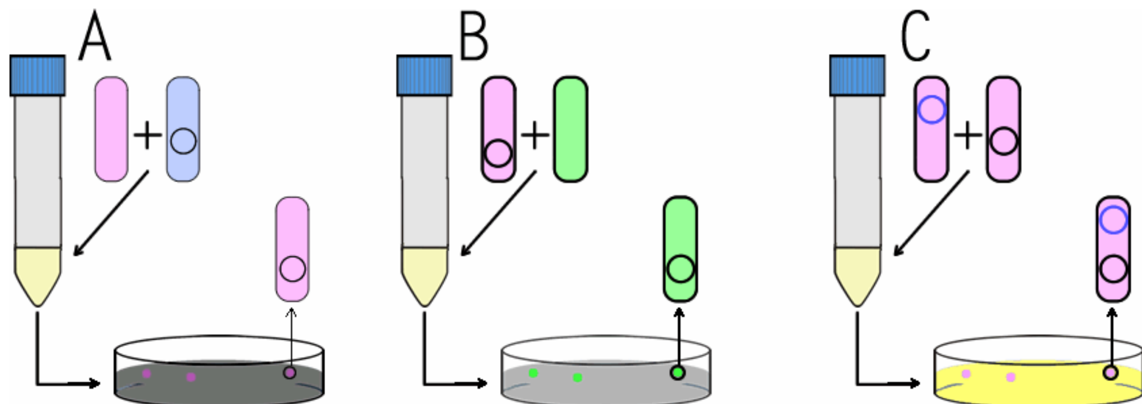
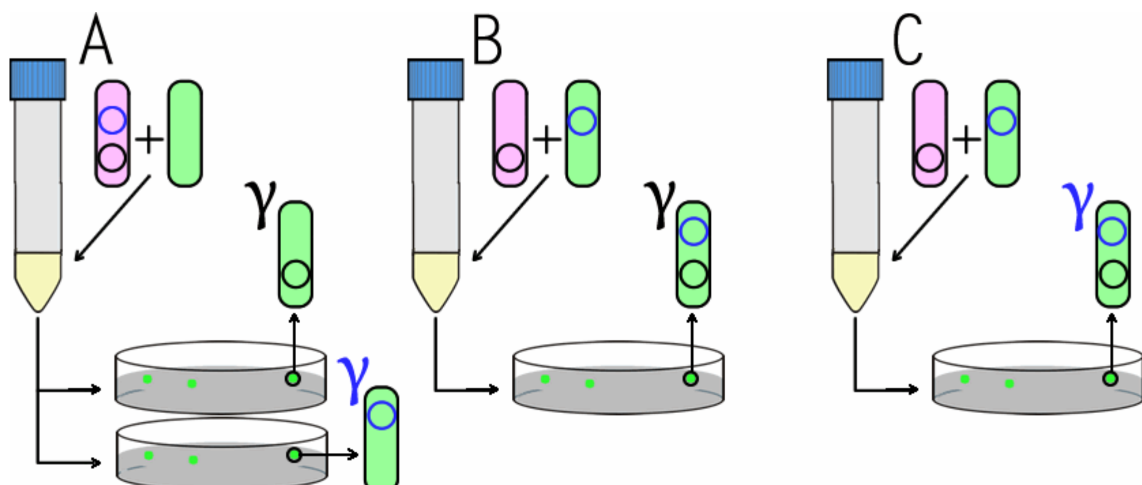
We also thank Doctor Karina Xavier for providing vectors pKD46, pKD3 and pCP20 used to delete the *traG* gene in plasmid F.

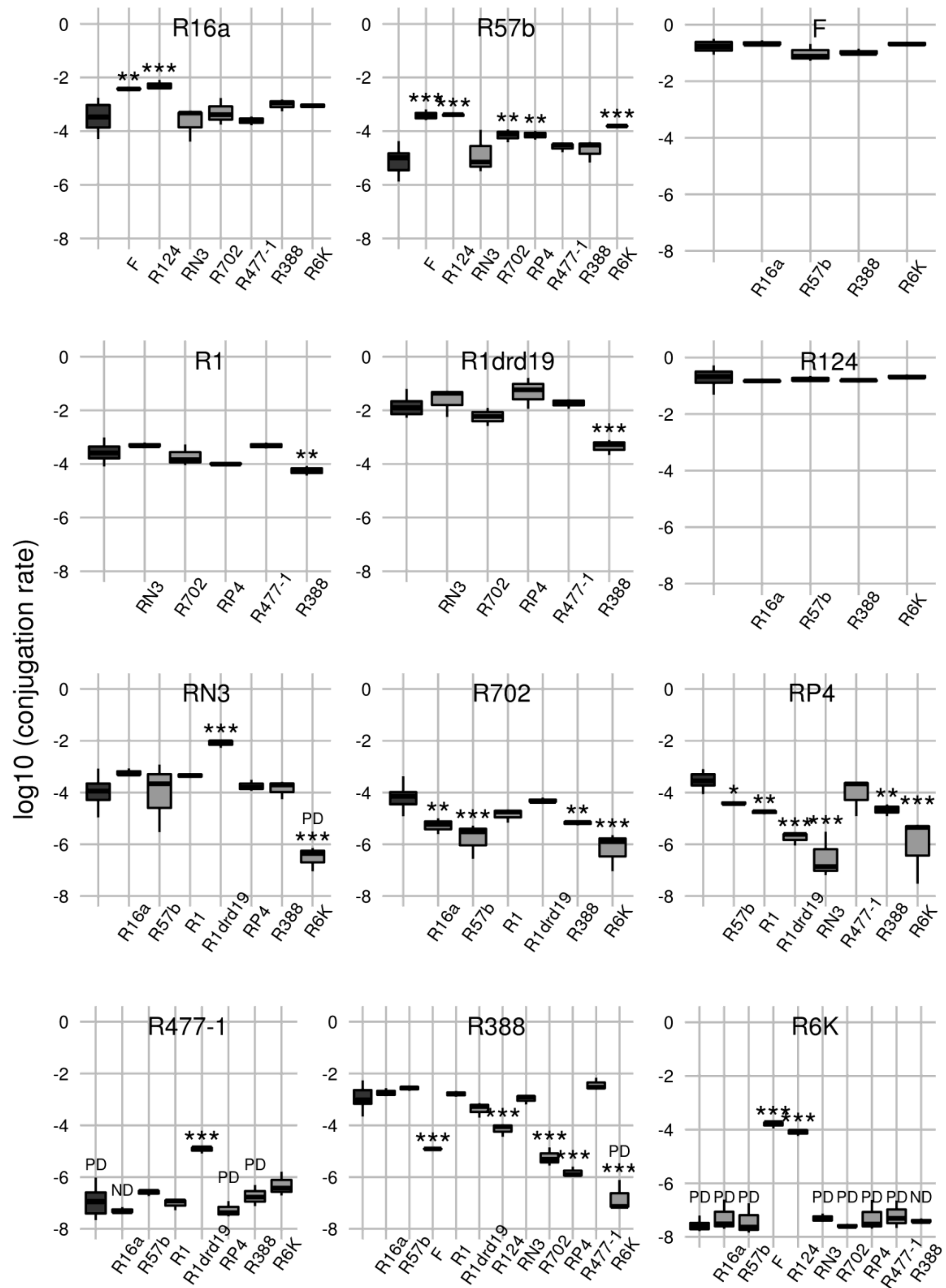
Supplementary Information



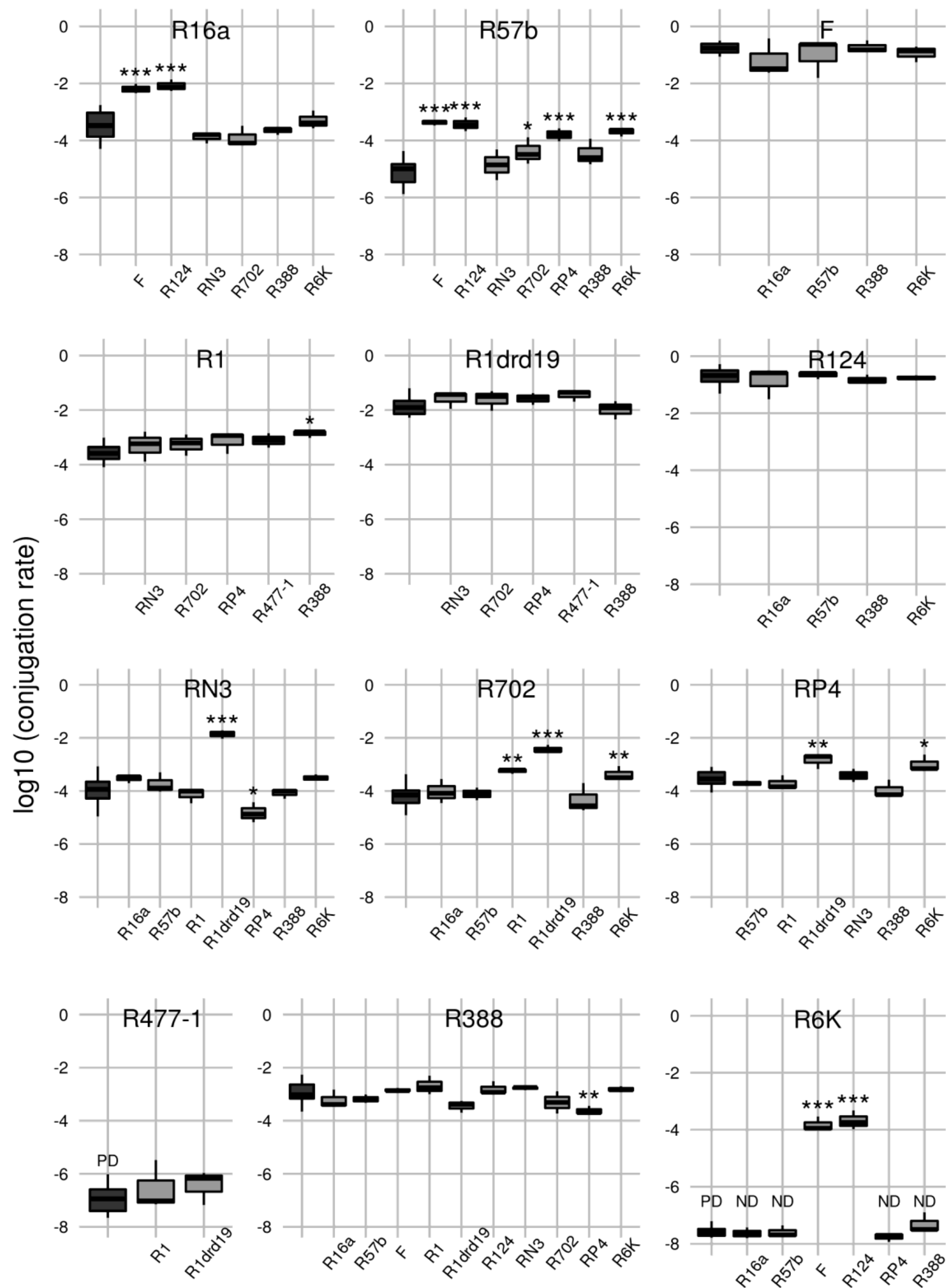
Supplementary Figure S3.1. Construction of strains by conjugation. Construction of single plasmid carrying strains of: A) *E. coli* K12 MG1655 *Δara* or *E. coli* K12 JW2669 *ΔrecA;* B) *E. coli* K12 MG1655 (*ara*+). C) Construction of double plasmid carrying strains of *E. coli* K12 MG1655 *Δara.* Blue cells represent the original plasmid donor strains, which are auxotrophic for two amino acids or, auxotrophic for one amino acid and unable to metabolize maltose. Pink cells represent *E. coli* K12 MG1655 *Δara* strain (they also represent the *E. coli* K12 JW2669 *ΔrecA* strain). Green cells represent the *E. coli* K12 MG1655 (*ara*+) strain. Grey Petri dishes represent M9 minimal solid medium supplemented with maltose (dark grey) or arabinose (light grey) and the required antibiotics. Yellow Petri dishes represent LB solid medium supplemented with the required antibiotics.



Supplementary Figure S3.2. Selection of transconjugants to measure conjugation rates. A) Measurement of conjugation rates of two intracellular competing plasmids. B and C) Measurement of conjugation rates of intercellular competing plasmids. Pink and green cells represent respectively *E. coli* K12 MG1655 *Δara* and *E. coli* K12 MG1655 (*ara*+) strains. Gamma letters represent the conjugation rate of the plasmid of the same colour. Measurement of conjugation rates of plasmids in the absence of competitor plasmids is depicted in Supp.Fig. S3.1.



Supplementary Figure S3.3. Effect of a co-resident plasmid. Titles indicate the focal plasmid and the horizontal axis indicate the co-resident plasmid. The first box of each plot represents the conjugation rate of the focal plasmid in the absence of co-residents (sample size indicated in Fig. 3.1). 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.



Supplementary Figure S3.4. Effect of a plasmid present in the recipient strain. Titles indicate the focal 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 focal plasmid to a plasmid free recipient strain (sample size indicated in Fig. 3.1). 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.

Chapter 4

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

Abstract

Bacterial cells may be colonized by conjugative plasmids, DNA elements capable of transfer among bacteria through conjugation. In Chapter 3, we have shown that plasmids may interact within cells, sometimes repressing the transfer of the co-resident plasmid, sometimes increasing their own transfer rate. In the present chapter, we compared the transfer rate of a plasmid in different conditions: i) when alone in a cell, ii) when in a cell with two other plasmids, iii) when in the same cell with either one or the other of those two plasmids. Here we analysed the nature of the interactions between plasmids, namely how the transfer rate of a plasmid is affected by the simultaneous interaction of two other co-resident plasmids. With these values, we concluded that plasmids interact in 59 out of 84 possible interactions. Moreover, although there are exceptions, we mostly observed that if a co-resident plasmid was inhibitory towards the focal plasmid, it remained inhibitory in the presence of a third plasmid, and that if a plasmid increased its conjugation rate in the presence of another, its transfer rate also increased even if there is a third plasmid in the cell. For both cases, it is 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, suggesting that plasmids are prone at joining efforts to repress a focal plasmid.

Introduction

Organisms can be simultaneously infected by more than one parasite. In multiple infections, parasites, whether they belong to the same species or not, might interact and compete, which will define how they ultimately affect the host. Parasites in multiple infections can face three types of competition (reviewed in (Mideo, 2009)): i) exploitation competition happens when parasites compete for host resources; ii) apparent competition happens in situations in which a common enemy exists, such as the host's immune system, and the abundance of each parasite impacts the other; iii) interference competition happens when the parasites employ specific strategies to inhibit the growth, reproduction or transmission of their competitors.

Several works have already shown that bacteria engage in interference competition, mediated for example by toxins (Chao & Levin, 1981), growth inhibition systems (Aoki et al., 2005; Hayes et al., 2014), low molecular-weight toxic compounds (Selva et al., 2009) and viruses (Gama et al., 2013). Yet, also bacteria are themselves colonized by parasites, such as plasmids, viruses and transposable elements. Interference competition is observable even when these parasites interact.

This work is focused on plasmids, which are double stranded DNA elements often found in natural isolated bacteria. Conjugative plasmids are a

subset of plasmids capable of transferring to other cells, a process called conjugation, which is encoded by plasmid genes (Cabezon et al., 2015). In general, conjugative plasmids start by expressing certain conjugative pili (appendages exposed on the cell-surface), which promote the attachment between donor and recipient cells, thus forming the mating pair. Finally, a single-stranded plasmid copy is transferred to the recipient cell, where the complementary strand will be synthesized. Thus, both cells become carriers of the plasmid, since the recipient cell acquired a plasmid copy while the donor cell kept its own copy.

Natural isolated bacteria are often colonized by more than one plasmid type. Not surprisingly, some mechanisms of competition between plasmids have been observed. Indeed, plasmids employ several strategies to compete with other plasmids and such strategies act to prevent competitors from entering or persisting in the same host cells or to prevent them to disseminate to nearby hosts.

Generally, conjugative plasmids – those encoding all the machinery genes required for horizontal transfer – contain an exclusion system (reviewed in (Garcillan-Barcia & de la Cruz, 2008)), which prevents their host cell from becoming recipient for a related plasmid. There are two kinds of exclusion systems: surface exclusion systems prevent the formation of the mating pair whereas entry exclusion systems inhibit conjugation on a later phase. While exclusion prevents plasmids from entering in an occupied cell, incompatibility precludes two related plasmids from persisting in the same host cell. Two given plasmids are said to be incompatible if they are unable to coexist stably over a number of generations in the same bacterial cell line. Usually, they are incompatible when they are closely related. Different plasmids, however, tend to be compatible. Incompatibility arises from two different mechanisms, either replication or partition (reviewed in (Novick, 1987)). Such systems regulate plasmid copy-number (through replication) and their distribution to daughter cells, respectively, but they fail to discriminate related plasmids. Persistence of related plasmids therefore is unstable and incompatible plasmids segregate upon cell division, originating single-plasmid lines.

Plasmids may encode Toxin-Antitoxin (TA) loci, also known as post-segregational (psk) systems, which 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 & Heinemann, 2000b).

Plasmids are also known to 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). However, plasmids can also reduce the transfer of unrelated competitors, which have been observed in several instances (Datta et al., 1971; Hochmannova et al., 1982; Olsen & Shipley, 1975; Pinney & Smith, 1974; Sagai et al., 1977; Tanimoto & Iino, 1983; Willetts & Skurray, 1980; Winans & Walker, 1985).

Plasmids do not compete solely against plasmids. Instead they are subject to compete against other parasites occupying the same niche. Indeed several works have already reported competitive interference between plasmids and

bacteriophages (viruses that infect bacteria, also known as phages) (for some examples see (Bannister & Glover, 1968; Marrero et al., 1981; Marrero & Lovett, 1982; Pecota & Wood, 1996; Taylor & Grant, 1976; Taylor & Summers, 1979; Watanabe et al., 1966) and the reviews (Labrie et al., 2010; van Houte et al., 2016) and references therein). These include cases where plasmids lead to prophage elimination, phage (entry) exclusion and abortive infections. It has also been recently shown that phages can competitively interfere with other genetic elements (Seed et al., 2013).

Plasmid F, one of the best studied plasmids, is a very interesting case because it possesses mechanisms used simultaneously to compete with plasmids and phages. Among other functions, this plasmid encodes the proteins PifA and TraT. TraT is responsible for surface exclusion, therefore preventing the host cell already inhabited by F from receiving other F-like plasmids through conjugation (Achtman et al., 1977). Additionally, TraT is also responsible for altering the conformation of the cellular lipoprotein OmpA, which is a common phage receptor (Riede & Eschbach, 1986). Thus, TraT is simultaneously able to exclude not only F-like plasmids but also OmpA dependent phages. PifA is also used to interfere both with plasmids and phages (Miller et al., 1985; Rotman et al., 1983). This protein reduces the horizontal transmission of plasmid RP4 and confers resistance to phages such as T7.

In this chapter we will focus solely on plasmids and study how they interact with each other. In Chapter 3 we have shown that transferable plasmids interact mostly negatively within bacterial cells, that is, some plasmids decrease the transfer rate of co-resident plasmids; in some cases, however, no interaction was observed, and in other cases a plasmid increases its transfer rate when in the presence of another one. In this chapter, we aim to understand the outcome of multiple interactions, because as the number of infecting parasites increases, so should the number of interference interactions observed between them. That is, when three plasmids are in the same cell, how does the interaction between two of the plasmids affects the transfer rate of the third plasmid?

To answer to this question, we compared the conjugation rates of the focal 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. We expected to observe 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.

Materials & Methods

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 a total of 11 natural conjugative plasmids, summarized in Table 1.1.

Generation of plasmid harbouring-strains

We produced a total of 28 strains of *E. coli* K12 MG1655 Δ*ara* carrying combinations of three plasmids. These strains resulted from overnight matings between two strains of *E. coli* K12 MG1655 Δ*ara* (produced in Chapter 3), 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.

Conjugation assays

After overnight growth at 37ºC 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 37ºC for 90 minutes without agitation. To quantify donor and recipient bacteria, we plated suitable culture dilutions (in $MgSO_4$ 0.01M) in Tetrazolium Arabinose (TA) medium, where, due to differences in arabinose metabolism, the donor strain grows as red colonies and the recipient strain grows as white colonies. To quantify transconjugants, we plated suitable culture dilutions in M9 minimal solid medium supplemented with arabinose (0.4%) and adequate antibiotics. Conjugation rates (γ) were 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.

Determination of distorting interactions in plasmid triplets

Classification of plasmid interactions in triads was performed according to Supp.Fig. 4.1 (explained in supplementary information). We considered that the co-resident plasmids did not interact with the focal plasmid if the focal's conjugation rate was not affected in either the double or in the triplet. Non-distorting interactions occurred when the effect observed in the triplet was identical to the stronger effect observed in the doubles. We identified distorting interactions when the effect observed in the triplet differed from the stronger 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 focal plasmid was alone.

Statistics

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

Results

To interact or not to interact, that is the question

In this chapter we studied whether the interaction between two plasmids shaped the conjugation rate of a third plasmid. For that, we measured the conjugation rates of each plasmid present in bacteria harbouring three plasmids simultaneously. We analysed a total of 28 combinations of three plasmids involving eleven different naturally-occurring plasmids. For each combination (of three plasmids), we studied the transfer rate of each plasmid, allowing the study of 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? Therefore, with 28 combinations of three plasmids, we can study a total of 84 possible interactions. We detected plasmid interactions in 59 of the 84 cases (70,2%). With the exception of combinations R16a/R388/RN3 and R388/R57b/RN3 (Fig. 4.1), there are interactions in all the other 26 combinations (Supp.Fig. S4.2).

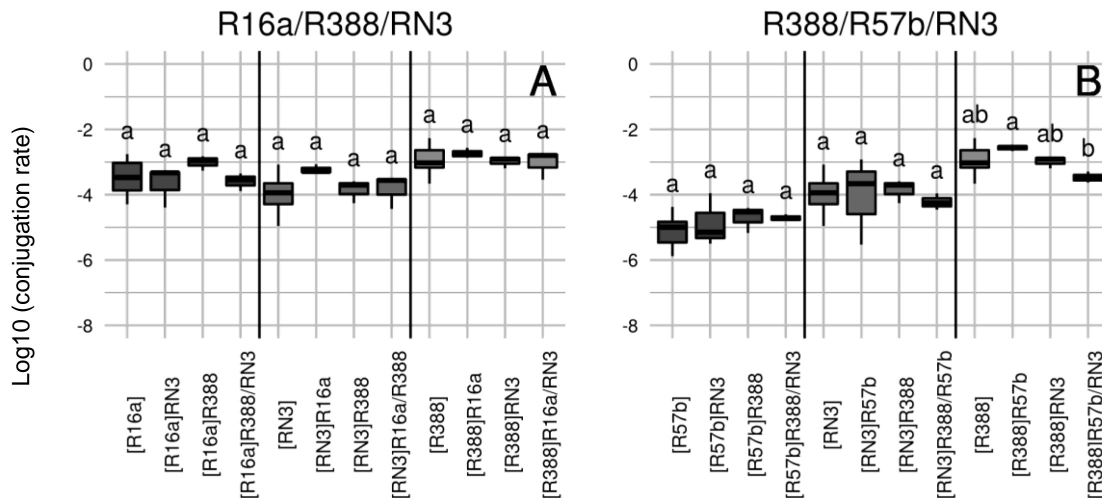


Figure 4.1. Plasmid combinations without interactions. Titles indicate the combination of three plasmids. In these combinations, plasmids do not seem to interact. Analysis for each focal plasmid are separated by vertical lines. The horizontal axis indicates the combinations of one, two or three plasmids; the focal plasmid is indicated in brackets. Conjugation rates for combinations of three plasmids were measured in triplicate. ND – not detected (no transconjugant colonies in any replicates). PD – partially detected (no transconjugant colonies in some replicates). For the sample sizes of the other combinations see Chapter 3. Annotations above the boxes represent the results of Tukey's multiple comparison test; plasmids with different letters are significantly different (p-value < 0.05).

Through the analysis of the transfer rates in each combination, one can see that if a co-resident plasmid was inhibitory towards the focal plasmid (negative interaction), it remained inhibitory in the presence of a third plasmid. For instance, in Chapter 3, we observed inhibition of plasmid RP4 in combinations with another plasmid, and now we still observed its inhibition when in the presence of two co-resident plasmids (Supp.Fig. S4.2I). 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 S4.2E). Despite this general trend, there were 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. Therefore, we tested if the effects between plasmid interactions could distort the conjugation rate of a focal plasmid. Thus, we compared the conjugation rates of the focal plasmid when alone, in the presence of both co-resident plasmids and in the presence of each one of them. Then, we classified the interactions, based on the groups resulting from the Tukey multi-comparison test, as outlined in supplementary information. Thus, we were able to identify distorting (intensifying or attenuating) interactions.

Distorting interactions: are they positive?

Among the observed distorting interactions, two were positive, which means that the focal plasmid increased its conjugation rate in the presence of co-resident plasmids. These interactions occurred in the combinations displayed in Fig. 4.2. Considering the combination of plasmids R388, R1drd19 and RN3, we observed a positive interaction between plasmids R1drd19 and RN3 (Fig. 4.2A). That is, the conjugation rate of plasmid RN3 was higher when plasmid R1drd19 was the only co-resident plasmid in the host cell. By contrast, the presence of R388 as a third plasmid suppressed this positive interaction, as the conjugation rate of RN3 was no longer different from that observed when RN3 occupied the host cell alone. When only R388 and RN3 inhabited the same host cell, they did not interact, however R388 decreased the conjugation rate of plasmid R1drd19 when these two plasmids occupied the host cell simultaneously. Therefore, the

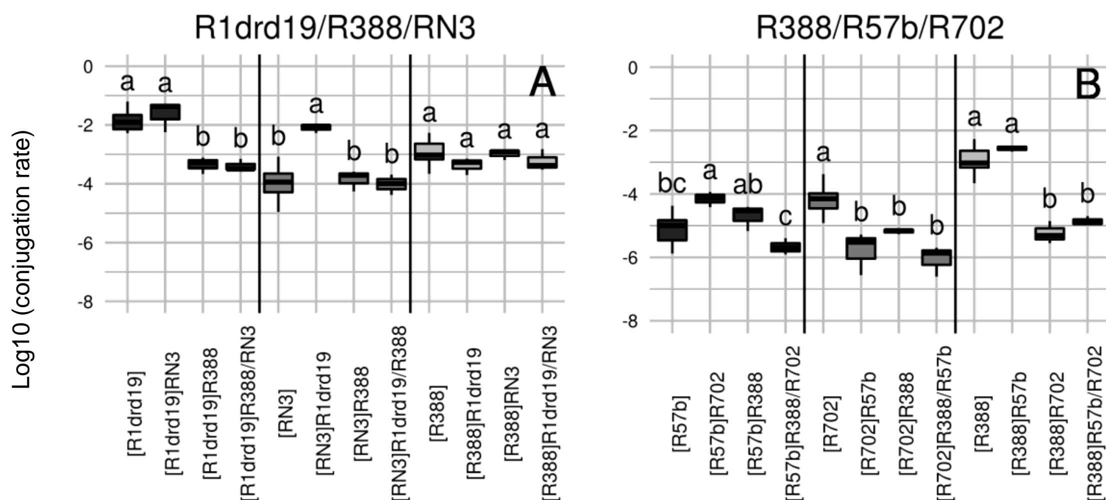


Figure 4.2. Plasmid combinations exhibiting distorting positive interactions. Distorting positive interactions are those in which a focal plasmid increases its conjugation rate in the presence of co-resident plasmids. Explanation of this figure is as in Fig. 4.1.

suppressive action of R388 on the conjugation rate of RN3 was an indirect effect resulting from the inhibition of R1drd19 by R388, which in turn prevented the positive effect of R1drd19 on RN3. We can follow the same reasoning for the

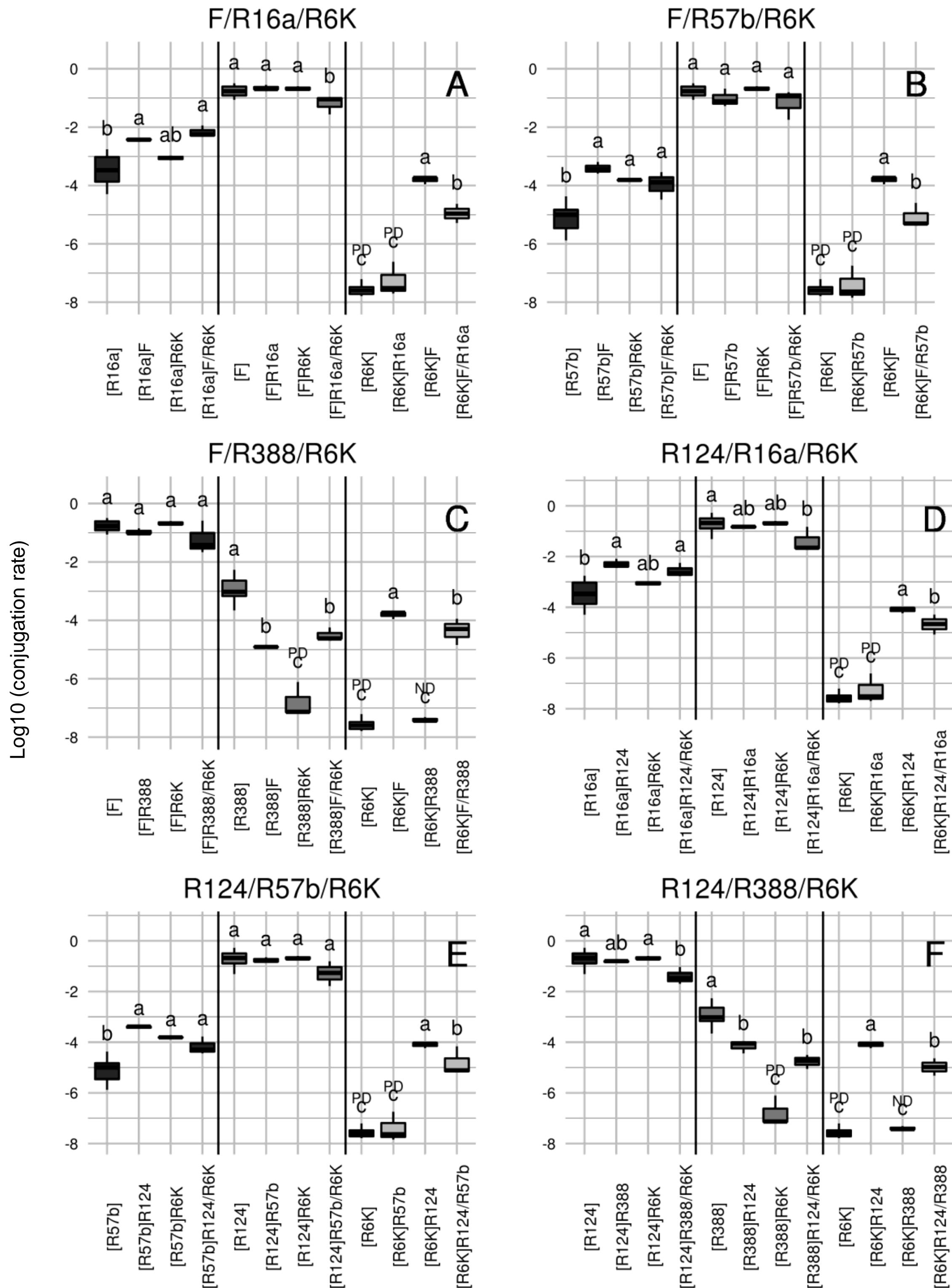


Figure 4.3. Distorting interactions regarded as non-distorting negative interactions. These are the interactions in which the positive effect directed to a focal plasmid decreased when this plasmid shared the cell with two other plasmids. Alternatively, these interactions can be regarded as non-distorting negative interactions because they could represent inhibitory effects, which we did not detect in Chapter 3 due to experimental limitations. Explanation of this figure is as in Fig. 4.1

combination comprising plasmids R388, R57b and R702 (Fig. 4.2B). In this case, we observed the inhibitory action of plasmid R388 towards R702 preventing the positive effect of R702 towards R57b.

We could account for six additional positive distorting interactions, since the positive effect directed to plasmid R6K decreased when this plasmid shared the host cell with another two plasmids (Fig. 4.3). However, in our view these might represent non-distorting negative interactions. Such cases will be further explored in the discussion section.

Distorting interactions: How low can plasmids go?

We will now focus on negative interactions, that is to say, inhibitory interactions that result in decreased conjugation rates. We observed a total of 18 negative distorting interactions. These interactions are depicted in Figs. 4.3, 4.4 and 4.5. We can see in Figs. 4.3C, 4.3F and 4.4 that plasmid R388 is the target of distorting negative interactions in all these five combinations.

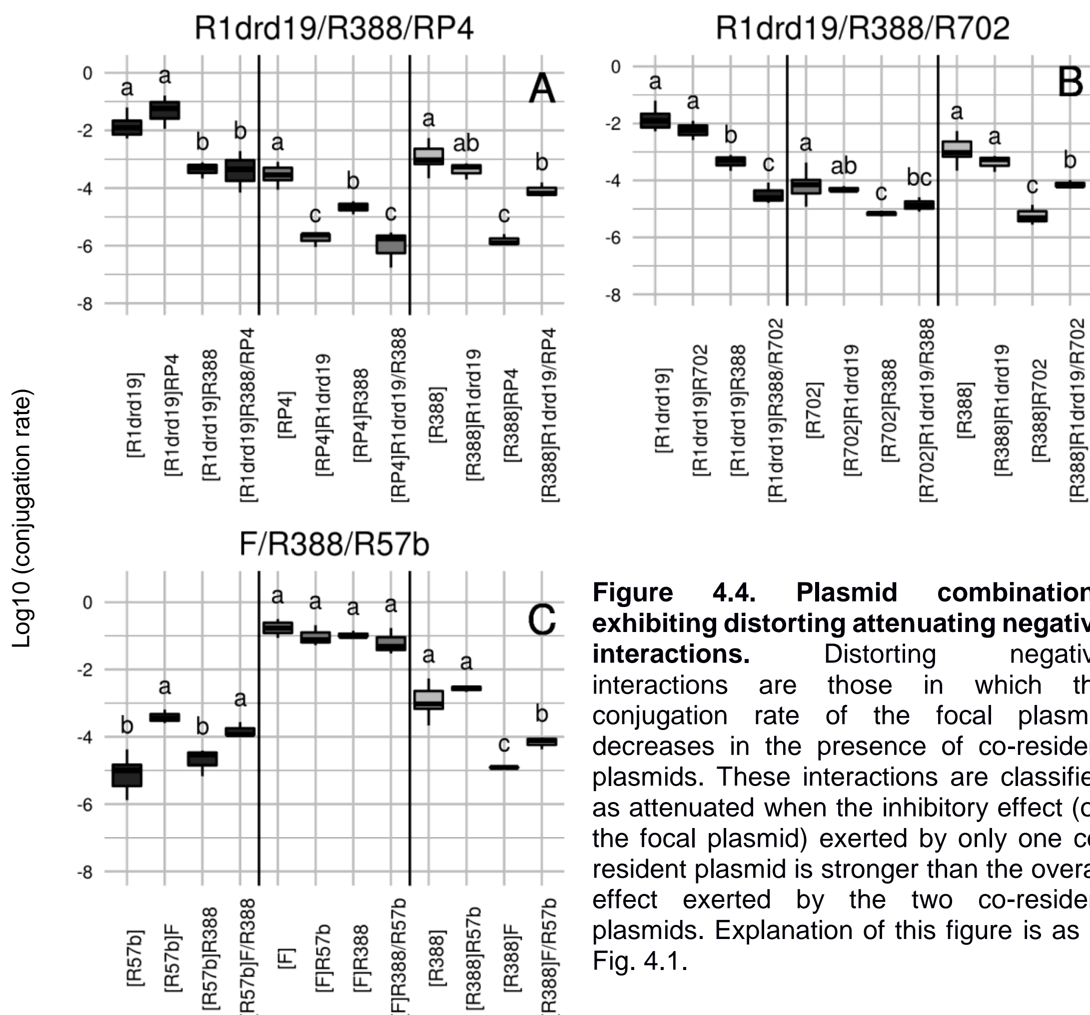


Figure 4.4. Plasmid combinations exhibiting distorting attenuating negative interactions. Distorting negative interactions are those in which the conjugation rate of the focal plasmid decreases in the presence of co-resident plasmids. These interactions are classified as attenuated when the inhibitory effect (on the focal plasmid) exerted by only one co-resident plasmid is stronger than the overall effect exerted by the two co-resident plasmids. Explanation of this figure is as in Fig. 4.1.

In the following two combinations: F/R388/R6K and R124/R388/R6K, plasmid R388 was inhibited by both the other two plasmids of the combination (Figs. 4.3C, 4.3F). When plasmid R388 co-resided with plasmid R6K, the latter provided a strong inhibition over R388. If either F or R124 was also present in the

cell, the inhibitory effect of R6K was not so strong. These are interesting cases of negative interactions because the overall inhibition of plasmid R388 when either F or R124 co-resided with R6K was similar to the inhibitory power of plasmids when either F or R124 were alone with R388. Therefore, the inhibition by plasmid R6K became negligible. However, one cannot infer that the inhibition by plasmid R6K was totally suppressed since it could only be reduced to a level equal or less than the level of the inhibition provided by the other co-resident plasmid.

On the three combinations depicted in Fig. 4.4, only one of the co-resident plasmids inhibited plasmid R388. However, the inhibition of R388 was attenuated when all three plasmids were present simultaneously in the same host cell. This suggests that the third plasmid partially blocked the inhibitory effect inflicted on R388.

The five combinations mentioned above, as the previous three combinations concerning positive interactions, illustrate attenuating interactions, which means that the effect of a plasmid on another is alleviated. Conversely, other combinations (Figs. 4.3A, 4.3D, 4.3E, 4.4B and 4.5), involve intensifying interactions, that is, the overall effect is heightened. For instance, one can observe that in both combinations R1/R388/R702 and R1drd19/R388/R702 the interaction between plasmids R702 and R388 magnifies the inhibitory effect of R388 towards either R1 and R1drd19, although plasmid R702, *per se*, did not inhibit either of the two plasmids in the absence of R388 (Figs. 4.5F, 4.4B).

One can also observe complementation of inhibitory mechanisms, which results in intensifying negative interactions, that is, when both co-resident plasmids inhibit the focal plasmid, the overall inhibitory effect increases. This differs from the previous cases where only one of the plasmids inhibited the focal, although the effect could be somewhat intensified. Focusing on the intensified inhibition of plasmid RP4, we can observe complementation between the mechanisms of inhibition encoded by plasmids R57b, R6K and R388, for the overall inhibition caused by two co-resident plasmids was stronger than the individual inhibitory effects (Fig. 4.5A-C).

Distorting interactions: how frequent?

Overall, we observed 59 interactions among the possible 84 interactions: 20 distorting interactions and 39 non-distorting interactions. Among the 59 observed interactions, we discerned 33.9% of distorting interactions and about twice as much of non-distorting interactions (66.1%). We focused on the prevalence of each type of distorting interaction: positive versus negative and attenuating versus intensifying. Among the 20 distorting interactions, we observed 7 attenuating interactions, 2 of which concerning positive interactions and 5 concerning negative interactions. The remaining 13 interactions were all negative intensifying interactions. We analysed if the frequencies of these interactions deviated from what would be expected and we found that they did (Barnard's test: p-value < 0.05, Fisher's exact test: p-value < 0.05). Thus, this suggests a bias towards negative intensifying interactions.

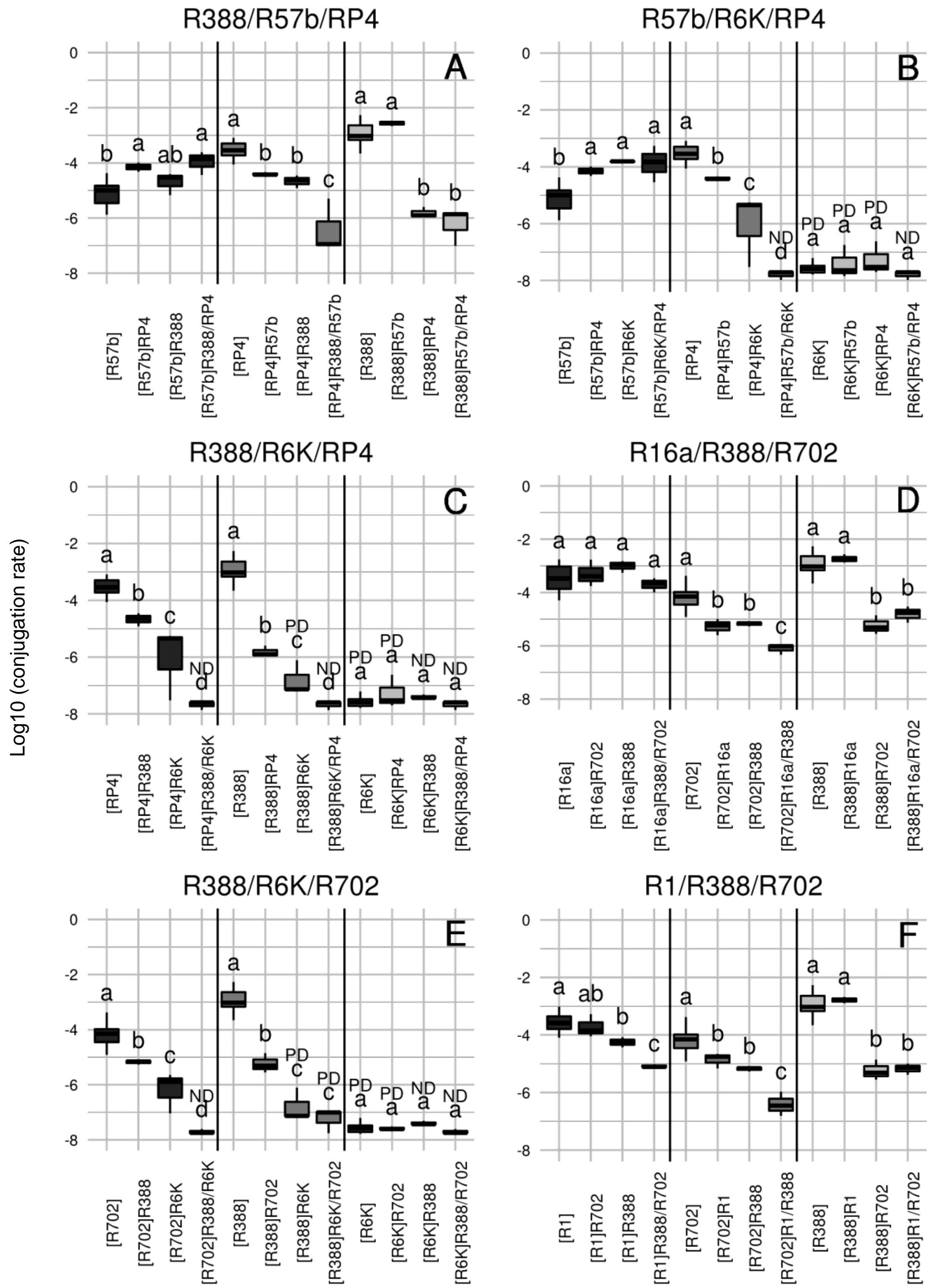


Figure 4.5. Plasmid combinations exhibiting distorting intensifying negative interactions.

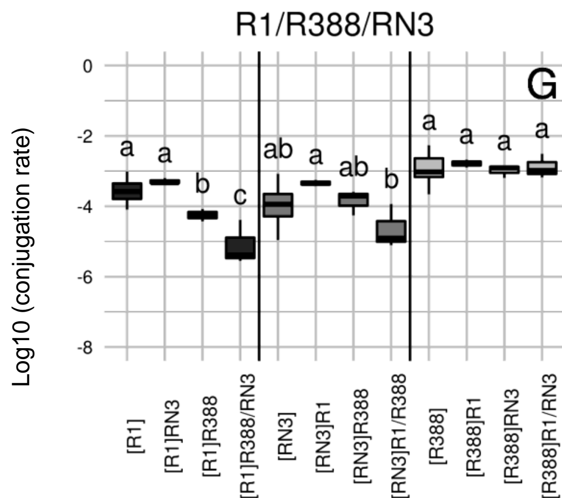


Figure 4.5. Plasmid combinations exhibiting distorting intensifying negative interactions (continued)**.** Distorting negative interactions are those in which the conjugation rate of the focal plasmid decreases in the presence of co-resident plasmids. These interactions are classified as intensifying when the overall inhibitory effect (on the focal plasmid) exerted by the two co-resident plasmids is stronger than the individual effect of any of the co-resident plasmids. Explanation of this figure is as in Fig. 4.1.

Discussion

In mixed infections parasites may interact and compete and plasmids are no exception to this rule. Indeed, several works have reported interactions between two plasmids (Chapter 3, and references therein). The present work focused on multiple infections where hosts were infected by three plasmids simultaneously. Here we showed that plasmids interacted in most combinations. Then we analysed if the interactions between two plasmids would influence a third co-residing plasmid. We termed such kind of interactions as distorting interactions and detected 20 cases in a total of 59 interactions.

Distorting interactions could be further categorized as intensifying or attenuating. In intensifying interactions, the overall effect on a focal plasmid is greater than the effect of any of the individual interactions. By opposition, in attenuating interactions, the overall effect is lesser than the strongest individual interaction. Most of the observed distorting interactions were intensifying, representing about 2/3 of all distorting interactions. Furthermore, all intensifying interactions exerted an inhibitory effect on the focal plasmid. Such result suggests that the inhibitory mechanisms detected might have evolved independently and in turn block different steps of conjugative transmission, which ultimately allows them to complement each other. The absence of positive intensifying distorting interactions may point out to the opposite conclusion, that the mechanisms leading to the increase of the conjugation rate of a focal plasmid appear not to have evolved independently, thus not complementing each other. This reasoning is compatible with the observation in Chapter 3 that the positive effect of stabilizing the cellular contact of the mating pair only increases the efficiency of DNA transfer. Therefore, once the mating pair stabilization maximizes DNA transfer of the focal plasmids, additional cell contact would not provide further positive effect.

Contrary to intensifying distorting interactions, the observed attenuating interactions were either positive or negative. Positive attenuating interactions were observed when a plasmid inhibited a second, thus not allowing the second plasmid to increase the conjugation rate of the remaining third plasmid. We only considered two cases of positive attenuating interactions, while the six cases involving plasmid R6K (Fig. 4.3) were instead regarded as non-distorting negative interactions. The conjugation rate of plasmid R6K, when occupying the host cell

alone, is near the experimental limit of detection, which made it impossible to recognize any negative interactions targeting R6K. We argue that the lower conjugation rate of plasmid R6K observed in Fig. 4.3, when it co-resided the host cell with another two plasmids, was due to negative interactions that we could not detect previously. Furthermore, we argue that negative interactions are dominant over positive interactions. In Chapter 3, we showed that plasmids R388 and R1drd19 simultaneously had positive and negative effect on plasmids R1 and RP4, respectively, and that the negative effects prevailed when cells harboured two plasmids. Therefore, we did not consider these six observations involving plasmid R6K, as distorting interactions.

As discussed above, for several combinations of three plasmids, one can find a reasonable explanation for the observed distorting interactions. Some cases, however, could not be as much intuitive, such as the intensifying inhibition of plasmids F and R124 (Fig. 4.3A, 4.3D, 4.3F). The present results (Figs. 4.3 and 4.4C, Supp.Fig. S4.3 A-C), and those in Chapter 3, show that neither F nor R124 were inhibited in cells harbouring only two plasmids. By comparing the conjugation rates when these plasmids inhabited the host cell with two co-resident plasmids with those when no co-resident plasmid existed (Supp.Fig. S4.2), one detected no inhibitory interactions. However, in Figs. 4.3A, 4.3D and 4.3F inhibition of plasmids F and R124 was detected. Therefore, the inhibitory effect on these plasmids was small, since its detection was not consistent. Since observations of such interactions are not consistent, they may result from stochastic experimental fluctuations. These facts prompt us to speculate that a destabilizing phenomenon arises as the number of different plasmids present in the host cell increases. Such unspecific interactions between the plasmids would provide the stochastic noise observed. Furthermore, considering the near-identical plasmids R1 and R1drd19, one should expect plasmid R1drd19 to be less affected by these fluctuating interactions, since its higher level of expression of conjugation could buffer the stochastic noise. Differences observed between the combinations R1/R388/RN3 and R1drd19/R388/RN3 could reflect such hypothesis (Figs. 4.2A, 4.5G).

It is important to note that the number of interactions might have be underestimated because some conjugation rates fell under the experimental limit of detection, making it impossible to detect negative interactions. Nonetheless, we can conclude the existence of a bias towards negative intensifying distorting interactions. Such a bias, lead us to conclude that these inhibitory mechanisms may complement each other, resulting in stronger inhibition. This points towards interference competition in triple plasmid carriage.

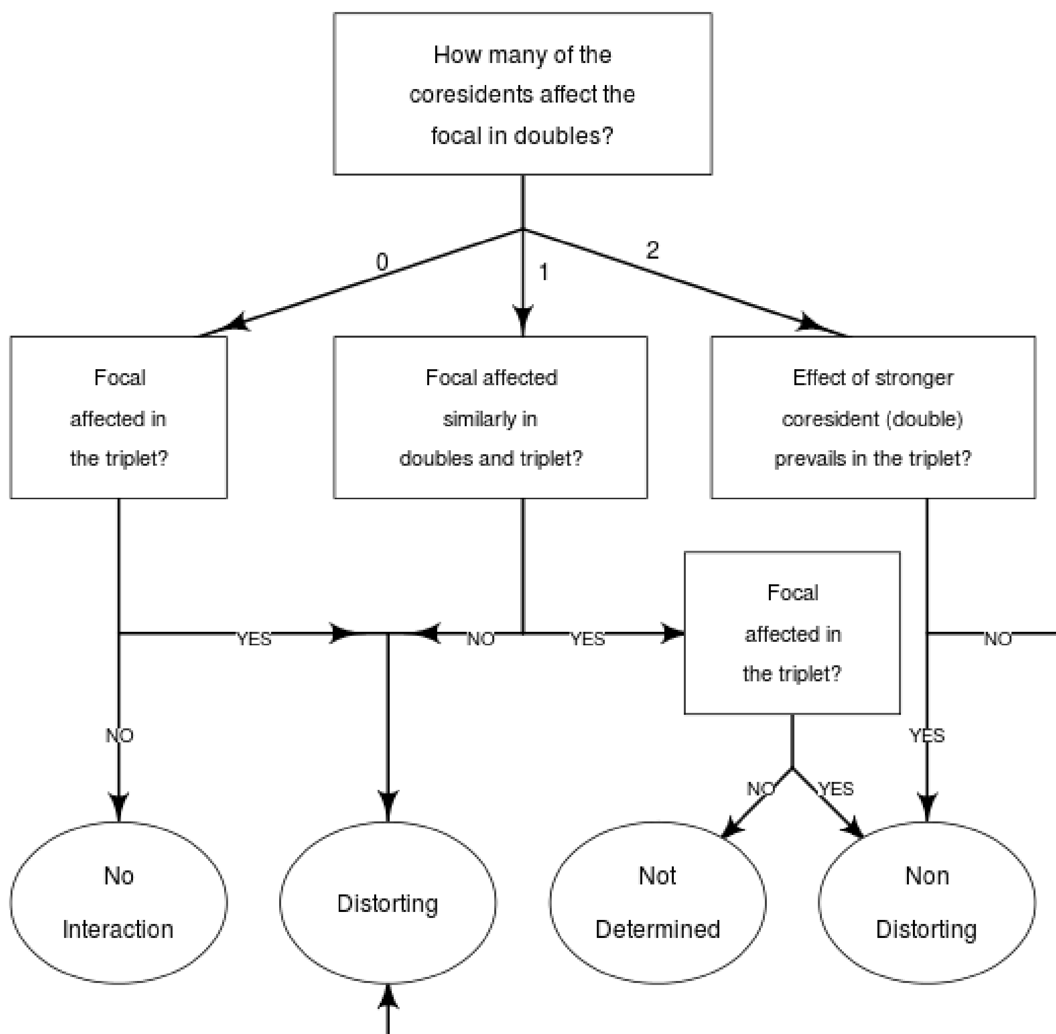
Author Contributions

Conceived and designed the experiments: João Alves Gama, Francisco Dionísio. Performed the experiments: João Alves Gama. Analysed the data: João Alves Gama, Francisco Dionísio, Rita Zilhão. Wrote the chapter: João Alves Gama, with contributions of Francisco Dionísio, Rita Zilhão.

Supplementary Information

Classification of interactions

We compared the conjugation rates of a focal plasmid in four conditions: i) when alone in the host cell, ii) when in the presence of two co-resident plasmids (generically X and Y), iii) when in the presence of only one co-resident plasmid (X), iv) or the other (Y). Classification of interactions is based on the results of Tukey's multiple comparison tests - where conditions having different letters are significantly different (p-value < 0.05). From these results, we proceed as indicated in the following flowchart (Supp.Fig. S4.1.):



Supplementary Figure S4.1. Flowchart for classification of interactions. Decisions are based on the results of Tukey's multiple comparison tests, where conditions having different letters are significantly different (p-value < 0.05).

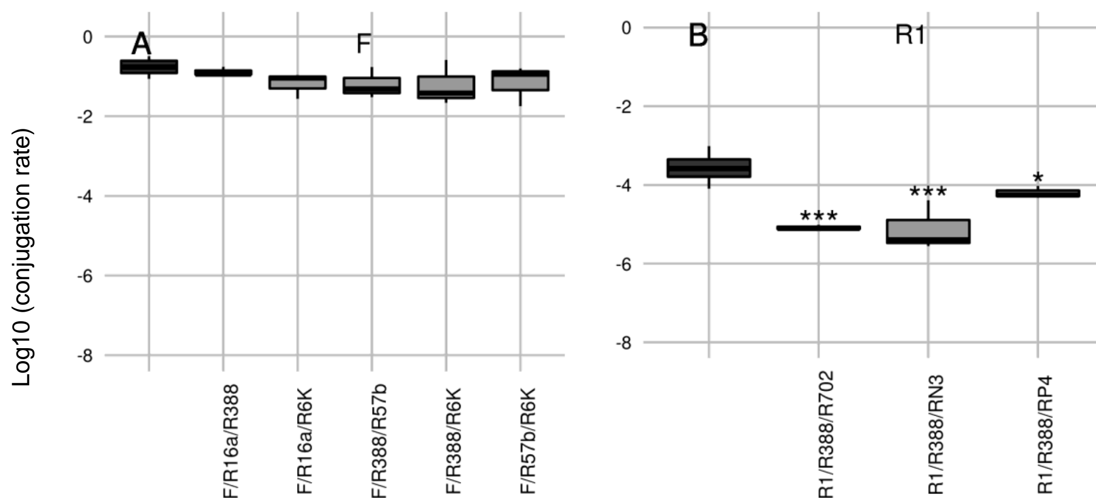
First, we ask how many co-resident plasmids affect the conjugation rate of the focal plasmid. The answer varies from zero to two co-resident plasmids. We say that the focal plasmid is affected when the letter shown, above the situation in which the plasmid is alone in the host cell, differs from the letter shown in other conditions. Taking Fig. 4.5-D as an example, we observe that plasmid R16a is

affected by zero plasmids, plasmid R388 is affected by one plasmid, plasmid R702 is affected by two plasmids.

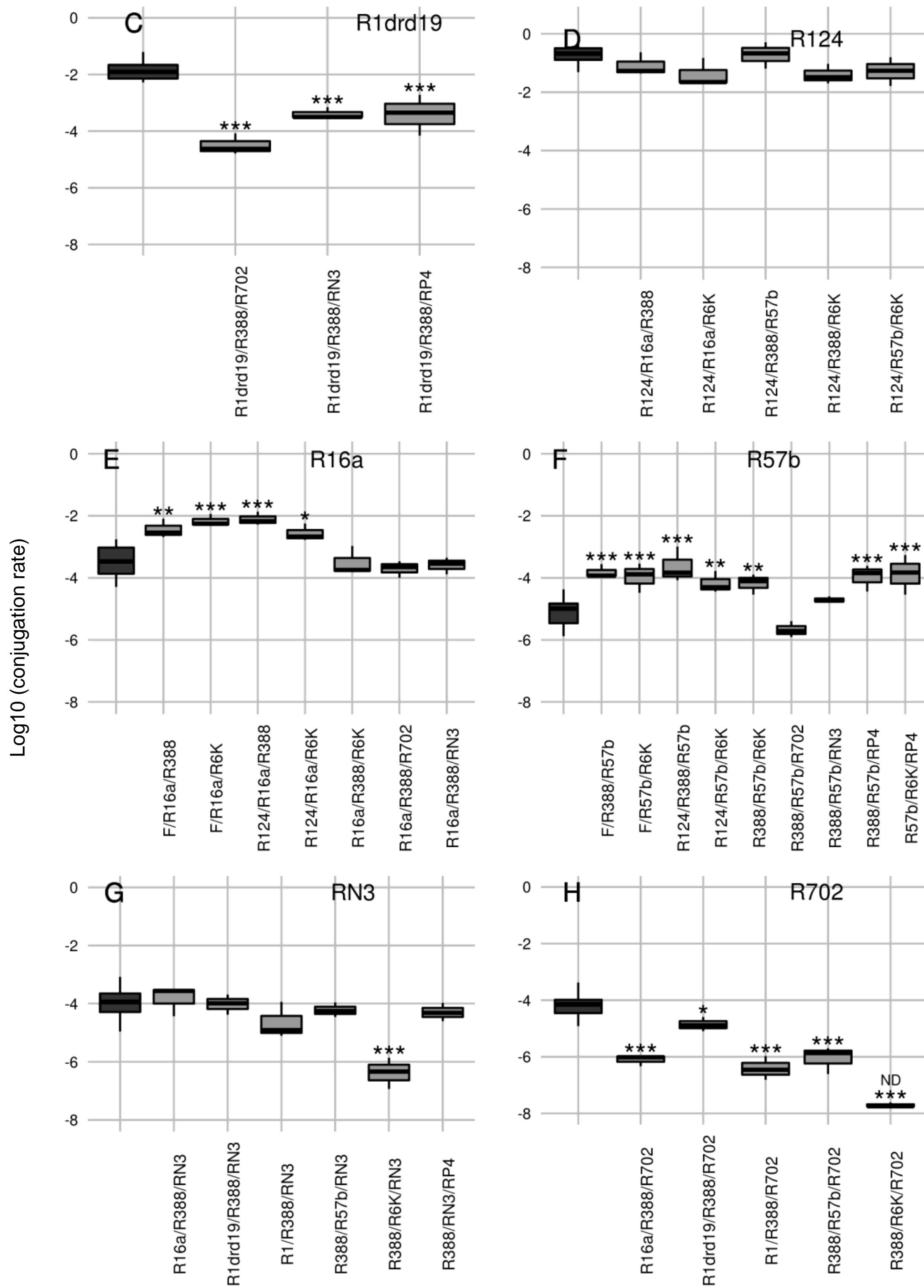
We can reach two outcomes when zero plasmids affect the focal: i) no interaction, or ii) distorting. Fig 4.1-A reveals cases of no interactions, for either focal plasmid. As we can see, the conjugation rate of the focal plasmid when it is alone in the host cell does not differ from any other condition. Regarding plasmid F, Fig 4.3-A reveals a distorting interaction. In this case, the conjugation rate of plasmid F, when it is alone in the host cell, does not differ from when it shares the host with either plasmid R16a or R6K. However, when plasmid F co-resides simultaneously with both plasmids R16a and R6K, its conjugation rate decreases.

When only one plasmid (let us say plasmid X) affects the conjugation rate of the focal plasmid, we ask if plasmid X exerts identical effects when it is the only co-resident and when plasmid Y is also present in the host cell. Let us analyse Fig. 4.2-A. Regarding plasmid RN3, we observe that it is affected by plasmid R1drd19. However, the effect of this interaction is no longer observed when plasmid R388 is also present in the same cell. This is classified as a distorting interaction, because the effect of plasmid R1drd19 was altered (distorted) due to the addition of plasmid R388. On the other hand, when we consider R1drd19 as the focal plasmid, we observe that the effect of interacting with plasmid R388 is not altered by the presence/absence of plasmid RN3. Thus, this is classified as a non-distorting interaction.

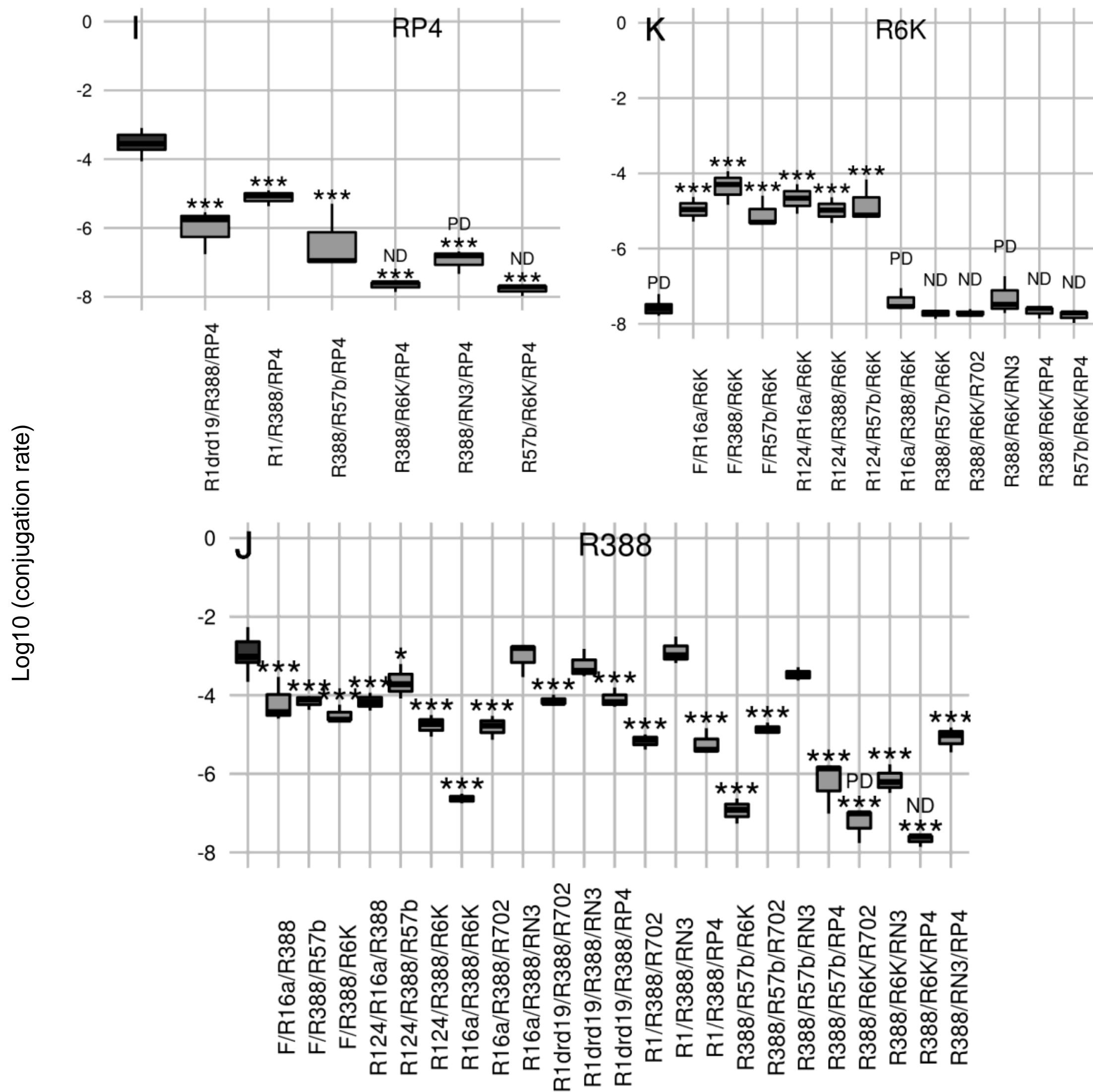
When two plasmids affect the focal plasmid, we ask if the overall effect is identical to the stronger effect exhibited by plasmids X and Y. In Fig. 4.5-E we observe that plasmid R6K exerts a stronger effect on R702 (focal palsmid) than does plasmid R388. Thus, we must compare the effect of plasmid R6K in the two conditions when plasmid R388 is i) present and ii) absent. The effect of plasmid R6K differs between the two conditions, being stronger when plasmid R388 is also present in the same cell. This is a distorting interaction. We can analyse the same figure in terms of plasmid R388. We observe that plasmid R6K exerts a stronger effect than plasmid R702. However, the effect of plasmid R6K on plasmid R388 does not differ whether plasmid R702 is present in the same cell or not. This should be classified as a non-distorting interaction.



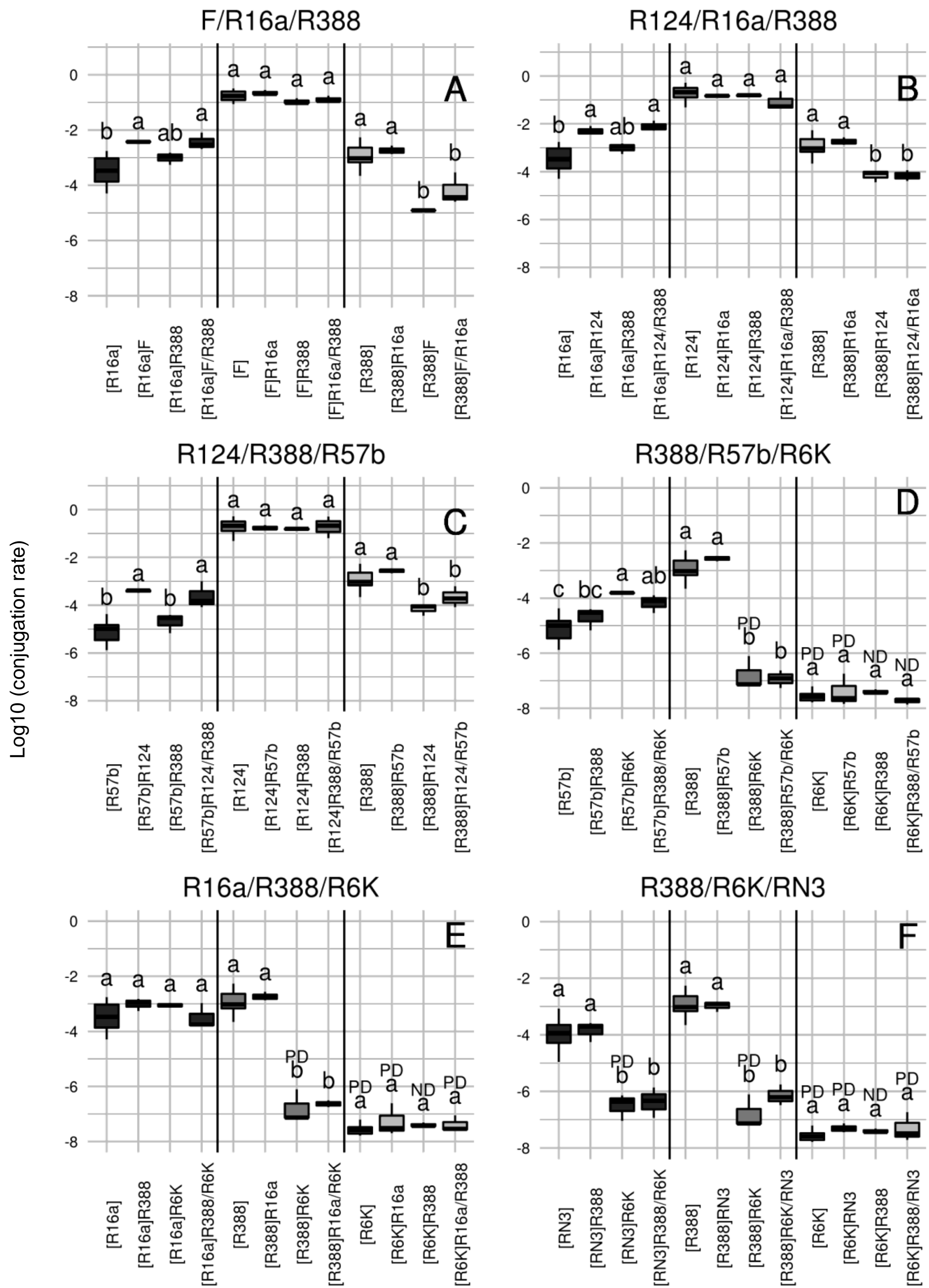
Supplementary Figure S4.2. Conjugation rate variation in combinations of three plasmids.



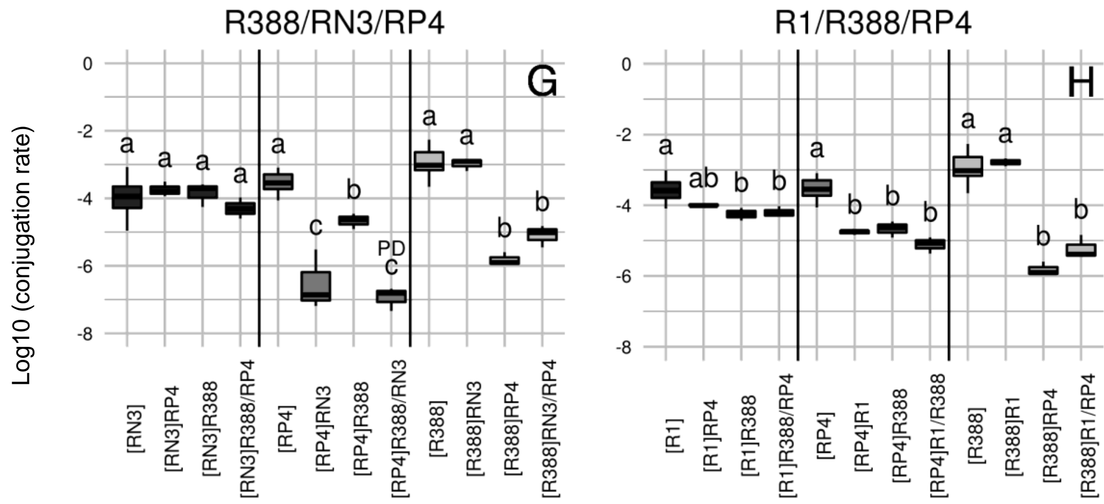
Supplementary Figure S4.2. Conjugation rate variation in combinations of three plasmids (continued).



Supplementary Figure S4.2. Conjugation rate variation in combinations of three plasmids (continued)**.** Titles indicate the focal plasmid and the horizontal axis indicate the combinations of three plasmids. The first box of each plot represents the conjugation rate of the focal plasmid in the absence of co-residents (sample size indicated in Chapter 3). Conjugation rates for combinations of three 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.



Supplementary Figure S4.3. Plasmid combinations exhibiting non-distorting interactions.



Supplementary Figure S4.3. Plasmid combinations exhibiting non-distorting interactions (continued)**.** In these combinations, the interactions between two plasmids are not affected by the presence of a third plasmid. Explanation of this figure is as in Fig. 4.1.

Chapter 5

Searching for fertility inhibition proteins – A preliminary study

Abstract

Plasmids can be transferred between bacterial cells through conjugation. Their conjugative efficiency depends on various factors, one of which is the presence of other conjugative plasmids in the same host cell. A plasmid can decrease the conjugative efficiency of its co-resident if it encodes fertility inhibition (FIN) proteins. A few of these proteins have already been characterized. In this chapter, we analyse the sequences of plasmids studied in previous chapters to analyse their potential to encode FIN homologues. We found that plasmid R16a encodes a protein exhibiting 27% of amino acid identity with the FipA protein of plasmid pKM101. We also studied plasmid sequences retrieved from GenBank database for their ability to encode FIN proteins. FipA homologues were found more frequently than homologues for other FIN proteins. Most of these FipA homologues were found in IncN plasmids and exhibit highly conserved sequences.

Introduction

Plasmids are double-stranded DNA molecules commonly carried by bacteria, yet they can also be found in archaea and eukaryotes (Heinemann, 1991). Plasmids replicate independently of chromosomes and encode some elements required for replication, although they require the replicative machinery of the host cells (del Solar et al., 1998). Plasmids are classified in incompatibility groups (Inc), a classification system based on similarity of the replicative elements. Related plasmids are classified into the same incompatibility group due to the similarity of their replicative elements (Novick, 1987). Such similarity does not allow these mechanisms to distinguish related plasmids when they co-reside in the same host cell. Consequently, plasmid replication is unbalanced and one plasmid replicates more than the other. Eventually, after a few generations, the two plasmids are no more segregated together. Thus, related plasmids are classified into the same Inc groups, as result of their inability to persist in the same host for long periods. In other words, such plasmids are said to belong to the same incompatibility group due to their inability to co-exist.

Plasmids are inherited vertically, during cell division, from mother to daughter cells. Some plasmids, however, are also able to be transmitted infectiously, between cells, in a horizontal process termed conjugation (for a review of the steps involved in conjugation see (Cabezon et al., 2015)). Conjugative plasmids encode all the machinery required for conjugation. In Gram negative bacteria, plasmids synthesize conjugative pili, which are assembled by a type IV secretion system (T4SS), termed transferosome. These pili are appendages expressed on the surface of donor cells and promote the essential cellular contact between donor and recipient cells, in a process termed mating pair formation. Retraction of pili facilitates the fusion between the membranes of

donor and recipient cells. Additionally, the transferosome also provides the signal for DNA processing. The relaxosome, constituted by a relaxase and other auxiliary proteins, is responsible for DNA processing. Plasmids contain a region, *oriT*, where the relaxosome binds. The relaxase cleaves on a *nic* site within *oriT*, resulting in the formation of a single stranded DNA that will be transferred to the recipient cell. Plasmids also encode a coupling protein (T4CP) that drives the relaxosome to the secretion channel.

Various studies have shown that the conjugative efficiency of a plasmid can be reduced if a different conjugative plasmid inhabits the same host cell (Hochmannova et al., 1985; Hochmannova et al., 1982; Olsen & Shipley, 1975; Tanimoto & Iino, 1983; Willetts & Skurray, 1980). Plasmids F and pKM101 encode, respectively, the proteins PifC and FipA that diminish the conjugative transfer of plasmid RP4 (Santini & Stanisich, 1998). These proteins do not prevent pilus assembly. Instead, they target the TraG coupling protein of RP4 (Miller et al., 1985; Santini & Stanisich, 1998; Winans & 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 & Shipley, 1975). The PifC protein of plasmid F has additional roles as it is also regulates the expression of genes involved in plasmid replication and inhibition of viral infection (Blumberg et al., 1976; Miller & Malamy, 1983; Miller & Malamy, 1984; Morrison & Malamy, 1971; Rotman et al., 1983; Tanimoto & Iino, 1984).

Plasmid RP4 encodes two regions: *fiwA* and *fiwB*, responsible for inhibiting the conjugative transfer of IncW plasmids R388 and Sa (Fong & Stanisich, 1989; Olsen & Shipley, 1975; Yusoff & Stanisich, 1984). The region *fiwB* comprises the *kilA* operon, which contains three genes encoding proteins KlaA, KlaB and KlaC (Goncharoff et al., 1991). All three proteins are required to prevent plasmid R388 from expressing conjugative pili and to confer tellurite resistance (Fong & Stanisich, 1989; Goncharoff et al., 1991). Moreover, overexpression of any of the three proteins inhibits the growth of the host cell (Figurski et al., 1982; Turner et al., 1994). Besides RP4, other IncP-1 plasmids retain the ability to reduce the conjugative transfer of plasmid R388, but not all IncP-1 plasmids do so (Yusoff & Stanisich, 1984). Indeed, IncP-1 plasmids diverged into two groups IncP-1-α and IncP-1-β (Lanka et al., 1985; Villarroel et al., 1983). IncP-1-β do not encode the *kilA* operon, which is consistent with their inability to inhibit the conjugation of plasmid R388 or to express tellurite-resistance (Bradley, 1985; Thomas et al., 1995; Yusoff & Stanisich, 1984). Tellurite-resistance is associated with IncP-1 and IncH plasmids, although more recently an IncP-7 plasmid has been shown to encode the three *kla* genes (Bradley, 1985; Taylor & Summers, 1979; Yano et al., 2007). Furthermore, the *kilA* operon is part of transposon Tn521 (Grewal, 1990).

The product of *fiwA* shares some homology with the Osa protein of plasmid Sa, which interacts with plasmid Ti of *Rhizobium radiobacter* (former *Agrobacterium tumefaciens* (Young et al., 2001)) (Chen & Kado, 1994; Close & Kado, 1991). Plasmid Ti encodes two T4SSs, one for its own transmission and another to transfer T-DNA (a portion of the Ti plasmid required for pathogenicity in plants) (Christie, 2004; Li et al., 1998). Plasmid Sa prevents the oncogenic effects on plants, because the Osa protein prevents the transfer of T-DNA (Chen & Kado, 1994; Chen & Kado, 1996; Close & Kado, 1991; Farrand et al., 1981; Loper & Kado, 1979; Vlasak & Ondrej, 1985). Osa seems to degrade T-DNA prior to its interaction with the coupling protein VirD4 (Cascales et al., 2005; Maindola

et al., 2014). The product of gene *p1056.10c*, sharing some homology with Osa and FiwA was identified in plasmid ICEhin1056 (Juhas et al., 2007). Osa homologues (FiwA and P1056.10c) all inhibited T-DNA transfer, possibly through a similar mechanism (Maindola et al., 2014). FipA and PifC were also shown to prevent the oncogenic effect of plasmid Ti (Maindola et al., 2014).

Plasmid R6K is able to reduce the conjugative transfer of IncN, IncP-1 and IncW plasmids (Hochmannova et al., 1985; Hochmannova et al., 1982; Nesvera & Hochmannova, 1983). The mechanisms responsible for this phenotype seem to be encoded near the *finO* region. To our knowledge, this mechanism was not further characterized.

In Chapter 3, we showed that conjugative transfer of plasmid R6K decreases if plasmids R16a, R57b or R388 are present in the same cell. Plasmid R388 has been previously shown to reduce the horizontal transfer of plasmid R6K, although this effect was not observed among all replicates (Olsen & Shipley, 1975). Moreover, plasmid RP4, also exhibited an inhibitory effect towards plasmid R6K (Olsen & Shipley, 1975). We also showed in Chapter 3, that plasmids R16a and R57b decreased the horizontal transfer of plasmid RP4. Moreover, we showed that both plasmids F and R124 were able to reduce the conjugative efficiency of plasmids RP4 and R388. PifC of plasmid F was previously shown to interact with the coupling protein of RP4 but not with that of R388 (Santini & Stanisich, 1998).

In Chapter 3, we also showed that, in cells carrying three conjugative plasmids, two inhibitory mechanisms can complement each other, resulting in stronger inhibition of a common target plasmid. Such complementation suggests that these inhibitory mechanisms act independently. This supports the hypothesis that the mechanism of FiwA homologues differs from that of FipA and PifC (Maindola et al., 2014).

The sequences of some of the plasmids studied in Chapter 3 are available. Thus, our first aim was to screen them for homologues of previously identified FIN (Fertility INhibition) proteins, which reduce the conjugative horizontal of other plasmids. Our second aim was to extend such screen to all complete prokaryotic plasmid sequences available in the GenBank database.

Materials & Methods

Identification of FINs in plasmids studied in previous chapters

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 Table 5.1), 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.

Identification of FINs in plasmid sequences in the GenBank database

We retrieved a total of 4543 prokariotic plasmid complete sequences from GenBank (ftp://ftp.ncbi.nih.gov/genomes), available on February 2016. To these, we added the following sequences: R16a (KX156773), RP4 (BN000925), R388

(BR000038) and R6K (ftp://ftp.sanger.ac.uk/pub/pathogens/Plasmids/R6K.dbs). We used the BLASTP algorithm (BLAST+ suite 2.2.28) to search for homology between plasmid proteins and the FIN proteins provided in Table 5.1 and also the putative FIN identified for R16a. We considered the following parameters: identity > 35%, query coverage > 0.5 and e-value < 10^{-4}. These parameters, used for identity and query coverage, were chosen based on the results retrieved from comparisons between FiwA and its two homologues Osa and P1056.10c. If one protein matched more than one FIN, the match with lower e-value was considered. For plasmid R6K, the BLASTX algorithm (with the same parameters) was used instead.

Table 5.1. Accession numbers of FIN protein sequences

FIN protein	Accession no.
FipA	AAC63100.1
PifC	CAA28641.1
FiwA	CAJ85704.1
Osa	AAA75248.1
P1056.10c	CAF29018.1
KlaA	CAJ85667.1
KlaB	CAJ85666.1
KlaC	CAJ85665.1

Classification in incompatibility groups

Classification in incompatibility groups was performed as described in Chapter 2.

Data Analysis

Data analysis was performed in R version 3.2.0, available at http://www.rstudio.com/ (R Core Team, 2015).

Results

Identification of FINs in plasmids studied in previous chapters

We searched for homologues of known FIN proteins in the genomes of the plasmids studied in Chapters 3 and 4. We found a potential FIN homologue in plasmid R16a. Plasmid R16a 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 in the sequence of R16a encodes this putative FIN protein. Henceforth, we will refer this putative FIN as Aip (for Alternative inhibition of IncP-1). 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 a 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.

Identification of FINs in plasmid sequences in the GenBank database

The three proteins FiwA, Osa and P1056.10c shared at least 36% of identity and 53% of coverage. Thus, we used 35% and 50% cut-off values, respectively, for identity and coverage, to search for homologues among

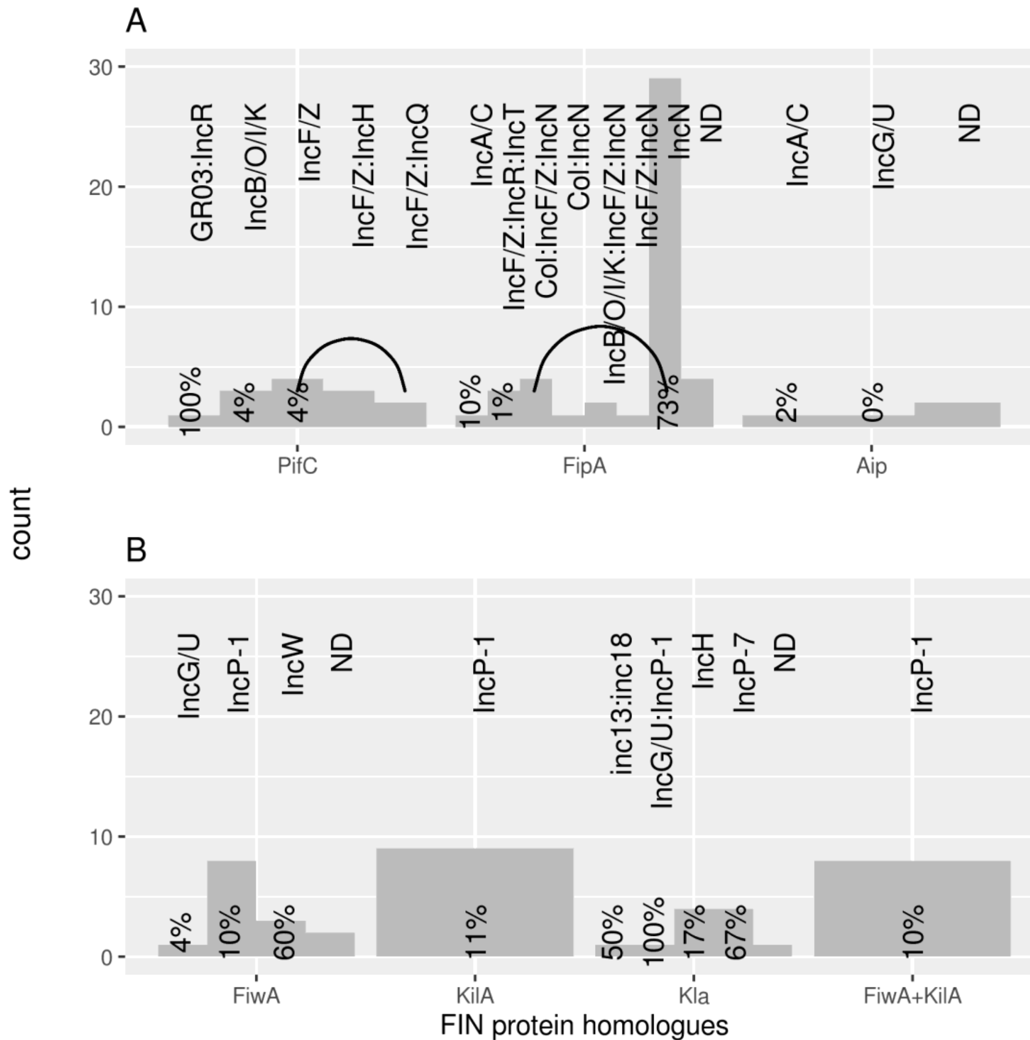


Figure 5.1. Distribution of plasmids encoding homologues of FIN proteins. The number of plasmids is indicated on the y axis. The x axis indicates the FIN homologues: PifC (of plasmid F), FipA (of plasmid pKM101), Aip (of plasmid R16a), FiwA [FiwA (of plasmid RP4) or Osa (of plasmid Sa) or P1056.10c (of plasmid ICEhin1056)], KilA (all three KlaA, KlaB and KlaC of plasmid RP4), Kla (only one or two of the following: KlaA, KlaB or KlaC of plasmid RP4), FiwA+KilA [FiwA (or Osa or P1056.10c) and all three KlaA, KlaB and KlaC]. Annotations indicate the plasmids' incompatibility groups (multiple groups are separated by a ":" sign) and the relative frequency of plasmids encoding FIN homologues per incompatibility group. Bars enclosed by curved lines share a common incompatibility group: all plasmids encoding IncF/Z replicons comprise 4% of all plasmids encoding IncF/Z replicons; all plasmids encoding IncN replicons comprise 73% of all plasmids encoding IncN replicons. The frequencies of GR03:IncR, inc13:inc18 and IncG/U:IncP-1 plasmids are relative to plasmids simultaneously exhibiting both incompatibility groups.

prokaryotic plasmid genomes retrieved from the GenBank database. In addition to homologues of the known FIN proteins, we also searched for homologues of Aip.

Besides plasmid F, we found PifC homologues in 12 plasmids (Fig. 5.1A, Supp.Table S5.1). The average identity shared with PifC was 84% with standard deviation of 23%. Among these plasmids, nine (including plasmid F) possessed IncF replicons. FipA homologues were found in a total of 45 plasmids (Fig. 5.1A, Supp.Table S5.1). Among these, 36 (including plasmid RN3) possessed IncN replicons. The average identity shared with FipA was 92% with standard deviation of 18%. Considering only plasmids having IncN replicons, average identity shared with FipA was 96% with standard deviation of 11%, thus, showing little variation. Considering only plasmids having IncA/C replicons, average identity shared with FipA was 88% with standard deviation of 25%. This shows that FipA is less identical to homologues carried in IncA/C plasmids than to those encoded in IncN plasmids. Besides plasmid R16a, Aip homologues were detected in one IncG/U plasmid (58% identity) and in two plasmids not assigned to any incompatibility groups (47% and 58% identity) (Fig. 5.1 A, Supp.Table S.5.1).

Homologues of either FiwA, Osa or P1056.10c, were found in a total of 14 plasmids (including RP4 and R388) (Fig. 5.1B, Supp.Table S5.1). Among these, eight possessed IncP-1 and three possessed IncW replicons. Homologues found in IncP-1 plasmids shared an average identity with FiwA of 89% with standard deviation of 23%. Homologues found in IncW plasmids shared 100% identity with Osa. Nine IncP-1 plasmids encoded complete *kilA* operons encoding all three homologues of KlaA, KlaB and KlaC. Moreover, all IncP-1 plasmids encoding FiwA homologues encoded simultaneously complete *kilA* operons. Alternatively, 11 plasmids carried incomplete *kilA* operons. In 10 cases, no KlaC homologue was found under the experimental conditions described. Further inspection showed that 9 of these 10 plasmids could potentially encode KlaC homologues in regions adjacent to those encoding KlaA and KlaB (Supp.Table S5.1). The inc13:inc18 plasmid encoded the only a KlaC homologue, but not KlaA nor KlaB.

Table 5.2. Number of plasmids assigned per incompatibility group.

Incompatibility Group	Number of plasmids
IncA/C	41
IncB/O/I/K	76
IncF/Z	295
IncG/U ‡	28
IncH	23
IncN	49
IncP-1 ‡	80
IncP-7	6
IncR *	18
IncW	5
GR01_05 *	39
inc13 º	140
inc18 º	28

‡ only 1 plasmid encoded simultaneously IncG/U and IncP-1 replicons

* only 1 plasmid encoded simultaneously GR3 and IncR replicons

º only 2 plasmids encoded simultaneously inc13 and inc18 replicons

We assigned as many plasmids as possible into incompatibility groups (Table 5.2). Plasmids encoding PifC homologues represented less than 5% of either all plasmids assigned to IncF/Z or IncB/O/I/K groups. Of all assigned IncP-1 plasmids, only 10% encoded FiwA homologues and *kilA*-like operons. Contrastingly, plasmids encoding FipA homologues comprised more than 70% of all assigned IncN plasmids and 10% of all assigned IncA/C plasmids. Thus, this seems to be a common trait among IncN plasmids. The frequency of FIN encoding plasmids belonging to other incompatibility groups was not estimated because few plasmids were assigned to those groups.

Discussion

Conjugative plasmids can encode FIN proteins which are responsible for reducing the conjugative efficiency of plasmids residing in the same host cell. The FIN proteins, PifC and FipA, of plasmids F (IncF) and pKM101 (IncN) inhibit the conjugation of plasmid RP4 (Santini & Stanisich, 1998). Osa from plasmid Sa (IncW) prevents the horizontal transfer of T-DNA from plasmid Ti (Chen & Kado, 1994; Close & Kado, 1991). FiwA is a homologue of Osa encoded by RP4 (IncP-1) and targets plasmid R388 (Fong & Stanisich, 1989; Olsen & Shipley, 1975; Yusoff & Stanisich, 1984). RP4 encodes another function to inhibit the conjugation of plasmid R388, by preventing the expression of the transferosome (Fong & Stanisich, 1989; Olsen & Shipley, 1975; Yusoff & Stanisich, 1984). This function is encoded in the operon kilA, comprising three genes: klaA, klaB and klaC (Goncharoff et al., 1991). Simultaneous expression of the three genes also confers tellurite resistance. Plasmid R6K encodes an uncharacterized system that reduced the conjugative efficiency of IncN, IncP-1 and IncW plasmids (Hochmannova et al., 1985; Hochmannova et al., 1982; Nesvera & Hochmannova, 1983).

In previous chapters, we detected further inhibitory interactions between conjugative plasmids. The present work constitutes a preliminary analysis of other potential FIN proteins. DNA sequences of six of the plasmids studied in previous chapters are currently available (plasmids F, R16a, R388, R6K, RN3 and RP4). We used the BLAST algorithms to search, in those sequences, for proteins with homology with known FIN proteins. We found that plasmid R16a encodes a potential FIN protein, exhibiting homology with FipA. Like FipA, this protein, here termed Aip, could be responsible for the observed inhibition of IncP-1 plasmids. Further experimental work is required to determine if Aip inhibits the conjugation of IncP-1 plasmids. All other FIN homologues identified in these plasmids have already been described.

We showed in previous chapters that plasmids could inhibit the transfer of distinct co-resident plasmids. For instance, plasmids R388 and R16a could inhibit both IncP-1 and IncX plasmids. Thus, it is possible that they encode various FIN proteins to target different plasmids. However, it is also possibly that these proteins could exhibit enough plasticity so to interact with different plasmids. In fact, all PifC, FipA and FiwA interact with plasmid Ti (Maindola et al., 2014). Additionally, PifC and FipA also interact with IncP-1 plasmids, while FiwA interacts with IncW plasmids. Thus, it is possible that, for instance, the Osa protein of R388 could target both IncP-1 and IncX plasmids. Further studies are required to test the role of these FIN proteins in the inhibition of distinct plasmids.

The PifC protein of plasmid F that targets the coupling protein of IncP-1 plasmids has been shown not to interact with the coupling protein of IncW plasmids (Santini & Stanisich, 1998). Therefore, it is expected that plasmid F encodes at least another inhibitory system. Therefore, the quest to search for additional Fin proteins should continue.

We explored the genomes of plasmids in the GenbBank database to examine the presence of FIN homologs in sequenced plasmids. The most frequent proteins exhibited homology with FipA. These homologs were mainly found in IncN plasmids, and their sequences were highly conserved. Contrastingly, PifC was found in less than 5% of all identified IncF/Z plasmids. Given that this protein is also involved in the regulation of the replication of plasmid F, we expected a higher frequency of homologs among other plasmids belonging to the same incompatibility group (Blumberg et al., 1976; Miller & Malamy, 1983; Miller & Malamy, 1984; Morrison & Malamy, 1971; Rotman et al., 1983; Tanimoto & Iino, 1984). In addition to inhibiting conjugative transfer, the kilA operon is also responsible for tellurite resistance (Goncharoff et al., 1991). The finding of identical operons in IncH and IncP-7 plasmids was not unexpected, since plasmids belonging to these incompatibility groups have been shown to encode such resistance (Bradley, 1985; Taylor & Summers, 1979; Yano et al., 2007). However, the ability of IncH and IncP-7 plasmids to inhibit conjugation remains to be proven.

Future works may identify additional FIN homologs and test their function. Knowledge concerning such proteins and their mechanisms of action could contribute to diverse fields. For instance, they could be useful to counter-act the dissemination of antibiotic-resistance or to prevent infection in plant species.

Author Contributions

Conceived and designed the experiments: João Alves Gama. Performed the experiments: João Alves Gama. Analysed the data: João Alves Gama, Francisco Dionísio, Rita Zilhão. Wrote the chapter: João Alves Gama, with contributions of Francisco Dionísio, Rita Zilhão.

Supplementary Information

Supplementary Table S5.1. FIN protein homologues detected.

Plasmid Accession no.	Incompatibility group	Match	Protein Identity (%)	E-value	Sequence Coverage (%)	Position of *kla*-like genes	
BN000925	IncP-1	KlaC	100	0	100	59512	369
		KlaB	100	0	100	366	1502
		KlaA	100	0	100	1520	2293
		FiwA	100	1×10^{-140}	81		
BR000038	IncW	Osa	100	5×10^{-141}	100		
KX156773	IncA/C	Aip	100	2×10^{-154}	100		
NC_002483	IncF/Z	PifC	100	0	100		
NC_003292	IncN	FipA	100	1×10^{-156}	100		

Supplementary Table S5.1 (continued)

Plasmid Accession no.	Incompatibility group	Match	Protein Identity (%)	E-value	Sequence Coverage (%)	Position of *kla*-like genes	
NC_004444	IncP-7	KlaA	65	1×10^{-111}	100	60265	61026
		KlaB	71	0	97	61055	62155
		KlaC	29	3×10^{-6}	44	62287	63543
NC_005014	IncB/O/I/K	PifC	99	9×10^{-143}	80		
NC_00521	IncH	KlaC	23	4×10^{-4}	72	230635	231528
		KlaB	56	6×10^{-151}	98	231518	232618
		KlaA	45	1×10^{-69}	98	232627	233412
NC_005249	IncF/Z:IncH	PifC	85	0	100		
NC_006352	IncP-1	FiwA	100	1×10^{-140}	81		
NC_006352	IncP-1	KlaC	100	0	100	63770	64723
		KlaB	100	0	100	64720	65856
		KlaA	100	0	100	65874	66647
NC_006625	IncF/Z:IncH	PifC	85	0	100		
NC_007682	IncN	FipA	100	4×10^{-158}	100		
NC_007950	ND	FipA	38	4×10^{-32}	90		
NC_008275	IncP-7	KlaA	64	6×10^{-112}	100	35186	35959
		KlaB	71	0 x10	96	35970	37088
		KlaC	29	3×10^{-6}	39	37088	38476
NC_008357	IncP-1	KlaB	100	0	100	366	1502
		KlaA	100	0	100	1520	2293
		FiwA	100	1×10^{-140}	81		
		KlaC	100	0	100	88563	369
NC_008612	IncA/C	FipA	100	3×10^{-69}	94		
NC_009132	IncN	FipA	100	4×10^{-158}	100		
NC_009141	IncA/C	FipA	100	3×10^{-69}	94		
NC_009228	ND	FiwA	39	2×10^{-15}	92		
		FiwA	51	2×10^{-66}	99		
NC_009349	IncA/C	FipA	51	1×10^{-71}	91		
NC_009651	GR03:IncR	PifC	86	0	99		
NC_009982	IncW	Osa	100	5×10^{-141}	100		
NC_010716	IncW	Osa	99	4×10^{-141}	100		
NC_010870	IncH	KlaC	23	4×10^{-4}	72	205763	206656
		KlaB	56	2×10^{-150}	98	206646	207746
		KlaA	45	1×10^{-69}	98	207755	208540
NC_010886	IncF/Z:IncR:IncT	FipA	53	8×10^{-14}	73		
NC_011383	IncN	FipA	98	2×10^{-95}	100		
NC_011385	IncN	FipA	98	1×10^{-81}	95		
		FipA	100	1×10^{-27}	100		
NC_011409	ND	p1056.10c	100	6×10^{-143}	100		

Supplementary Table S5.1 (continued)

Plasmid Accession no.	Incompatibility group	Match	Protein Identity (%)	E-value	Sequence Coverage (%)	Position of *kla*-like genes	
NC_011838	IncP-7	KlaA	65	1×10^{-111}	100	60265	61026
		KlaB	71	0	97	61055	62155
		KlaC	29	3×10^{-6}	44	62287	63543
NC_011964	IncF/Z:IncQ	PifC	100	0	100		
NC_013285	IncF/Z	PifC	35	1×10^{-63}	99		
NC_014208	IncN	FipA	98	1×10^{-81}	95		
NC_014231	IncN	FipA	100	4×10^{-158}	100		
NC_014385	IncF/Z:IncN	FipA	98	8×10^{-96}	100		
NC_015599	IncN	FipA	99	4×10^{-155}	100		
NC_015872	Col:IncN	FipA	53	8×10^{-20}	64		
NC_016839	IncA/C	FipA	100	1×10^{-27}	100		
NC_017541	IncF/Z:IncH	PifC	85	0	100		
NC_017659	IncF/Z:IncQ	PifC	100	0	100		
NC_017961	inc13:inc18	KlaC	40	4×10^{-5}	82	28086	28253
NC_019020	IncP-1	FiwA	100	1×10^{-140}	81		
		KlaC	100	0	100	46817	47770
		KlaB	100	0	100	47767	48903
NC_019020	IncP-1	KlaA	100	0	100	48921	49694
NC_019021	IncP-1	FiwA	100	1×10^{-140}	81		
		KlaC	100	0	100	50987	51940
		KlaB	100	0	100	51937	53073
		KlaA	100	0	100	53091	53864
NC_019022	IncP-1	FiwA	100	1×10^{-140}	81		
		KlaC	100	0	100	49300	50253
		KlaB	100	0	100	50250	51386
		KlaA	100	0	100	51404	52177
NC_019033	IncN	FipA	100	4×10^{-158}	100		
NC_019087	IncN	FipA	100	4×10^{-158}	100		
NC_019098	IncN	FipA	100	4×10^{-158}	100		
NC_019124	IncN	FipA	100	4×10^{-158}	100		
NC_019166	IncN	FipA	98	1×10^{-81}	95		
NC_019166	IncN	FipA	100	1×10^{-27}	100		
NC_019888	IncN	FipA	100	5×10^{-59}	98		
NC_020086	Col:IncF/Z:IncN	FipA	98	1×10^{-81}	95		
NC_020088	IncN	FipA	98	1×10^{-81}	95		
NC_020261	IncF/Z	PifC	86	0	100		
NC_020277	ND	FipA	37	5×10^{-43}	83		
NC_021356	ND	FipA	98	2×10^{-95}	100		

Supplementary Table S5.1 (continued)

Plasmid Accession no.	Incompatibility group	Match	Protein Identity (%)	E-value	Sequence Coverage (%)	Position of *kla*-like genes	
NC_021506	IncP-7	KlaA	64	6×10^{-112}	100	58987	59760
		KlaB	71	0	96	59771	60889
		KlaC	29	3×10^{-6}	39	60889	62277
NC_021622	IncN	FipA	98	1×10^{-81}	95		
		FipA	100	3×10^{-69}	94		
NC_021660	IncN	FipA	98	2×10^{-95}	100		
NC_021664	IncN	FipA	98	2×10^{-95}	100		
NC_021845	IncH	KlaA	46	8×10^{-70}	95	110098	110907
		KlaB	57	5×10^{-152}	97	110904	112016
		KlaC	25	2×10^{-4}	40	112006	112899
NC_021997	ND	FipA	98	2×10^{-95}	100		
NC_022375	IncN	FipA	98	1×10^{-81}	95		
NC_022437	ND	Aip	47	1×10^{-58}	64		
NC_022539	ND	Aip	58	9×10^{-43}	58		
NC_022650	IncP-1	KlaB	58	1×10^{-153}	100	11621	12733
		KlaA	54	5×10^{-98}	95	12730	13515
		KlaC	36	5×10^{-8}	53	24491	24874
NC_022885	IncB/O/I/K: :IncF/Z:IncN	FipA	98	1×10^{-81}	95		
NC_022995	ND	KlaA	42	5×10^{-68}	86	274116	274928
NC_022995	ND	KlaB	46	6×10^{-108}	88	276269	277450
		KlaC	25	3×10^{-3}	41	278682	279794
NC_023025	IncF/Z	PifC	35	1×10^{-63}	99		
NC_023275	IncB/O/I/K	PifC	100	0	100		
NC_023276	IncB/O/I/K	PifC	100	0	100		
NC_023907	IncG/U:IncP-1	KlaB	58	1×10^{-153}	100	1395	2507
		KlaA	56	5×10^{-102}	95	2504	3289
NC_023909	IncN	FipA	99	2×10^{-122}	100		
		FipA	81	2×10^{-28}	95		
NC_023910	IncN	FipA	99	2×10^{-122}	100		
		FipA	81	2×10^{-28}	95		
NC_024954	Col:IncN	FipA	53	8×10^{-20}	64		
NC_024967	IncN	FipA	100	3×10^{-69}	94		
		FipA	98	1×10^{-81}	95		
NC_024974	IncN	FipA	100	4×10^{-158}	100		
NC_024983	IncH	KlaC	23	4×10^{-4}	72	264904	265797
		KlaB	56	7×10^{-151}	97	265787	266899
		KlaA	45	1×10^{-69}	98	266896	267681
NC_025059	IncN	FipA	100	4×10^{-158}	100		

Supplementary Table S5.1 (continued)

Plasmid Accession no.	Incompatibility group	Match	Protein Identity (%)	E-value	Sequence Coverage (%)	Position of *kla*-like genes	
NC_025060	IncN	FipA	100	4×10^{-158}	100		
NC_025061	IncN	FipA	100	4×10^{-158}	100		
NC_025062	IncN	FipA	100	4×10^{-158}	100		
NC_025082	IncP-1	FiwA	99	5×10^{-140}	81		
		KlaC	100	0	100	55601	56554
		KlaB	100	0	100	56551	57687
		KlaA	100	0	100	57705	58478
NC_025091	Col:IncN	FipA	98	2×10^{-150}	87		
NC_025141	IncF/Z:IncN	FipA	100	4×10^{-158}	100		
NC_025163	IncP-1	FiwA	99	5×10^{-140}	81		
		KlaC	100	0	100	62309	63262
		KlaB	100	0	100	63259	64341
		KlaA	100	0	100	64412	65185
NC_025164	IncG/U	Aip	58	2×10^{-87}	100		
NC_025164	IncG/U	FiwA	41	5×10^{-41}	87		
NC_025183	IncN	FipA	100	1×10^{-27}	100		
		FipA	98	1×10^{-81}	95		
NC_025186	IncN	FipA	100	3×10^{-69}	94		
		FipA	98	1×10^{-81}	95		

Chapter 6

Co-resident plasmids travel together

Abstract

Some plasmids can transfer between host cells through a process termed conjugation. Expression of genes involved in conjugation is often repressed and the ability of a plasmid to transfer horizontally depends on its transient de-repression. Due to such transient de-repression only a small proportion of plasmid harbouring bacteria engages in conjugation. If host cells harbour multiple plasmids, their simultaneous transfer depends on simultaneous transient de-repression of all plasmids. This is assumed to be a rare event because de-repression of both plasmids was thought to be independent. Some observations support this hypothesis, while others show that co-transfer of plasmids is more frequent. Here, we show that co-transfer of multiple plasmids does not seem to result from independent events and found a simple model for the probability of co-transfer: co-transfer seems to be limited by the plasmid having the lowest conjugation rates. In this sense, cells receiving the plasmid with the lower transfer rate also receive the other plasmid. In spite of the transiently de-repressed conjugative machinery, our results suggest that de-repression happens simultaneously on co-resident plasmids. Therefore, common cues may stimulate de-repression of distinct plasmids.

Introduction

Conjugative plasmids are genetic elements able to transfer horizontally between hosts, through a process termed conjugation (Cabezon et al., 2015). Conjugative plasmids disseminate among bacteria through conjugation, which requires cellular contact between donor and recipient cells. Such contact is provided by appendages (pili) included in the conjugative machinery encoded by the conjugative element.

In general, plasmids do not express the conjugative machinery constitutively (for a review of the conditions affecting such expression, see (Frost & Koraimann, 2010)). Fertility inhibition systems (FIN), encoded by the plasmid, prevent the expression of its own conjugative machinery. However, de-repression occurs transiently allowing the horizontal transfer of the plasmid briefly. Paradoxically, although this feature reduces their frequency of horizontal transfer, it may enhance their ability to persist in nature (Lundquist & Levin, 1986).

Expression of the conjugation machinery is presumed to impose a fitness cost on the plasmid's host. Such cost would lead plasmid free bacteria to displace plasmid harbouring bacteria. Observations of reduced conjugation rates and loss of accessory genes in conditions favouring vertical transmission confirmed the trade-off hypothesis between horizontal and vertical transmission (Dahlberg & Chao, 2003; Turner, 2004; Turner et al., 1998). Moreover, it was shown in populations of bacteria harbouring two types of plasmids, FIN^+ and FIN^-, that hosts harbouring the FIN^+ variant grew faster than hosts harbouring the FIN^-, therefore outcompeting them (Haft et al., 2009).

Male-specific phages are viruses that specifically infect bacteria harbouring conjugative elements, and target them via surface exposed conjugative pili. Therefore, in the presence of this type of virus, expression of the conjugative machinery tends to be counter selected. This outcome may result in either bacteria losing the plasmid or in plasmids unable to express their conjugative machinery (Jalasvuori et al., 2011), which in turn greatly reduce the plasmid's frequency in bacterial populations (Ojala et al., 2013). Since plasmid de-repression is only transitory, male-specific phages rarely target their host cells. Therefore, this feature plays an important role in plasmid maintenance when male-specific phages are present, as shown in computer simulations (Dionisio, 2005).

Another consequence of the expression the conjugative machinery is lethal zygosis (Alfoldi et al., 1957). This form of lethality occurs due to the cellular contact mediated by pili during the conjugation process. Such contacts lead to excessive permeability of the membrane of the recipient cells (Gross, 1963; Skurray & Reeves, 1973).

Although fertility inhibition proves beneficial, the question remains: when should a plasmid express the conjugative machinery? Some signals that trigger the expression of such machinery have already been identified and are reviewed in (Frost & Koraimann, 2010; Koraimann & Wagner, 2014). One of such signals is the presence of recipient cells in the bacterial population, but others exist, such as environmental, nutritional and stressful stimuli. Upon such signals, expression of the conjugative machinery is turned on and plasmid transfer occurs.

Bacteria frequently harbour multiple plasmid (Caugant et al., 1981; Sherley et al., 2003). The question here is: how frequently do these plasmids transfer together? To our knowledge, few studies addressed this question. Thus, the aim of the present work is to answer it.

Due to transient de-repression only a small proportion of plasmid harbouring bacteria engages in conjugation. This fact led previous authors to hypothesize that the simultaneous transfer of two plasmids from the same donor cell would be a rare event, because it relies on the independent transfer of each plasmid (Romero & Meynell, 1969). Some of their observations agreed with this hypothesis: using strains harbouring two natural conjugative plasmids, they observed that co-transfer rates of the plasmids were identical to the product of individual transfer rates (two out of three cases). They further studied de-repressed plasmid variants and if cells harboured a pair of such plasmids, co-transfer was still independent. They reached the same conclusion when cells harboured simultaneously a repressed and a de-repressed plasmid. Additionally, they also found for another pair of plasmids that the co-transfer rate was low, but not as much as the product of individual transfer rates (one out of three cases). Another study focused on a strain harbouring simultaneously three conjugative plasmids (Bouanchaud & Chabbert, 1969) and found that the simultaneous co-transfer of either two or three plasmids occurred more frequently than it would be expected if plasmid transfer was independent.

In this chapter we will study co-transfer of plasmids, using a larger experimental sample, to unveil how frequently it occurs.

Materials and Methods

Bacterial strains and plasmids

We used *E. coli* K12 MG1655 and *E. coli* K12 MG1655 Δ*ara* (unable to metabolize arabinose), respectively as recipient and donor strains. Donor strains harbouring combinations of two and three plasmids were produced in Chapters 3 and 4. The plasmids used are summarized in Table 1.1.

Co-transfer assays

We inoculated 10^8 total bacteria, in a 1:1 donor:recipient ratio, into a 15 mL tube containing 5 mL of Lysogeny Broth. Tubes were incubated at 37ºC without agitation for 90 minutes. We diluted the cultures in $MgSO_4$ 0.01M and plated them in Tetrazolium Arabinose medium to quantify donor and recipient bacteria (respectively red and white colonies due to their differential arabinose metabolism); and in M9 minimal solid medium supplemented with arabinose (0.4%) and suitable antibiotics to quantify transconjugants for two and three plasmids. We calculated conjugation rates (γ) as: $\gamma = log_{10}\left(\frac{T}{\sqrt{D*R}}\right)$, where D, R and T represent, respectively, the number of donors, recipients and transconjugants per millilitre.

Models of plasmid co-transfer

We calculated the expected values of plasmid co-transfer according to different models. We will consider γ1, γ2, γ3 as the respective conjugation rates of plasmids 1, 2 and 3, and γ1:2, γ1:3 and γ2:3 as the co-transfer rates of pairs of plasmids.

Independent transfer model (ε)

As mentioned earlier, there are some observations in which co-resident plasmids transfer independently of each other. In this case, the rate of co-transfer should be identical to the product of the conjugation rates of each plasmid. Since we consider log-transformed conjugation rates, the expected co-transfer rate is calculated as the sum of the conjugation rates of the plasmids, instead of the product.

$\varepsilon = \gamma1 + \gamma2$ or $\varepsilon = \gamma1 + \gamma2 + \gamma3$

Least conjugative plasmid model (τ)

Due to some observations of co-transfer rates higher than those predicted if plasmid co-transfer was independent, we propose another model. We propose that the number of recipients receiving both co-resident plasmids is limited by the number of recipients receiving the plasmid having the lowest conjugation rate. This implies that the expected rate of co-transfer should be equal to the conjugation rate of the lowest conjugative plasmid.

$\tau = \min(\gamma1, \gamma2)$ or $\tau = \min(\gamma1, \gamma2, \gamma3)$

Average model (μ)

Co-transfer of two plasmids was not always explained by previous models as we will see in the results section. For some pairs of plasmids, the rate of plasmid co-transfer corresponds to an intermediate value that falls between the values expected according to models ε and τ. Therefore, we considered another model in which we calculate the rate of co-transfer as the average of the values estimated according to models ε and τ.

$$\mu = \text{average}(\varepsilon, \tau)$$

Least conjugative pair model (π)

We will also see in the results section that when analysing co-transfer of three plasmids, again, neither model ε nor τ explained the values observed. Since co-transfer of three plasmids logically depends on the co-transfer of two plasmids, co-transfer of three plasmids should, at most, be identical to the rate of co-transfer of the least conjugative pair of plasmids of the combination. Therefore, we propose another model in which the number of recipients receiving the three plasmids is limited by the number of recipients simultaneously receiving the two plasmids having the lowest co-transfer rate. Then, the expected rate of co-transfer should be equal to the co-transfer rate of the lowest co-transferred pair, instead of the lowest conjugative plasmid *per se*.

$$\pi = \min(\gamma 1{:}2, \gamma 1{:}3, \gamma 2{:}3)$$

Statistics

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

Results

Simultaneous transfer of plasmid pairs

In this work we studied the co-transfer of plasmids from donor strains harbouring multiple plasmids. A model has been proposed to explain plasmid co-transfer (Romero & Meynell, 1969). It assumes that plasmid transfer is independent from the transfer of other co-resident plasmids. According to this model, one should expect co-transfer rates to result from the product of the transfer rate of each individual plasmid. Note however that in this work we calculated log-transformed transfer rates, thus, the expected co-transfer value is the sum of each of these two log-transformed rates. We propose an alternative model, where we assume co-transfer to be limited by the plasmid having the lowest conjugation rate. That is to say that all recipient cells receiving the plasmid having the lowest conjugation rate also receive all the other co-resident plasmids. Therefore, according to this model, co-transfer rates should be identical to the transfer rate of the plasmid having the lowest conjugation rate. In the present work, we test these models, in order to understand plasmid co-transfer.

We measured the conjugation rates of plasmids in matings where donors harboured two plasmids. We determined the number of transconjugants receiving both plasmids. We computed the estimated values according to each model,

based on experimental data from Chapter 3, where we measured the transfer rates of each plasmid in all the different combinations. In the following analysis, we considered only pairs where plasmid co-transfer was detected in at least one of the three replicates, thus considering only 34 of the 40 combinations of two plasmids.

For each pair of plasmids, we calculated the expected rate of co-transfer either if the transfer of each plasmid was independent of the transfer of the other (model ε) or if the co-transfer of both plasmids was limited by the plasmid having the lowest conjugation rate (model τ). Then we computed the linear regression between the values observed experimentally and those estimated according to each model. In Fig. 6.1 the observed co-transfer rates are plotted against the rates estimated by models ε and τ. Fig. 6.1 also includes the identity line y = x (the line in which observed values are identical to the estimated). Both regression lines produce R^2 values higher than 75%. The regression line produced by model τ is more similar to y = x than the regression line produced by model ε, as corroborated bellow.

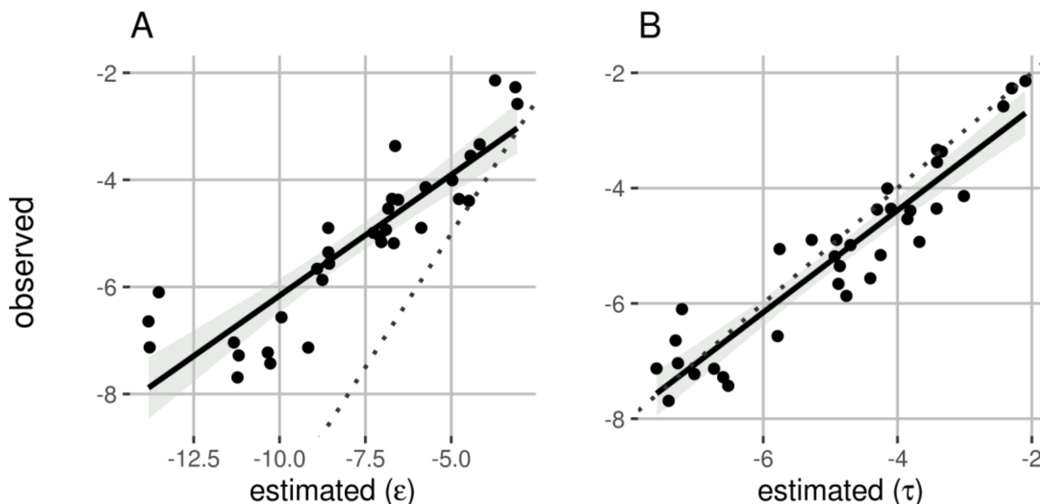


Figure 6.1. Co-transfer of combinations of two plasmids from hosts harbouring two plasmids. Horizontal axis represents the co-transfer values estimated according to: A) Independent Transfer Model – ε; B) Least Conjugative Plasmid Model – τ. Vertical axis represents the observed co-transfer values. Each data point represents the mean value of three independent measurements. The grey dotted line represents the identity line y = x. The dark continuous line represents the regression line (and the grey shade its 95% confidence interval): A) y = 0.45 x – 1.6, R^2 = 0.78, σ^2 = 0.52; B) 0.89 x – 0.84, R^2 = 0.87, σ^2 = 0.31.

Next, we calculated the difference between the observed and the estimated values (for each model) and tested if the difference values were equivalent to zero. From a significant p-value we can infer that the mean difference value is equivalent to 0 +/- Δ, where Δ is the equivalence threshold. We considered a margin of 0.5. This means that the mean difference values were considered equivalent to zero if they were comprised in the interval [-0.5, 0.5].

The difference values obtained from model τ were equivalent to zero (TOST test, threshold value = 0.5, p-value = 0.02) and were also normally distributed (Shapiro test, p-value = 0.64). However, the ones obtained from model ε were not equivalent to zero (TOST test, threshold value = 0.5, p-value ~ 1), neither normally distributed (Shapiro test, p-value = 0.002). This suggests that co-transfer of the two plasmids when the donor cell contains two plasmids follows

model τ. In other words, if a recipient cell received the plasmid having the lowest conjugation rate, it is likely to have received the other plasmid.

As mentioned above, in six of the 40 combinations, co-transfer was not detected in any of the three replicates and, consequently, this group was not included in the previous analysis. In four of these six combinations, we did not detect transfer of one of the two plasmids; therefore, for these four combinations, it is impossible to decide between models ε and τ. In the two remaining combinations, R388/R702 and R388/RP4, we detected transfer of each plasmid but not co-transfer (no cell received both plasmids). Thus, model τ does not explain the results for these two combinations of plasmids and model ε was not testable.

Simultaneous transfer of combinations of two plasmids from hosts carrying plasmid triplets

We then studied matings involving bacterial cells harbouring three plasmids. With these triplets as donors, we determined the number of transconjugants receiving all three possible combinations of two plasmids. We then calculated the transfer rate of each pair of plasmids. The 28 triple-plasmid combinations originated 84 possible combinations of two plasmids, from which we considered the 61 pairs where co-transfer was detected in at least one of the three replicates. We computed the co-transfer rates estimated by models ε and τ, based on the individual conjugation rates obtained in Chapter 4. In Fig. 6.2A we can observe that y = x is different from the regression line produced by model ε. On the other hand, in Fig. 6.2B we can observe that the line y = x is parallel to the regression line produced by model τ but the two lines seem to be too far apart.

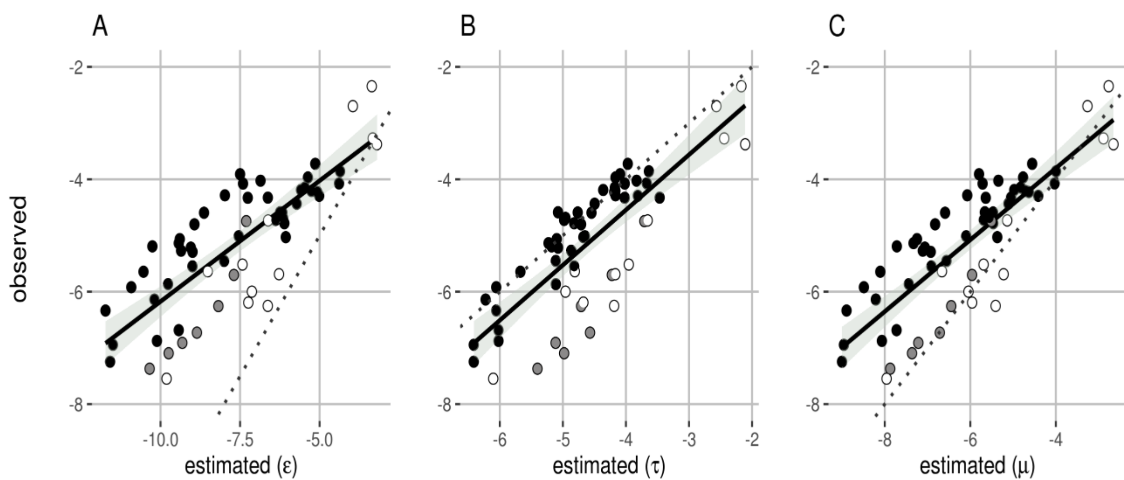


Figure 6.2. Co-transfer of combinations of two plasmids from hosts harbouring three plasmids. Horizontal axis represents the co-transfer values estimated according to: A) Independent Transfer Model – ε; B) Least Conjugative Plasmid Model – τ; C) Average Model – μ. Vertical axis represents the observed co-transfer values. Each data point represents the mean value of three independent measurements. The grey dotted line represents the identity line y = x. The dark continuous line represents the regression line (and the grey shade its 95% confidence interval): A) y = 0.43 x – 1.9, R^2 = 0.65, σ^2 = 0.49; B) 0.98 x – 0.62, R^2 = 0.64, σ^2 = 0.49; C) 0.64 x – 1.2, R^2 = 0.69, σ^2 = 0.43. Black points follow the trend line in B, while grey and white points follow the trend line in C. White points represent pairs of plasmids that include plasmid R16a.

We tested a third model, μ, which is computed as the average between the values estimated by the previous models ε and τ. In Fig. 6.2C we can observe that the regression line produced by this model is also different from y = x.

Next, we calculated the difference between the observed and the estimated values (for each model) and tested if the difference values were equivalent to zero. The values were not equivalent to zero in any case (TOST test, threshold value = 0.5, p-value > 0.05) and the difference values relative to model τ were not even normally distributed (Shapiro test, p-value < 0.001).

Examining again Fig. 6.2, one can observe that the points in grey and in white clearly deviate from the trend. Therefore, we reanalysed the data after splitting the points in two groups: one containing all the points in black and another containing the remaining points. Fig. 6.3A represents the group of points in black and Fig. 6.3B represents the remaining points. We can now observe that both regression lines are identical to y = x and produce R^2 values higher than 85%. Considering these two groups, we retested if the values of the difference between observed and estimated were equivalent to zero: they were (TOST test, threshold value = 0.5, p-value < 0.001) and were also normally distributed (Shapiro test, p-value > 0.05). Therefore, the first group, containing the 42 black points, seems to follow model τ, that is, their co-transfer is limited by the plasmid having the lowest conjugation rate. The second group, containing the remaining 19 points seems to follow model μ, which means that co-transfer of these pairs of plasmids is not entirely independent but it is also not limited by the least conjugative plasmid, lying somewhere in between.

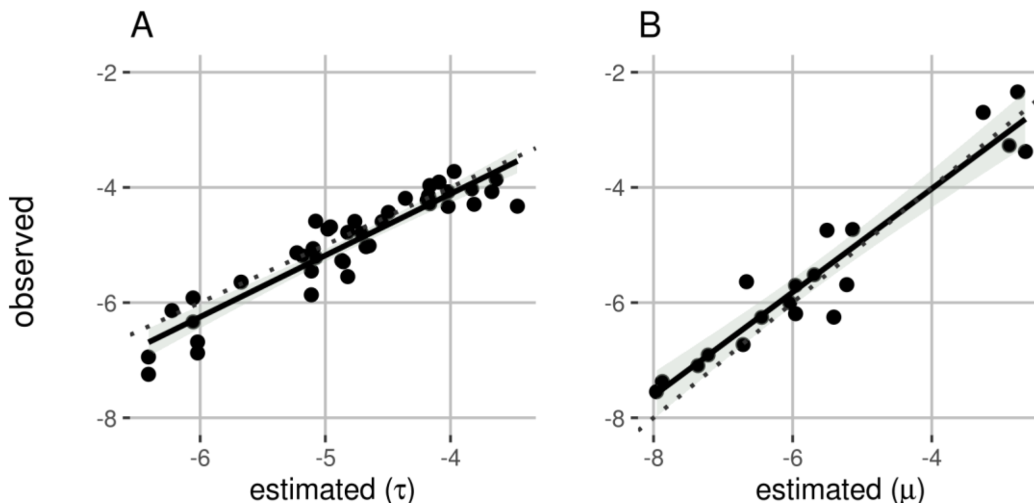


Figure 6.3. Co-transfer of the groups of combinations of two plasmids from hosts harbouring three plasmids. A) Points previously not deviating from the trend line; B) points previously deviating from the trend line. Horizontal axis represents the co-transfer values estimated according to: A) Least Conjugative Plasmid Model – τ; B) Average Model – μ. Vertical axis represents the observed co-transfer values. Each data point represents the mean value of three independent measurements. The grey dotted line represents the identity line y = x. The dark continuous line represents the regression line (and the grey shade its 95% confidence interval): A) y = 1.1 x + 0.16, R^2 = 0.86, σ^2 = 0.12; B) 0.9 x – 0.43, R^2 = 0.91, σ^2 = 0.22.

From these results we can infer that, when considering plasmid donors harbouring three plasmids, co-transfer of combinations of the two plasmids can be limited by the plasmid having the lowest conjugation rate (model τ) but can also have an additional effect of independent plasmid transfer (model μ).

Simultaneous transfer of plasmid triplets

Finally, we determined the number of transconjugants receiving all three plasmids and calculated the respective transfer rates. Here, we considered only combinations where simultaneous co-transfer of three plasmids was detected in at least one of the three replicates. Therefore, we considered 19 of the 28 triple-plasmid combinations.

We computed the co-transfer rates of triplets estimated according both models ε and τ. Linear regressions between the observed values and expected values calculated according to each model are represented in Fig. 6.4. It also shows the identity line y = x. The regression line produced by model τ is more similar to y = x than the regression line produced by model ε. However, the regression line produced by model τ and the line y = x are parallel but far apart. Furthermore, the R^2 values of these regression lines are only near 0.3. Therefore, co-transfer of three plasmids does not seem to follow either of these two models.

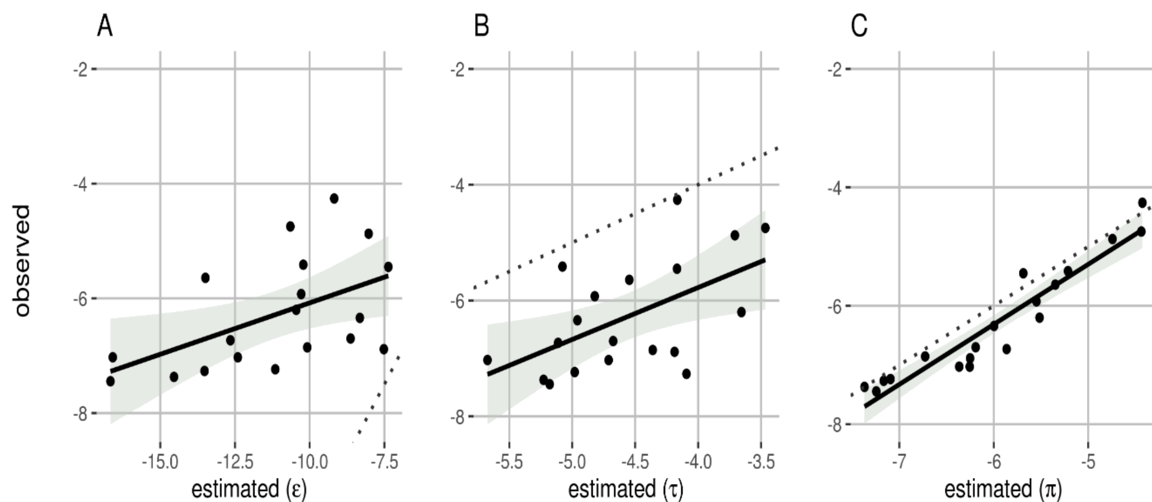


Figure 6.4. Co-transfer of combinations of three plasmids from hosts harbouring three plasmids. Horizontal axis represents the co-transfer values estimated according to: A) Independent Transfer Model – ε; B) Least Conjugative Plasmid Model – τ; C) Least Conjugative Pair Model – π. Vertical axis represents the observed co-transfer values. Each data point represents the mean value of three independent measurements. The grey dotted line represents the identity line y = x. The dark continuous line represents the regression line (and the grey shade its 95% confidence interval): A) y = 0.18 x – 0.43, R^2 = 0.27, σ^2 = 0.73; B) 0.9 x – 2.2, R^2 = 0.31, σ^2 = 0.69; C) x – 0.23, R^2 = 0.9, σ^2 = 0.1.

Co-transfer of three plasmids is logically dependent on the co-transfer of two plasmids. Thus, since model τ could not explain co-transfer of all the pairs resulting from combinations of two plasmids, it is not surprising that it fails to explain co-transfer of three plasmids. In other words, we observed that for some cases, the rate of co-transfer of two of the plasmids was lower than the rate of transfer of the least conjugative plasmid. Therefore, the co-transfer of three plasmids must also be lower than the rate of transfer of the least conjugative plasmid. Co-transfer of three plasmids should, at most, be identical to the rate of co-transfer of the least conjugative pair of plasmids of the combination. We considered this third model, π, which assumes co-transfer of three plasmids to be limited by the plasmid pair having the lowest co-transfer rate (instead of the plasmid having the lowest transfer rate as in model τ). We can observe in Fig. 6.4

that the regression line obtained by this model is almost identical to y = x and produces an R^2 value of 0.9.

We further compared the difference between the observed values and the values expected according to each of the three models ε, τ and π. Difference values relative to models ε and τ were not equivalent to zero (TOST test, threshold value = 0.5, p-values ~ 1) while difference values relative to model π were (TOST test, threshold value = 0.5, p-value = 0.002). Therefore, co-transfer of the three plasmids followed model π, that is to say, co-transfer of three plasmids was limited by the plasmid pair having the lowest co-transfer rate.

In nine of the twenty-eight combinations of three plasmids, co-transfer of the three plasmids was not detected in any of the three replicates. In six of these nine combinations, we did not detect transfer of at least one of the three plasmids alone and consequently we could not detect plasmid co-transfer. In the three remaining combinations (R388/R702/R16a, R388/RP4/R57b and R388/RP4/RN3), we detected transfer of each plasmid but not co-transfer of all the three pairs. Thus, the observed co-transfer rates seem to be lower than the expected rates calculated according to model τ. However, we do not know if models ε or π could predict co-transfer rates, since, for these cases, the values are under the experimental limit of detection.

Discussion

Plasmids frequently inhabit their hosts along with unrelated plasmids and consequently they can interact, thus interfering with the transfer of each other, as seen in Chapters 3 and 4. Since, the expression of genes involved in conjugation is generally repressed, the ability of a plasmid to transfer horizontally depends on its transient de-repression. Therefore, one could assume the transfer of co-resident plasmids to be an independent event. Previous works showed that co-transfer of plasmids is not always independent (Bouanchaud & Chabbert, 1969; Romero & Meynell, 1969). Our aim in this work was to understand the general trend of plasmid co-transfer. To do so, we analysed the co-transfer of combinations of two and three plasmids using mainly repressed plasmids.

We found that when using hosts harbouring combinations of two plasmids, co-transfer of both plasmids tends to be limited by the plasmid having the lowest conjugation rate. We also analysed the rates of co-transfer of combinations of two plasmids when hosts harboured three plasmids. In this case results did not follow a single trend. Co-transfer of most combinations of two plasmids, 42 out of 61 (69%), was limited by the conjugation rate of the least conjugative plasmid. Therefore, these combinations of plasmids seem to follow the same rule just established. However, co-transfer of the 19 (31%) remaining combinations exhibited an intermediate value between the transfer rate of least conjugative plasmid and the value expected if transfer of both plasmids was independent.

Briefly, we observed that co-transfer of two plasmids was mostly, but not always, limited by the plasmid having the lowest conjugation rate. Exceptions to this rule were observed when hosts harboured simultaneously three plasmids. Due to the occurrence of such exceptions, one should therefore expect co-transfer of all the three plasmids not to follow that rule, that is, to be limited by the plasmid having the lowest co-transfer rate. Since co-transfer of three plasmids depends on the co-transfer of two plasmids, we speculated it to be instead limited

by the combination of two plasmids having the lowest co-transfer rate instead. We indeed verified this hypothesis. Therefore, we suggest a generalization of our observations, proposing that when a host bacterium harbours simultaneously n conjugative plasmids, co-transfer of all n plasmids tends to be limited by the (n - 1) combination of plasmids having the lowest co-transfer rate. This model could be tested in future studies, for example, by studying the co-transfer of plasmids from donors harbouring a greater number of plasmids.

We observed that, out of the 19 combinations of two plasmids whose co-transfer followed model μ, 12 involved plasmid R16a. None of the remaining 42 combinations involved this plasmid. However, in the case of co-transfer of two plasmids from a host harbouring three plasmids, we could not establish a clear rule indicating when to consider a null or partial impact of the independent-transfer effect.

We conclude that in most cases of plasmid combinations, either of two or of three plasmids, co-transfer seems to be limited by the plasmid having the lowest conjugation rates. These results suggest that, despite plasmids being only transiently de-repressed, de-repression happens simultaneously on co-resident plasmids. Thus, de-repression of unrelated plasmids could respond to a common stimulus. In fact, at least for IncF plasmids, regulation of conjugation responds to environmental, nutritional and stressful stimuli (Frost & Koraimann, 2010) . Bacteriophages also regulate their genes based on such kind of stimuli (Knoll, 1979; Kourilsky, 1973; St-Pierre & Endy, 2008; Yen & Gussin, 1980). Therefore, it would not be surprising that plasmids, in general, could respond to common cues, which would explain their non-independent co-transfer. We postulate that upon a certain common trigger all co-resident plasmids activate expression of genes involved in conjugation, which in turn allows that multiple plasmids transfer to the same recipient cell at once. However, the genetic conjugation system of each specific plasmid would determine the susceptibility and rate of response to such stimuli. Moreover, other more specific stimuli would affect only some plasmids. Additionally, some plasmids remain de-repressed for longer periods after induction of the conjugative machinery, which leads to their epidemic transfer (Frost & Koraimann, 2010). Together, these last factors could be responsible for the heterogeneity of conjugations rates observed for different plasmids.

Author Contributions

Conceived and designed the experiments: João Alves Gama, Francisco Dionísio. Performed the experiments: João Alves Gama. Analysed the data: João Alves Gama, Francisco Dionísio, Rita Zilhão. Wrote the chapter: João Alves Gama, with contributions of Francisco Dionísio, Rita Zilhão.

Chapter 7

Co-infecting plasmids affect biofilm formation in a combination-dependent way

Abstract

Conjugative plasmids have been previously shown to enhance the bacterial ability to form biofilms. This ability is related to the expression of conjugative pili, which can function as adhesion factors promoting biofilm formation. Additionally, previous works have shown that different plasmids may interact synergistically, further enhancing biofilm formation. It is still unclear, however, in which cases plasmids and in what conditions two plasmids interact synergistically in the biofilm formation. Here, we aim to test how combinations of two plasmids interact regarding biofilm formation. We tested it in two conditions: intracellular – both plasmids being carried in the same host cell and intercellular – each plasmid being carried in different cells of the same bacterial population. We observed that the outcome of plasmid interactions varied among combinations, thus being combination specific. It was commonly observed that the ability to form biofilm did not differ between the strain carrying both plasmids and one of the strains carrying only one plasmid. Thus, one of the plasmids seemed to have a dominant effect over the other. However, cases of co-dominance were also observed. Moreover, some combinations exhibited synergism. We also found that the condition in which both plasmids were present in the population (intracellular versus intercellular plasmid interactions) affected biofilm formation. However, none of the conditions favoured consistently biofilm formation over the other, rather such effect seemed to be combination specific.

Introduction

Regardless of their unicellular nature, bacteria may live as complex communities. Biofilms are examples of such communities and can be defined as an aggregate of microorganisms, generally embedded in a polymeric matrix (for a review in biofilms see (Hall-Stoodley et al., 2004)). Biofilm formation primarily requires cellular adhesion to a surface and subsequently biofilms develop into macroscopic structures as the bacterial population increases and produces an extracellular matrix. These complex structures may have a medical impact because they often confer protection to bacteria from harmful agents such as antibiotics (Gilbert et al., 2002; Mah & O'Toole, 2001; Stewart & Costerton, 2001). This protection is due to low diffusion inside the biofilm, which prevents the detrimental agents from targeting the core population whereas only the outer layer is subject to damage. Thus, biofilms constitute a source wherefrom microorganisms can disperse, representing an important impact in medical and industrial environments (Flint et al., 1997; Maukonen et al., 2003; Sihorkar & Vyas, 2001).

Close cellular contact and high cell densities in biofilms have been suggested as factors favouring horizontal gene transfer (this subject is reviewed in (Molin & Tolker-Nielsen, 2003; Sorensen et al., 2005; Stalder & Top, 2016)).

More specifically, these conditions allow increased conjugative transfer of conjugative plasmids and transfer of non-conjugative plasmids and naked DNA through transformation. On the other hand, genetic elements such as plasmids can encode adhesion factors and DNA itself can promote cellular contact, favouring biofilm formation. Some observations however, indicate that genetic material might be transferred only at outer layers of the biofilm and unable to penetrate its inner core.

The role of plasmids as enhancers of biofilm formation has been reported in several works. *Escherichia coli* K12 MG1655 is a poor biofilm former strain but when it harbours a plasmid de-repressed for conjugation its ability to form biofilms increases (Ghigo, 2001). Such observations suggest that sex-pili, which mediate the contact required for conjugative transfer, may act as adhesion factors during biofilm formation. Moreover, when the same strain carried repressed plasmids it could also enhance biofilm development when invading a population of plasmid-free cells. Thus, this ability does not seem to be restricted to a specific group of plasmids since of the 29 natural plasmids tested, which express different pilus types and belong to different incompatibility groups, 90% (9 de-repressed and 17 repressed plasmids) induced biofilm development. Another work, in which 403 *E. coli* strains were screened, confirmed the role of plasmids as biofilm developers (Reisner et al., 2006). Almost 50% of these plasmid-harbouring strains developed better biofilms when co-cultured with plasmid-free cells than when cultured alone. Moreover, for a sample of strains, co-culture biofilm formation values were higher than the sum of the values measured for each strain monoculture, showing a synergistic effect. This sample of strains was shown to carry conjugative plasmids. Indeed, cells of the plasmid-free strain that acquired plasmids during co-culture also became better biofilm formers. However, co-culture with a strain harbouring a related plasmid decreased biofilm formation, due to surface exclusion, a process by which plasmid-carrying cells become less capable to engage in conjugation with cells carrying a related plasmid (in terms of conjugative machinery). Overall, these results imply that plasmid transfer can act as a factor promoting biofilm development, instead of being just a consequence of favourable conditions experienced in this type of communities.

Additionally, with plasmid R1 and its de-repressed mutant R1drd19, it was shown that, for repressed plasmids to enhance biofilm formation, a population of plasmid-free cells, already attached to a surface, was required *a priori*. When cells harbouring plasmid R1 invaded a population of plasmid-free bacteria, biofilm formation occurred to an identical extent to that formed by cells carrying plasmid R1drd19 and, most of the biofilm cells had acquired plasmid R1 through horizontal gene transfer. However, if cells carrying plasmid R1 were inoculated first, the extent of plasmid transfer and biofilm formation decreased. Such difference may originate from the phenomenon of epidemic transfer, by which plasmids remain transiently de-repressed after recent transfer events (Lundquist & Levin, 1986). Therefore, recent transconjugant cells present in the biofilm outer layers should better recruit planktonic cells through increased sex-pili expression than cells in which expression of genes responsible for conjugative transfer occurs less frequently (Ghigo, 2001).

The impact of plasmids in biofilm development seems, however, to depend on additional factors. Although sex-pili promote cellular contact in early phases of biofilm formation, the conjugative machinery of plasmid F (instead of pilus synthesis specifically) stimulates synthesis of colonic acid and curli, which play a

role on biofilm maturation (May & Okabe, 2008). Thus, plasmids may contribute to biofilm development not only through pilus synthesis but also due to the expression of other adhesion factors. Other studies, focused on the de-repressed plasmid R1drd19, showed that the expression of several chromosomal genes changes upon the addition of this plasmid (Barrios et al., 2006; Yang et al., 2008). By affecting the expression of those genes, R1drd19 increases cell aggregation and quorum sensing AI-2 signalling and decreases motility, thus resulting in enhanced biofilm formation. As another example, plasmid pMAS2027, encodes type 3 fimbriae which mediate attachment to both biotic and abiotic surfaces (Ong et al., 2009). Deletion of this operon resulted in decreased biofilm formation and increased conjugation transfer. Deletion of genes required for pilus formation also decreased biofilm formation, although type 3 fimbriae were still expressed. These effects of type 3 fimbriae and sex-pili on bacterial co-cultures suggest that sex-pili are only required for efficient transfer of type 3 fimbriae which in turn are essential for biofilm formation.

Not only plasmids, but also host cells affect biofilm formation. The ability of plasmid pKJK5 to promote biofilm development was shown to be host dependent (Roder et al., 2013). When carried in *E. coli* and *Kluyvera sp.*, pKJK5 increased biofilm formation non-significantly relatively to the plasmid-free strains, yet when carried in *Pseudomonas* putida the plasmid decreased the ability to form biofilms. Formation of a multispecies biolfilm by the three plasmid-free species was shown to be synergistic relatively to the biofilm formation of each species alone. When plasmid pKJK5 was present in the three-species community, biofilm formation varied, depending in which species harboured the plasmid: when in *Kluyvera sp.* biofilm formation increased, in *P. putida* it decreased and, remained unaltered when carried in *E. coli*. Additionally, another IncP plasmid, RP4, also decreased the ability of *P. putida* to develop biofilms. Moreover, a knockout mutant of pKJK5 not expressing pilus synthesis did not alter the ability of *P. putida* to develop biofilms, showing sex-pili to play a negative role in biofilm formation. In another work, plasmid pOLA52, which encodes type 3 fimbriae, increased biofilm formation in *Salmonella enterica* serovar Typhimurium, *Kluyvera sp.* and *Enterobacter aerogenes*. In *Klebsiella pneumoniae*, however, biofilm formation only increased to a lesser extent, as this species also encodes type 3 fimbriae on the chromosome (Burmolle et al., 2008). Indeed, type 3 fimbriae are commonly encoded by IncX plasmids, to which pOLA52 belongs, and were presumably acquired from *Klebsiella pneumoniae (Burmolle et al., 2012)*.

Bacterial strains may harbour more than one type of plasmid, as is the case of *E. coli* strain C1096, which, among others, carried plasmids pSERB1 and pSERB2 (Dudley et al., 2006). pSERB1 is a conjugative plasmid, related to IncI1 plasmids, thus encoding two different types of pili. The thick pilus is required for conjugation while the thin (type IV) pilus is only required in liquid medium where it optimizes transfer efficiency. pSERB2 is a small cryptic plasmid. *E. coli* strain C1096 was able to produce biofilms in Pyrex glass slides but not in polystyrene plates, thus showing that the ability to enhance biofilm formation depended on the type of surface. Both plasmids were shown to contribute to this phenotype. The type IV pilus was shown to play a role in adherence to epithelial cells and in abiotic surfaces. Moreover, the two plasmids seem to cooperate, as they promote biofilm formation synergistically. As stated before, plasmids can also act

antagonistically, decreasing biofilm formation when they encode related surface exclusion systems (Reisner et al., 2006).

In Chapter 3, we studied the interactions between plasmids in terms of conjugative transfer. In most interactions, one of the plasmids decreased the conjugative transfer of the other, although we have also observed cases where transfer of a plasmid increased. Given the role of conjugation in biofilm development and some evidence of synergistic and antagonistic effects on plasmid-mediated biofilm formation, in this chapter we will study how plasmid interactions affect biofilm formation.

Materials and Methods

Bacterial strains and plasmids

We used the following bacterial strains: plasmid-free *E. coli* K12 MG1655 *Δara*, *E. coli* K12 MG1655 *Δara* containing each of the 11 natural conjugative plasmids (summarized in Table 1.1) and *E. coli* K12 MG1655 *Δara* containing the 33 possible combinations (due to incompatibility or selective markers) of two plasmids. These strains were produced in Chapter 3.

Biofilm formation assays

We measured biofilm formation in four conditions: i) the plasmid-free strain was inoculated alone (reference values); ii) single-plasmid donor strains were co-inoculated with the plasmid-free (recipient) strain; iii) double-plasmid donor strains were co-inoculated with the plasmid-free (recipient) strain; iv) two different single-plasmid donor strains were co-inoculated together (each strain served simultaneously as donor and recipient).

To assay biofilm formation, we followed the protocol by (Christensen et al., 1985). Briefly, the strains were grown in 96-well plates (U-bottom, polystyrene) containing 200 µL of Lysogeny broth (LB) per well. After overnight incubation at 37ºC with agitation (50 rpm), 5 µL of the donor and 5 µL of the recipient strain were mixed in 190 µL of LB in a new 96-well plate. 5 µL of each mix were inoculated in 195 µL of LB in a new plate and incubated for 24 hours, at 37ºC without agitation, inside plastic bags to prevent evaporation.

Biofilm formation quantification

Plates were washed to remove unattached cells and stained with crystal violet (0.1%) for 15 min (adapted from (Christensen et al., 1985)). Ethanol (96%) was added to recover the crystal violet attached to the wells and absorbance was measured at 595 nm. These measurements were normalized by dividing each value by the average value measured for the monoculture of the plasmid-free strain to all 96 wells of the same plate.

Epistasis measurement

We measured epistasis (ε) of biofilm formation according to an additive model, as: $\varepsilon = \mathrm{BF_{p1\,p2}} - (\mathrm{BF_{p1}} + \mathrm{BF_{p2}} - 1)$, where BFp1 and BFp2 represent respectively biofilm formation of the strains carrying either plasmid p1 or p2 and BFp1 p2 represents biofilm formation when both plasmids were present in the bacterial population (either both in the donor cells or each in different cells). We considered epistasis when $0 \notin [\varepsilon - SE\,, \varepsilon + SE\,]$, where SE represents the standard error associated with these calculations, which was determined according to the error propagation method as: SE = $\sqrt{SE\left(BF_{p1\,p2}\right)^2 + SE\left(BF_{p1}\right)^2 + SE\left(BF_{p2}\right)^2}$, where SE represents the standard error.

Co-dominance measurement

Here we defined a variable of plasmid co-dominance (ς) of biofilm formation, using an approach identical to epistasis. We considered an additive model, as: $\varsigma = \mathrm{BF_{p1\,p2}} - \frac{\mathrm{BF_{p1}} + \mathrm{BF_{p2}}}{2}$, where the definitions of BFp1, BFp2 and BFp1 p2 are the same used for epistasis. We considered co-dominance when $0 \notin [\varsigma - SE\,, \varsigma + SE\,]$. Standard error was determined according to the error propagation method as: SE = $\sqrt{SE\left(BF_{p1\,p2}\right)^2 + \frac{SE\left(BF_{p1}\right)^2}{2^2} + \frac{SE\left(BF_{p2}\right)^2}{2^2}}$.

Statistics

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

Results

Plasmidic biofilm formation ability

We measured the ability of eleven natural plasmids, belonging to six incompatibility groups, to affect biofilm formation (Fig. 7.1). Compared with the plasmid-free strain, seven plasmids increased the strain's ability to form biofilms. Cells carrying plasmid R124 exhibited the highest biofilm formation. In contrast, plasmid R16a decreased the strain's ability to form biofilms. Three plasmids did not affect biofilm formation significantly. Results of the post hoc Tukey multiple comparison analysis showed the biofilm formation abilities of the eleven plasmids to be clustered in seven groups (Fig. 7.1).

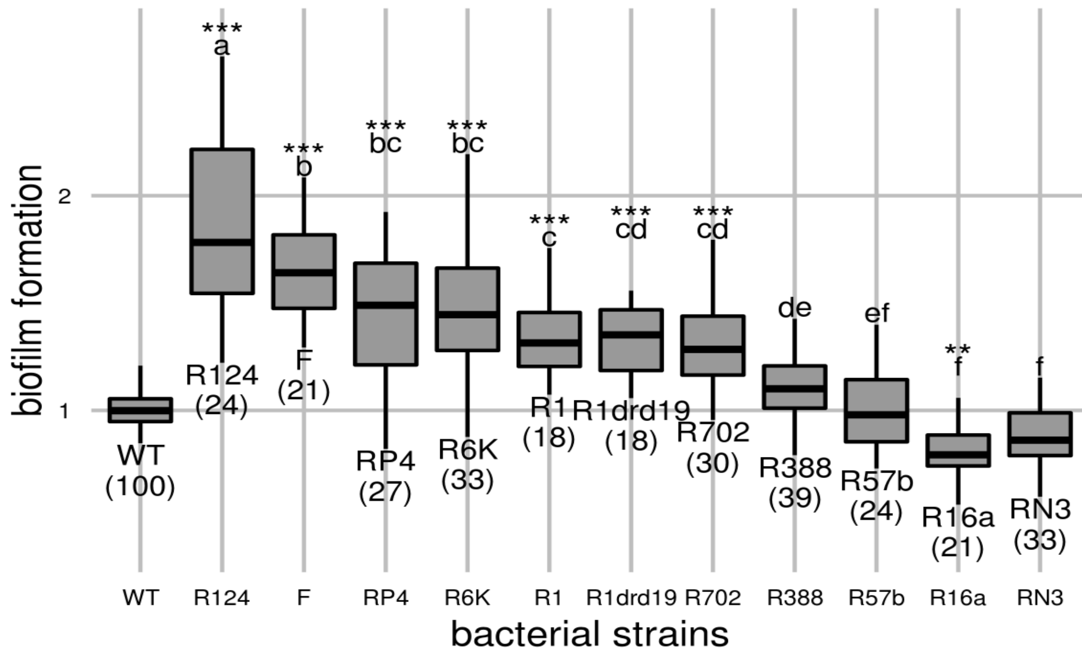


Figure 7.1. Plasmidic effect on biofilm formation. Annotations under the boxes indicate the identity of the plasmids and, in parenthesis, the sample size. Letter annotations above the boxes represent the results of Tukey's multiple comparison test, plasmids with different letters are significantly different (p-value < 0.05). Results of Dunnett's multiple comparison against the plasmid-free strain (WT) are indicated above the boxes as: * - p-value < 0.05; ** - p-value < 0.01; *** - p-value < 0.001. WT – wild-type (plasmid-free) strain.

Since plasmids can enhance biofilm formation via conjugative pili, we checked whether a correlation existed between their conjugation rates (measured in Chapter 3) and the ability to form biofilms. Correlation between the two

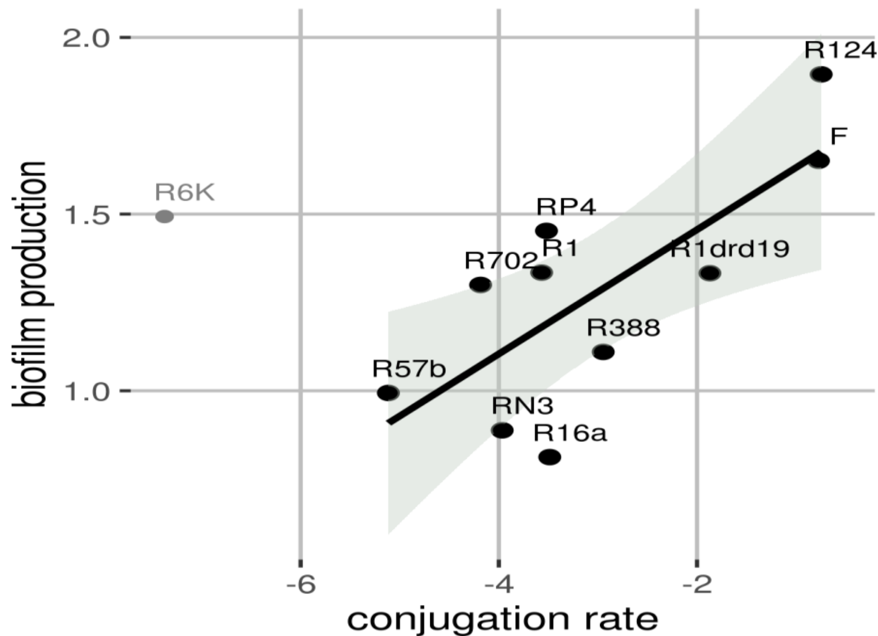


Figure 7.2. Correlation between biofilm formation and conjugation rate. Each data point represents the mean value for each plasmid. The dark line represents the regression line ($y = 0.18x + 1.8$, $R^2 = 0.57$) for black points. The shade represents the 95% confidence interval. The grey point, R6K, represents an outlier. Significant correlation: Pearson method, $\rho = 0.75$, p-value = 0.01.

variables was significant (Pearson method, ρ = 0.75, p-value = 0.01) if plasmid R6K was excluded from the analysis (Fig. 7.2).

Intracellular plasmid interactions

We measured biofilm formation of the 33 strains carrying simultaneously two plasmids. We then compared the ability of all 33 strains to form biofilms simultaneously against the ability of the plasmid-free strain (Dunnett's multiple comparison test). When compared with the plasmid-free strain, 17 of the strains carrying two plasmids exhibited increased biofilm formation (Fig. 7.3A). In opposition, the ability to form biofilms decreased in one strain and did not significantly change in the remaining 15 (Fig. 7.3A).

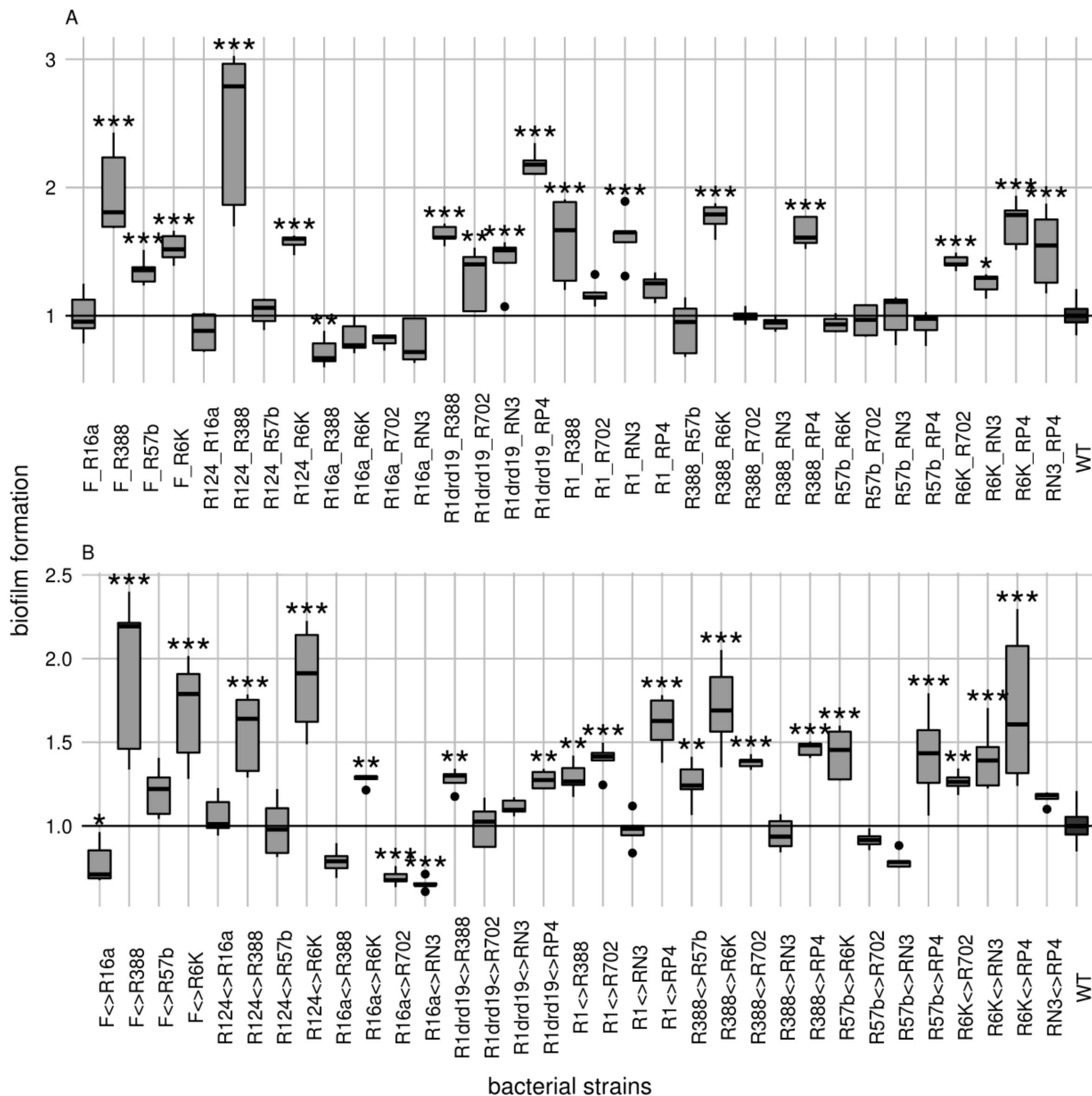


Figure 7.3. Effect of two plasmids on biofilm formation. A) Intracellular effect. B) Intercellular effect. Results of Dunnett's multiple comparison against the plasmid-free strain are indicated as: * - p-value < 0.05; ** - p-value < 0.01; *** - p-value < 0.001. Each box comprehends the values of five replicates for each plasmid combination (indicated in the x axis). WT – wild-type (plasmid-free) strain.

To understand how plasmid interactions shaped biofilm formation, we compared the results of the strain carrying both plasmids against those of the two strains carrying only one plasmid (Dunnett multiple comparison analysis). These results are summarized in Fig. 7.4. We observed that, in approximately half of the combinations, one of the plasmids had a dominant effect over the other. This means that the biofilm produced by the two-plasmid carrying strain was not significantly different from one of the two single-plasmid carrying strains. Considering the two-plasmid carrying strain as the focal strain, we considered a plasmid to have a dominant effect when only the biofilm produced by the strain carrying such plasmid alone did not differ from that produced by the focal strain. Thus, we observed a dominant effect of the plasmid leading to greater biofilm formation in four cases while in the remaining twelve the plasmid leading to lesser biofilm formation had a dominant role. Plasmids R16a and R57b were the main plasmids having a dominant effect decreasing biofilm formation. In nine cases, the biofilm produced by the focal strain did not significantly differ from those produced by either of the two single-plasmid carrying strains.

	F	R124	RN3	R702	RP4	R388	R6K
R16a							
R57b	~ ^{c}						
R1			↑$^{+}$			↑	
R1drd19					↑$^{+}$	↑$^{+}$	
RP4							
R388	↑$^{+}$	↑$^{+}$					
R6K						↑$^{+}$	

Figure 7.4. Intracellular effects of plasmid combinations in biofilm formation. Light grey – combinations exhibiting dominance by the plasmid producing less biofilm; dark grey – combinations exhibiting dominance by the plasmid producing more biofilm; blank – combinations not significantly different from any of the single plasmids; black – combinations not tested (due to incompatibility or absence of selective markers). Upward arrow – combinations exhibiting greater biofilm formation than each plasmid alone (synergy represented with $^{+}$). ~ – combinations exhibiting greater biofilm formation than one of the single-plasmid strains but lesser than the other (co-dominance represented with ^{c}).

In the eight remaining cases, biofilm produced by the focal strain differed from both of those produced by each single-plasmid carrying strain. Interestingly, the focal strain produced more biofilm than the single-plasmid carrying strains in seven of these cases. Thus, we tested these combinations for epistatic effects. We identified synergy (positive epistasis) in six cases, that is, the overall effect was greater than the sum of individual effects, while the other combination was not epistatic. In the remaining combination, the focal strain exhibited greater biofilm formation than one of the single-plasmid strains but lesser than the other. We tested such combination for co-dominance. Thus, we compared the observed values of the focal strain against the average of the values of the two single-plasmid carrying strains. We found no significant difference. Therefore, the two

plasmids contributed with identical strength to the overall effect, but in opposite directions, which resulted in the antagonistic effect observed.

Intercellular plasmid interactions

Plasmids can be present simultaneously in the same bacterial population but carried in different host cells instead of sharing the same host. In this situation, cells harbouring one of the plasmids serve as its donors and additionally as recipients for plasmids carried in other cells. This should result in more cellular interactions since both cells engage in conjugation to transfer the harboured plasmid. We measured again the ability conferred by two plasmids to form biofilms but now the plasmids were not carried in the same strain. Under these experimental conditions the ability to form biofilms increased in 19 cases, decreased in three and in the remaining 11 was not significantly different from the ability of the plasmid-free population (Fig. 7.3B).

To understand the effect of intercellular plasmid interactions, we compared the results of the co-culture of two single-plasmid carrying strains against the co-cultures between each single-plasmid carrying strain with the plasmid-free strain (Dunnett multiple comparison analysis). These results are summarized in Fig. 7.5 and were analysed following the same reasoning as before. We observed that a plasmid had a dominant effect over the other in a total of 19 combinations. We observed a dominant effect of the plasmid leading to greater biofilm formation in ten cases while in the remaining nine the plasmid leading to lesser biofilm formation had a dominant role. Eight of the later nine combinations involved plasmids IncA/C R16a and R57b.

F	R124	RN3	R702	RP4	R388	R6K	
		↓$^-$					R16a
							R57b
							R1
		~^{c}	↓$^-$				R1drd19
		~^{c}					RP4
↑	~^{c}						R388
							R6K

Figure 7.5. Intercellular effects of plasmid combinations in biofilm formation. Light grey – combinations exhibiting dominance by the plasmid producing less biofilm; dark grey – combinations exhibiting dominance by the plasmid producing more biofilm; blank – combinations not significantly different from any of the single plasmids; black – combinations not tested (due to incompatibility or absence of selective markers). Upward arrow – combinations exhibiting greater biofilm formation than each plasmid alone (not synergistic); downward arrow – combinations exhibiting lesser biofilm formation than each plasmid alone (negative epistasis represented with $^-$); ~ – combinations exhibiting greater biofilm formation than one of the single-plasmid strains but lesser than the other (co-dominance represented with ^{c}).

In eight cases, the biofilm produced by the focal co-culture (two single-plasmid carrying strains) did not significantly differ from those produced by either of co-cultures of the plasmid-free strain with each of the two single-plasmid carrying strains. However, the focal co-culture differed from both the other co-cultures in the remaining six cases. We observed that in one of such combinations the focal co-culture exhibited the highest biofilm formation but it was not a synergistic case. Following the same reasoning explained in the previous results' section, we observed three cases of co-dominance. In the two remaining cases, the focal exhibited the lowest biofilm formation of the three compared co-cultures. We tested such combinations for epistasis and observed negative effects.

Difference between intracellular and intercellular effects

As stated previously, plasmid combinations in intracellular interactions enhanced biofilm formation in 17 cases and weakened it in one case. In intercellular interactions, the figures are not that dissimilar, 19 combinations increased biofilm formation and three decreased it. These results suggested that, in general, the ability to form biofilm did not depend on the type of interaction (intra or intercellular). To better understand the impact of the type of interaction, we re-analysed this data. For each combination of plasmids, we compared biofilm formation under the two types of interaction (Table 7.1). In 17 of the 33 combinations, biofilm formation did not differ significantly between conditions (Student's t-test, p-values > 0.05). In nine combinations biofilm formation was greater in intercellular interactions, while in the remaining seven combinations biofilm formation was greater in intracellular interactions. These results show that biofilm formation is not consistently favoured by one of the two types of cellular interactions.

Table 7.1. Comparison between intra and intercellular effects.

	Intracellular		Intercellular		p-value
	mean	standard deviation	mean	standard deviation	
R1drd19/RP4	2,19	0,10	1,28	0,05	0,000002
R388/R702	1,00	0,05	1,38	0,04	0,000004
R1drd19/R388	1,63	0,07	1,28	0,06	0,00004
R16a/R6K	0,83	0,12	1,28	0,04	0,001
R57b/R6K	0,94	0,06	1,43	0,15	0,001
R1/RN3	1,62	0,21	0,98	0,10	0,001
R6K/R702	1,42	0,06	1,26	0,06	0,003
R1/RP4	1,22	0,10	1,61	0,17	0,004
R1/R702	1,17	0,09	1,40	0,09	0,01
R16a/R702	0,81	0,05	0,69	0,05	0,01
R57b/RP4	0,93	0,11	1,42	0,28	0,01
R388/R57b	0,91	0,21	1,26	0,13	0,02
R388/RP4	1,65	0,12	1,46	0,04	0,02
R1drd19/RN3	1,42	0,20	1,11	0,05	0,03

Table 7.1. Comparison between intra and intercellular effects. (continued)

	Intracellular		Intercellular		p-value
	mean	standard deviation	mean	standard deviation	
R124/R388	2,47	0,64	1,56	0,24	0,03
R57b/RN3	1,01	0,17	0,79	0,05	0,04
R1drd19/R702	1,29	0,24	1,01	0,13	0,06
R124/R16a	0,87	0,15	1,06	0,12	0,06
F/R16a	1,00	0,18	0,78	0,13	0,06
RN3/RP4	1,52	0,30	1,17	0,04	0,06
R124/R6K	1,57	0,06	1,88	0,32	0,10
R1/R388	1,59	0,33	1,29	0,10	0,12
F/R57b	1,35	0,11	1,21	0,15	0,13
R16a/RN3	0,79	0,17	0,65	0,04	0,15
R6K/RN3	1,25	0,08	1,41	0,20	0,16
R16a/R388	0,72	0,12	0,79	0,08	0,28
F/R6K	1,53	0,11	1,69	0,31	0,34
R57b/R702	0,96	0,12	0,92	0,05	0,45
R124/R57b	1,03	0,11	0,99	0,17	0,67
R388/R6K	1,76	0,11	1,71	0,27	0,70
R388/RN3	0,94	0,05	0,95	0,10	0,83
F/R388	1,97	0,34	1,92	0,49	0,86
R6K/RP4	1,72	0,18	1,71	0,47	0,94

Discussion

Plasmids encode diverse genetic traits, providing bacteria a provision of mechanisms, which allow them to resist and thrive in adverse conditions. The role of conjugative plasmids as agents enhancing biofilm formation has already been recognised (Ghigo, 2001; Reisner et al., 2006). Such ability is related with the expression of sex pili to promote the cellular contact required for conjugative transfer because cellular contact is also an essential factor in biofilm development. We screened the ability of natural conjugative plasmids to enhance biofilm formation. Although most plasmids behaved accordingly, there are exceptions. Interestingly, one plasmid was able to decrease biofilm formation. This is, however, not the first report of a negative plasmidic contribution as it has also been demonstrated that the effect of a plasmid on the ability to produce biofilms is host dependent (Roder et al., 2013). Additionally, we tested if the effect of a plasmid on biofilm formation was correlated with its conjugation rate. Such correlation was verified if IncX plasmid R6K was excluded from the analysis. It is known that plasmids can carry traits, other than sex pili, relevant for the establishment of biofilms. Curly and type 3 fimbriae are among such traits. In fact, it has been shown previously that type 3 fimbriae are a trait commonly found in IncX plasmids (Burmolle et al., 2012). This could explain why plasmid R6K behaved as an outlier in the correlation analysis, having a disproportional strong

effect in biofilm formation that is not accompanied by a corresponding high conjugation rate. We, however, cannot guarantee that this plasmid encodes type 3 fimbriae, since its sequence has not yet been annotated.

There is already evidence that plasmid enhancement of biofilm formation varies with the experimental design and the materials employed (Dudley et al., 2006). In addition, several works have been devoted to study the effect of biofilms in conjugative transfer. Thus, we must point out that, in this work, biofilm formation and conjugation rates were not measured under the same experimental conditions, which could also contribute to a lack of correlation. Furthermore, experimental differences between our work and that by (Ghigo, 2001) could explain the different effects of plasmids such as R16a, RN3 and R388, which enhanced biofilm formation in the work by (Ghigo, 2001) but not in the present work.

Two plasmids can affect biofilm formation synergistically, as observed in some instances. This means, that the observed ability to form biofilms exceeded what would have been expected if the plasmids had additive effects. We studied how a plasmid affected biofilm formation when another plasmid was additionally present in the same host population. To do so, we measured the ability of strains carrying simultaneously two plasmids to produce biofilms. Although we observed combinations of plasmids exhibiting positive epistasis, the trend was not ubiquitous. Overall, we observed that one of the two plasmids had a dominant effect on the host's ability to form biofilm. In some combinations of two plasmids, biofilm formation was identical to that of the better biofilm-forming plasmid of the two. We observed the opposite in other combinations, which often comprised either plasmid R16a or R57b. In such combinations, biofilm formation was identical to that produced by the worst biofilm-producing plasmid of the two. Thus, in these combinations, one of the plasmids prevented its co-resident from enhancing biofilm formation. Co-dominance was observed only in a minority of combinations.

As observed in Chapters 3 and 4, conflicts between conjugative plasmids exist which can lead to the inhibition of conjugative transfer. These conflicts could result in reduced expression of conjugative traits such as sex pili. If plasmids, however, inhabited the same bacterial population, but in different host cells, such inhibition of conjugation could be minimized. Moreover, since plasmids could trigger conjugation independently and each host cell could act simultaneously as plasmid donor and recipient for the other plasmid, a greater number of cellular contacts would be expected. Therefore, we studied the effect of two plasmids in bacterial populations comprising cells carrying each of the plasmids. The overall results from this experiment differed only slightly from the previous one as we again observed patterns of dominance and co-dominance. However, this time we observed fewer cases of synergistic effects, which was unexpected given that conjugation rate increased in many cases when the two plasmids were in different cells (Chapter 3). It is also relevant to stress out that, in almost half of the combinations, the way plasmids were maintained in the population (whether co-residing in the same host cell or not) affected biofilm formation. We could not establish a trend to explain this effect, since it seemed combination specific.

The effect of plasmids on biofilm development does not seem to follow a straight forward tendency. These observations might result from complex interactions between cells and the habitat, the cells and the plasmids and also between different plasmids. Sex-pili and conjugation rates are not the only plasmidic factors involved in biofilm formation as other adhesion factors are carried in plasmids. Furthermore, plasmids also interfere with the expression of chromosomal genes, which affect biofilm formation. Not only the cross-talk between plasmid and host cell, but also the interaction between different plasmids has a role to play in this phenotype. If a clear trend of how plasmids affect biofilm formation exists, then future work is required to evaluate the interplay between these and possible additional factors.

Author Contributions

Conceived and designed the experiments: João Alves Gama, Ana Maria Reis. Performed the experiments: João Alves Gama. Analysed the data: João Alves Gama, Ana Maria Reis, Francisco Dionísio, Rita Zilhão. Wrote the chapter: João Alves Gama, with contributions of Ana Maria Reis, Francisco Dionísio, Rita Zilhão.

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Chapter 8

Negative epistasis among co-infecting plasmids

Abstract

It has been frequently reported that plasmids imposed fitness costs in bacteria carrying them. In this chapter, we confirm that some plasmids decrease host's fitness, but that others can increase it, thus being beneficial. We also show that plasmids exhibiting higher conjugation rates impose higher fitness costs, thus revealing a trade-off between fitness costs and plasmid transfer rates. Moreover, we observed that, among 33 pairs of plasmids, 23 displayed additive epistasis, that is, plasmids interact and so their combined fitness effect on hosts is not the sum of the fitness effects of each plasmid separately. We observed a prevalence of negative epistasis (16 out of 23 cases), where the costs of harbouring the two plasmids is higher than the sum of both costs separately, and 7/23 cases only of positive epistasis (costs are lower than the sum of costs). By performing computer simulations and joining together the trade-off observed here, epistatatic interactions between plasmids, and inter-cellular and intra-cellular interactions between plasmids observed in previous chapters, we may conclude that in some cases chemostats containing bacteria harbouring more two plasmids may increase the stability (or decrease instability) of each plasmid when alone. This may explain the maintenance of costly conjugative plasmids in nature, even when plasmids are not directly selected.

Introduction

Plasmids are mostly acknowledged as beneficial to bacteria due to their ability to promote adaption through the dissemination of genes that encode useful traits, such as resistance to antibiotics and heavy metals, virulence factors or catabolism of xenobiotics (Foster, 1983; Gyles & Boerlin, 2014; Heuer & Smalla, 2012; Nojiri et al., 2004). However, plasmids also impose metabolic and physiological costs on their hosts, which are associated with the expression of genes involved, for instance, in plasmid replication and maintenance (reviewed in (Baltrus, 2013)). Such costs reduce the growth rates of plasmid-bearing bacteria, thus resulting in decreased fitness (Bouma & Lenski, 1988; Dahlberg & Chao, 2003; Dionisio et al., 2005). Additionally, conjugative plasmids may increase the susceptibility of host bacteria to viral infection, because the sex pili required for plasmidic horizontal transmission, also act as viral receptors (Dionisio, 2005; Jalasvuori et al., 2011; Ojala et al., 2013).

Fitness costs imposed by plasmids can however disappear after a period of co-evolution with their hosts (Bouma & Lenski, 1988; Dahlberg & Chao, 2003; Dionisio et al., 2005). Mechanisms involved in the amelioration of fitness costs have been reviewed in (Harrison & Brockhurst, 2012). These include the loss or altered expression of plasmid genes and changes in conjugation rates. For instance, silencing antibiotic resistance genes can reduce the cost of harbouring plasmids (Humphrey et al., 2012). The mechanisms involved in horizontal transfer also impose a fitness cost, for instance, due to synthesis of conjugative

pili. Indeed, the association between these two features has been demonstrated: plasmids exhibiting greater conjugative rates impose grater fitness costs, and vice versa (Turner, 2004; Turner et al., 1998).

Furthermore, interactions between a plasmid and distinct hosts is variable. Indeed, carriage of plasmid F determines distinct patterns of chromosomal gene expression in different *Escherichia coli* strains (Harr & Schlotterer, 2006). Due to such genotype-dependent interactions, the cost of harbouring plasmids may vary in function of host cells, which in turn can contribute to plasmid maintenance. This was corroborated in a study that measured the cost of plasmid carriage in *E. coli* hosts exhibiting different genotypes (resulting from point mutations conferring antibiotic resistance) (Silva et al., 2011). Non-additive (or epistatic) interactions were observed in 60% of the strains. This means that the total fitness cost was different than the sum of the individual costs of carrying the plasmid or the mutation. Interestingly, 50% of the strains experienced lesser fitness costs than those expected by the sum of costs, thus exhibiting positive epistasis. Therefore, in many strains, point mutations reduced the cost associated with plasmid carriage, which in turn may increase plasmid stability.

Epistasis was also observed between plasmids (Morton et al., 2014; San Millan et al., 2014; Silva et al., 2011). A small non-mobilizable plasmid exhibited predominantly positive epistasis when co-residing in the host cells with bigger plasmids that could be either conjugative or mobilizable (San Millan et al., 2014). Regulation of copy numbers was shown to have impact on fitness costs, as such, greater copy numbers imposed greater metabolic burdens on the host (Harrison et al., 2012; Patnaik, 2000). However, plasmid size does not seem to be correlated with fitness cost (Vogwill & MacLean, 2015). This may be due to the dependence of small plasmids on higher copy numbers (than bigger plasmids) to correctly segregate to daughter cells, which may result in small and big plasmids exhibiting identical fitness costs (Millan et al., 2010). Nonetheless, plasmid interactions minimizing copy numbers was not consistent with positive epistasis (San Millan et al., 2014). Moreover, repression of plasmid encoded genes conferring antibiotic resistance could not account for positive epistasis either (San Millan et al., 2014).

Epistasis was also observed with pairs of conjugative plasmids, however, no clear trend favouring positive or negative epistasis was detected (Silva et al., 2011). The authors of the later work, suggest that a small sample of plasmid combinations might have been responsible for the inability to detect such a trend and encourage further works to analyse a larger sample (Silva et al., 2011). That is the first aim of the present work, to study epistasis in larger sample of pairwise combinations of conjugative plasmids. An additional work reported that the combination of plasmids pAt and Ti from *Rhizobium radiobacter* (former *Agrobacterium tumefaciens* (Young et al., 2001)) exhibited positive epistasis. The minimized cost of simultaneously harbouring these plasmids might by related to interactions that decrease horizontal transfer (Lang et al., 2013; Morton et al., 2014). In this sense, our second aim is to test for a correlation between epistasis and the ability of plasmids to repress horizontal transfer (which was determined in Chapter 3). Given that an association between fitness cost and horizontal transfer was already demonstrated, we will check if our results support previous reports (Turner, 2004; Turner et al., 1998).

Positive epistasis may have a role in plasmid persistence in nature. Indeed, strains harbouring multiple plasmids are overrepresented, as it would be

expected if positive epistasis prevailed in nature (San Millan et al., 2014). The existence of conditions for the persistence of plasmids have been demonstrated mathematically relying on parameters reflecting plasmid fitness cost, horizontal transfer and segregational loss (Stewart & Levin, 1977). The latter and other works modelling the conditions for plasmid persistence usually consider bacterial populations harbouring one sole plasmid (Bergstrom et al., 2000; Lili et al., 2007; Ponciano et al., 2007; Simonsen, 1991). Our fourth aim is to computationally simulate if plasmid persistence could be attained based on the experimental parameters determined in this (fitness costs) and previous Chapters (conjugation rates).

Materials and Methods

Bacterial strains and plasmids

We used 44 *E. coli* K12 MG1655 *Δara* strains harbouring different plasmids: eleven strains carried each of the natural conjugative plasmids summarized in Table 1.1, 33 strains carried each of the possible combinations (due to incompatibility or selective markers) of two plasmids. All these plasmids carrying strains were the same produced in Chapter 3. Additionally, we used the *Staphylococcus aureus* Newman (as reference) strain and the plasmid-free *E. coli* K12 MG1655 *Δara* strain.

Fitness assays

We co-inoculated the reference *S. aureus* strain with each of the *E. coli* strains in a ratio of 1:1 (10^4 total bacteria) into 10 mL of Lysogeny broth (LB). Tubes were incubated at 37ºC for 24 hours with agitation (170 rpm). Co-cultures were then diluted in buffer (MgSO4 0.01M) and plated in differential media. *E. coli* cells were quantified in Lysogeny medium (1.5% agar) supplemented with crystal violet (1 mg/L) while *S. aureus* cells were quantified in Lysogeny medium supplemented with NaCl (7.5%). Plates were incubated at 37ºC for 24h.

Epistasis measurement

We calculated the relative fitness of *E. coli* strains as: $\log_2 \frac{E.coli\,(final) / E.coli\,(initial)}{S.aureus\,(final) / S.aureus\,(\text{initial})}$. We normalised fitness values by dividing them by the average fitness of the plasmid-free *E. coli* strain. Using the normalised values, we calculated epistasis according to an additive model and to a multiplicative model. Considering Wp1 and Wp2 as the respective fitness of strains carrying either plasmid p1 or p2 and Wp1 p2 as the fitness of strains carrying both plasmids, epistasis (ε) was calculated as: $\varepsilon = \mathrm{W_{p1\,p2}} - \mathrm{W_{p1}} \times \mathrm{W_{p2}}$ (multiplicative model), or as: $\varepsilon = \mathrm{W_{p1\,p2}} - (\mathrm{W_{p1}} + \mathrm{W_{p2}} - 1)$ (additive model). The standard error (SE) associated with these calculations was estimated by the error propagation method as: i) multiplicative model:

$SE = \sqrt{\left(SE\left(W_{p1\,p2}\right)\right)^2 + \left(SE\left(W_{p1}\right)\right)^2 \times \overline{W_{p2}}^2 + \left(SE\left(W_{p2}\right)\right)^2 \times \overline{W_{p1}}^2}$, or ii) additive model: $SE = \sqrt{\left(SE\left(W_{p1\,p2}\right)\right)^2 + \left(SE\left(W_{p1}\right)\right)^2 + \left(SE\left(W_{p2}\right)\right)^2}$. We considered epistatic effects when $0 \notin [\varepsilon - SE\,,\ \varepsilon + SE\,]$.

Model

We employed a computational model to simulate continuous cultures. The differential equations 1-5 describe, respectively, the kinetics of nutrients (R) and of the following strains: plasmid-free (Ø), harbouring both plasmids (XY) and carrying each of the plasmids (X and Y). All parameters are defined in Supp.Tables S8.1 and S8.2 and Supp.Fig. S8.1.

(Eq.1)

$$\frac{dR}{dt} = \Omega \times (R_0 - R) - \mu \times \Psi \times \frac{R}{R+Q} \times (\text{Ø} + X \times \omega_X + Y \times \omega_Y + XY \times \omega_{XY})$$

(Eq.2)

$$\frac{d\text{Ø}}{dt} = \text{Ø} \times \Psi \times \frac{R}{R+Q} - \Omega \times \text{Ø} + \alpha \cdot (X+Y) + \alpha^2 \cdot XY - \gamma_X \cdot \text{Ø} \cdot X - \gamma_Y \cdot \text{Ø} \cdot Y - \left(\gamma_{XY} + \left(\gamma_{X(y)} - \gamma_{XY}\right) + \left(\gamma_{Y(x)} - \gamma_{XY}\right)\right) \cdot \text{Ø} \cdot XY$$

(Eq.3)

$$\frac{dXY}{dt} = XY \times \Psi \times \omega_{XY} \times \frac{R}{R+Q} - \Omega \times XY - 2 \cdot \alpha \cdot XY + \gamma_{XY} \cdot \text{Ø} \cdot XY + (\gamma_{X \to Y} + \gamma_{Y \to X}) \cdot X \cdot Y + \gamma_{Y(x)} \cdot X \cdot XY - \gamma_{X(y)} + \gamma_{X(y)} \cdot Y \cdot XY$$

(Eq.4)

$$\frac{dX}{dt} = X \times \Psi \times \omega_X \times \frac{R}{R+Q} - \Omega \times X + \alpha \cdot (XY - X) + \gamma_X \cdot \text{Ø} \cdot X + \left(\gamma_{X(y)} - \gamma_{XY}\right) \cdot \text{Ø} \cdot XY - \gamma_{Y \to X} \cdot X \cdot Y - \gamma_{Y(x)} \cdot X \cdot XY$$

(Eq.5)

$$\frac{dY}{dt} = Y \times \Psi \times \omega_Y \times \frac{R}{R+Q} - \Omega \times Y + \alpha \cdot (XY - Y) + \gamma_Y \cdot \text{Ø} \cdot Y + \left(\gamma_{Y(x)} - \gamma_{XY}\right) \cdot \text{Ø} \cdot XY - \gamma_{X \to Y} \cdot X \cdot Y - \gamma_{X(y)} \cdot Y \cdot XY$$

Statistics

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

Results

Fitness cost due to plasmid carriage

We measured the fitness of strains carrying each of the eleven natural plasmids. As observed in Fig. 8.1, when compared with the plasmid-free strain, five plasmids affected the fitness of the host strain (p-value < 0.05, Dunnett's multiple comparison test). Four of these plasmids imposed a fitness cost on the host strain while only one, R6K, increased the host's fitness by approximately 5% (fitness of 105%). Plasmids F and R57b imposed the highest fitness costs, an average of approximately 10% (fitness of 90%). The remaining six plasmids did not impose a statistically significant fitness cost on the host.

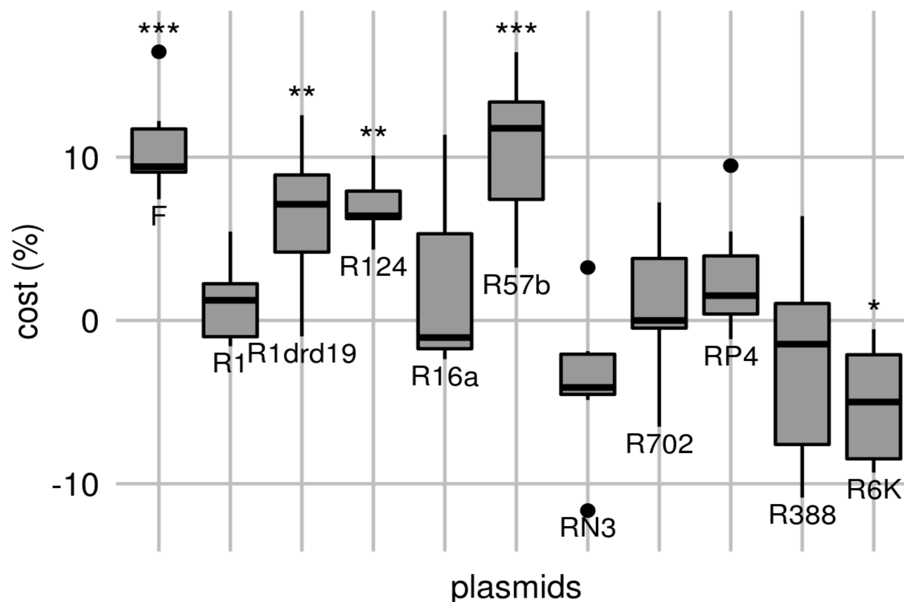


Figure 8.1. Plasmid-imposed fitness costs. Annotations under the boxes indicate the identity of the plasmids. Values were determined with seven replicates per plasmid. Annotations above the boxes represent the results of Dunnett's multiple comparison test: * p-value < 0.05; ** p-value < 0.01; *** p-value < 0.001.

Additionally, we measured the fitness of strains carrying each of the 33 possible combinations of two plasmids. The fitness of these strains varied from 0.78 to 1.18, with an average of 0.93 and a median of 0.92 (Fig. 8.2). A majority of plasmid combinations imposes a burden on the host and, the fitness of their hosts seems to be normally distributed approximately between 0.8 and 1. A minority of plasmid combinations confers a fitness advantage to the host, up to almost 0.2.

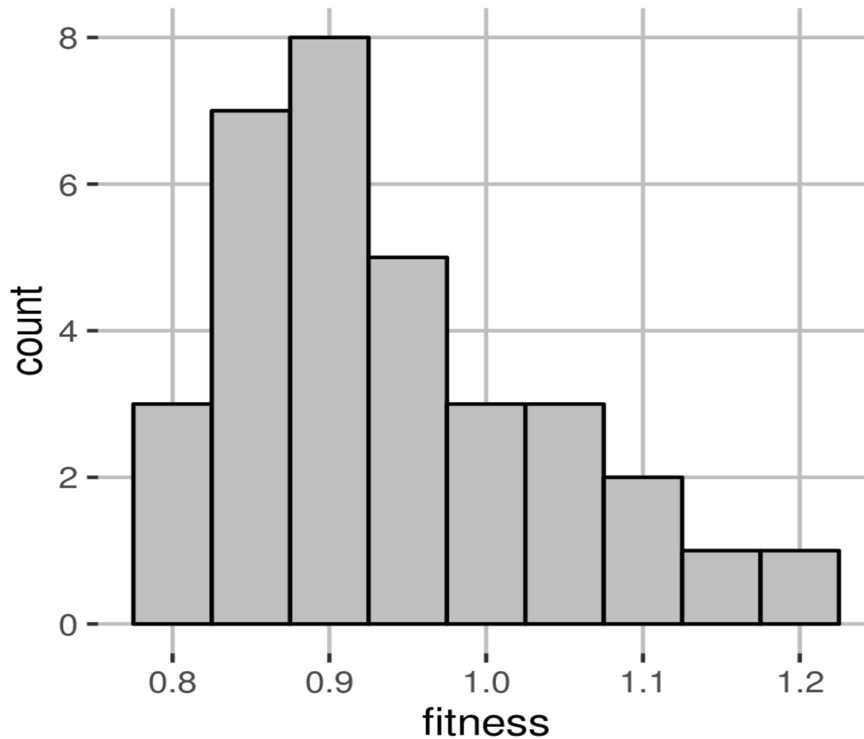


Figure 8.2. Distribution of the fitness of 33 plasmid combinations. The value for each combination represents the average of three replicates.

Trade-off between fitness and conjugation rates

On Fig. 8.3 we show the mean conjugation rates of each plasmid (measured in Chapter 3) plotted against the mean fitness of the strains harbouring them. The two variables are significantly correlated (Pearson method, p-value = 0.002, ρ = -0.84). We observed qualitatively equivalent results when conjugation rates were estimated according to the end-point method (Simonsen et al., 1990). Such results agree with the hypothesis that conjugation is costly to the host, since we observe that conjugation rates are negatively correlated with hosts' fitness. For instance, plasmid F exhibits one of the highest conjugation rates as well as one of the highest fitness costs, while conjugation rate of plasmid R6K is barely detectable but the plasmid confers a fitness advantage to its host. However, exceptions to this trend do exist, exemplified by plasmid R57b, which is more costly to the host than expected according to its conjugation rate. We also tested for a correlation between the size of a plasmid and the cost it imposes. Such correlation was however not observed (Pearson method, p-value > 0.05).

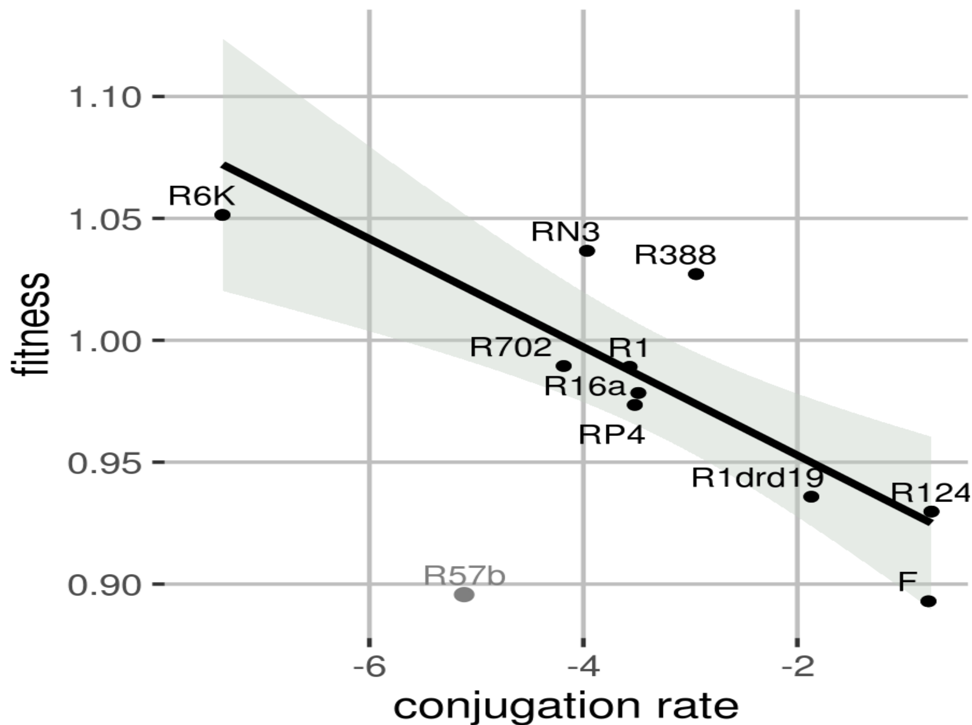


Figure 8.3. Correlation between fitness and horizontal transmission. Fitness values were determined in this work while conjugation rates were determined in Chapter 3. Each point represents the average of fitness and conjugation rates (number of replicates indicated in the respective publication). Linear regression line ($y = -0.022x + 0.91$, $R^2 = 0.71$) and 95% confidence interval shown. Significant correlation (Pearson method, p-value = 0.002, $\rho = -0.84$), when R57b is excluded from the analysis.

Epistasis

We calculated the epistatic effect of two plasmids on the fitness of their hosts, according to an additive and a multiplicative model. According to the additive model, we detected 23 cases of epistasis and 10 of non-epistasis (Supp.Table S8.3). Among the cases of epistasis there were only seven cases of positive epistasis, meaning that the cost of harbouring both plasmids is lower than the sum of the cost of each plasmid alone. However, there were 16 cases of negative epistasis, where the total fitness cost imposed on the host is greater than the sum of the individual plasmidic costs.

We obtained qualitatively equivalent results using the multiplicative model (data not shown). There was only one exception, the combination of plasmids R57b and R124, which exhibited positive epistasis according to the additive model, while according to the multiplicative model it was not epistatic. The 33 values of epistasis, estimated by the additive model, were normally distributed (Shapiro-Wilk test, p-value = 0.49) and varied between -0.21 and 0.13, with an average of -0.032 (Fig. 8.4A). The results from the multiplicative model followed the same trend (Fig. 8.4A). Altogether, these results suggest a greater tendency towards negative epistasis.

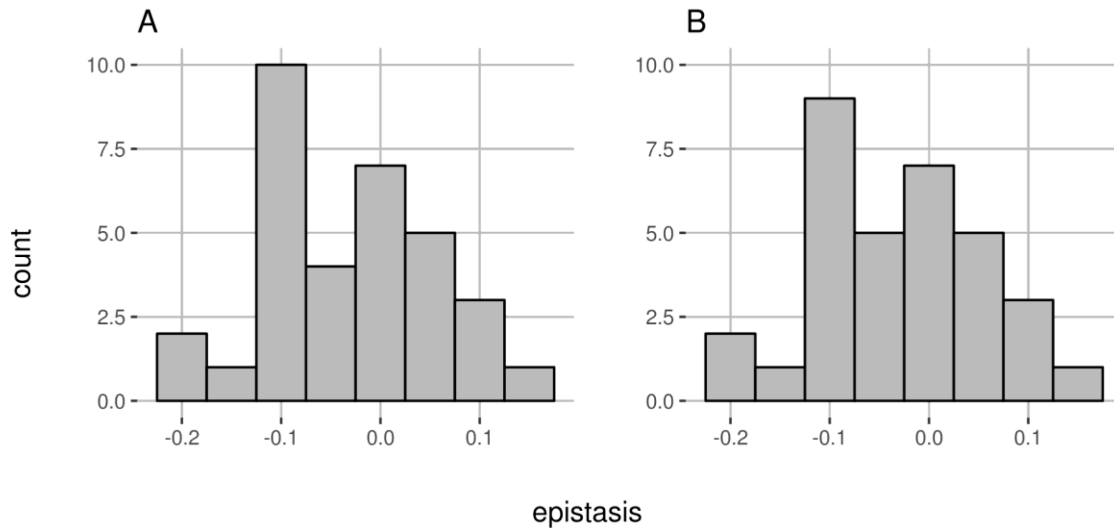


Figure 8.4. Distribution of epistastic effects. A – Additive model; B – Multiplicative model. The value for each combination represents the average of three replicates. Values are normally distributed (Shapiro-Wilk test, p-value > 0.05).

Next, we checked for cases of sign epistasis, that is, when the total fitness cost imposed on the host is less than the cost imposed by one of the individual plasmids. We observed that among the seven cases of positive epistasis, four combinations exhibited sign epistasis (Fig. 8.5). Moreover, the combinations of plasmid RN3 with R388 or R6K exhibited reciprocal sign epistasis, that is, the fitness of the strains carrying two plasmids was higher than the fitness of the strains carrying each plasmid. Thus, despite the low number of cases of positive epistasis, approximately half of them exhibited sign epistasis.

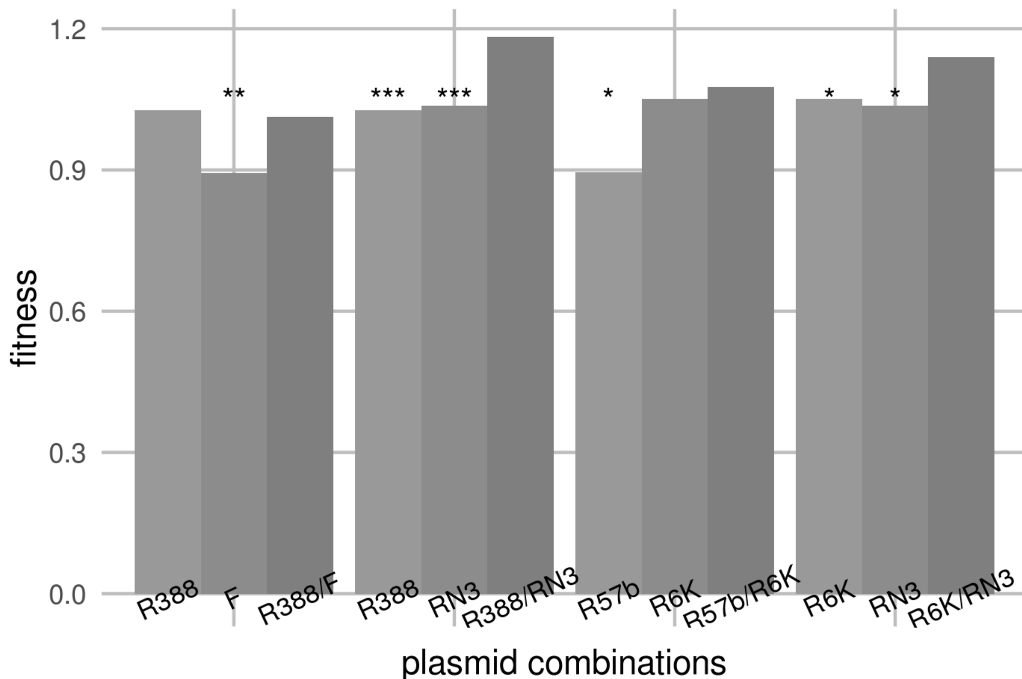


Figure 8.5. Sign epistasis. Bars represent the average fitness of the strains (three replicates for plasmids combinations, seven replicates for each plasmid alone). Significant difference (t-test) between each strain carrying only one plasmid and the strain harbouring both plasmids are shown: * p-value < 0.05; ** p-value < 0.01; *** p-value < 0.001.

We evaluated the average epistasis displayed by each plasmid in each combination. To estimate this value, we considered all the combinations comprising a focal plasmid and calculated the average of their values of epistasis (per combination). In general, the epistasis of each plasmid was not significantly different from zero (Fig. 8.6). The only exception was plasmid R702, which showed a tendency towards negative epistasis.

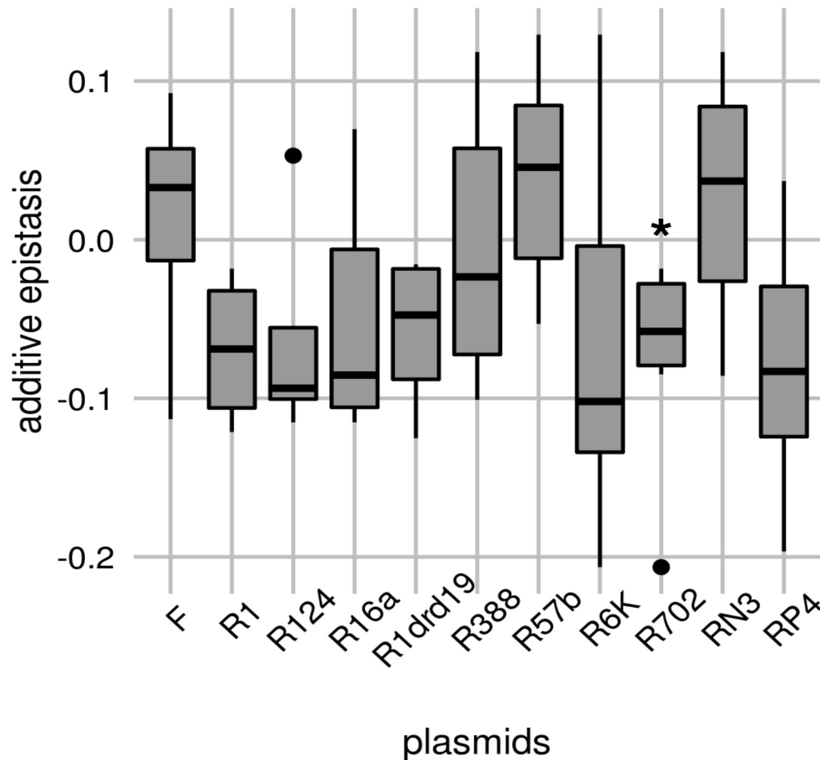


Figure 8.6. Epistasis per plasmid. Annotations under the boxes identify plasmids. For each plasmid, the values represent the average epistasis of three replicates of each combination where it is present. Annotations above the boxes represent the results of t-test: * p-value < 0.05.

Effect of conjugative transfer

We hypothesized that plasmid interactions, affecting conjugative transfer, could affect the fitness of strains harbouring combinations of two plasmids. Following this reasoning, we would expect that combinations exhibiting inhibition of conjugation could experience reduced fitness costs and possibly display positive epistasis. To check this hypothesis, we resorted to results obtained in Chapters 3 and 4, to classify plasmids interactions in terms of conjugative transfer. These phenotypes were classified in the following five categories: I) conjugative efficiency of a plasmid increased, or; II) decreased; III) conjugative efficiencies of both plasmids decreased or; IV) did not change, and; V) the conjugative efficiency of one plasmid increased but the conjugative efficiency of the other plasmid decreased. We compared the variance of fitness among the five categories and did not observe any significant effect (ANOVA, p-value > 0.05; Kruskal-Wallis test, p-value > 0.05). We also compared the variance of epistasis and again found no significant differences between the categories (ANOVA, p-value > 0.05; Kruskal-Wallis test, p-value > 0.05).

Simulations

We have shown that there is a negative correlation between conjugation rate and plasmid cost. Therefore, it is possible that even in cases of strong cost or of negative epistasis, plasmids may be able to maintain themselves in a bacterial population. To test this hypothesis, we simulated a chemostat initially containing only plasmid-harbouring bacteria and nutrients for their growth. We aim to understand if plasmids spread fast enough to compensate for their costs and synergistic interactions. To do so, we employed a computational model of differential equations. This methodology simulates continuous cultures (Stewart & Levin, 1977), with nutrient concentrations being estimated by equation 1; equations 2 to 5 describe the kinetics of the concentration (per millilitre) of four bacterial strains: of plasmid-free cells (Ø), of cells harbouring both plasmids (XY) and of cells carrying each of the plasmids (X and Y). We considered three main parameters regarding plasmid biology: the effect on fitness and the rates of conjugation and segregation. These parameters are described in Supp.Table S8.1. Fitness costs were measured in this work. Conjugation rates were estimated according to the end-point method using values obtained in our previous works (Chapters 3 and 5). Due to the unavailability of some values, we could only analyse 26 of the 33 plasmid combinations. We considered a segregation rate of $1x10^{-4}$ as natural plasmids have been described to be stably maintained (Gordon, 1992; Nordstrom & Austin, 1989; Sayeed et al., 2000).

We simulated the behaviour of populations harbouring only a plasmid (Fig. 8.7), before studying plasmid combinations. The space of parameters used allows the stable maintenance of almost all plasmids. Plasmid R57b, which was shown previously not to exhibit a trade-off between conjugation and fitness cost, was again the exception, being too costly and having a relatively low conjugation rate, hence not being able to be maintained in the chemostat.

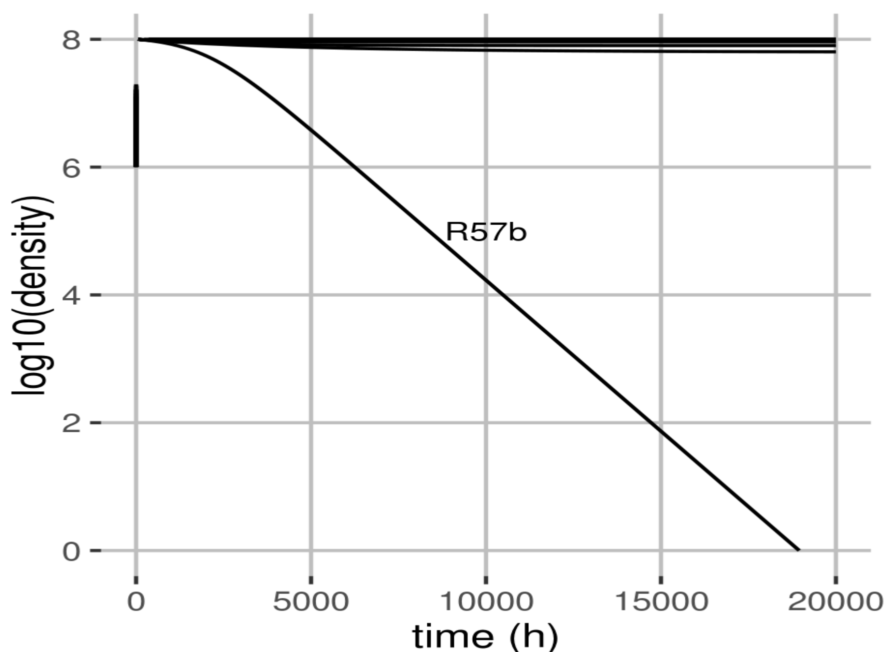


Figure 8.7. Simulations of populations harbouring each of the 11 plasmids. The number (log-transformed) of cells harbouring the plasmid is indicated in the y axis, while time (in hours) is represented in the x axis.

To study plasmid combinations, we started by considering an initial pure culture of $1x10^6$ cells simultaneously harbouring two plasmids. Then, we proceeded to test a second condition, starting with a total of $2x10^6$ cells, half harbouring one of the plasmids and the other half harbouring the other plasmid. The first condition, considering $1x10^6$ initial cells harbouring both plasmids, mimics events after a selective sweep (such as antibiotic treatment) that led to the sole survival of bacteria carrying both plasmids. Strains exhibiting positive epistasis persisted stably in the population (Fig. 8.8A). Maintenance of strains not exhibiting epistasis varied among combinations (Fig. 8.8B). In most cases, the frequency of strains exhibiting negative epistasis declined over time (Fig. 8.8C).

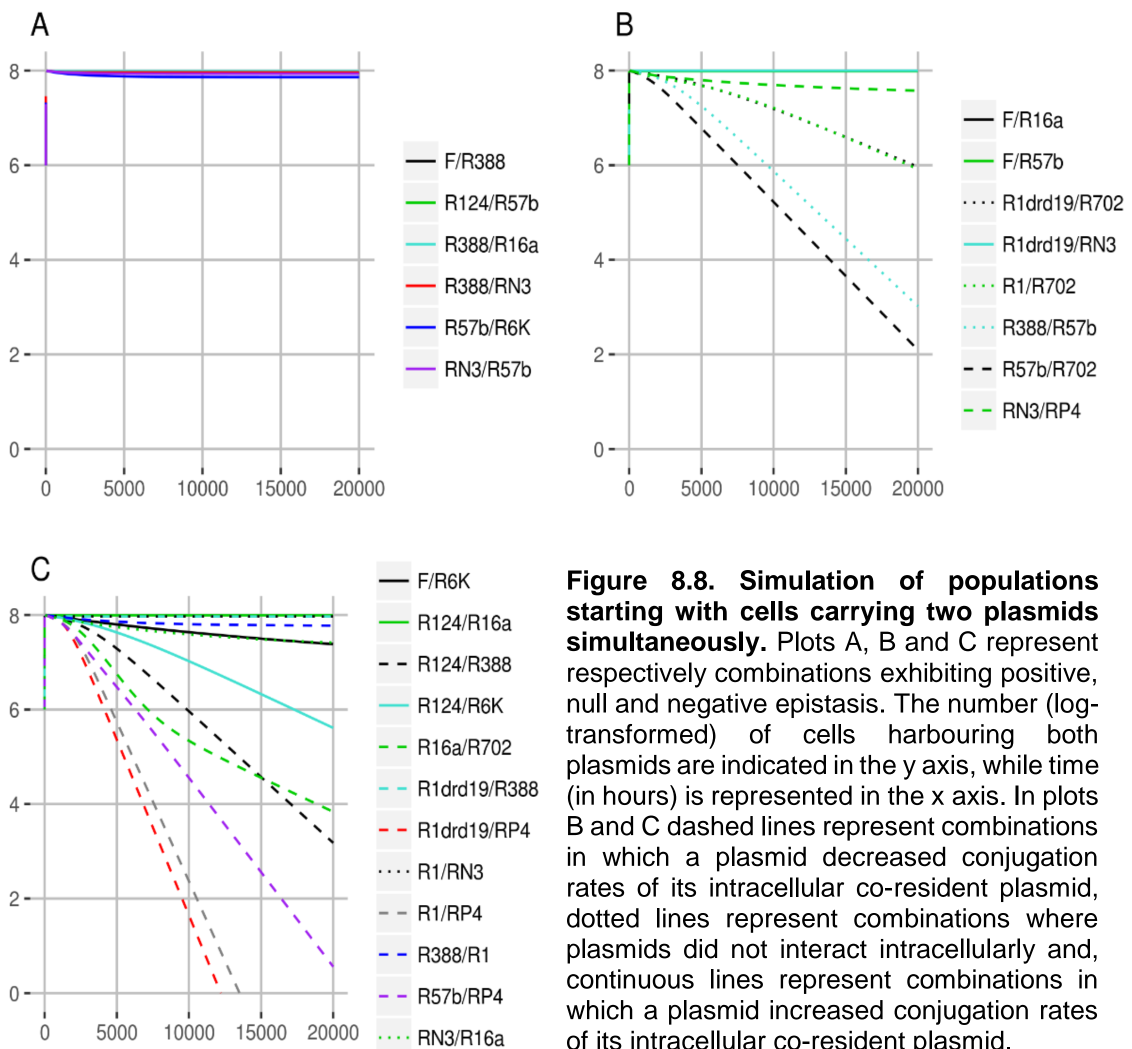


Figure 8.8. Simulation of populations starting with cells carrying two plasmids simultaneously. Plots A, B and C represent respectively combinations exhibiting positive, null and negative epistasis. The number (log-transformed) of cells harbouring both plasmids are indicated in the y axis, while time (in hours) is represented in the x axis. In plots B and C dashed lines represent combinations in which a plasmid decreased conjugation rates of its intracellular co-resident plasmid, dotted lines represent combinations where plasmids did not interact intracellularly and, continuous lines represent combinations in which a plasmid increased conjugation rates of its intracellular co-resident plasmid.

Interestingly, some combinations were stably maintained, even if exhibiting negative epistasis. Indeed, the combination of plasmids R124 and R16a persisted at high frequencies over time, despite exhibiting an average cost of 21%. Its success results from two factors: first, the high conjugation rate of plasmid R124 that easily disseminates; second, R124 also increases the efficiency of transfer of R16a (see Chapter 3). Altogether, these effects lead to the formation of strains harbouring both plasmids. Contrastingly, five of the strains exhibiting greater declining rates harbour plasmids that reduce the conjugation

rate of their co-resident plasmids. Results were identical when the initial bacterial population consisted on cells carrying either of the plasmids.

We tested the effect of epistasis on plasmid maintenance in an additional way. First we extracted the final values from all simulations done before (Fig. 8.7 and Fig. 8.8). Secondly, for each combination (Fig. 8.8), we computed the sum of the values of single-plasmid and of double-plasmid harbouring strains. Such values represent the total cells harbouring a specific plasmid. Thirdly, we plotted (Fig. 8.9) these values against those obtained from simulations where only a plasmid was present (Fig. 8.7). Analysing Fig. 8.9, one can see that generally when

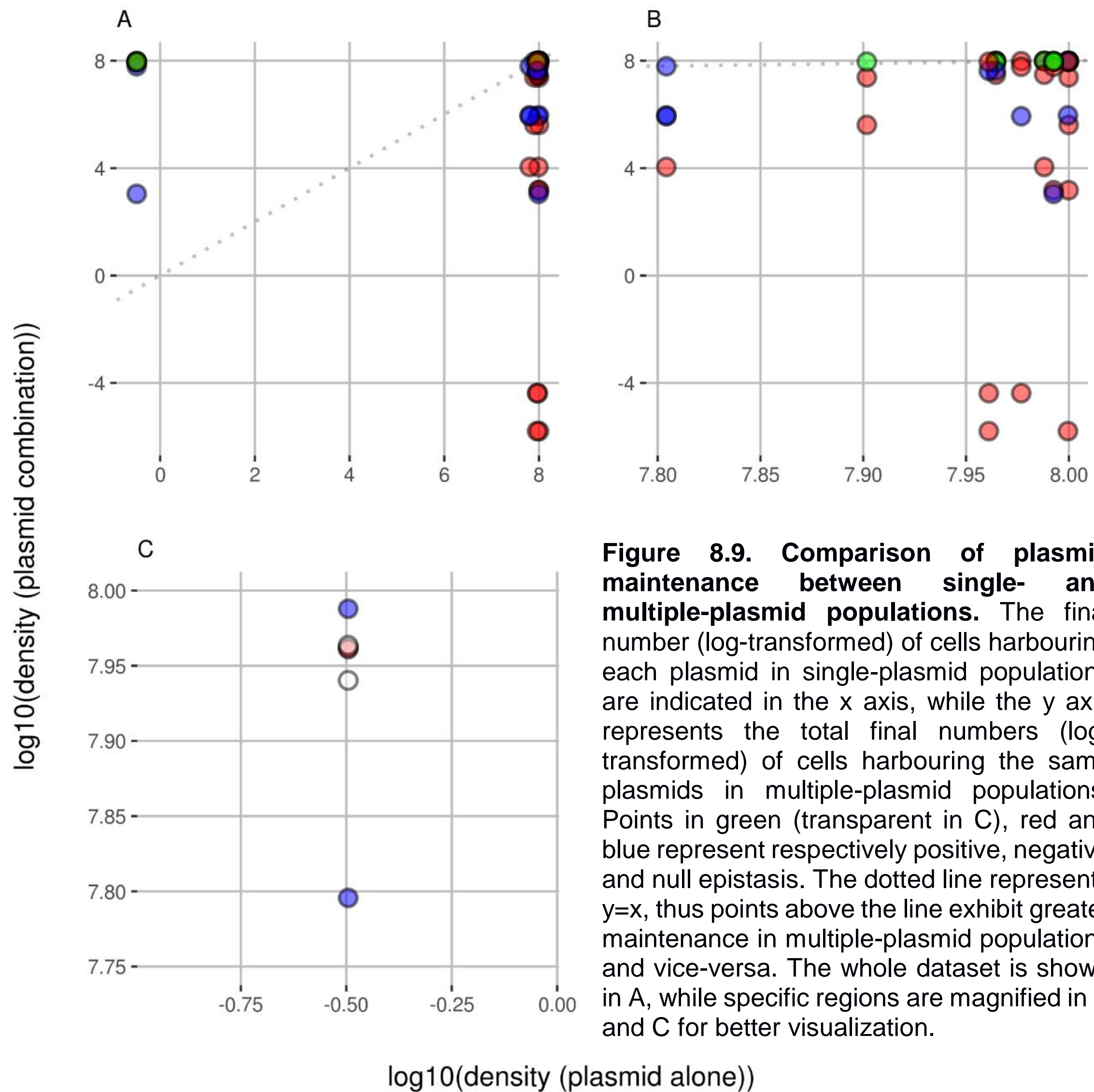


Figure 8.9. Comparison of plasmid maintenance between single- and multiple-plasmid populations. The final number (log-transformed) of cells harbouring each plasmid in single-plasmid populations are indicated in the x axis, while the y axis represents the total final numbers (log-transformed) of cells harbouring the same plasmids in multiple-plasmid populations. Points in green (transparent in C), red and blue represent respectively positive, negative and null epistasis. The dotted line represents y=x, thus points above the line exhibit greater maintenance in multiple-plasmid populations and vice-versa. The whole dataset is shown in A, while specific regions are magnified in B and C for better visualization.

exhibiting positive epistasis, plasmids maintained their frequency in bacterial populations. However, there were some cases (all concerning plasmid R57b) in which plasmid frequency increased. In two of these combinations, plasmid R57b co-inhabited the bacterial population with plasmids R124 or R6K. As seen in chapter 3, the transfer rate of R57b increases in the presence (either in the same or in different cells) of either R124 or R6K. Moreover, the combination of plasmids R57b and R6K exhibited sign epistasis and higher fitness (1.08±0.04, Supp.Table

S8.3) than the plasmid-free strain. The fitness of a strain carrying both these plasmids is not different from that of a strain carrying only plasmid R6K (Fig 8.5). Therefore, cells already harbouring plasmid R6K receive plasmid R57b at a greater rate than plasmid-free cells but do not incur a fitness cost. The remaining case where plasmid R57b increased its frequency was when con-inhabiting the host population with plasmid RN3. Such plasmid combination of plasmids does not impose a fitness cost on the host (1,05 ± 0,05, Supp.Table S8.3).

When exhibiting negative epistasis, plasmid frequencies tended to maintain or to decrease. There was one exception: the combination of plasmids R57b and RP4 increased the frequency of plasmid R57b in the host population. As seen in Chapter 3, the presence of RP4 increases the transfer rate of R57b in two conditions: i) from cells only harbouring R57b to cells only harbouring RP4, and ii) from cells carrying both plasmids to plasmid-free cells. In such cases, the transfer rate of plasmid R57b becomes high enough to compensate the host's fitness cost. In non-epistatic combinations, all three possible outcomes were observed. Again, R57b was the plasmid increasing its frequency in the bacterial population.

Overall these results show that positive epistasis can increase plasmid maintenance, as does the ability of plasmids to increase their own transfer rate in the presence of rival plasmids.

Discussion

Plasmid-imposed fitness burdens have been reported in numerous works (Bouma & Lenski, 1988; Dahlberg & Chao, 2003; Dionisio et al., 2005). Several factors can be pointed as the cause of such burden (Baltrus, 2013). Here, we started to estimate fitness burdens and observed that they are relatively low, in most instances difficult to detect. Despite that, we observed an association between the conjugation rates and the fitness cost conferred by each plasmid. Such result supports the hypothesis of a trade-off between horizontal transfer and fitness (Turner, 2004; Turner et al., 1998).

Recently, epistasis among plasmids has been the subject of several works (Morton et al., 2014; San Millan et al., 2014; Silva et al., 2011). It has been shown that among conjugative plasmids, positive and negative epistasis occur with identical frequencies, resulting in a null net effect (Morton et al., 2014; San Millan et al., 2014; Silva et al., 2011). On the other hand, positive epistasis between conjugative, or mobilizable, and non-conjugative appears to be rather frequent and thus been thought to play an important role in plasmid persistence (Morton et al., 2014; San Millan et al., 2014; Silva et al., 2011).

Here, we too observed cases of positive epistasis. Furthermore, among those seven cases, four combinations exhibit sign epistasis. Two of such cases even exhibited reciprocal sign epistasis, showing that by harbouring both plasmids, hosts possess a higher fitness than by carrying each plasmid alone. Surprisingly, our results contrasted those obtained in other works, by revealing a greater tendency towards negative epistasis. Such result was rather surprising.

A recent work showed that positive epistasis could result from decreased expression of horizontal transfer (Morton et al., 2014). We concluded in Chapters 3 and 4 that plasmids tended to interact negatively, that is, by inhibiting the horizontal transfer of competitors. Then, we expected that an effect reducing

plasmid transfer would result in decreased fitness costs. Therefore, we would expect a bias towards positive epistasis. Results obtained in the present work however, do not support such hypothesis. Mechanisms causing the inhibition of horizontal transfer could account for the differences between this and previous works. For instance, fitness costs could be reduced due to mechanisms repressing the expression of genes responsible for horizontal transfer. Alternatively, mechanisms that promote degradation of DNA (that is about to be transferred) decrease the efficiency of transfer without altering the expression of genes involved in horizontal transfer, which would not result in a drastic reduction of fitness costs.

Plasmid maintenance is affected by the costs imposed on their host's fitness. Thus, plasmid maintenance is harder to explain if negative epistasis prevails, because this results in greater fitness costs. Using experimental data obtained in this and previous works, we examined plasmid maintenance using computer simulations of a chemostat system. Results obtained therefrom, showed that cells carrying two negatively epistatic plasmids can persist for some time. The frequency of most combinations exhibiting negative epistasis decreased over time. However, in some cases, each plasmid persisted for greater periods in populations carrying both plasmids than in populations harbouring only one plasmid. We observed such trend in two different conditions, considering initial populations containing either: i) only cells harbouring both plasmids or ii) cells carrying only one or the other plasmid.

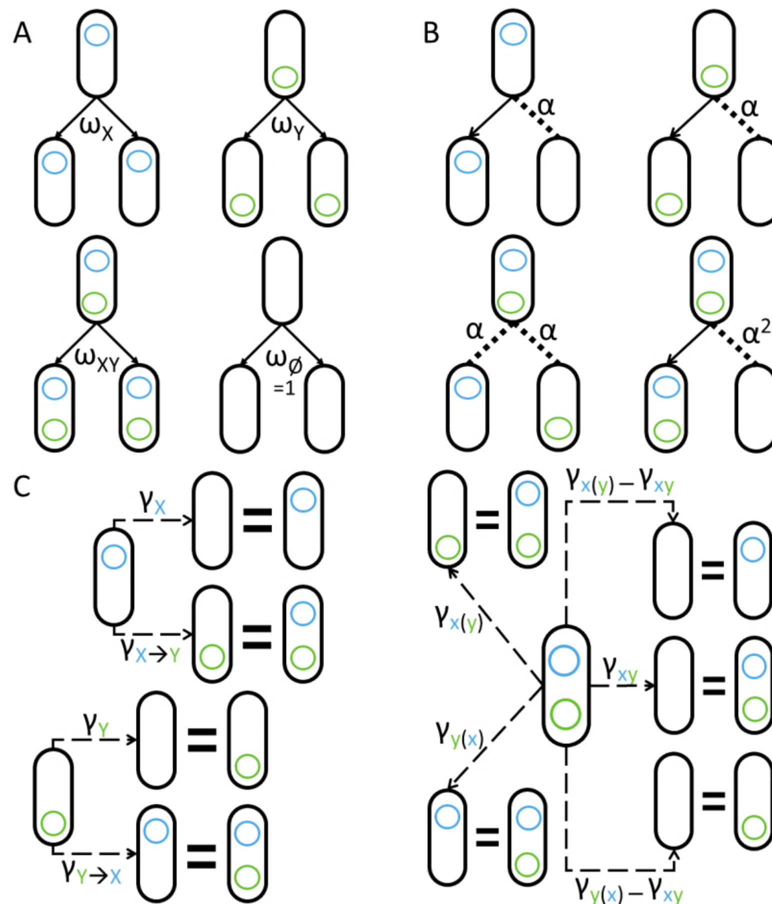
Given the trade-off between cost and transmission observed for each plasmid, plasmid persistence could result from plasmids imposing null or positive fitness effects or if their rates of horizontal transmission were high enough to compensate plasmid loss (Stewart & Levin, 1977). We observed that most plasmids could be stably maintained in populations initially carrying only one plasmid. The only exception was plasmid R57b, which was also an exception for the trade-off hypothesis. Thus, this plasmid imposes a cost that is too high to be compensated by its horizontal transfer rate. However, this plasmid could persist for longer times if another plasmid was also present in the population. In fact, some combinations involving plasmid R57b exhibited positive epistasis. Other combinations of this plasmid did not exhibit epistasis, but R57b was still able to persist for longer times than when it was the sole plasmid in the population. In Chapter 3, we showed that plasmid R57b exhibits a strong potential to compete with other plasmids in terms of conjugation rates, given that it was able to decrease conjugation rates of rival plasmids, but was also able to exploit them to increase its own transfer rate. Such abilities could therefore result in increased maintenance.

In this chapter, we experimentally showed the existence of a trade-off between plasmid burden and horizontal transmission and a tendency towards negative epistasis. Additionally, we showed with computer simulations that despite negative epistasis, plasmids can persist for quite some time, and in some cases for longer time than when alone (e.g. plasmid R57b). In this chapter we performed simulations mimicking chemostat environments with a strict set of parameters. In this sense, deeper studies concerning maintenance of plasmid combinations are required. Therefore, further works are required to improve our knowledge of plasmid maintenance.

Author Contributions

Conceived and designed the experiments: João Alves Gama, Francisco Dionísio. Performed the experiments: João Alves Gama. Analysed the data: João Alves Gama, Francisco Dionísio, Rita Zilhão. Wrote the chapter: João Alves Gama, with contributions of Francisco Dionísio, Rita Zilhão.

Supplementary Information



Supplemental Figure S8.1. Parameters used in computational simulations. A) Growth rates (ω), B) Segregation rates (α), C) Conjugation rates (γ). See Supp.Table S8.1 for a description of the parameters.

Supplementary Table S8.1. Parameters used in computational simulations.

Ø	plasmid-free strain	
X	strain harbouring plasmid X	
Y	strain harbouring plasmid Y	
XY	strain harbouring both plasmid X and Y	
R	resource concentration	
Ω	chemostat turnover rate	
Ro	initial resource concentration	100
μ	resource required per cell division	10^{-6}
Q	Monod constant	5
Ψ	standart growth rate	3
α	segregation rate	10^{-4}
ω_X	relative fitness of strain X	Supp. Table S8.2
ω_Y	relative fitness of strain Y	
ω_{XY}	relative fitness of strain XY	
γ_X	conjugation rate of plasmid X from strain X to strain Ø	
γ_Y	conjugation rate of plasmid Y from strain Y to strain Ø	
γ_{XY}	co-transfer rate of plasmids X and Y from strain XY to strain Ø	
$\gamma_{X(y)}$	conjugation rate of plasmid X from strain XY to strain Ø	
$\gamma_{Y(X)}$	conjugation rate of plasmid Y from strain XY to strain Ø	
$\gamma_{X \to y}$	conjugation rate of plasmid X from strain X to strain Y	
$\gamma_{Y \to x}$	conjugation rate of plasmid Y from strain Y to strain X	

See Supp.Fig. S8.1 for a better understanding of the parameters.

Supplementary Table S8.2. Empirically determined parameters.

X/Y	ωX	ωY	ωXY	γX	γY	γXY	γX(y)	γY(X)	γX→y	γY→x
F/R16a	0,89	0,98	0,89	$9,65x10^{-9}$	$3,91x10^{-11}$	$3,78x10^{-11}$	$3,00x10^{-9}$	$5,20x10^{-11}$	$3,14x10^{-8}$	$4,63x10^{-10}$
F/R57b	0,89	0,90	0,83	$9,65x10^{-9}$	$5,48x10^{-13}$	$3,18x10^{-11}$	$1,21x10^{-8}$	$4,38x10^{-11}$	$5,90x10^{-9}$	$1,70x10^{-11}$
F/R388	0,89	1,03	1,01	$9,65x10^{-9}$	$5,69x10^{-11}$	$2,41x10^{-13}$	$2,10x10^{-9}$	$2,28x10^{-13}$	$9,12x10^{-9}$	$1,25x10^{-10}$
F/R6K	0,89	1,05	0,83	$9,65x10^{-9}$	$7,92x10^{-16}$	$1,98x10^{-12}$	$9,30x10^{-9}$	$7,68x10^{-12}$	$2,00x10^{-9}$	$6,60x10^{-12}$
R124/R16a	0,93	0,98	0,79	$1,23x10^{-8}$	$3,91x10^{-11}$	$9,73x10^{-11}$	$2,53x10^{-9}$	$9,65x10^{-11}$	$3,68x10^{-9}$	$2,83x10^{-10}$
R124/R57b	0,93	0,90	0,88	$1,23x10^{-8}$	$5,48x10^{-13}$	$2,07x10^{-11}$	$6,75x10^{-9}$	$1,78x10^{-11}$	$3,12x10^{-9}$	$1,42x10^{-11}$
R124/R388	0,93	1,03	0,86	$1,23x10^{-8}$	$5,69x10^{-11}$	$2,69x10^{-12}$	$3,63x10^{-9}$	$1,90x10^{-12}$	$3,15x10^{-9}$	$1,18x10^{-10}$
R124/R6K	0,93	1,05	0,89	$1,23x10^{-8}$	$7,92x10^{-16}$	$1,05x10^{-12}$	$5,10x10^{-9}$	$2,00x10^{-12}$	$3,46x10^{-9}$	$7,22x10^{-12}$
R1drd19/RN3	0,94	1,04	0,96	$1,16x10^{-9}$	$9,22x10^{-12}$	$1,96x10^{-10}$	$8,33x10^{-10}$	$2,10x10^{-10}$	$1,11x10^{-9}$	$8,71x10^{-10}$
R1drd19/R702	0,94	0,99	0,91	$1,16x10^{-9}$	$3,76x10^{-12}$	$8,24x10^{-13}$	$1,36x10^{-10}$	$9,80x10^{-13}$	$7,14x10^{-10}$	$1,96x10^{-10}$
R1drd19/RP4	0,94	0,97	0,78	$1,16x10^{-9}$	$1,44x10^{-11}$	$1,78x10^{-13}$	$1,69x10^{-9}$	$3,96x10^{-14}$	$1,35x10^{-9}$	$3,56x10^{-11}$
R1drd19/R388	0,94	1,03	0,89	$1,16x10^{-9}$	$5,69x10^{-11}$	$2,26x10^{-12}$	$2,59x10^{-11}$	$2,18x10^{-11}$	$1,33x10^{-9}$	$1,88x10^{-11}$
R1/RN3	0,99	1,04	0,99	$2,04x10^{-11}$	$9,22x10^{-12}$	$2,79x10^{-11}$	$3,25x10^{-11}$	$2,94x10^{-11}$	$1,91x10^{-11}$	$2,98x10^{-12}$
R1/R702	0,99	0,99	0,96	$2,04x10^{-11}$	$3,76x10^{-12}$	$7,94x10^{-14}$	$4,40x10^{-12}$	$2,46x10^{-13}$	$3,13x10^{-11}$	$1,45x10^{-11}$
R1/RP4	0,99	0,97	0,84	$2,04x10^{-11}$	$1,44x10^{-11}$	$2,32x10^{-14}$	$1,67x10^{-12}$	$2,92x10^{-13}$	$1,41x10^{-10}$	$5,19x10^{-12}$
R388/R1	1,03	0,99	0,92	$5,69x10^{-11}$	$2,04x10^{-11}$	$2,77x10^{-12}$	$3,91x10^{-10}$	$1,38x10^{-11}$	$3,54x10^{-10}$	$9,82x10^{-11}$
R388/R16a	1,03	0,98	1,08	$5,69x10^{-11}$	$3,91x10^{-11}$	$3,48x10^{-12}$	$9,04x10^{-11}$	$4,58x10^{-11}$	$1,52x10^{-11}$	$2,71x10^{-11}$
R388/R57b	1,03	0,90	0,94	$5,69x10^{-11}$	$5,48x10^{-13}$	$1,18x10^{-12}$	$1,79x10^{-10}$	$2,45x10^{-12}$	$1,14x10^{-11}$	$7,59x10^{-12}$
R388/RN3	1,03	1,04	1,18	$5,69x10^{-11}$	$9,22x10^{-12}$	$6,88x10^{-13}$	$2,38x10^{-11}$	$3,92x10^{-12}$	$3,02x10^{-11}$	$2,47x10^{-12}$
RN3/R16a	1,04	0,98	0,93	$9,22x10^{-12}$	$3,91x10^{-11}$	$3,11x10^{-13}$	$1,66x10^{-11}$	$1,07x10^{-11}$	$4,57x10^{-12}$	$9,50x10^{-12}$
RN3/R57b	1,04	0,90	1,05	$9,22x10^{-12}$	$5,48x10^{-13}$	$1,14x10^{-12}$	$7,50x10^{-11}$	$6,85x10^{-12}$	$3,35x10^{-12}$	$1,14x10^{-12}$
RN3/RP4	1,04	0,97	1,05	$9,22x10^{-12}$	$1,44x10^{-11}$	$2,05x10^{-15}$	$5,74x10^{-12}$	$7,81x10^{-14}$	$2,84x10^{-13}$	$6,48x10^{-12}$

Supplementary Table S8.2. Empirically determined parameters. (continued)

X/Y	ωX	ωY	ωXY	γX	γY	γXY	γX(y)	γY(X)	γX→y	γY→x
R57b/R702	0,90	0,99	0,83	$5{,}48x10^{-13}$	$3{,}76x10^{-12}$	$3{,}34x10^{-14}$	$1{,}14x10^{-11}$	$4{,}74x10^{-13}$	$3{,}65x10^{-12}$	$1{,}31x10^{-12}$
R57b/RP4	0,90	0,97	0,82	$5{,}48x10^{-13}$	$1{,}44x10^{-11}$	$2{,}28x10^{-13}$	$5{,}10x10^{-12}$	$2{,}64x10^{-12}$	$3{,}97x10^{-12}$	$2{,}68x10^{-12}$
R57b/R6K	0,90	1,05	1,08	$5{,}48x10^{-13}$	$7{,}92x10^{-16}$	$4{,}19x10^{-16}$	$2{,}22x10^{-12}$	$8{,}63x10^{-16}$	$5{,}16x10^{-12}$	$8{,}50x10^{-16}$
R16a/R702	0,98	0,99	0,88	$3{,}91x10^{-11}$	$3{,}76x10^{-12}$	$1{,}87x10^{-12}$	$1{,}57x10^{-10}$	$5{,}99x10^{-13}$	$5{,}17x10^{-12}$	$8{,}00x10^{-12}$

Fitness values were determined in 24h experiments (this work).

Conjugation rates were estimated by the endpoint method using values determined in 1,5h experiments (Chapters 3 and 5).

Supplemental Table S8.3. Epistasis according to an additive model.

Combination	Fitness (observed)	Fitness (expected)	Epistasis	
R6K/R702	0,83 ± 0,04	1,04	-0,21 ± 0,04	negative
R6K/RP4	0,83 ± 0,04	1,02	-0,20 ± 0,04	
R1drd19/RP4	0,78 ± 0,04	0,91	-0,13 ± 0,04	
R1/RP4	0,84 ± 0,04	0,96	-0,12 ± 0,04	
R16a/R124	0,79 ± 0,02	0,91	-0,12 ± 0,03	
R6K/F	0,83 ± 0,05	0,94	-0,11 ± 0,06	
R16a/R6K	0,92 ± 0,06	1,03	-0,11 ± 0,06	
R1/R388	0,92 ± 0,04	1,02	-0,10 ± 0,04	
R388/R124	0,86 ± 0,04	0,96	-0,10 ± 0,04	
R6K/R124	0,89 ± 0,08	0,98	-0,09 ± 0,08	
R16a/RN3	0,93 ± 0,02	1,02	-0,09 ± 0,04	
R16a/R702	0,88 ± 0,06	0,97	-0,08 ± 0,06	
R1drd19/R388	0,89 ± 0,02	0,96	-0,08 ± 0,03	
R388/R702	0,95 ± 0,04	1,02	-0,06 ± 0,05	
R57b/RP4	0,82 ± 0,02	0,87	-0,04 ± 0,03	
R1/RN3	0,99 ± 0,01	1,03	-0,04 ± 0,02	
R6K/RN3 **	1,14 ± 0,02	1,09	0,05 ± 0,03	positive
R57b/R124‡	0,88 ± 0,04	0,83	0,05 ± 0,05	
R16a/R388	1,08 ± 0,06	1,01	0,07 ± 0,06	
R388/F *	1,01 ± 0,02	0,92	0,09 ± 0,03	
R57b/RN3	1,05 ± 0,05	0,93	0,12 ± 0,05	
R388/RN3 **	1,18 ± 0,02	1,06	0,12 ± 0,03	
R57b/R6K *	1,08 ± 0,04	0,95	0,13 ± 0,05	
R57b/R702	0,83 ± 0,07	0,89	-0,05 ± 0,08	null
R388/RP4	0,98 ± 0,04	1,00	-0,02 ± 0,05	
R388/R6K	1,06 ± 0,02	1,08	-0,02 ± 0,04	
R1drd19/R702	0,91 ± 0,05	0,93	-0,02 ± 0,05	
R1/R702	0,96 ± 0,02	0,98	-0,02 ± 0,03	
R1drd19/RN3	0,96 ± 0,04	0,97	-0,02 ± 0,04	
R16a/F	0,89 ± 0,03	0,87	0,02 ± 0,04	
R57b/R388	0,94 ± 0,03	0,92	0,02 ± 0,04	
RP4/RN3	1,05 ± 0,05	1,01	0,04 ± 0,06	
R57b/F	0,83 ± 0,05	0,79	0,05 ± 0,06	

‡ only according to the additive model.

* sign epistasis; ** reciprocal sign epistasis.

Chapter 9

General Discussion

"A plasmid is known by the hosts it inhabits"

Plasmids are double-stranded DNA molecules commonly isolated from bacterial populations but also found in archaea and eukaryotes (Heinemann, 1991). Plasmids replicate inside their host cells in a chromosome independent fashion. A diversity of replicative mechanisms exists among plasmids (del Solar et al., 1998). They can also encode mechanisms to enhance their maintenance in host cells (Bahl, 2009; Zielenkiewicz & Ceglowski, 2001). Plasmids are inherited by daughter cells after cell division, and can encode mechanisms to prevent being lost by segregation during this process and/or to prevent plasmid-free cells from prospering. Furthermore, some plasmids are able to transfer between host cells (Frost et al., 2005). Conjugation is the predominant mechanism for horizontal transfer and is plasmid encoded (Cabezon et al., 2015). These mechanisms shape how plasmids interact with their host cells and ultimately define in which hosts plasmids can prosper, that is, their host range (delSolar et al., 1996; Jain & Srivastava, 2013).

The definition of host range varies, depending on which trait is the focus of analysis (discussed in (Suzuki et al., 2010)). Typically, it is understood as replication host range, the set of hosts in which a plasmid is able to replicate. Given that plasmids can transfer horizontally, transfer host range includes all the hosts into which plasmids can transfer. Alternatively, long-term host range defines in which hosts plasmids can be stably maintained. Host range is narrower if one considers long-term host range, but expands, when considering replication and transfer (the widest) host range.

In Chapter 2, we focused on the evolutionary host range of plasmids, that is, the group of hosts where plasmids spread and evolved. We studied mechanisms involved in replication, maintenance and horizontal transfer, to understand how they contributed to the evolutionary host range of plasmids. Of these three components, we observed that replication was the main feature defining evolutionary host range, followed by transfer. Altogether, these mechanisms explained more than 2/3 of the host range variation.

Some of the mechanisms responsible for plasmid maintenance showed significant effects when studied alone. However, they did not seem to further contribute to evolutionary host range, when all factors were considered. We only studied some of the mechanisms involved in plasmid maintenance, which could account for our inability to observe a greater explanatory power associated with the maintenance component. Moreover, replication and transfer components were analysed as single variables, while maintenance was split in several variables, such as palindrome avoidance and addiction systems, which could also contribute to weaker explanatory power. Furthermore, plasmids possibly code for maintenance mechanisms not yet identified.

This work focused mainly in mechanisms involved in interactions between plasmids and their hosts. It is possible, however, that interactions between plasmids may play a role in host range. Indeed, we showed in Chapters 3, 4 and 6 that interactions among plasmids contributed to their dissemination across

hosts, thus shaping, at least partially, transfer host range. In Chapters 7 and 8, we showed that interactions between plasmids may further define (long-term) host ranges because they affect host's fitness and ability to form biofilms.

"All covet, all lose"

Conjugative plasmids transfer horizontally by conjugation, a process that requires physical contact between donor and recipient cells. The machinery essential this process is plasmid encoded and determines, among other features, the synthesis of conjugative pili. Pili are responsible for the cellular contact between donor and recipient cells, thus forming a mating pair (Cabezon et al., 2015). Not all pili are the same, some are thick and flexible while other are rigid (Bradley, 1980). Pili morphology affects the efficiency of conjugation. Plasmids encoding thick pili transfer more efficiently in solid than in liquid media, while plasmids coding for thick flexible pili transfer with identical efficiency in both media (Bradley et al., 1980). Some plasmids synthesise secondary thin flexible pili, which maximizes their transfer in broth (Bradley, 1984; Komano et al., 1994).

In Chapter 3 we analysed how interactions between cells harbouring different plasmids affect the efficiency of conjugation. We observed several instances in which the rates of plasmid transfer increased. We provided evidence that pili synthesised by the plasmid present in the recipient cell were involved in such behaviour, thus concluding that a plasmid can better transfer if another plasmid contributes to mating pair formation, stabilizing it. In this sense, a plasmid may take advantage of rival plasmids to disseminate. However, greedy plasmids can end up in a bad situation. That happens if the plasmid already residing in the recipient cell has the ability to inhibit the conjugative transfer of the incoming plasmid. Therefore, limiting further dispersion.

"You shall not pass"

In Chapter 3 we also analysed how interactions between two plasmids residing in the same host cell affect their conjugative efficiency. We observed a tendency towards negative interactions, that is, a plasmid decreasing the efficiency of transfer of its companion. Moreover, these negative interactions were observed predominantly when plasmids inhabited the same cell, but were rather rare when they were present in different cells. Thus, inhibition of transfer could be a strategy to limit the invasion of rival plasmids. As stated before, due to its own conjugation, a plasmid can inadvertently favour the transfer of an incoming plasmid, as it may be hard to know if the other cell carries unrelated plasmids. However, by encoding a mechanism that inhibits the conjugation of a rival plasmid after it settles in the host cell, the previously sole resident has a way of saying: "stay out of my territory".

"The enemy of my enemy is my friend"

In Chapter 4 we studied the contribution of a third plasmid, thus studying the variation of conjugative efficiency when three plasmids inhabited simultaneously the same host cell. We observed that the conjugative efficiency of a given plasmid is affected by the interactions observed among the other two.

It was common to observe that two plasmids inhibited a third one to a greater extent in a three-plasmid-host than in a 2-plasmid-host. This suggests that inhibitory systems can complement each other, resulting in a combined reduction of a rival's conjugative efficiency. We also observed a few cases where a plasmid by inhibiting directly one its rivals would also indirectly inhibit the other. This happened when the inhibited plasmid was responsible for increasing the conjugative efficiency of another plasmid. The inhibitory plasmid would nip the problem in the bud, because by decreasing the conjugative transfer of a rival it would consequently prevent this rival from enhancing the conjugative transfer of a second rival. In such situation, a plasmid can kill two birds with one stone.

"I am not locked in here with you... you are locked in here with me"

In Chapter 6 we studied the simultaneous efficiency of conjugation of plasmids, in other words, how plasmids co-transfer. Expression of genes involved in conjugation is generally repressed and the ability of a plasmid to transfer horizontally depends on its transient de-repression (Frost & Koraimann, 2010). Therefore, one could expect the simultaneous transfer of co-resident plasmids to be an independent event (Romero & Meynell, 1969). However, co-transfer of plasmids is not always independent (Bouanchaud & Chabbert, 1969).

Despite some exceptions, results obtained in Chapter 6 seemed to obey to general trends. When host cells carry two plasmids, co-transfer of both plasmids tends to be limited by the plasmid having the lowest conjugative efficiency. Alternatively, when cells harboured three plasmids, co-transfer of all three was instead limited by the combination of two plasmids exhibiting the lowest efficiency of co-transfer. Generalizing these observations, when a host cell harbours simultaneously n conjugative plasmids, the (n – 1) combination of plasmids exhibiting the lowest efficiency of co-transfer tends to define the extent of co-transfer of all n plasmids. Therefore, simultaneous transfer of plasmids does not seem to be independent and may respond to common regulatory signals.

Focusing on the results involving combinations of two plasmids and the inhibitory interactions observed in previous Chapters, one may speculate that the inhibitory plasmid has the higher ground. Consider a combination of two plasmids in which one inhibits the other. In this situation, the inhibitory plasmid may have a higher efficiency of transfer (although this was not always confirmed). Thus, this plasmid can transfer to the same extent whether it is alone in the host cell or not. In opposition, the inhibited plasmid transfers less when inhabiting the cell with a rival than when alone. Moreover, since co-transfer of both plasmids does not tend to be independent, hosts acquiring the inhibited plasmid will also acquire the inhibitory plasmid. Therefore, the inhibited plasmid is somewhat trapped, because it will end up inhibited. Contrastingly, the inhibitory plasmid has the chance to be transmitted to cells where it will reside alone (without the inhibited plasmid at least).

“The high cost of harbouring a plasmid”

Expression of the conjugative machinery imposes a fitness cost on plasmid hosts because it requires energetic resources, alters cell physiology and determines viral susceptibility (Baltrus, 2013). In Chapter 8 we observed a negative correlation between conjugative efficiency and host’s fitness, as such, plasmids exhibiting higher conjugation rates reduced their hosts’ fitness, that is imposed greater fitness costs. We also observed that the cost of simultaneously carrying two plasmids tends (2/3 of the cases) to be greater than the sum of the individual cost of each plasmid (negative epistasis). Thus, these bacteria harbouring multiple plasmids should be counter selected in nature, unless, for example, these plasmids have very high transfer rates to compensate the fitness costs they impose.

Moreover, given that a trade-off between conjugation and fitness exists, we hypothesised that pairs of plasmids where one of the plasmids reduced the conjugative efficiency of the other, would impose reduced costs on their hosts. However, this hypothesis was not proved correct by our experimental results. We can speculate that the mechanisms employed to inhibit the transfer of rival plasmids may be the source of this failure. For instance, mechanisms acting on the transferosome would be major contributors to decrease fitness costs because it is involved in pilus synthesis (which requires the expression of many genes) and mating-pair formation but also signals that DNA processing should start (de la Cruz et al., 2010). Contrastingly, mechanisms acting in latter phases might not have such a strong impact in fitness because so many resources had already been wasted.

Some mechanisms employed to reduce the efficiency of conjugation of rival plasmids have been characterized. The few known mechanisms act in distinct steps of conjugation. The *kilA* operon of plasmid RP4 inhibits the expression of conjugative pili, thus preventing the first step (Fong & Stanisich, 1989; Goncharoff et al., 1991). The Osa protein and their homologues, degrade plasmid DNA prior to its interaction with coupling protein, which is responsible for driving the DNA to the secretion channel (Cascales et al., 2005; Maindola et al., 2014). The FipA and PifC proteins of plasmids pKM101 and F, respectively, interact with the coupling protein, presumably at later phases (Santini & Stanisich, 1998). Therefore, some of these mechanisms act at time steps where the cell already wasted some resources.

In Chapter 5, we searched for genes coding for homologues of fertility inhibition (FIN) proteins in plasmid genomes available in the GenBank database. Most of the homologues identified resembled the FipA protein. This suggests interactions in later phases of conjugation, which, according to our reasoning, would not lead to drastically reduce fitness costs. However, it is important to keep in mind that little is known about FIN proteins. Many might remain to be found. This means, that FipA-like proteins may not be the most prevalent FINs. Furthermore, the hypothesis relating the fertility inhibition mechanism and fitness cost has yet to be proved experimentally.

“What plasmids do in the (bio)films”

As stated before, we have not found many cases of positive epistasis, regarding host fitness, nor pairs of plasmids exhibiting inhibition of conjugation

and imposing lower fitness costs. Furthermore, some fertility inhibition mechanisms do not prevent the expression of conjugative pili. Altogether, these results may indicate that pilus synthesis confers some kind of advantage. Indeed, it has been shown that conjugative pili contribute to the ability of bacteria to form biofilms (Ghigo, 2001; Reisner et al., 2006).

We confirmed this phenomenon in Chapter 7. Furthermore, we observed that conjugation efficiency was positively correlated with biofilm formation. Therefore, we could expect that the presence of two plasmids in a bacterial population could enhance biofilm formation. However, we did not observe a tendency towards synergistic effects in such conditions. Instead, we observed that one of the plasmids tends to have a dominant effect over the other.

Future Directions

A substantial part of the results obtained in this thesis revealed that fertility inhibition systems may play an important role in plasmid dissemination. Inhibition of conjugation has already been suggested as a prophylactic strategy to prevent the dissemination of plasmids encoding antibiotic-resistance (reviewed in (Williams & Hergenrother, 2008)). This approach, once implemented could also be directed towards plasmids carrying virulence factors, and be useful to attenuate plant and animal pathogens. Indeed, bacterial and fungal extracts as well as chemical have been screened for their ability to inhibit conjugation. We therefore suggest that conjugation inhibitors may be identified in natural plasmids.

Knowledge of plasmid host ranges, as well as the factors shaping it, would allow a better design of vectors used in molecular biology. Furthermore, construction of vectors for bioremediation would also benefit from studies concerning plasmid maintenance, either by studying the interactions between plasmids and host but also among different plasmids (Ikuma & Gunsch, 2012). Finally, conjugative plasmids have also been envisioned as toxic delivery systems (reviewed in (Filutowicz et al., 2008)). In this view, engineered conjugative plasmids, once transmitted into target bacteria would produce a toxic effect leading to cell death. In this sense, future studies would be beneficial not only to generate knowledge but also because of the applicability of such information in diverse areas, such as health and bioremediation.

Chapter 10

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Appendix A

Symbiosis between non-transferable plasmids and prokaryote cells

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Abstract

Plasmids are common in the prokaryotic world, both in bacteria and archaea. Most of these extrachromosomal DNA molecules do not code for essential genes. One may expect that the replication of plasmids and the expression of plasmidic genes implicate a fitness cost to their host. Given this cost, and given that plasmid-free cells often arise, it is striking that so many non-transferable plasmids are able to maintain themselves inside prokaryotic cells without being counter-selected in favor of plasmid-free cells. A solution to this paradox would be the evolution of controlling mechanisms to regulate rivalry between plasmids for the stability of these symbiotic relationships. In this chapter, we discuss the evolutionary selective conditions for such mechanisms to evolve.

João Alves Gama contributed to the writing of the manuscript.

Appendix B

Social behaviour involving drug resistance: antibiotic-degrading enzymes produced by some bacteria protect other bacteria from antibiotics

Iolanda Lopes Domingues, João Alves Gama, Luís M. Carvalho, &
Francisco Dionisio

Article manuscript submitted to Heredity

Abstract

Bacteria sometimes cooperate with co-inhabiting cells. Pathogenic bacteria, for example, often produce and excrete virulence factors, eventually benefitting both producer and non-producer cells. The role of social interactions involving antibiotic resistance, however, has been more elusive. Enzymes that inactivate β-lactam antibiotics such as ampicillin or penicillin (β-lactamases) are good candidates as public goods. Nonetheless, it has been claimed that bacteria harbouring plasmids of natural origin coding for β-lactamase almost do not protect sensitive bacteria. This does not fit with the fact that ampicillin-sensitive bacteria can be isolated from subjects undergoing ampicillin treatment. We hypothesized that there are two non-exclusive explanations for the discrepancy between previous works: (1) the range of values of demographic conditions (such as initial strain frequency, initial total cell density, or habitat structure) has not been broad enough to include most scenarios, or, (2) there are interactions between some of these factors. We performed experiments with *Escherichia coli* bacterial cells to measure the degree of protection of sensitive cells when co-cultured with cells harbouring RP4, R16a or the R1 plasmids, all of natural origin and coding for β-lactamase, and in presence of ampicillin. In these co-cultures, performed in structured and non-structured environments, both the initial total cell density and the initial frequency of sensitive cells spanned four orders of magnitude. We found protection of sensitive cells in 63% of tested conditions. All factors (plasmid, structure, frequency, and density) significantly affect levels of protection. Moreover, all factors interact, with interactions revealing large or very large effect sizes.

João Alves Gama contributed to data analysis and the writing of the manuscript.

Appendix C

Harming behavior mediated by plasmids

Iolanda Lopes Domingues, João Alves Gama, & Francisco Dionisio

Article manuscript in preparation

Abstract

Plasmids are known for decades and they seem to be ubiquitous in the bacterial world, but it is still unclear which forces maintain them among bacterial populations. Some plasmids carry helpful genes, but it is unclear why these genes are maintained in the plasmid and not recruited by the chromosome. Possibly, plasmids are maintained as parasites, however, plasmid transfer rates between bacteria seems to be too low. Here we show that bacteria use plasmids to harm plasmid-free cells, being able to slow down their growth. We performed growth experiments in a liquid environment while taking advantage of membranes with 0.4 µm pores. Using this pore size we block direct contact and bacterial conjugation between cells placed in the two opposite sides of the membrane, but allow the exchange of culture media and free metabolites between the two sides. We show that, in many cases, the growth rate of plasmid-free *Escherichia coli* cells is lower when mixed with *E. coli* cells harbouring a plasmid of natural origin (RP4, R16a or R1) than when the two populations are separated by the membrane. This was observed under six initial ecological conditions (density and frequency of cells with and without plasmid), and concluded that plasmids may be used as harming agents even in some cases where plasmid transfer was almost absent. To our knowledge, this is the first report demonstrating that conjugative plasmids may be used as biological weapons.

João Alves Gama performed a few experiments and contributed to data analysis and the writing of the manuscript.

Appendix D

The Survival of the Luckiest or the Inoculum Effect – Explaining the Survival of Susceptible Bacteria in the Presence of Antibiotics

Iolanda Lopes Domingues, João Alves Gama, Diana Adão & Francisco Dionisio

Article manuscript in preparation

Abstract

Some enzymes confer antibiotic resistance by inactivating antibiotic molecules. This is the case, for example, of β-lactamases, enzymes that neutralise β-lactam antibiotics such as penicillin or ampicillin by hydrolysing the β-lactam ring of these molecules. Sometimes detoxification is so effective that nearby susceptible cells are able to survive and grow. With experiments involving *Escherichia coli* bacterial cells growing in the presence of ampicillin, a simple theoretical model, and computer simulations, we show that the probability of survival of susceptible cells is proportional to the frequency of beta-lactamase producer cells (as expected), but proportional to, at least, the sixth power of the frequency of susceptible cells. The biological meaning is that, for a susceptible cell to survive, at least six of them have to be near the β-lactamase producer. We argue that, while ampicillin is still not destroyed by β-lactamase, an significant fraction of antibiotic molecules attach to the cellular structures of susceptible cells. As such, these susceptible cells partially detoxify the environment. This gives the opportunity to a few, fortunate, susceptible cells to survive. As such, killed cells end up having an altruistic death. Meanwhile, β-lactamase completes detoxification, allowing the growth of the surviving susceptible cell. The clinical implications of this finding are worrisome, because it means that both resistant and sensitive bacteria, including non-pathogenic cells living in the same habitat, may collaborate to rescue susceptible pathogenic cells.

João Alves Gama contributed to the experimental design, data analysis and the writing of the manuscript.