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Study of the biological cost of antibiotic resistance.

Rui Francisco Ribeiro de Almeida e Silva

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ABSTRACT

Multi-drug resistance is caused by the accumulation of chromosomal mutations, by the acquisition of mobile genetic elements (mostly conjugative plasmids), or both. With few exceptions, the acquisition of drug-resistance mutations or of conjugative plasmids is costly to the bacterial cell in the absence of drugs. Recently, it has been found that, in *Escherichia coli* bacterial cells, a mutation conferring resistance to an antibiotic can be advantageous to the bacterial cell if another antibiotic-resistance mutation is already present, a phenomenon called sign epistasis. Here we show that sign epistasis is even more common in the interaction between antibiotic-resistance chromosomal mutations and conjugative plasmids, as well as an overall antagonistic interaction between mutations and plasmids. This implies that the acquisition of an additional resistance plasmid or of a resistance mutation often increases the fitness of a bacterial strain already resistant to antibiotics. These results further complicate expectations of resistance reversal by interdiction of antibiotic use.

KEYWORDS

Antibiotic Resistance, Epistasis, Conjugative Plasmid, Resistance Mutation, Fitness, Bacterial Evolution.

RESUMO

Os antibióticos são essenciais na medicina moderna (Martinez *et al.*, 2009). A sua utilização permite a prevenção e tratamento de infecções bacterianas. Desde o inicio da era dos antibióticos que se observa a emergência e disseminação de estirpes bacterianas resistentes (Levy & Marshall, 2004). Actualmente, o ritmo de descoberta de novas drogas com actividade antimicrobiana é muito lento, logo, a sociedade moderna enfrenta novamente um desafio no combate contra as doenças infecciosas de origem bacteriana.

A resistência a antibióticos pode resultar de dois processos distintos: i) mutação espontânea de genes cromossomais (Martinez *et al.*, 2009) ou ii) aquisição de genes de resistência localizados em elementos genéticos móveis (Martinez *et al.*, 2009). Este estudo foca-se em mutações em três genes de *housekeeping*: *rpsL*, *gyrA* e *rpoB*. Mutações no gene *rpsL* podem modificar a proteína S12, um dos constituintes da subunidade ribossomal 30S, conferindo resistência à estreptomicina (Schrag *et al.*, 1997). O gene *gyrA* codifica a enzima ADN girase, envolvida na síntese de ADN e mutações neste gene resultam em resistência a quinolonas como o ácido nalidíxico (Emmerson & Jones, 2003). A resistência à rifampicina pode resultar de mutações no gene *rpoB* que modificam a subunidade β da polimerase de ARN, envolvida na transcrição de ADN (Trinh *et al.*, 2006). Sistemas celulares de detecção e correcção de erros no ADN permitem manter a taxa de mutação em níveis baixos. No entanto, ambientes com uma forte pressão selectiva (como uma infecção crónica sujeita a tratamento com antibióticos), contribuem para a selecção de fenótipos com uma elevada taxa de mutação (LeClerc *et al.*, 1996; Oliver *et al.*, 2000; Vulic *et al.*, 1997).

As mutações de resistência afectam genes "essenciais" (normalmente denominados de *housekeeping*, termo utilizado doravante) que codificam alvos de antibióticos (Andersson & Levin, 1999; Higgins *et al.*, 2003). Logo, a resistência está frequentemente associada a um desvio da fisiologia óptima da célula. Devido a isso, estirpes resistentes a antibióticos frequentemente exibem uma taxa de crescimento menor que as estirpes ancestrais susceptíveis (Andersson & Levin, 1999; Andersson, 2006; Lenski, 1998; Nilsson *et al.*, 2003). Esta diferença corresponde ao custo biológico (normalmente denominado de custo de *fitness*, termo utilizado doravante) da resistência a antibióticos. A grandeza do custo de fitness varia entre estirpes e mutações e, em alguns casos, a mutação de resistência pode não criar qualquer custo mensurável. O custo de *fitness* afecta a frequência de uma determinada estirpe bacteriana na população, observando-se que estirpes com menor custo de fitness exibem uma maior frequência que estirpes resistentes com um elevado custo de *fitness* (O'Neill *et al.*, 2006; O'Sullivan *et al.*, 2005). Num ambiente não sujeito à pressão selectiva de um determinado antibiótico, é espectável que as estirpes susceptíveis exibam uma taxa de crescimento superior à das estirpes resistentes, conduzindo à eliminação do fenótipo resistente. Logo, uma possível estratégia para eliminar a resistência a antibióticos consiste em banir a utilização de um

determinado antibiótico. Infelizmente, vários fenómenos podem minar a eficácia deste tipo de estratégia.

A evolução de estirpes resistentes conduz frequentemente à redução ou eliminação do custo biológico associado com a mutação de resistência (Andersson & Levin, 1999; Bjorkman *et al.*, 2000; Emmerson & Jones, 2003; Gagneux *et al.*, 2006; Maisnier-Patin *et al.*, 2002; Schrag *et al.*, 1997). Este processo é o resultado da ocorrência de mutações espontâneas compensatórias, que podem ocorrer noutros locais do cromossoma. A ocorrência de mutações compensatórias é muito mais frequente que a ocorrência de mutações de reversão para a susceptibilidade devido ao muito maior alvo para mutações compensatórias (todo o cromossoma).

A resistência produzida pela aquisição de genes de resistência está relacionada com a troca horizontal de elementos genéticos como integrões, transposões e plasmídeos (Martinez *et al.*, 2009). Os integrões exibem um arranjo modular que permite a introdução e expressão de genes. Integrões são recrutados por transposões, que por sua vez são recrutados por plasmídeos (Carattoli, 2001). Os plasmídeos conjugativos são capazes de mobilizar o seu ADN (e a de outros plasmídeos - plasmídeos mobilizáveis) de uma célula dadora para uma célula recipiente através do processo da conjugação bacteriana (Amabile-Cuevas & Chicurel, 1992). A conjugação permite a troca de genes de resistência entre estirpes bacterianas diversas contribuindo para a disseminação do fenótipo resistente no ambiente. A manutenção, replicação e expressão do plasmídeo de resistência também pode produzir um custo biólogico, expresso numa menor taxa de crescimento da estirpe contendo o plasmídeo em relação à mesma estirpe sem o plasmídeo. Logo, também na resistência a antibióticos produzida pela disseminação de plasmídeos de resistência, é possível conceber como estratégia para a eliminação da resistência, a suspensão da utilização de um determinado antibiótico. Este cenário é, no entanto, tornado mais complexo devido a vários fenómenos.

A evolução conjunta de um plasmídeo e da sua estirpe hospedeira conduz frequentemente à redução ou eliminação do custo de *fitness* (Bouma & Lenski, 1988; Dionisio *et al.*, 2005). Por outro lado, a presença no plasmídeo de outros genes seleccionados pelo ambiente (por exemplo, resistência a metais pesados) impede a eliminação do plasmídeo da população (Martinez & Baquero, 2002). Adicionalmente, a existência de estirpes bacterianas com uma capacidade dadora mais elevada permite que estas estirpes tenham um efeito amplificador, conduzindo a uma rápida disseminação do plasmídeo (Dionisio *et al.*, 2002). Finalmente, a presença de sistemas estabilizadores de plasmídeos, como por exemplo o sistema de morte pós-segregacional, também dificulta a sua eliminação de uma população (Engelberg-Kulka & Glaser, 1999).

Recentemente, um estudo revelou um fenómeno adicional que complica a reversão para a susceptibilidade em estirpes multi-resistentes: a ocorrência de uma interacção antagonística entre alelos de resistência conduzindo a um custo de *fitness* menor que a soma independente do custo de *fitness* associado com cada um dos alelos de resistência, quando

isolados (Trindade *et al.*, 2009). A observação deste fenómeno de epistasia positiva entre mutações deletérias sugere que a interacção entre alelos de resistência pode ser um dos fenómenos contribuindo para a manutenção da resistência a antibióticos em populações bacterianas.

O presente estudo pretende averiguar se ocorrem interacções entre mutações cromossomais de resistência e plasmídeos conjugativos e entre plasmídeos conjugativos co-existindo na mesma célula. Nesse sentido, o custo de *fitness* associado a seis plasmídeos conjugativos de resistência foi determinado utilizando o mesmo ensaio de *fitness* utilizado no estudo de Trindade e tal (Trindade *et al.*, 2009). De seguida, através de conjugação, foram produzidas todas as combinações possíveis entre 10 mutações e 5 plasmídeos e todas as combinações possíveis entre 6 plasmídeos. O *fitness* de cada estirpe resultante foi medido e utilizado para detectar e medir possíveis interacções epistásticas.

Os resultados mostram que 52% (26/50) das combinações entre mutação e plasmídeo exibem epistasia positiva. Adicionalmente, 16 destas 26 estirpes exibindo epistasia positiva (62%), também exibem epistasia de sinal. Tal significa que o custo de *fitness* associado aos 2 determinantes de resistência em conjunto é menor que o custo de *fitness* associado a um dos determinantes isolados. Do mesmo modo, observou-se a ocorrência de epistasia positiva em 6 das 14 combinações entre plasmídeos co-existindo na mesma célula. Os resultados revelam também que mutações mais deletérias tendem a ser mais epistáticas que mutações com menor custo de *fitness*. Por outro lado, a comparação entre o efeito das mutações e dos plasmídeos no nível de epistasia, sugere que as mutações são mais determinantes do nível de epistasia que os plasmídeos.

O presente trabalho mostra que as interacções epistáticas não se limitam a mutações mas também envolvem plasmídeos. A ocorrência de epistasia de sinal entre mutações e plasmídeos sugere que em determinados casos, a aquisição de um determinante de resistência adicional pode conduzir a um aumento da taxa de crescimento da estirpe (aumento de *fitness*). Dado o papel fundamental desempenhado pelos plasmídeos como vectores na disseminação da resistência a antibióticos, os resultados aqui descritos mostram a necessidade de implementar estratégias para a reversão para a susceptibilidade que tenham em conta a complexidade da ecologia evolutiva das estirpes resistentes. Sugerimos a implementação de medidas para a reversão que estejam direccionadas contra as vulnerabilidades dos plasmídeos conjugativos. Três estratégias foram já identificadas (Williams & Hergenrother, 2008): i) inibição da conjugação bacteriana; ii) inibição da replicação do plasmídeo e iii) exploração dos sistemas toxina-antitoxina codificados pelo plasmídeo.

PALAVRAS-CHAVE

Resistência a Antibióticos, Epistasia, Plasmídeo Conjugativo, Mutação de Resistência *Fitness*, Evolução Bacteriana.

INDEX

I. INTRODUCTION

1. ANTIBIOTIC RESISTANCE

Antibiotics are of extreme importance in modern medicine, where they are heavily used to treat infection by bacterial pathogens (Bertino, 2009) and prevent the establishment of infection associated with many commonplace medical practices (Martinez *et al.*, 2009). In addition, antibiotics are also widely used in non-clinical settings such as in animal farming as growth promoters in animal feed and in the prevention of animal infection (Edrington *et al.*, 2009). The antibiotic era has started six decades ago, which means that bacterial populations have been under a selective pressure for a very long period. In fact, human antibiotic usage can be regarded as a large scale evolution experiment. The major result of such experiment is the emergence of resistance to all commercially available antimicrobial drugs (Johnsen *et al.*, 2009) in a diverse array of bacterial populations, including clinical and environmental isolates (Martinez *et al.*, 2009). The introduction of a new antibiotic is soon followed by the detection of resistant strains (Levy & Marshall, 2004). Furthermore, the rate of discovery of new antibiotics has slowed significantly (Projan, 2003). Hence, we are now facing the worrying prospect of unavailability of effective antibiotics to treat common infections.

1.1. RESISTANCE MUTATIONS

Bacteria can become resistant to antibiotics by two distinct processes: i) spontaneous mutation of chromosomal genes (Martinez *et al.*, 2009) and ii) acquisition of resistance genes located in horizontally mobile genetic elements (Martinez *et al.*, 2009). Spontaneous mutation of antibiotic resistance mutations usually affect housekeeping genes that are drug targets (Martinez *et al.*, 2009). In the present work, mutations in 3 different genes will be studied: *rpsL*, *gyrA* and *rpoB*. Mutations in the *rpsL* gene alter the 30S ribosomal protein S12, and can confer resistance to streptomycin (Schrag *et al.*, 1997). Depending on the effect on protein translation fidelity, *rpsL* mutants can be divided in two phenotypes: i) restrictive, which have augmented translation fidelity and ii) non-restrictive which exhibit no change on protein translation fidelity. Quinolone resistance can be due to alterations in the target enzymes DNA gyrase (coded by *gyrA*) and topoisomerase IV, involved in DNA synthesis (Emmerson & Jones, 2003; Higgins *et al.*, 2003; Maisnier-Patin *et al.*, 2002; Truong *et al.*, 1995). Rifampicin resistance can result from alterations in the β-subunit of the target RNA polymerase (coded by *rpoB*) involved in RNA synthesis (Trinh *et al.*, 2006).

1.1.1. MUTATION RATE

The rate of spontaneous mutation to resistance varies with the antibiotic (Martinez *et al.*, 2009) and the cell genotype (LeClerc *et al.*, 1996; Oliver *et al.*, 2000; Vulic *et al.*, 1997). DNA error correction systems ensure the mutation rate is kept low. However, stressful environments with a strong selective pressure, such as a chronic infection, select for phenotypes with a

higher-than-normal mutation rate (Oliver *et al.*, 2000). The presence of these hypermutators in a population increases the probability of resistance emergence (LeClerc *et al.*, 1996; Oliver *et al.*, 2000; Vulic *et al.*, 1997). In addition, some antibiotics can increase the mutation rate. For example, quinolones (Phillips *et al.*, 1987) and β-lactams (Miller *et al.*, 2004) induce the SOS response and can, therefore, facilitate mutation to resistance.

1.1.2. MUTATION FITNESS COST

Besides conferring resistance to antibiotics, mutations can have other effects in the cell. Since most resistance mutations are located in housekeeping genes, they can interfere with essential cell processes, such as DNA replication and transcription, protein translation and bacterial wall synthesis (Andersson & Levin, 1999; Dahlberg & Chao, 2003; Higgins *et al.*, 2003). Hence, resistant cells physiology is deviated from an optimal state and these cells may grow slower than their drug-susceptible ancestrals. Thus, in the absence of the antibiotic selective pressure, resistance mutations are often deleterious and confer a biological fitness cost to the cell (Andersson & Levin, 1999; Andersson, 2006; Lenski, 1998; Nilsson *et al.*, 2003). This fitness cost can vary widely with the mutation and the strain genetic background (Gagneux *et al.*, 2006).

Despite the generally accepted principle that resistance mutations are deleterious, recent studies have suggested that, in specific environments and/or strains, the metabolic rewiring produced by resistance mutations can lead to greater fitness (Andersson, 2006; Yu *et al.*, 2005). For example, resistance to ciprofloxacin in *Campylobacter jejuni* is due to a single mutation in *gyrA*. In the absence of antibiotic this mutation increases the fitness of the resistant strain (Luo *et al.*, 2005). Furthermore, there is also evidence for no-cost mutations (Andersson & Levin, 1999; Andersson, 2006). It is possible that these no-cost mutations do in fact decrease fitness, but our fitness cost determination methods fail to detect such cost. One apparent exception is the $Lys_{42} \rightarrow Arg$ mutation in the *rpsL* gene. This mutation confers resistance to streptomycin and it appears to produce no cost in fitness (Andersson & Levin, 1999). Therefore, a certain degree of specificity in the fitness effect of a resistance mutation should be expected.

The fitness cost caused by a mutation should affect the frequency of the resistant strains carrying such mutation. It is expected that mutations with reduced cost will be the ones more frequently isolated from clinical environments. This prediction has been confirmed by several studies (O'Neill *et al.*, 2006; O'Sullivan *et al.*, 2005). For example, in *Mycobacterium tuberculosis* it has been shown that the most frequent rifampicin-resistant clinical isolates have mutations with small fitness cost (O'Sullivan *et al.*, 2005). Similarly, the rifampicin-resistance mutations with the smaller cost are the most common in clinical isolates of *S. aureus* (O'Neill *et al.*, 2006).

In the absence of the antibiotic selective pressure, it is logical to expect that, when a resistance mutation imposes a fitness cost, the resistant strains will be outcompeted by the

susceptible ones. This assumption suggests that a possible procedure to eliminate antibiotic resistance is to ban the use of the respective antibiotic. It is also natural to assume that the higher the fitness cost, the more effective the ban would be. This policy has been applied in different countries with varying results. For example, in Finland, a 50% decrease in the frequency of macrolide-resistant group A streptococci was observed after a deliberate reduction in the prescription of macrolides (Seppala *et al.*, 1997). However, in the UK, a 98% decrease in the consumption of sulfonamides was accompanied by an increase of 6,2% in the frequency of sulfonamide resistance in *Escherichia coli (Enne et al., 2001)*. Clearly, there are other factors affecting the reversal to susceptibility besides the fitness cost.

1.1.3. COMPENSATORY EVOLUTION

Resistant-bacteria evolution often involves second-site mutations that can ameliorate the fitness cost of resistance (Andersson & Levin, 1999; Bjorkman *et al.*, 2000; Emmerson & Jones, 2003; Gagneux *et al.*, 2006; Maisnier-Patin *et al.*, 2002; Schrag *et al.*, 1997). Compensatory mutations can significantly reduce or even eliminate the fitness cost associated with resistance. Evidence for compensatory evolution has been obtained in HIV (Borman *et al.*, 1996), *M. tuberculosis* (Gagneux *et al.*, 2006), *Salmonella typhimurium* (Maisnier-Patin *et al.*, 2002) and *E. coli* (Schrag *et al.*, 1997). Moreover, it has been shown that the environment affects the path of compensatory evolution in *S. typhimurium* (Bjorkman *et al.*, 2000). Resistant bacteria evolution was apparently mediated by different mechanisms within and outside a host (Bjorkman *et al.*, 2000). In resistant clinical isolates with no or a low fitness cost it is difficult to determine if this reduced cost is the outcome of compensatory evolution or if the resistance mutation produced no cost. (Andersson & Levin, 1999)

The evolution of resistant bacteria through compensatory mutations can result in a fitness landscape where resistant strains are fitter than the susceptible revertants (Levin *et al.*, 2000). Consistent with this, in *E. coli*, adaptation to the fitness cost associated with a streptomycin-resistance mutation was shown to be very effective, resulting in a resistant strain fitter than the susceptible (Schrag & Perrot, 1996; Schrag *et al.*, 1997). This fitness landscape made up of an adaptive valley means that it is very unlikely that resistant strains carrying compensatory mutations will revert to susceptibility (Schrag *et al.*, 1997).

1.1.4. REVERSAL MUTATIONS

Resistant bacteria evolution can involve spontaneous reversal mutations, which reestablish the susceptible phenotype. However, there are many more loci for compensatory than for reversal mutations. Thus, compensatory evolution has a much larger target and therefore is thought to be more likely to occur than the reversal to susceptibility (Schrag *et al.*,

1997). Moreover, in a strain well adapted to resistance, the occurrence of reversion mutations may result in lower host fitness (Levin *et al.*, 2000; Schrag *et al.*, 1997).

1.1.5. REACQUISITION OF RESISTANCE MUTATIONS

Another factor that can prevent the reversal to susceptibility is the spontaneous reacquisition of resistance mutations. Generally, the mutation rate is too low to undermine the reversal to susceptibility in an antibiotic-free environment (LeClerc *et al.*, 1996). Despite that, and as discussed earlier, the presence of hypermutators in the population can play a role in pathogenicity and resistance development (LeClerc *et al.*, 1996; Oliver *et al.*, 2000; Vulic *et al.*, 1997).

1.2. MOBILE GENETIC RESISTANCE DETERMINANTS

Antibiotic resistance can also result from the acquisition of novel genes located in mobile genetic elements such as integrons, transposons and plasmids (Martinez *et al.*, 2009). These accessory genetic elements make bacterial genomes more flexible. Their frequent exchange (Boucher *et al.*, 2003; Cohen & Pupko, 2009; Denamur *et al.*, 2000), between related and phylogenetically distant microbes (Amabile-Cuevas & Chicurel, 1992; Cohen & Pupko, 2009; Denamur *et al.*, 2000), has consequences for microbial evolution, including the emergence, dissemination and persistence of antibiotic resistance (Martinez & Baquero, 2002).

Integrons have a structure that facilitates recombination and gene recruitment and expression. They contain a gene coding for a site-specific recombinase, an attachment site and a strong promoter (Carattoli, 2001). Each gene is inserted separately as a *gene cassette* (Carattoli, 2001). Eventually, multiple resistance determinants can be found within an integron enabling multidrug resistance. Integrons are generally contained within transposons (Carattoli, 2001). Following a Chinese-box scheme transposons are, in turn, recruited by plasmids (Carattoli, 2001). This modular arrangement of horizontally mobile genetic elements facilitates the acquisition and dissemination of antibiotic resistance determinants. Moreover, transposons are capable of moving between plasmids and between a plasmid and the chromosome (Sota *et al.*, 2006).

Conjugative plasmids are able to mobilize their own transfer and the transfer of other plasmids from a donor to a recipient cell (Amabile-Cuevas & Chicurel, 1992). Conjugation allows resistance determinants located in plasmids to be exchanged among even distantly related bacteria (Amabile-Cuevas & Chicurel, 1992) Thus, the resistant phenotype can quickly spread among the population. There is almost no difference, other than the presence of resistance determinants, between plasmids from the pre-antibiotic era and plasmids found in strains isolated 40 years after humans started using antibiotics (Datta & Hughes, 1983; Hughes & Datta, 1983). This finding supports the notion that plasmids contribution to antibiotic

resistance dissemination is due solely to their activity as vectors for gene recruitment and transfer.

1.2.1. PLASMID-ENCODED RESISTANCE

Plasmid-encoded resistance to multiple antibiotics, including β-lactams, aminoglycosides, tetracyclines, macrolides and glycopeptides is widespread in an array of pathogenic microorganisms including vancomycin-resistant enterococci (VRE) and methicillin-resistant *Staphylococcus aureus* (MRSA) (Williams & Hergenrother, 2008). Recently, it has been reported the transfer of plasmids from VRE to MRSA, resulting in the vancomycin-resistant *S. aureus* (VRSA) strain, which worryingly exhibits a high level or multidrug resistance (Weigel *et al.*, 2003).

Plasmid-encoded antimicrobial resistance is generally the result of the activity of efflux pumps or agent-modifying enzymes (Andersson & Levin, 1999). For instance, aminoglycoside resistance in bacteria is primarily mediated by the presence of plasmid-encoded modifying enzymes (Shaw *et al.*, 1993) and resistance to β-lactam antibiotics frequently results from plasmid-borne β-lactamases (Weldhagen, 2004). Drug resistance through modification and/or protection of the target can also be mediated by plasmid-borne enzymes (Bennett, 2008). For example, resistance to macrolides, streptogramins and lincosamides is often the result of the methylation of an adenine of 23S rRNA (Galimand *et al.*, 2003). This modification is accomplished by *erm*-encoded methyltransferases (Galimand *et al.*, 2003). *erm* genes are most often acquired on plasmids (Galimand *et al.*, 2003). Resistance to fluoroquinolones in *Enterobacteriaceae* can be due to the extrusion activity of the plasmid-encoded efflux pump QepA (Cattoir & Nordmann, 2009).

1.2.2. PLASMID FITNESS COST

Similarly to the fitness cost produced by resistance-determining mutations in housekeeping genes, harboring mobile genetic elements also creates a cost to the host. Firstly, there is a cost associated with the replication and maintenance of the genetic element. Secondly, the expression of its genes, including the antibiotic resistance determinants, produces an additional load in the cell´s transcription and translation machinery. Such cost has been experimentally demonstrated in a number of resistance-encoding plasmids (Bouma & Lenski, 1988; McDermott *et al.*, 1993; Smith & Bidochka, 1998).

If carriage of a resistance plasmid produces a fitness cost in the host cell, then in an environment without the antibiotic selective pressure, the resistant cells are expected to be outcompeted by the susceptible ones. This assumption also suggests that to eliminate plasmid-borne resistance one must ban an antibiotic. Unfortunately, as it happens with chromosomal

resistance-mutations, the elimination of plasmid-borne resistance becomes a challenge due to several factors.

1.2.3. PLASMID AND CELL ADAPTATION

The fitness cost created by harboring a plasmid can be compensated (Bouma & Lenski, 1988; Dionisio *et al.*, 2005). For instance, transformation of *E. coli* cells with the non-conjugative resistance plasmid pACYC184 initially creates a fitness cost, in the absence of antibiotic. However, by evolving the transformed cells for 500 generations, the host chromosome undergoes adaptive changes that eliminate the cost associated with plasmid infection (Bouma & Lenski, 1988). Coevolving, for 800 generations, *E. coli* cells and the plasmid pBR322 resulted in amelioration of the initial deleterious effect of plasmid carriage. The adaptation was shown to be due to genetic changes in the host chromosome and in the plasmid that resulted in a decrease in plasmid copy number and an increase in the rate of segregational plasmid loss (Modi *et al.*, 1991). Recently, it has been shown that *E. coli* adapts to the plasmid R1 in as little as 420 generations, fully eliminating the fitness cost (Dionisio *et al.*, 2005). This adaptation appears to be the result of genetic change by the host and the plasmid. When the evolved R1 plasmid was inserted into the ancestral *E. coli* strain or into a new host, *Salmonella enterica*, it was able to increase their relative fitness (Dionisio *et al.*, 2005).

In addition, it has been reported that harboring some plasmids does not create a fitness cost or it can even give a fitness advantage to the host. For instance, the sulfonamide-resistance p9123 plasmid can confer a small fitness advantage on *E. coli* (Enne *et al.*, 2004). It seems this effect might be responsible for the maintenance of sulphonamide resistance, even in the absence of the antibiotic selective pressure.

1.2.4. GENE LINKAGE

Another factor that can contribute to the persistence of plasmid-borne resistance, even in the absence of the antibiotic, is the physical linkage between resistance determinants and other genes being selected for. Mobile genetic elements activity as platforms for gene recruitment and exchange has resulted in the inclusion in them of many genes, other than resistance determinants, that can confer a selective advantage. For example, virulence determinants are often found in plasmids and transposons (Martinez & Baquero, 2002). Heavy metal resistance determinants are also typically found in plasmids and other mobile genetic elements (Johnsen *et al.*, 2009). This linkage allows the *genetic hitch-hiking* of unselected resistance determinants when the environment selects for other linked traits (O'Brien *et al.*, 2002).

1.2.5. HORIZONTAL GENE TRANSFER

The reacquisition of resistance through ongoing horizontal gene transfer (HGT) must also be considered when dealing the reversal to susceptibility. A high rate of HGT may undermine the reversal of resistance by directly supplying resistance determinants to susceptible cells within the same population. Cells may lose plasmids through segregation. Therefore, the segregation rate must also be considered. When the transfer and segregation rates compensate each other, the fate of a plasmid in a population is dependent on its fitness cost only. Recently, experimental evidence of the existence of *E. coli* strains with a higher-than-normal donor ability has been gathered (Dionisio *et al.*, 2002). These strains have an amplifying effect, allowing a conjugative plasmid (the R1 plasmid) to quickly disseminate among the population (Dionisio *et al.*, 2002). Such finding supports the notion that the influence of recurring HGT on reversal of resistance may be substantial.

1.2.6. PLASMID STABILITY SYSTEMS

Plasmids have evolved systems that promote their persistence within a population. For example, the postsegregation killing (PSK) system promotes plasmid stability by selectively killing cells that have lost the plasmid (Engelberg-Kulka & Glaser, 1999). This effect is the result of a plasmid-encoded toxin. In a plasmid-containing cell, the toxin activity is blocked by the expression of an antitoxin, also encoded in the plasmid (Engelberg-Kulka & Glaser, 1999). The plasmid loss results in the death of the plasmid-free cell because the toxin is more stable in the cytoplasm than the antitoxin (Engelberg-Kulka & Glaser, 1999). The presence of a PSK system in a resistance plasmid will influence the reversal to susceptibility, even in the absence of the drug-selective pressure.

1.3. MULTI-DRUG RESISTANCE

There are several reports of multiresistant pathogens accumulating mutations and resistance plasmids (Minarini *et al.*, 2008; Morgan-Linnell *et al.*, 2009; Strateva & Yordanov, 2009; Zhang *et al.*, 2008). For instance, in *Pseudomonas aeruginosa*, an important agent of nosocomial infections, all possible mechanisms of antimicrobial resistance have been found and can be the product of mutations in housekeeping genes and/or be coded in integrons (chromosome and/or plasmid-borne (Strateva & Yordanov, 2009). A recent epidemiological survey of 257 nalidixic acid-resistant enterobacterial clinical isolates from Brazil showed that 2.3% accumulated mutations in *gyrA* or *parC* with *qnr*-like genes involved in plasmid-mediated quinolone resistance (Minarini *et al.*, 2008). In this study, three *E. coli* and two *Klebsiella pneumonia*e strains harbored *qnrB2* and a single *Citrobacter freundii* isolate had the *qnrB8* gene. The *qnrB2* genes were shown to be located in large (55-154kb) plasmids, three of them capable of conjugative transfer (Minarini *et al.*, 2008). The *qnrB8* gene of the *C. freundii* appeared to be located in the chromosome. A single *K. pneumoniae* isolate also carried an extended-spectrum β-lactamase $bla_{CTX\text{-}M\text{-}2}$ plasmid-borne gene (Minarini *et al.*, 2008).

Another recent large-scale survey of fluoroquinolone-resistant clinical isolates revealed that all isolates were *gyrA* mutants, 85% also had *parC* mutations and 23% harbored the plasmid-borne *aac(6´)-Ib-cr* gene (Morgan-Linnell *et al.*, 2009). Moreover, the ciprofloxacin and norfloxacin MICs (minimal inhibitory concentration) for isolates who accumulated chromosomal mutations with the *aac(6´)-Ib-cr* plasmid-borne resistance determinant were significantly higher (Morgan-Linnell *et al.*, 2009). This suggests that simultaneously harboring a resistance plasmid and a resistance mutation can produce a phenotype significantly more resistant than having a single or even several resistance mutations. This is a worrying scenario because the frequent horizontal gene transfer can easily spread plasmid-borne resistance genes in a chromosome-mutation resistant population. Genetic analysis of a multi-resistant strain of *Klebsiella oxytoca*, an opportunistic pathogen, recently isolated from a patient in China, revealed the presence of a mutation in *gyrA* and a 55kb conjugative plasmid carrying three resistance determinants: *armA*, $bla_{\text{CTX-M-15}}$ and $bla_{\text{TEM-1}}$ (Zhang *et al.*, 2008).

An in-depth study of an *E. coli* environmental-isolate displaying outstanding levels of resistance to multiple drugs, revealed the simultaneous presence of both classes of resistance determinants: chromosomal mutations and mobile genetic elements (Fricke *et al.*, 2008). Several of the latter were mapped to a 130kb plasmid (pSMS35_130). pSMS35_130 individual resistance gene cassettes show significant sequence conservation with isolates from multiple phylogenetic and geographic origins (Fricke *et al.*, 2008). This finding supports both the role of environmental bacteria in the dissemination of resistance determinants and the co-existence of both classes of resistance determinants within strains with high-levels of antibiotic resistance.

Multi-resistance can also come as a result of the simultaneous coexistence in the same cell of several plasmids. This situation has been reported in clinical strains of *Pasteurella multocida* carrying two or three small plasmids, conferring resistance to tetracyclines, streptomycin and sulfonamides (San Millan *et al.*, 2009). Worryingly, one of these small plasmids was successfully mobilized into *E. coli* cells by the conjugation machinery of an IncP plasmid (San Millan *et al.*, 2009). This finding suggests that small plasmids carrying resistance determinants can coexist within the same host and be successfully transferred by horizontal gene transfer to other cells. The coexistence of several plasmids was also reported in a clinical isolate of *Borrelia burgdorferi* (Casjens *et al.*, 2000). The above cited study of quinolone-resistant clinical isolates (Minarini *et al.*, 2008) seems to also support the notion that the coexistence of several plasmids within the same host may be a common phenomenon, since all the isolates carrying *qnrB*-like genes had three to seven plasmids. Interestingly, analysis of the *K. pneumoniae* isolate with the $bla_{\text{CTX-M-2}}$ and *qnrB2* genes revealed that these two resistance determinants were located in distinct plasmids, which is another report for a pathogenic strain carrying multiple resistance plasmids. A study of extended-spectrum β-lactamase(ESBL)-producing *Enterobacteriacea* and *Pseudomonadacea* clinical isolates, mapped their several resistance determinants to resistance plasmids and several cases of co-existence of plasmids within the same isolate were identified (Chanawong *et al.*, 2001). Interestingly, for *Klebsiella pneumoniae*

Chanawong et al further gathered evidence supporting the occurrence of horizontal gene transfer within the hospital as a means of adding resistance determinants to an already resistant strain (Chanawong *et al.*, 2001).

2. EPISTASIS

Epistasis or gene interaction occurs when the phenotypic effect of a mutation in a locus depends on mutations present at other loci. Recently, a study of the interaction between resistance-determining chromosomal mutations, responsible for resistance to nalidixic acid, rifampicin and streptomycin in *E. coli* found that, in the majority of the cases, the combined fitness cost of double resistance is smaller than what one would expect if they were independent (Trindade *et al.*, 2009). This finding suggests that the occurrence of an epistastic interaction between deleterious resistance mutations is a driving force in the acquisition, dissemination and persistence of multidrug resistance (Trindade *et al.*, 2009).

Epistasis is generally accepted as being relevant for the understanding of the evolution and dynamics of complex genetic systems (Phillips, 2008). More specifically, it is currently debated if the occurrence of epistasis between deleterious mutations has contributed to the emergence of genetic recombination (Kouyos *et al.*, 2007). Epistasis can vary in strength and form. When epistasis affects fitness, one can expect two possible outcomes. A positive epistastic interaction has an antagonistic effect on deleterious mutations. In this case, the double mutant has a higher fitness than the sum of the effect of each mutation separately. Negative epistasis between deleterious mutations creates a synergistic effect. Here, the double mutant is less fit than the sum of the effect of each the individual mutations separately.

Different studies of epistasis gathered evidence for both antagonistic and synergistic gene interaction. Positive epistasis between random deleterious mutations has been experimentally detected in phage ΦX174 (Silander *et al.*, 2007), HIV-1 (Parera *et al.*, 2007), RNA virus Φ6 (Burch & Chao, 2004), *Salmonella typhimurium* (Maisnier-Patin *et al.*, 2005) and in the yeast *Saccharomyces cerevisae* (Jasnos & Korona, 2007). Other studies have found no evidence of epistastic interactions within the HIV-1 transcriptional promoter (van Opijnen *et al.*, 2006), in RNA virus (Crotty *et al.*, 2001; Elena, 1999) and in *Saccharomyces cerevisae* (Jasnos & Korona, 2007), or evidence that positive epistasis occurs as often as negative epistasis in RNA virus (Sanjuan *et al.*, 2004) and in *E. coli* (Elena & Lenski, 1997). Interestingly, an *in silico* study of phage T7 mutants suggests that the form and strength of the interaction depends on environment and mutation severity (You & Yin, 2002). In their study, You and Yin found that severely deleterious mutations always interacted antagonistically. Mildly deleterious mutations also interacted synergistically in resource-poor environments. However, in resource-rich environments, mildly deleterious mutations interacted antagonistically (You & Yin, 2002). Thus, available data suggests that there is no general rule for predicting the nature and form of epistasis.

1. BACTERIAL STRAINS AND GROWTH CONDITIONS

We used six natural conjugative plasmids, R124, R702, R16, R831, RP4 and R16a, given by the Institute for Health, Environment and Safety of the Belgian Nuclear Research Centre. Plasmid characteristics are listed in Table 1. We introduced these plasmids in wild-type *E. coli* K12 MG1655 and in a set of 10 spontaneous antibiotic-resistant clones derived from the wild-type strain (Table 2). These mutations have been previously mapped to *gyrA*, *rpoB* and *rpsL* resulting in resistance to nalidixic acid, rifampicin and streptomycin (Trindade et al. 2009).

In the competition assays, we used *E. coli* K12 MG1655 Δara as "reference strain". Due to a deletion in the arabinose operon this strain produces red colonies when grown in tetrazolium arabinose (TA) indicator agar, allowing it to be distinguished from its competitor, which produces white colonies.

All bacterial strains were grown in liquid Luria-Bertani (LB) medium at 37ºC with agitation. Solid media was obtained by the addition of agar (15 g/L). For growth and transconjugant selection, antibiotics were added as follows: 40 μg/mL of nalidixic acid, 100 μg/mL of rifampicin, 100 μg/mL of streptomycin, 20 μg/mL of tetracycline, 100 μg/mL of kanamycin and 100 μg/mL of ampicilin. For the construction of bacterial strains with conjugative plasmids, transconjugant selection was performed in M9 minimal medium (56.4 g/L M9 minimal salts, 2 mM magnesium sulfate, 4 g/L sugar (see below), 15 g/L agar), supplemented with the appropriate antibiotics.

Table 1 – Donor strains. List of the *Escherichia coli* donor strains used in the plasmid transfer experiments. All strains are auxotrophic for specific amino-acids and/or sugars, due to deletions in essential genes/operons, as indicated in chromosomal markers: Mal^-: maltose; Lac^-: lactose; Trp^-: tryptophan; Met^-: methionine and Pro^-: proline. Plasmid features listed include incompatibility grouping and antibiotic resistance markers (Tc: tetracycline; Km: kanamycin; Sm: streptomycin; Ap: ampicilin; Su: sulphonamides). All plasmids were obtained from the Belgian Nuclear Research Centre (S.C.K. – C.E.N.).

Bacterial strain	Plasmid harbored	Chromosomal markers	Plasmid incompatibility group	Plasmid markers
E. coli CM317	**R124**	Mal^- Trp^-	IncF4	Tc
E. coli CM319	**R831**	Mal^- Trp^-	IncL	Km, Sm
E. coli CM312	**R16**	Mal^- Trp^-	IncB	Ap
E. coli CM597	**R702**	Met^- Pro^-	IncP	Tc, Km, Sm, Su
E. coli CM140	**RP4**	Met^- Pro^-	IncP1	Ap, Km, Tc
E. coli CM517	**R16a**	Lac^- Trp^-	IncA/C	Ap, Km, Su

Table 2 – Spontaneous antibiotic resistant mutants and resistance fitness cost. List of the *Escherichia coli* K12 MG1655 spontaneous mutants used in the present study and the fitness cost associated with each resistance allele (Nal: nalidixic acid; Rif: rifampicin; Sm:

streptomycin). Mutations were mapped through target-gene amplification (*gyrA*, *rpoB* and *rpsL*) and sequencing. The same primers were used for sequencing straight from the PCR product and their complete nucleotide sequence is available in Trindade et al. (Trindade *et al.*, 2009).

Mutation	**Gene**	**Genotype** **amino acid change; nucleotide change**	**Fitness cost (2*standard error) %**	**Resistance**
1N	*gyrA*	D 87 G ; GAC to GGC	3.7 (1.5)	Nal
2N		S 83 L; TCG to TTG	3.3 (1.2)	
3N		D 87 Y; GAC to TAC	3.1 (1.9)	
2R	*rpoB*	H 526 N; CAC to AAC	1.4 (1.1)	Rif
5R		I 572 F; ATC to TTC	14.6 (1.2)	
8R		R 529 H; CGT to CAT	26.2 (4.9)	
1S	*rpsL*	K 43 R; AAA to AGA	0.5 (1.4)	Sm
3S		K 88 E; AAA to GAA	27.5 (2.8)	
4S		K 43 N; AAA to AAC	18.0 (1.9)	
5S		K 88 R; AAA to AGA	6.1 (1.2)	

2. PLASMID TRANSFER

Conjugation assays were performed to insert the six naturally conjugative resistance plasmids used in this study (Table 1) into antibiotic-susceptible *E. coli* K12 MG1655. This allows the estimation of the fitness cost created by the plasmid carriage of each plasmid. Conjugation was also used to produce the strains corresponding to all possible combinations between five conjugative resistance plasmids (R124, R831, R16, R702 and RP4) and all antibiotic-resistant chromosomal-mutants (Table 2). A final set of conjugations between plasmid-carrying strains was performed to produce all possible pair-wise combinations between six plasmids (Table 1). Since plasmids RP4 and R702 belong to the same incompatibility group, the resulting set of transconjugant strains included only 14 strains, representing all possible pair-wise combinations of the six plasmids used.

Three different protocols for the conjugation experiments were used: conjugation in liquid media; conjugation in membrane; and conjugation in solid media. All plasmid transfers were first attempted in liquid media. The two other protocols were only employed when conjugation in liquid media failed to produce transconjugants.

2.1. CONJUGATION IN LIQUID-MEDIA

Donor and recipient strains were grown in 10mL of Luria-Bertani (LB) liquid media supplemented with the appropriate antibiotics. Strains were incubated at 37ºC, in an orbital shaker (150 rpm), for 24 hours. From the resulting over-night cultures, 200 μL were transferred to 10 mL of fresh LB and the tubes incubated for 5 hours, at 37ºC, with aeration (150 rpm). To enable plasmid transfer, we mixed 200 μL of the donor and recipient strains in one 50 mL screw-cap tube containing 10 mL of fresh LB and incubated the mixed culture for 24 hours, at 37ºC, with no agitation. Agitation was prevented to facilitate contact between donor and recipient cells and attachment of the sex-pili, and hence maximize the rate of conjugation. To screen for transconjugants, i.e. recipient cells that received the plasmid, 100 μL of the overnight conjugation culture was plated on selective minimal media plates supplemented with agar and the appropriate antibiotics for selection of transconjugant cells only, and incubated for 48 hours, at 37ºC. To ensure that colonies growing on these selective minimal media plates were indeed transconjugants and not spontaneous mutants of the donor or recipient strain, a negative control was performed in every conjugation experiment. This negative control consisted in plating, individually, the donor and the recipient strains in selective minimal media plates supplemented with agar and the appropriate antibiotics. This minimal media selects against donor cells, even in the absence of antibiotics, due to the nutritional auxotrophy of the donor strains (Table 1). Despite that, antibiotics which correspond to the resistance markers of the recipient cells were added to the media. Similarly, recipient cells were counter-selected by using minimal media containing the antibiotics corresponding to the resistance markers of the plasmid present in the donor cell. The negative control plates were incubated for 48 hours, at 37ºC. When colonies were recovered in the negative control plates, the conjugation assay was repeated and the colonies present on the plates selective for transconjugants discarded.

When the negative control was successful, i.e. when control plates contained no colonies after incubation in 37ºC for 24 hours, we pursued the conjugation experiment, by obtaining a pure culture of a transconjugant clone. To that end, a single colony (or an area of confluent growth) was re-streaked in a new plate of selective M9 minimal media (supplemented with agar and antibiotics) and incubated for 48 hours, at 37ºC. After incubation, an isolated colony was selected and re-streaked onto to another plate of selective M9 minimal media and incubated for another 48 hours. From the resulting plate, an isolated colony was chosen and stock of that individual clone produced, following the protocols previously described in Methods - Bacterial Strains and Growth Conditions.

2.2. CONJUGATION IN SOLID-MEDIA

As in liquid-media conjugation, donor and recipient strains were grown in 10 mL of Luria-Bertani (LB) broth supplemented with the appropriate antibiotics. Strains were incubated at

37ºC, in an orbital shaker (150 rpm), for 24 hours. From the resulting over-night cultures, 200 μL were transferred to 10 mL of fresh LB broth in 50 mL screwcap tubes and incubated for 5 to 6 hours, at 37ºC, with aeration (150 rpm). To enable plasmid transfer, 200 μL of the donor and the recipient strains were mixed in an eppendorf and 100μL of the mixture were plated on LB supplemented with agar (1.5%). This plate was incubated for 24 hours, at 37ºC. After incubation, confluent bacterial growth was visible on top of the agar. Since the media was non-selective, one can assume that this bacterial lawn resulted from the growth of both donor and recipient cells, and these cells should have been able to conjugate in the plate as there is no physical disruption and there are suitable conditions for growth (appropriate temperature and nutrient availability). Thus, transconjugant cells should have also been part of the visible confluent growth. To isolate transconjugants, the bacterial growth found on one fourth of the plate surface was recovered and resuspended in 500 μL $MgSO_4$ 0.01 M. From this very dense cell suspension, 100 μL were plated on selective minimal media supplemented with agar and the appropriate antibiotics to select for transconjugants. Transconjugant-selective plates were incubated for 48 hours, at 37ºC. To obtain a pure culture of the transconjugant clones, the same techniques for isolating a single bacterial clone previously described for liquid conjugation were followed and stock produced.

To ensure that cells growing on the transconjugant-selective minimal media plates were indeed transconjugants and not spontaneous mutants of the donor or recipient cells, a negative control was performed with every solid-media conjugation experiment. To that end, 100 μL of the donor and recipient strains were individually plated onto LB plates supplemented with agar and incubated for 24 hours, at 37ºC. The bacterial growth of one fourth of the plate was recovered and resuspended in 500 μL $MgSO_4$ 0.01 M. From this suspension, 100 μL were plated on minimal media plates supplemented with agar and with the appropriate antibiotics to select against either donor or recipient cells. Control plates were incubated for 48 hours, at 37ºC. Transconjugants were only purified when no growth was recovered on the negative control plates.

2.3. CONJUGATION IN MEMBRANE

This conjugation protocols started by inoculating 50 mL screw-cap tubes with 10 mL of fresh LB broth supplemented with the appropriate antibiotics, with either the donor or the recipient bacterial strains. Both strains were incubated overnight, at 37ºC, with aeration (150 rpm). From the dense over-night culture, 50 μL were transferred to a 50 mL screw-cap tube containing 10 mL of fresh LB broth. The tubes were incubated in an orbital shaker (150 rpm), at 37ºC. At regular time intervals, a 800 μL sample was taken from the growing cultures and its optical density (OD) at a wavelength of 670 nm determined using a Thermo Scientific Genesys 10UV spectrophotometer. Donor and recipient strains incubation was pursued until cultures reached

an optical density between 0.8 and 1.0. When the required OD was reached, growth was inhibited by immediately placing the culture tubes in ice. This procedure ensured that the donor and recipient strains cultures had the appropriate average cell density to allow the use of the membrane filter. Then, in a 5 mL screw-cap tube, 1mL of the donor strain was mixed with 1 mL of the recipient strain. The total volume of 2 mL of this mixed culture was introduced into a 10 mL sterile needless syringe. The syringe was then attached to an apparatus consisting of a membrane filter holder and, in its interior, a Schleicher & Schuell 0.45 μm pore size membrane filter. The 2 mL volume of donor and recipient cells was filtered through this membrane filter and the supernatant discarded. If further growth of the cultures had been allowed, the high cell density reached could saturate the membrane filter, preventing the culture flow. The membrane filter was then transferred onto a LB plate supplemented with agar (1.5%) and incubated for 24 hours, at 37ºC. This protocol ensures that donor and recipient cells are kept in close proximity, with minimal disturbance, therefore optimizing DNA transfer rates. After incubation, the membrane filter was resuspended in 5 mL of $MgSO_4$ 0.01 M and cells released by swirling in a vortex mixer during 2 minutes. To identify transconjugants, 100 μL of the resulting cell suspension were plated on selective minimal media supplemented with agar and the appropriate antibiotics to select for transconjugants. The plates were incubated for 48 hours, at 37ºC and screened for growth.

To ensure that the growth in the plates selective for transconjugants was, in fact, due to recipient cells who received the plasmid, a negative control was performed with every membrane conjugation experiment. To that end, 100 μL of the donor and recipient strains cultures were plated in solid selective minimal media appropriately supplemented with antibiotics which would select against either the donor or recipient strains. These plates were then incubated for 48 hours, at 37ºC. The remaining of the protocol is identical to the two previously described methods (liquid and solid-media conjugation) and it included performing a negative control and obtaining a pure culture a transconjugant clone.

3. FITNESS ASSAYS

Competition assays were performed to determine the fitness cost of the resistance determinants, either the plasmid carriage alone, the coexistence of both plasmid and mutation and the carriage of two plasmids. The method used has been previously described by Trindade et al. 2009. The strains carrying resistance determinants were competed against a susceptible reference strain, *E. coli* K12 MG1655 Δara, in an approximate proportion of 1:1 and in the absence of antibiotic selective pressure. First, both strains were grown in liquid LB medium for 24 hours at 37ºC with aeration. Secondly, values of the each strain initial ratio were estimated by plating a dilution of the mixture in TA agar. Competitions were performed in 50 mL screw-cap tubes containing 10 mL of LB liquid medium by a period of 24 hours at 37ºC with aeration. At

the end of the competition, appropriate dilutions were plated onto TA agar plates to obtain the final ratios of both competitors. If a high fitness cost precluded the resistant strain of being recovered in the TA plates, a smaller dilution was plated onto minimal medium supplemented with arabinose, which doesn't allow the growth of the reference strain. The fitness cost of each strain – i.e. the selection coefficient – was estimated as the per generation difference in Malthusian parameters for the being-tested and reference strains, discounted by the cost of the Δara marker (Lenski *et al.*, 1991). The fitness cost was estimated as an average of three independent competition assays.

Let *S* and *R* represent the susceptible and resistant strains respectively. The fitness of the antibiotic susceptible strain is set as 1. The relative initial (R_0 and S_0) and final frequencies (R_T and S_T) of the antibiotic-resistant and the susceptible strains allow the determination of the fitness of each resistant strain. One can calculate strain fitness by two distinct procedures (Lenski, 1991), as strain performance can be mathematically expressed differently: i) Malthusian fitness and ii) relative fitness. Relative fitness is a dimensionless quantity, which is calculated as the ratio of the growth rate of one strain relative to that of another during their direct competition:

$$W = \frac{Log_2(R_T / R_0)}{Log_2(S_T / S_0)} \qquad \text{(Equation1)}$$

Malthusian fitness involves calculating strain fitness from the selection rate (r) (or selection coefficient). This seglection rate is the per generation (or per unit of time) difference in the Malthusian parameters ($Log_2((R_T + S_T)/(R_0 + S_0))$) of the two strains. In this study, strain Malthusian fitness was calculated from the selection coefficient as follows:

$$W = 1 + Log_2\left[\frac{(R_T / R_0)}{(S_T / S_0)}\right] / Log_2((R_T + S_T)/(R_0 + S_0)) \qquad \text{(Equation 2)}$$

Both methods to estimate fitness produce the same (or approximate) result as they can be shown to be qualitatively equivalent and, quantitatively almost equivalent.

If one competitor is *not* much less fit than the other, e.g., if:

$Log_2((R_T + S_T)/(R_0 + S_0)) \approx Log_2(S_T / S_0)$

Now, one can introduce this simplification into Equation 2:

$W \approx 1 + Log_2\left[\frac{(R_T / R_0)}{(S_T / S_0)}\right] / Log_2(S_T / S_0)$, which is equivalent to:

$W \approx \frac{Log_2(S_T / S_0)}{Log_2(S_T / S_0)} + \frac{Log_2\left[\frac{(R_T / R_0)}{(S_T / S_0)}\right]}{Log_2(S_T / S_0)}$, or

$W \approx \frac{Log_2\left((S_T / S_0) \cdot \frac{(R_T / R_0)}{(S_T / S_0)}\right)}{Log_2(S_T / S_0)}$, which simplifies to:

$W = \frac{Log_2(R_T / R_0)}{Log_2(S_T / S_0)}$, and this is the same as Equation 1.

The only situations where the two methods yield different results are: i) when one competitor is much less fit than the other; or ii) when both competitors are declining in abundance, such as during competition assays under starvation conditions or in the presence of an antibiotic. In such cases, it is not appropriate to calculate Malthusian fitness and one should calculate relative fitness. None of these scenarios apply to the conditions on the competition assays performed in the present study. Hence, although strain fitness was calculated with both methods for all strains, the determination of fitness cost and all other calculations from then on were based on the Malthusian fitness only.

The fitness cost (c) associated with a single resistance determinant or a combination of determinants can be calculated from the strain fitness as follows:

$$c = 1 - W + c_{\Delta\text{ara}}$$

where $c_{\Delta\text{ara}}$ is the fitness cost associated with the marker (deletion on the arabinose operon) of the susceptible strain (3.6%) as determined by Trindade et al. (Trindade *et al.*, 2009). To test if the fitness costs experimentally determined were indeed different from zero, we performed a t-Student test.

Conjugative transfer of the plasmids to the reference strain during competition was monitorized for the wild type *E. coli* K12 MG1655 carrying each plasmid. At the end of the competition, one hundred isolated Δara colonies were tooth picked from the TA plates to LB medium supplemented with the appropriate antibiotics.

4. MEASURE OF EPISTASIS AND STATISTICAL SIGNIFICANCE

Pairwise epistasis, ε, can be measured assuming a multiplicative model in which: $\varepsilon = W_{\{-;-\}} W_{\{mut;pla\}} - W_{\{-;pla\}} W_{\{mut;-\}}$, where $W_{\{pla;mut\}}$ is the fitness of the strain carrying a chromosomal mutation and a plasmid and $_{\{-;-\}}$ represent the absence of the mutation and/or plasmid. We assume that the fitness of the wild-type strain ($W_{\{-;-\}}$) is equal to one. Therefore, epistasis can be calculated as $\varepsilon = W_{\{mut;pla\}} - W_{\{-;pla\}} W_{\{mut;-\}}$. Similarly, by following a multiplicative model to estimate pairwise epistasis between conjugative plasmids, we can define epistasis as $\varepsilon = W_{\{plasmid\ 1;plasmid\ 2\}} - W_{\{-;plasmid\ 2\}} W_{\{plasmid\ 1;-\}}$. Error ($\sigma_\varepsilon$) of the value of ε is then estimated by the method of error propagation:

(a) for pairwise combinations of mutation and plasmid:

$$\sigma_\varepsilon \sqrt{W_{\{mutation;plasmid\}}{}^2 \sigma_{W\{-;-\}}{}^2 + W_{\{-;-\}}{}^2 \sigma_{W\{mutation;plasmid\}}{}^2 + W_{\{mutation;-\}}{}^2 \sigma_{W\{-;plasmid\}}{}^2 + W_{\{-;plasmid\}}{}^2 \sigma_{W\{mutation;-\}}{}^2}$$

(b) for pairwise combinations of plasmids:

$$\sigma_{\boldsymbol{\varepsilon}} = \sqrt{\mathbf{W}\{plasmid1;plasmid2\}^2 \sigma W\{-;-\}^2 + \mathbf{W}\{-;-\}^2 \sigma W\{plasmid1;plasmid2\}^2 + \mathbf{W}\{plasmid1;-\}^2 \sigma W\{-;plasmid2\}^2 + \mathbf{W}\{-;plasmid2\}^2 \sigma W\{plasmid1;-\}^2}$$

If the value of ε was within the error, we considered that the two resistance determinants (mutation and plasmid or plasmid and plasmid) did not show any significant epistasis (indicated as white boxes labeled “no epistasis” in Figures 2 and 6). From the

distribution of values of ε, provided in Figures 3A and 7, we calculated the median value of ε and its 95% CI by bootstrap where we took 10.000 samples. We tested normality of both distributions of ε values by a Shapiro-Wilk normality test. The data regarding interactions between mutations and plasmids differs from a normal curve (p-value=0.000968), while the distribution of ε values between plasmids does not significantly differ from a normal curve (p-value=0.219).

In order to assess if there were significant differences between the mean epistastic value of the resistance mutations and that of the conjugative plasmids, a Kruskal-Wallis test was performed for both data sets, i.e. comparison between plasmids and comparison between mutations. Significant differences were only found in the mean epistastic value between conjugative plasmids (p-value=0.0016).

To test the presence of sign epistasis, we compared the fitness of the each strain carrying two resistance determinants (mutation and plasmid or plasmid and plasmid) and its corresponding single resistance-determinant strains. We used a Mann-Whitney U-test to assess if the fitness of the double-resistance-determinants strain was higher than the fitness of any of the single resistance-determinants strains. The p-values are indicated in Figure 8 for those combinations that provided significant results, at 5% confidence level.

It is notewhorthy to mention that epistasis can also be defined according to an additive model: $\varepsilon = W_{ab} - W_{Ab} + W_{aB}$. The additive and multiplicative models can be shown to be equivalent when the fitness costs are relatively small. According to the multiplicative model, epistasis is given by: $\varepsilon_{mult} = W_{AB}W_{ab} - W_{Ab}W_{aB}$

Given that the relative fitness of the wild type strain is unity, $W_{AB} = 1$, then: $\varepsilon = W_{ab} - W_{Ab}W_{aB}$.

Assuming that the resistance mutations create a fitness cost to the bacterial cell:

$W_{Ab} = 1 - c_b$; $W_{aB} = 1 - c_a$; $W_{ab} = 1 - c_{ab}$, Therefore, according to the multiplicative model, epistasis is given by:

$$\varepsilon_{mult} = 1 + W_{ab} - W_{Ab} - W_{aB} + c_a c_b =$$
$$= \varepsilon_{addit} + c_a c_b \approx \varepsilon_{addit}$$

The approximation is possible if the costs are low. For example, if costs as 3%=0.03, then the product is 9×10^{-4}, a number that is close to zero and can be discarded.

Hence, in the present study epistasis was only estimated according to the multiplicative model.

Statistical analyses performed using software Statistica 9.0 and MatLab R2009b.

III. RESULTS

1. INTERACTION BETWEEN ANTIBIOTIC RESISTANCE MUTATIONS AND RESISTANCE PLASMIDS

Pairwise epistasis, ε, between loci A and B can be measured as follows. If W_{AB} is the fitness of the wild-type, W_{Ab}, W_{aB} are the fitnesses of each of the single mutants and W_{ab} that of the double mutant, then multiplicative epistasis is given by: $\varepsilon = W_{AB}W_{ab} - W_{Ab}W_{aB}$. The aim of this study was to understand the epistasis between chromosomal alleles conferring antibiotic resistance and resistance plasmids in the evolution of multidrug resistance. For this, epistasis between plasmids and mutations was estimated and defined in a similar way. If $W_{(A;-)}$ is the fitness of the wild-type, $W_{(A;plasmid)}$ is the fitness of the wild-type strain containing a plasmid, $W_{(a;-)}$ is the fitness of the single mutants, and $W_{(a;plasmid)}$ is the fitness of the strain containing both the mutation and the plasmid, then one can define multiplicative epistasis between a plasmid and a chromosomal mutation as:

$$\varepsilon = W_{(A;-)}W_{(a;plasmid)} - W_{(A;plasmid)}W_{(a;-)}$$

Six naturally occurring conjugative resistance plasmids (Table 1) were introduced in *Escherichia coli* K12 MG1655 cells by bacterial conjugation. Then, the fitness cost due to the presence of each plasmid relative to plasmid-free *E. coli* K12 MG1655 cells was determined (Figure 1). This was performed using a competition assay, in the absence of antibiotics, against a marked strain (*E. coli* K12 MG1655 Δara). On average, plasmid load created a fitness cost of 5.6% (2*standard error=1.1%). No correlation between plasmid size or number of resistance markers and the fitness cost created by the insertion of each plasmid was found.

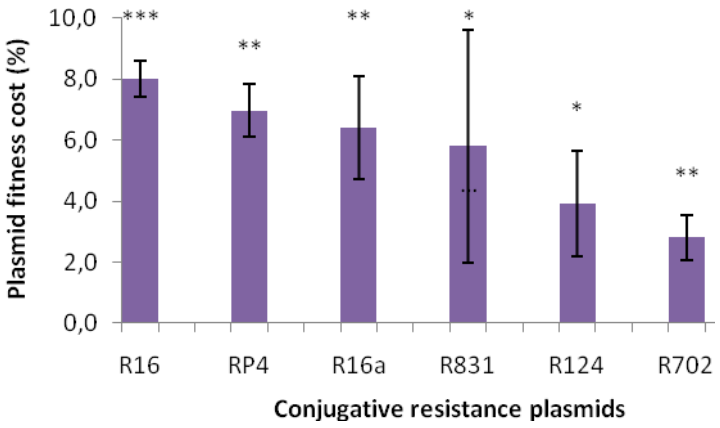

Figure 1 – Resistance conjugative plasmids fitness cost. Fitness cost created by inserting each resistance conjugative plasmid in *Escherichia coli* K12 MG1655. Error bars represent 2*standard error. On average, plasmid load created a fitness cost of 5.6% (2*standard error=1.1%). Plasmid fitness cost statistical significance was assessed by performing a Student

T-test for each plasmid. P-values are indicated on top of the bars (***:p-value<0.001; **:p-value<0.01 and *:p-value<0.05).

Table 3 – Monitoring of conjugative transfer of plasmids to reference strain. Total cell density and proportion of transconjugants for each plasmid studied.

	Beginning of stationary phase		**End of competition (24 hours)**	
	Total cell density (CFU/mL)	Transconjugants (% of Δara)	Total cell density CFU/mL	Transconjugants CFU/mL (% of Δara)
R124	$1,04X10^9$	1	$1,79X10^9$	18
R702	$6,90X10^8$	2	$1,43X10^9$	2
R16	$1,08X10^9$	0	$1,42X10^9$	1
R831	$6,90X10^8$	0	$1,28X10^9$	14
RP4	$1,30X10^9$	1	$1,30X10^9$	30

The fitness cost of ten *E. coli* K12 MG1655 spontaneous mutants was determined in Trindade *et al* (Trindade *et al.*, 2009). These clones carry single nucleotide changes in housekeeping genes involved in replication (*gyrA*), transcription (*rpoB*) and translation (*rpsL*). These genes are targeted by the following antibiotics: the quinolone nalidixic acid, the rifamycin rifampicin, and the aminoglycoside streptomycin, respectively. Table 2 shows the fitness costs of these mutations. Overall, the mean fitness cost of these mutations is 10% (2*standard error=1.1%).

To screen for epistastic interactions between chromosomal mutations and conjugative plasmids, 5 plasmids were selected from our plasmid set and, by conjugation, all possible 50 combinations between mutations and plasmids were constructed. By using the previously mentioned competition assay, the fitness of each of these 50 combinations was estimated. All the values of fitness cost are significantly different from zero ($p<0.05$), except the following strains: *rpsL* K43R (R16), *rpsL* K43R (R831) and *gyrA* D87G (R16). In these three *E. coli* strains it appears that having a mutation in a housekeeping gene and harboring a conjugative plasmid does not create a fitness cost. This value was used to estimate pairwise epistasis, ε, between mutation and plasmid.

In theory, the occurrence of plasmid transfer from the strain under study to the reference strain could bear a cost on the reference strain, artificially raising the resistant strain's fitness. Therefore, the appearance of transconjugants was monitored over time and was found to occur significantly only later in the competition, after maximum cell densities were reached (Table 3).

Epistasis was detected in 30 out of 50 combinations (60%) (Figure 2). Strikingly, the vast majority of the cases with epistasis corresponded to positive epistasis (26/30 or 87%), an antagonistic interaction that diminishes the fitness cost created by each isolated resistance determinant. Negative epistasis, a synergistic interaction where the fitness cost of the strain carrying both the mutation and the plasmid is higher than the independent sum of such costs,

was detected in only 4 of the 50 combinations studied (8%). Figure 2 further shows that the nature of the epistastic interaction is not gene but allele specific.

The same pattern had been observed for epistasis between deleterious resistance mutations in a previous study (Trindade *et al.*, 2009). In fact, the conjugative resistance plasmid influences how a specific allele interacts with plasmid-borne resistance determinants. That is, depending on the plasmid, the same allele can display no epistasis, positive or negative epistasis. For example, allele *gyrA D87G* exhibits no epistastic interaction with plasmid R124, however the same allele displays negative epistasis with plasmid R831 and positive epistasis with plasmids R16, R702 and RP4 (Figure 2).

			Conjugative resistance plasmids				
			R124	R831	R16	R702	RP4
Chromosomal mutations	*gyrA*	D 87 G		-	++	+	++
		S 83 L			+		++
		D 87 Y		-			++
	rpoB	H 526 N			++		++
		I 572 F		++		+	
		R 529 H	+	+	+	+	+
	rpsL	K 43 R	-		+		-
		K 88 E	++	++		++	++
		K 43 N	++	++	++	++	
		K 88 R		++	+		

-	Negative Epistasis (4)
+	Positive Epistasis (10)
++	Positive (Sign) Epistasis (16)
	No Epistasis (20)

Figure 2. Interaction between antibiotic resistance mutations and conjugative plasmids exhibits positive epistasis. Distribution of the types of epistastic interaction found between different alleles of *gyrA*, *rpoB* and *rpsL* and conjugative resistance plasmids (positive epistasis in green, negative red and no epistasis in white). Combinations of mutation and plasmid where sign epistasis was found are indicated with ++. Note how epistasis appears to be both allele and plasmid dependent.

Supporting the pervasive nature of antagonistic interactions between mutations and plasmids, the distribution of the ε values shown in Figure 3A has a significant positive median (median=0.037, Bootstrap 95% CI [0.021; 0.065]). Strikingly, very strong positive epistasis appears not to be rare as our data shows 8 strains (out of 50) with ε above 0.200 (Figure 3A). This proportion is higher than the frequency of strains exhibiting negative epistasis. In addition, plotting the observed fitness against the fitness expected when assuming no epistasis (ε=0)

further supports the pervasive nature of antagonistic interactions between conjugative plasmids and resistance mutations (Figure 3B).

The same pattern had been observed for epistasis between deleterious resistance mutations in a previous study (Trindade *et al.*, 2009). In fact, the conjugative resistance plasmid influences how a specific allele interacts with plasmid-borne resistance determinants. That is, depending on the plasmid, the same allele can display no epistasis, positive or negative epistasis. For example, allele *gyrA D87G* exhibits no epistastic interaction with plasmid R124, however the same allele displays negative epistasis with plasmid R831 and positive epistasis with plasmids R16, R702 and RP4 (Figure 2).

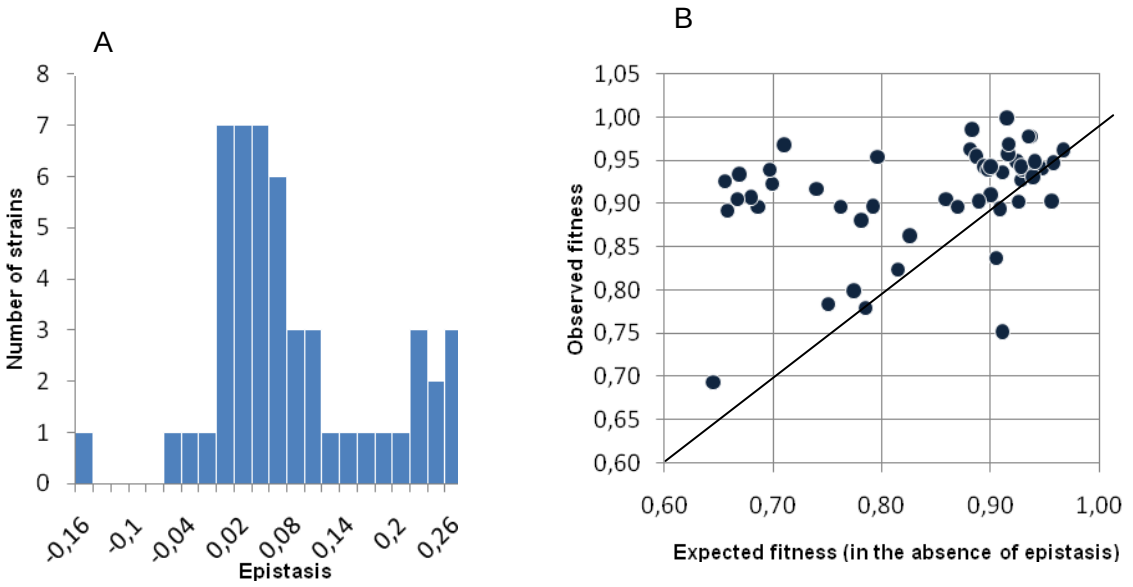

Figure 3. Evidence for positive epistasis in the interaction between antibiotic resistance mutations and resistance plasmids. (A) Distribution of the epistasis level, ε, whose median is clearly positive (0.037) with bootstrap 95% confidence interval [0.021; 0.065], showing an overall level of positive epistasis between chromosomal resistance mutations and conjugative resistance plasmids. According to a Shapiro-Wilk W-test, the ε values do not follow a normal distribution (p-value=0.000968). (B) Relation between the observed fitness of the strains carrying a resistance mutation and a conjugative plasmid and the expected fitness under the assumption of no epistasis (represented by the line). Note how most points (52%) are significantly above the line.

Focusing on the resistance mutations, it is noticeable that the mean epistastic value significantly varies amongst them (Kruskal-Wallis p-value=0.0016). Figure 4A suggests that it is possible to subdivide our set of 10 mutations in 3 classes, based on the strength of their interaction with a plasmid. The three classes are i) *rpoB R529H* with the highest mean ε value ii) *rpsL K88E* and *rpsL K43N* with high mean ε values; iii) all remaining mutations, which show the lowest mean ε values. The differences between these groups are statistically different according

to Mann-Whitney U-tests: the first class is different from the remaining classes with a p-value=0.00109; the second class is different from the third class with a p-value=0.000156 and the third class differs from the other two classes with a p-value=0.000002.

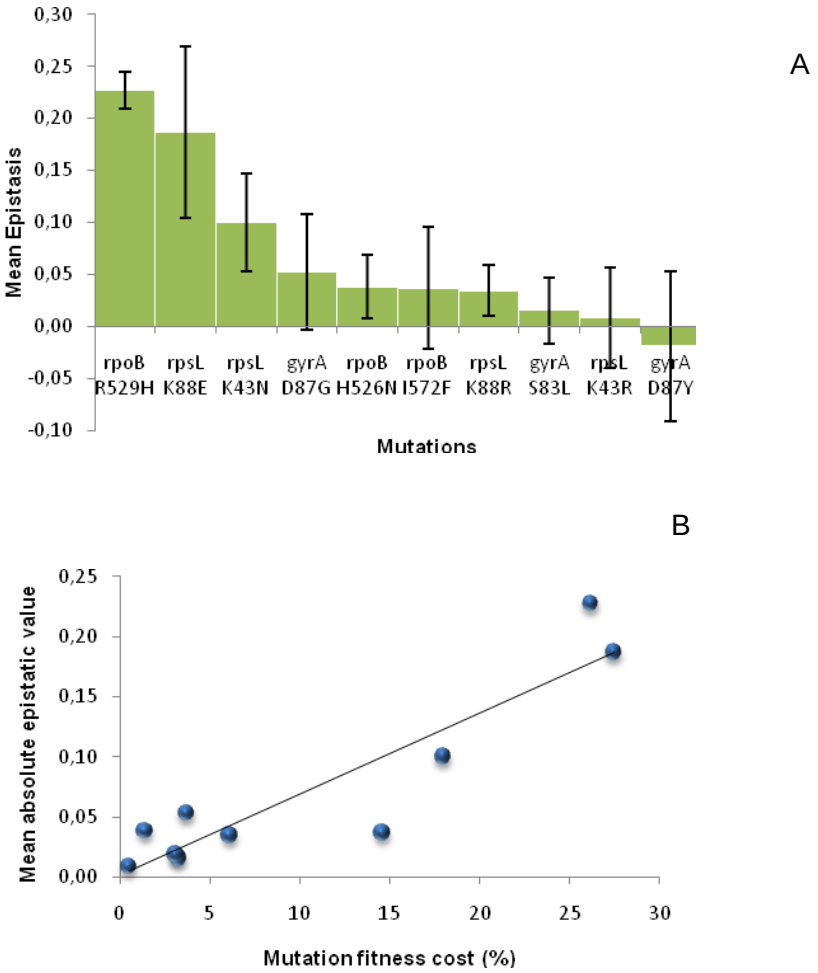

Figure 4. Mutation effect on the mean epistastic value. (A) Mean epistastic value for each mutation. Error bars indicate 2*standard error. Note how the mean epistastic effect significantly differs between mutations (Kruskal-Wallis p-value=0.0016). (B) Mutations become increasingly epistastic as mutational severity increases. Note how the mean absolute epistastic effect correlates with the fitness cost associated with each mutation (Pearson p-value=0.0002).

Figure 4B shows that there is a significant correlation between the fitness cost created by a mutation and its mean epistastic value (deviation from zero in absolute value) (Pearson p-value=0.0002). That is, mutations with a more deleterious effect on the cell tend to be more epistastic. This relationship had been initially proposed after *in silico* studies of digital organisms and theoretical modeling of RNA secondary structures (Wilke & Adami, 2001). The results from

this study are in accordance with previous experimental data from studies of epistasis amongst antibiotic resistance alleles in *E. coli* (Trindade *et al.*, 2009), and from a study of enzymes involved in gene expression and protein synthesis in *Pseudomonas aeruginosa* (MacLean, 2010).

Focusing on conjugative plasmids, Figure 5A shows the mean values of epistatic values of epistatic interactions of each plasmid with all chromosomal mutations. There are no significant differences in the mean ε values between plasmids (Kruskal-Wallis p-value=0.6758). This suggests that all studied conjugative plasmids tend to interact in the same way with the chromosomal mutations. Moreover, comparison between Figures 4A and 5A seems to suggest that the mutation (and not the plasmid) may be the major factor determining the type and the strength of the epistastic interactions observed in the present study. In contrast to what was observed with the effect of mutations on epistasis, there is no significant correlation between the fitness cost created by a plasmid and its mean epistastic value (Figure 5B – Pearson p-value=0.2738).

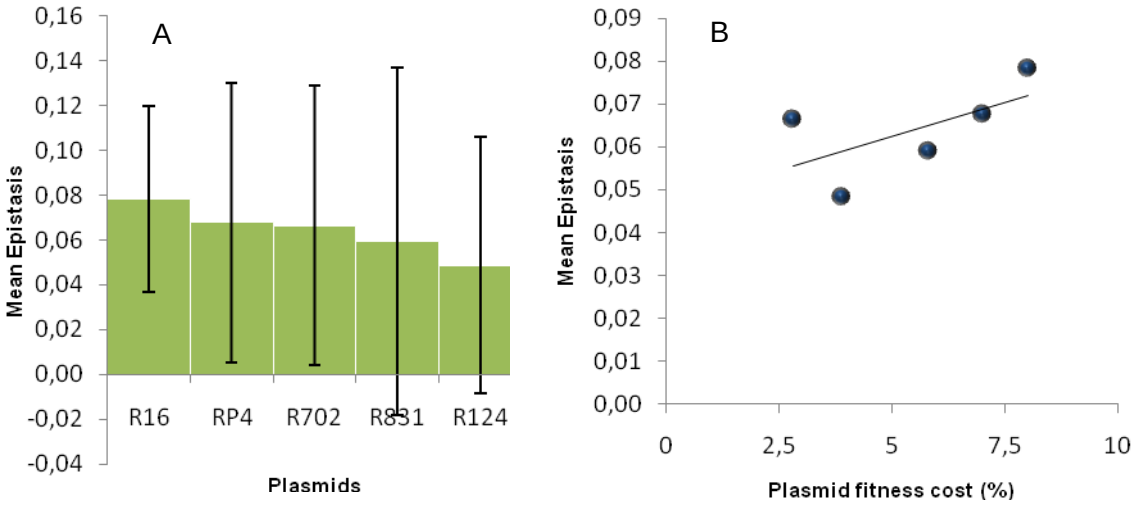

Figure 5. Plasmid effect on the mean epistastic value. (A) Mean epistastic value for each plasmid. Error bars indicate 2*standard error. Note how the mean epistastic effect does not significantly differs between plasmids (Kruskal-Wallis p-value=0.6758). (B) Evidence for the lack of correlation between the fitness cost associated with each plasmid and its mean epistastic value (Pearson p-value=0.2738).

2. Interaction between Conjugative Resistance Plasmids

Several examples of bacterial pathogens harboring multiple resistance plasmids have been reported (Minarini *et al.*, 2008; San Millan *et al.*, 2009). Such findings highlight the importance of better understanding antibiotic resistance determined by multiple plasmids coexisting in the same host. Hence, the present study investigates the interaction between 2 types of conjugative

resistance plasmids. To do so, all possible pairwise combinations between a set of 6 plasmids (Table 1) were constructed. Since plasmids R702 and RP4 belong to the same incompatibility group, the final data set contained only 14 different combinations of plasmids (instead of 15 combinations), each one carrying a different pair of conjugative plasmids. Their relative fitness was determined using the previously mentioned competition assay. Epistasis was estimated as:

$$\varepsilon = W_{(-;-)}W_{(plasmid1;plasmid2)} - W_{(\text{plasmid}1;\text{-})}W_{(-;plasmid2)}$$

where $W_{(-;-)}$ is the plasmid-free wilt-type strain, $W_{(-;plasmid2)}$ and $W_{(plasmid1;-)}$ are the fitnesses of single-plasmid strains and $W_{(plasmid1;plasmid2)}$ is the fitness of the strain carrying 2 types of plasmids.

Results show that 2 different plasmids inside the same bacterial cell can interact either antagonistically or synergistically (Figure 6). Epistastic interaction was found in 10 out of 14 (71%) strains. Positive epistasis (antagonistic interaction) appears to be nearly as frequent (6/14) as negative epistasis (4/14). Figure 7 shows the distribution of ε values for all pairwise combinations of plasmids. On average, plasmid pairwise epistasis is close to 0 (median=-0.007, bootstrap 95% CI [-0.061; 0.059]). However, most plasmid-plasmid interactions are epistastic (10/14).

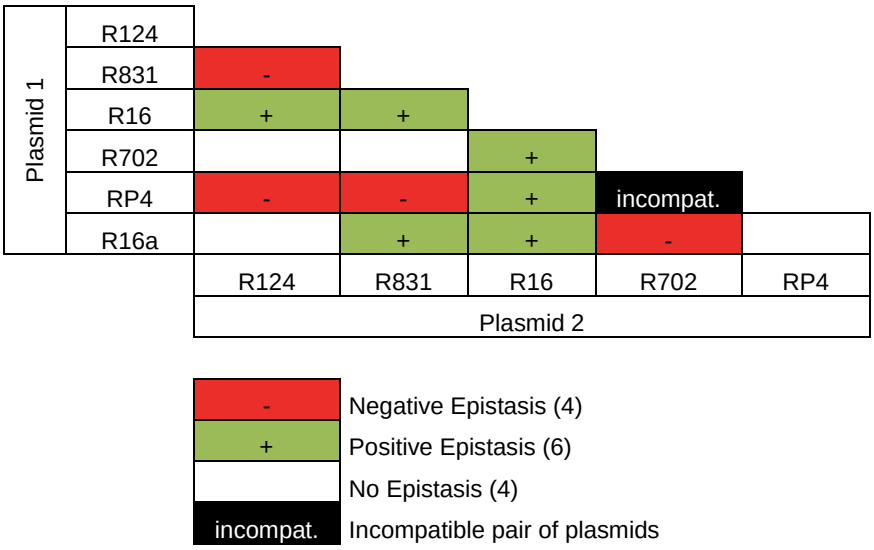

Figure 6. Evidence for epistasis between conjugative plasmids. Distribution of the types of epistastic interaction found between conjugative resistance plasmids (positive epistasis in green, negative red and no epistasis in white; black indicates a combination of incompatible plasmids).

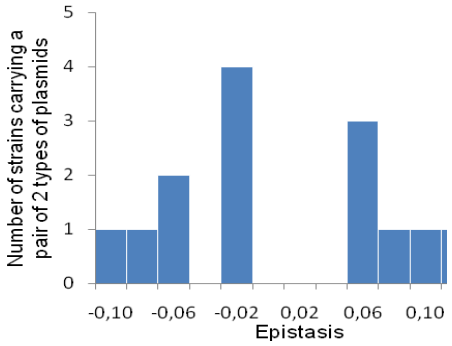

Figure 7. Evidence for epistasis between conjugative plasmids. Distribution of the epistasis level, ε, whose median is close 0 (-0.007) with bootstrap confidence interval [-0.061; 0.059], showing that there are several cases of strong positive and negative epistasis despite the near 0 median. According to a Shapiro-Wilk W-test, the distribution of ε values does not differ significantly from a normal distribution (p-value=0.219).

3. SIGN EPISTASIS

It is possible for a mutation to be deleterious on a particular genetic background and beneficial on others, a phenomenon known as sign epistasis (Weinreich *et al.*, 2005). In the context of antibiotic resistance, it has been shown that sign epistasis can allow an initially deleterious resistance mutation to become beneficial through the acquisition of another mutation. For example, in a study of multidrug resistance, 12% of the studied allelic combinations showed sign epistasis (Trindade *et al.*, 2009). In this study we found sign epistasis in 32% (16/50) of combinations between resistance chromosomal mutations and conjugative plasmids (Figure 2 where "++" indicates sign epistasis). These are cases where the mutant strain carrying a resistance plasmid was fitter than the strain carrying only the mutation or only the conjugative plasmid (Figure 8). Furthermore, the majority (62%) of the 26 interactions with positive epistasis identified in our data set, relative to the interaction between a mutation and a plasmid, are cases of sign epistasis.

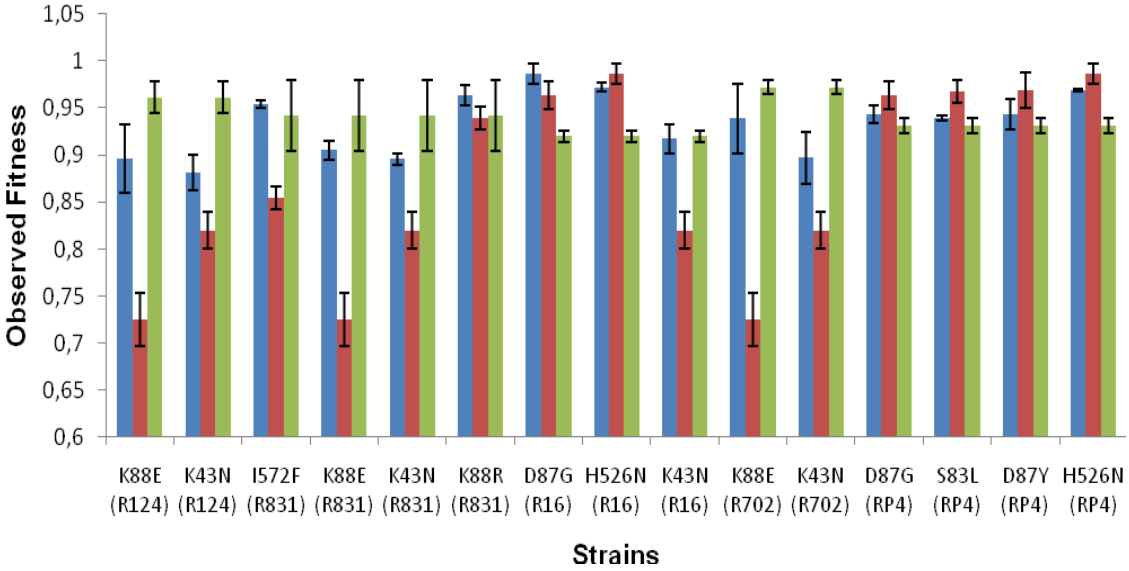

Figure 8. Evidence for sign epistasis between chromosomal mutations and conjugative plasmids. Sign epistasis occurs when the fitness of the strain carrying both resistance determinants, i.e. mutation and plasmid (blue bars), is greater than the fitness of at least one of the strains carrying a single resistance determinant (mutation - red bars; or plasmid – green bars). The genotype and the plasmid introduced in strains where significant sign epistasis was found are indicated below the bars (Mann-Whitney U-test p-values<0.05). Error bars represent 2*standard error.

We found examples of sign epistasis in almost all the resistance alleles studied, (Figures 5 and 8). Apparently, sign epistasis can occur regardless of the conjugative plasmid or chromosomal mutation. Accordingly, we failed to identify significant differences between alleles and between plasmids in their propensity to exhibit sign epistasis. It is possible, of course, that our sample was too small and by chance included only alleles and plasmids that exhibit sign epistasis. In contrast, we have not detected sign epistasis in any of the combinations between conjugative plasmids analyzed in this study (Fig. 6).

IV. DISCUSSION

This study show that 52% (26/50) of the combinations between antibiotic resistance mutations and resistance conjugative plasmids interact antagonistically. This is a remarkable result because the fitness cost of these strains that carry both resistance determinants is lower than the independent sum of the cost of each resistance determinant. Moreover, 16 out of these 26 antagonistic interactions (62%) exhibit sign epistasis, also an outstanding finding because it means that the fitness cost of harboring resistance determinants is lower than the fitness cost of bearing one of them. In other words, an initially deleterious antibiotic resistance mutation may become beneficial through the acquisition of a transferable antibiotic resistant plasmid; likewise, initially costly antibiotic resistant plasmid may become beneficial through the acquisition of a mutation conferring resistance to an additional antibiotic. The observed sign epistasis is a worrying result and it highlights the importance of a careful and informed choice of drugs when treating patients infected with resistant pathogens.

Plasmid-encoded resistance to multiple antibiotics, including β-lactams, aminoglycosides, tetracyclines, macrolides and glycopeptides is widespread in an array of pathogenic microorganisms including vancomycin-resistant enterococci (VRE) and methicillin-resistant *Staphylococcus aureus* (MRSA) (Williams & Hergenrother, 2008). Recently, it has been reported the transfer of plasmids from VRE to MRSA, resulting in the vancomycin-resistant *S. aureus* (VRSA) strain, which worryingly exhibits a high level or multidrug resistance (Weigel *et al.*, 2003). Many studies report the identification and characterization of plasmid-borne resistance and virulence elements in clinical isolates (Bennett, 2008; Cattoir & Nordmann, 2009; Morgan-Linnell *et al.*, 2009), allowing the monitoring of the emergence and dissemination of resistance determinants. However, typically few studies look at how to minimize such spread. This study focus on how conjugative resistance plasmids affect bacterial fitness, a trait with impact on the evolutionary fate of resistant microbes. It also investigates how bacterial fitness is affected by the interplay between conjugative resistance plasmids and chromosomal resistance determinants and also how bacterial fitness is affected by the interaction between conjugative plasmids coexisting within the same host.

Positive epistasis has been shown to occur between resistance alleles in multidrug resistant *E. coli*, (Trindade *et al.*, 2009), *P. aeruginosa* (Ward *et al.*, 2009) and *Streptococcus pneumoniae* (Rozen *et al.*, 2007). Such phenomena reduce the fitness cost expected to be associated with multidrug resistance and may drive its spread. This study aimed to detect the putative occurrence of epistastic interactions involving conjugative resistance plasmids. Such knowledge may help predict how a bacterial population will evolve after the introduction of plasmid-borne resistance determinants through horizontal gene transfer.

Strikingly, our data regarding the interaction between chromosomal mutations and conjugative plasmids suggests the pervasive occurrence of sign epistasis. Sign epistasis has been shown to have the power to constraint protein adaptation by limiting the number of possible mutational paths and thus it has relevance to the understanding of multidrug resistance emergence (Weinreich *et al.*, 2005). This means that the occurrence of a specific spontaneous

resistance mutation will, in part, determine what other additional resistance mutations are likely to emerge, due to the nature of the interaction between the two alleles, where an antagonistic interaction will favour such combination of mutations. Moreover, bacterial adaptation to the cost of mutation-determined resistance involves the acquisition of second-site mutations that compensate the fitness cost of the original mutation. The insertion of such compensatory mutations in the ancestral susceptible strain creates a cost showing that these mutations can be beneficial or deleterious, depending on the genetic background (Schrag *et al.*, 1997). Thus, compensatory mutations are an example of sign epistasis. The present finding of pervasive sign epistasis with conjugative plasmids is one of the worst possible scenarios for the current efforts to eradicate resistance through antibiotic-use bans, because sign epistasis allows strains carrying a resistance mutation and a plasmid to exhibit higher fitness, and thus able to outcompete, isogenic strains carrying only one of such genetic elements of resistance (either the plasmid or the mutation).

Also important in the context of antibiotic resistance is our finding of pervasive occurrence of positive epistasis between conjugative plasmids and chromosomal resistance mutations. If such antagonistic interaction is a common phenomenon, then multi-drug resistance determined by the simultaneous presence of plasmid-borne and chromosomal determinants will not create such a high fitness cost as one could predict based on the individual cost of each determinant. Hence, such multiresistant strains may be able to persist at significant frequencies in populations where the antibiotic selective pressure has been removed. As previously mentioned, positive epistasis amongst chromosomal resistance alleles has been previously described in *E. coli* (Trindade *et al.*, 2009), *P. aeruginosa* (Ward *et al.*, 2009) and *S. Pneumoniae* (Rozen *et al.*, 2007). The findings of the present work are in accordance with the results of a large-scale survey for genes of the *E. coli* chromosome that are affected by the presence of the conjugative F-plasmid (Harr & Schlotterer, 2006). Such study found 107 genes exhibiting epistastic effects with the F-plasmid. Although such effect was not found for *gyrA*, *rpoB* and *rpsL*, which, in this study, are the genes where our resistance mutations are located, other host genes involved in information transfer were reported to be affected by the presence of the F-plasmid (Harr & Schlotterer, 2006). Hence, the cross-talk between chromosomal and plasmid genes at an initial level and the cross-talk between host genes at a subsequent level may explain the epistastic effects reported here. It would be useful to perform additional whole-genome expression analysis using a broader range of conjugative plasmids and mutant host genotypes to better understand the pathways involved in fitness cost amelioration. In addition, a comparison between the mean epistastic effect of the mutations and conjugative plasmids suggests that it may not be the plasmid but the host genotype determining the type and strength of the epistastic interaction. This is supported by Harr and Schlotterer, who found that most of the genes identified as epistastic showed a response to the presence of the F-plasmid that depended on the genotype of the host chromosome (Harr & Schlotterer, 2006).

The present study also reports the occurrence of significant epistasis between conjugative plasmids within the same host. This has relevance for clinical isolates exhibiting multidrug resistance afforded by the co-existence of several plasmids, a situation which appears to be relatively common (San Millan *et al.*, 2009). Our data indicates that, on average, epistasis between conjugative plasmids is close to 0 (median=-0.007). However, we do not believe that our results suggest a tendency for no epistastic interactions between conjugative plasmids. In fact, our near 0 median level of epistasis between conjugative plasmids is the consequence of having a similar frequency of somewhat strong positive and negative epistastic interaction pairs. Present results may indicate that plasmid interaction follows an all-or-nothing type of response where the net epistastic effect is either strongly negative or strongly positive. However, future studies should use a broader sample of plasmids. Moreover, the average level of epistasis between conjugative plasmids is significantly below the average found for interactions between chromosomal mutations and plasmids. Thus, our results may suggest that positive epistasis between plasmids may not be as common as it is between plasmids and chromosomal mutations. Recently, *in silico* studies of *E. coli* and *Saccharomyces cerevisiae* metabolic networks have suggested that genes involved in essential reactions tend to interact antagonistically, while negative epistasis was mainly limited to non-essential gene pairs (He *et al.*, 2010). The accessory nature of plasmids versus the essential role of *gyrA*, *rpoB* and *rpsL* in information flow may explain why positive epistasis appears to be more frequent in the interaction between the studied chromosomal mutations and a plasmid than between two types of plasmids and it may also explain our failure to detect sign epistasis between plasmids. It is noteworthy to mention that plasmid R16 exhibited positive epistasis with all other conjugative plasmids studied, suggesting that it may have a higher propensity for antagonistic interaction with other plasmids not included in this study. Note that R16 had the highest fitness cost of our plasmid panel. Thus, this result supports the hypothesis that resistance plasmids that create a higher fitness cost may also be the ones where such cost is more easily ameliorated through epistatic interaction with additional plasmids or resistance mutations. Once again, further studies utilizing a broader sample of plasmids could contribute to confirm this assumption.

In theory, the occurrence of plasmid transfer from the strain under study to the reference strain could bear a cost on the reference strain, artificially raising the resistant strain's fitness. Therefore, the appearance of transconjugants was monitored over time and was found to occur significantly only later in the competition, after maximum cell densities were reached (Table 3). This was expected since plasmid transfer requires cell contact to occur and is therefore favored at higher cell densities (Levin *et al.*, 1979). These results show that plasmid transfer to the reference strain had little impact on the fitnesses measured because it occurred after the exponential growth phase, when the competitors ratio were already defined (Table 3).

Our finding of pervasive sign epistasis amongst mutations and conjugative plasmids raises serious concerns to the reversal of antimicrobial-drug resistance. Plasmid-borne multidrug resistance is widespread in microbial clinical, animal and environmental isolates.

Dissemination is facilitated by the conjugative plasmids ability to mobilize their own transfer (and of other plasmids) from their original host to a new cell. Many plasmids are even able to move between phylogenetically distant microbes. Furthermore, it is known that plasmids act as recruiting platforms for resistance genetic determinants, many of them able to transpose between the plasmid and the host chromosome (and vice-versa). Thus and given the widespread nature of horizontal gene transfer between close and distant prokaryotes some have suggested that microbes share a common gene pool. Based on all these factors and in our present results we predict that plasmid-borne resistance dissemination control through antibiotic use bans is not likely to be successful.

V. CONCLUSION

The present work aimed to gather a better understanding of the interplay between distinct antibiotic-resistance determinants, i.e. resistance mutations and conjugative plasmids and the consequences of such interaction in bacterial fitness. Such data may be helpful in the design of better strategies targeted at the reversal to susceptibility of bacterial pathogens.

Previous work (Trindade *et al.*, 2009) had shown the occurrence of epistastic interactions amongst resistance alleles. Such study gathered evidence for the occurrence of pervasive positive epistasis between mutations conferring resistance to nalidixic acid, rifampicin and streptomycin in *E. coli*. This antagonistic interaction diminished the fitness cost that one would expect to be associated with multi-drug resistance.

The present work adds an additional layer of complexity to this scenario by showing that such epistastic interactions are not limited to chromosomal mutations, but in fact, appear to also occur between mutations and plasmids in one hand and between conjugative plasmids, on the other hand.

The interaction between resistance mutations and conjugative plasmids is mostly antagonistic in nature. In other words, the fitness cost of harboring a resistance mutation and a conjugative plasmid is often smaller than the independent sum of such costs. There is no particular mutation or plasmid that tends to always interact in a certain way, which prevents any prediction making. However, when comparing the mean epistastic effect of mutations and plasmids, it appears that the mutations and not the plasmids are key in determining the sign and strength of the interaction. Furthermore, mutations that impose a higher fitness cost in the cell are the ones who tend to exhibit stronger epistasis, a pattern previously seen in other studies (MacLean, 2010; Wilke & Adami, 2001). On the contrary, the interaction between 2 types of conjugative plasmids in the same cell tends to be, on average, null. Despite that, the present work reports several cases of strong epistastic interaction between plasmids, both positive and negative in nature.

Unexpectedly, the current work found several cases of sign epistasis between resistance mutations and conjugative plasmids. These are situations where the fitness of the strain carrying both resistance determinants (mutation and plasmid) is higher than the fitness of at least one of the strains carrying only a single resistance determinant (either the plasmid or the mutation). In fact, these cases mean that the acquisition of a further resistance determinant by an already resistant strain can improve its fitness.

Given the fundamental role played by conjugative plasmids as vectors for dissemination of antibiotic resistance through a broad host-range, the findings here reported highlight the need to design and implement strategies for reversal to susceptibility that take into account the complexities We suggest that resistance reversal policies must target plasmids vulnerabilities. Three approaches have been suggested (Williams & Hergenrother, 2008): inhibition of plasmid conjugation, inhibition of plasmid replication, and exploitation of plasmid-encoded toxin-antitoxin systems.

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