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THE IMPACT OF GENETIC INTERACTIONS IN ANTIBIOTIC RESISTANCE

Sandra Isabel Gonçalves Trindade

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Doutora Isabel Gordo, Instituto Gulbenkian de Ciência

Professor Doutor Francisco Dionísio, Faculdade de Ciências da Universidade de Lisboa

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SUMMARY

Genetic interactions, both between genetic material and between this and the surrounding environment of a given individual are important factors for understanding the process of evolution of natural populations. The study of this interactions as well as their integration in evolutionary terms may have several applications such as, for example, understanding the observed prevalence of antibiotic resistance in natural populations. In this thesis the patterns of genetic interactions between multiple-resistances to antibiotics were explored. In particular, epistasis and genotype-by-environment interactions operating among antibiotic resistances were studied.

To measure levels of epistasis occurring between multiple-antibiotic-resistances in the complete absence of antibiotics, mutants resistant to three antibiotics commonly used in clinic were first generated: nalidixic acid, rifampicin and streptomycin. With these mutants double resistant mutants were created. By measuring the costs of each mutation individually and jointly the type of epistasis operating between different sets of resistance mutations was determined. The cost of double resistance was majorly lower than expected, revealing the presence of positive epistasis between mutations conferring resistance to the studied antibiotics. The same patterns were observed for interactions between a set of resistance mutations and conjugative plasmids with multiple-resistance factors. However, in this case, extreme cases of positive epistasis coined sign epistasis were observed for a large fraction of combinations. This type of interactions was also observed in the previous study yet less frequently.

Naturally occurring bacteria are often faced with a multitude of environments. The action of environmental changes in the effects of antibiotic resistance mutations was studied for a group of mutations resistant to four antibiotics in three environments differing in the

number of comprised environmental stresses. This study revealed, for most cases, a strong pattern of interactions between the different genotypes and the environment.

KEYWORDS

Antibiotics, Resistance, Epistasis, Genotype-by-environment, Mutation

RESUMO

As interacções genéticas, quer entre material genético quer entre este e o ambiente que rodeia um determinado organismo, são factores importantes para entender o processo de evolução de populações naturais. O estudo destas interacções bem como a sua integração em termos evolutivos pode ter diversas aplicações tais como, por exemplo, a compreensão da prevalência da resistência a antibióticos observada em populações naturais. Nesta tese exploraram-se os padrões de interacções genéticas entre múltiplas resistências a antibióticos nomeadamente, epistasia e as interacções genótipo-ambiente que ocorrem em resistências a antibióticos.

Para medir os níveis de epistasia entre múltiplas resistências a antibióticos na ausência dos mesmos, foram construídos mutantes resistentes a três antibióticos vulgarmente usados em clínica: ácido nalidíxico, rifampicina e estreptomicina. Com estes mutantes foram gerados duplos mutantes. Medindo os custos de cada uma das mutações individualmente e em conjunto determinou-se o tipo de epistasia a operar entre diferentes conjuntos de mutações de resistência. O custo da dupla resistência foi maioritariamente menor do que o esperado, revelando a presença de epistasia positiva entre mutações que conferem resistência aos antibióticos estudados. Os mesmos padrões foram observados para interacções entre um conjunto de mutações que conferem resistência e plasmídeos conjugativos com múltiplos factores de resistência. No entanto, neste caso, foram observados numa larga fracção de combinações casos extremos de epistasia positiva denominados epistasia de sinal, situação também observada anteriormente mas em menor frequência.

Na natureza, as bactérias enfrentam frequentemente uma multiplicidade de ambientes. A acção das alterações ambientais nos efeitos das mutações que conferem resistência a antibióticos foi

estudada num grupo de mutações resistentes a quatro antibióticos em três ambientes que diferem no número de factores ambientais prejudiciais comportados por cada um. Este estudo revelou, para a maioria dos casos, um forte padrão de interacções entre os vários genótipos e o ambiente.

PALAVRAS-CHAVE

Antibióticos, Resistência, Epistasia, Genótipo-ambiente, Mutação

RESUMO ALARGADO

A sobrevivência das populações naturais depende largamente da forma como estas enfrentam as adversidades que vão ocorrendo durante a sua existência. As condições ambientais que rodeiam estas populações sofrem constantes alterações. De modo a prevalecerem, as populações naturais necessitam de evoluir em conformidade; uma alteração nestas condições implica inevitavelmente um novo processo de adaptação de modo a que a sobrevivência seja garantida. Alterações na base genética de uma população são apontadas como o motor deste processo de evolução. O aparecimento sucessivo de várias mutações bem como o seu efeito conjunto são responsáveis pela perpetuação ou extinção de uma determinada população. Ser bem sucedida face ao processo de selecção depende exclusivamente da forma como a população lida com todas as condições stressantes que tem que enfrentar durante a vida.

O efeito fenotípico de uma mutação, em termos de fitness, depende de dois factores. O primeiro factor é intrínseco ao indivíduo: o fundo genético onde a mutação surge. A ocorrência de uma mesma mutação em diferentes fundos genéticos pode gerar alterações fenotípicas distintas devido ao facto de esta mesma mutação ser capaz de interagir com outras mutações presentes no genoma. Essas interacções são conhecidas como epistasia (Bateson 1909). O segundo factor é puramente ecológico no sentido em que se relaciona com as condições ambientais às quais o indivíduo está sujeito. Vários estudos levados a cabo em bactérias provaram que os efeitos fenotípicos das mutações em termos de fitness podem variar consideravelmente em função da alteração das condições ambientais (ver por exemplo Remold and Lenski (2001), Ward et al. (2009), Bataillon et al. (2011), MacLean and Buckling (2009)). Esta variação fenotípica em resposta às condições

ambientais é conhecida como interacção genótipo-ambiente (Falconer 1981).

Populações naturais de bactérias enfrentam diferentes condições ambientais no decorrer das suas vidas (Savageau 1983). Por exemplo, estas populações deparam-se muitas vezes com concentrações letais de antibióticos, em oposição à sua completa ausência. No entanto, e apesar da letalidade das referidas condições, as bactérias desenvolveram uma estratégia que lhes permite sobreviver. Esta implica alterações no genoma (por exemplo relacionadas com a modificação dos alvos dos antibióticos) de modo a evitar que os antibióticos aos quais são expostas sejam bem sucedidos. O sucesso desta estratégia está a questionar a eficácia dos actuais tratamentos de infecções bacterianas, particularmente devido à emergência de bactérias resistentes e multirresistentes.

O principal objectivo da pesquisa descrita nesta tese foi estudar os efeitos fenotípicos de mutações de resistência a antibióticos em termos de fitness e entender como esses efeitos podem variar dependendo da presença de mutações adicionais, ou mesmo de elementos genéticos acessórios, e das condições ambientais. Este conhecimento pode ser útil na compreensão do complexo processo de evolução de populações naturais bem como na concepção de novas terapias de erradicação ou atenuação da evolução da resistência a antibióticos.

Alguns estudos de resistência a antibióticos sugeriram a presença de interacções genéticas entre mutações de resistência e o fundo genético (Luo et al. 2005; Gagneux et al. 2006) e entre pares de mutações responsáveis pelo aumento da resistência a um dado antibiótico (Lindgren et al. 2005; Rozen et al. 2007). Contudo, nenhum destes estudos testou a hipótese de ocorrência de interacções epistáticas entre duas mutações conferindo resistência a dois antibióticos diferentes; situação que se sabe ocorrer com frequência na natureza. Este tipo de análise foi feito por exemplo por Ward et al. (2009) num estudo no qual

testaram o custo de múltiplas resistências em *Pseudomonas aeruginosa*. Apesar de terem encontrado epistasia positiva a operar entre mutações que conferem resistência a rifampicina e estreptomicina na total ausência de antibióticos a identidade das mutações que conferiram resistência à estreptomicina não foi definida. Como tal, a base mecânica das interacções genéticas relatadas não pôde ser determinada e foi impossível atribuir padrões de epistasia a diferentes mutações conferindo resistência ao mesmo antibiótico.

O estudo descrito no Capitulo 2 mostra claramente a presença de interacções epistáticas a operar entre mutações conhecidas que conferem resistência a antibióticos na ausência dos mesmos. Para medir os níveis de epistasia entre múltiplas resistências a antibióticos, foram construídos mutantes resistentes a três antibióticos vulgarmente usados em clínica: ácido nalidíxico, rifampicina e estreptomicina. Com estes mutantes criaram-se duplos mutantes. Medindo os custos de cada uma das mutações, individualmente e em conjunto, determinou-se o tipo de epistasia a operar entre diferentes pares de mutações de resistência. O custo da dupla resistência foi maioritariamente menor do que o esperado, revelando a presença de epistasia positiva entre mutações que conferem resistência aos antibióticos estudados. Adicionalmente, estimou-se uma preocupante fracção de casos extremos de epistasia positiva (12%) nos quais o aparecimento de uma segunda mutação de resistência a antibiótico é também capaz de compensar para o custo combinado da resistência de modo que o custo final é menor do que o custo de pelo menos uma das mutações (epistasia de sinal). Este tipo de interacções foi também reportado no Capitulo 3. Neste estudo, foram analisadas as interacções genéticas entre uma amostra das mutações de resistência usadas no Capitulo 2 e plasmídeos conjugativos bem como entre pares de plasmídeos conjugativos, tendo como base o supracitado procedimento experimental. No caso das interacções entre mutações cromossomais e plasmídeos conjugativos, também foi observada a

prevalência de epistasia positiva e em 40% dos casos a presença de epistasia de sinal. No caso das interacções genéticas entre pares de plasmídeos, em média, não foi observado qualquer tipo de epistasia. No entanto, a análise pormenorizada das interacções epistáticas revelou a presença de ambos os tipos de epistasia (positiva e negativa) a operar na mesma proporção o que em média dá uma ideia errada de ausência de epistasia. Esta situação tinha já sido reportada em estudos anteriores (de Visser et al. 1997; Elena and Lenski 1997).

Finalmente, no Capitulo 4, estudou-se a acção das condições ambientais no efeito das mutações de resistência a antibióticos, em termos de fitness. Esta análise foi elaborada num grupo de mutações resistentes a quatro antibióticos (as 19 mutações utilizadas no Capitulo 2 e um grupo adicional de 6 mutações de resistência a D-cicloserina) em três ambientes que diferem no número de factores ambientais prejudiciais. Este estudo revelou, para a maioria dos casos, um forte padrão de interacções entre os vários genótipos e o ambiente; a maioria das mutações relacionadas com processos essenciais da célula mostrou um forte padrão de interacções genótipo-ambiente (mutações que conferem resistência a rifampicina, estreptomicina e uma mutação de resistência a ácido nalidíxico) enquanto, mutações não essenciais permaneceram neutras em todos os três ambientes estudados (mutações que conferem resistência a D-cicloserina). Algumas mutações de resistência à rifampicina mudaram mesmo abruptamente de deletérias para benéficas, considerando determinados níveis de stress.

Os resultados apresentados têm consequências importantes para a evolução da resistência a antibióticos uma vez que, na maior parte dos casos, eles mostraram uma melhoria dos custos conferidos pela resistência em comparação ao esperado considerando os custos individuais. Este comportamento em particular pode ser suficiente para manter as bactérias resistentes em populações naturais e assim contribuir para os padrões de múltipla resistência que têm sido

observados. Adicionalmente, a compreensão de como elementos genéticos acessórios interagem com o restante genoma de uma célula e até mesmo entre si é crucial para definir o processo de prevalência da resistência em populações naturais uma vez que estes são tidos como factores importantes na proliferação de múltiplas resistências (Doucet-Populaire et al. 1992; McConnell et al. 1991; Salyers and Shoemaker 1996; Casjens et al. 2000; San Millan et al. 2009). O conhecimento dos supracitados padrões de interacções genéticas e de que alelos estão a contribuir para estas mesmas interacções, pode ajudar na compreensão do funcionamento da maquinaria molecular por detrás deste cenário e na consequente projecção de novas terapias.

Num contexto mais amplo, em última instância, este estudo acrescenta conhecimento ao que está descrito para epistasia. A maioria dos estudos feitos até à data baseou-se em experiências de acumulação de mutações e os poucos que não o fizeram, estudaram delecções ou mutações nocaute (ver por exemplo, Elena and Lenski (1997), Segrè et al.(2005), Jasnos and Korona (2007) e St Onge et al. (2007)). Com o trabalho descrito ao longo desta tese mostrou-se que a epistasia, mais do que específica do gene, é específica do alelo, o que acrescenta uma camada extra de complexidade à compreensão do processo. Adicionalmente, o facto de os objectos de estudo terem sido alterações ao nível do alelo possibilitou a compreensão daquilo que pode ser o resultado final das alterações em diferentes genes essenciais da célula, algo que não era possível nos estudos anteriores.

O estudo dos efeitos fenotípicos das mutações de resistência a diferentes antibióticos em vários ambientes é também crucial para a compreensão da prevalência de populações resistentes bem como para a explicação da diferencial evolução da resistência em clínica e na comunidade: o facto de uma mesma mutação apresentar diferentes efeitos fenotípicos em diferentes ambientes pode explicar os padrões observados.

Dado que na actualidade as infecções tendem a ser causadas por bactérias que comportam resistência a pelo menos um antibiótico é esperado que a melhor estratégia seja uma estratégia em que a próxima droga utilizada seja uma que leve simultaneamente a resistentes com maior custo inerente bem como com maior epistasia negativa. No entanto, esta abordagem deve ser feita de forma cuidadosa uma vez que, as condições ambientais também alteram os custos da resistência. Assim sendo, este conhecimento também deve ser tido em consideração aquando da projecção de novas terapias.

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CONTENTS

CHAPTER I

INTRODUCTION

Evolving Populations

Natural populations are known to be constantly evolving. Mutations are the basic source feeding this process. The acquisition of mutations supplies populations with different genotypes that translate into different phenotypes. Natural selection will ultimately act upon the entire panoply of phenotypes and populations will consequently evolve. Most of the appearing mutations are deleterious leaving for beneficial mutations only a small fraction in which lies the process of adaptation.

Once generated, the diversity could even be increased by several processes. Horizontal gene transfer is one of such processes. This seems to be an important force driving the evolution of bacteria. By adding mutations to the ones coded in the genome of a given organism, due to incorporation in the genome or simply by adding external genetic material to a given organism, this process might be able to raise even more the amount of variability available to be selected.

Environmental changes also have a strong influence on the way populations evolve. A good example is perhaps the change on the predominant coloration of the peppered moth. Most of the peppered moths had primarily a light coloration, key for the camouflage in their habitat, the light-colored trees and the lichens. With the Industrial Revolution, a major change occurred on those moths habitat and most of the ancient light colored moths died. Concomitantly with this decrease on the percentage of light-colored moths, an increase in the fraction of dark-colored moths was observed. But this is not the only way the environment alters how populations evolve. The effects of some mutations gathered on organism's genomes vary according to environmental changes implying different pathways for populations to evolve.

Obviously, this doesn't mean that there aren't other forces shaping, more or less directly, the evolution of populations such as, for instance, genetic drift where deleterious mutations are of extreme importance. Since mutations are being discussed, the extreme importance of mutation rates as well as their effects as predictors of how fast populations will evolve has to be mentioned. However, discussion on these factors and their effects on evolution will not be extended simply because they are beyond the scope of this thesis.

Genetic Interactions: Epistasis

When the discussion is the simultaneous existence of more than one mutation on the same background it is imperative to think about possible interactions between those mutations, especially if they are part of a common process. For instance, if mutation A and B are both players of the same biosynthetic pathway, one would expect two things: first that each would affect negatively the proper function of that pathway and second that they would interact to attenuate the detrimental combined effect. This process of interaction is termed epistasis.

Epistasis is by definition the interaction between genes at different loci, which means that the phenotypic effect of a mutation at a given locus depends on the genetic background in which it appears (Bateson 1909; Phillips 2008). In general terms, epistasis can be divided in magnitude and sign epistasis. Magnitude epistasis occurs when the effect of a given mutation depends on the genetic background but this mutation is unconditionally beneficial or deleterious. In that sense two mutations can act synergistically, if the combined effect of the mutations is bigger than expected by their individual effects, or antagonistically, if

the combined effect of the mutations is smaller than predicted by their individual effects. An extreme case of the former type of epistasis (synergistic epistasis) is synthetic lethality. This type of interaction, first discovered in Drosophila by Dobzhansky (1946) and Sturtevant (1956) and later in yeast by Novick et al. (1989), refers to cases where mutations are lethal only when occurring in combination. On the other hand, sign epistasis implies a change in the sign of mutation effect that is itself under epistatic control, i.e. a mutation is beneficial in the presence of other mutation but deleterious in a wild type background (Weinreich et al. 2005). Both types of epistasis will be discussed ahead on Chapters 2 and 3 which will also show that despite the fact that epistasis is commonly portrayed as a property of a given set of loci, it is more complex than that: it is a property of individual alleles at multiple loci.

Up to now, several studies analyzed the patterns of epistasis (every time it is present) in a variety of organism, as will be discussed afterwards. These studies used three main different methodological approaches to measure epistasis (reviewed on Kouyos et al. (2007)). On the explanation of the methods, and for the sake of simplicity, I will consider only deleterious mutations. A first approach relies on the comparisons between the parent fitness with offspring fitness by means of a cross experiment. When the mean fitness of the offspring is larger than the mean fitness of the parents, this implies antagonistic epistasis, same line of reasoning being applicable for the opposite (smaller mean fitness of the offspring, compared with the parents, revealing synergistic epistasis). A second approach, applied mostly in mutation accumulation experiments, determine epistasis by deviations to the predicted linearity between the log-fitness and the number of mutations in the absence of epistasis. A curvilinear relationship between log-fitness and mutations number unveil epistasis: concave distribution implies synergistic epistasis (accelerated fitness loss with increasing mutations number)

and a convex distribution implies antagonistic epistasis (decelerated fitness loss with the accumulation of mutations). The sign of epistasis can be mathematically determined through:

$$lnW_k = -\propto k - \beta k^2,$$

where W_k represents the fitness of mutants with k mutations, α represents the type of mutations (positive for deleterious mutations) and β defines the interaction between mutations (if positive implies synergistic interactions if negative implies antagonistic interactions) being zero the absence of genetic interactions (Elena and Lenski 1997). A third method implies the gathering of a double mutant and the respective single mutants, having as a starting point a wild type organism. This method determines epistasis by analyzing the deviations to the predicted linearity between the observed and the expected fitness of the double mutants in the absence of epistasis: concavity means antagonistic epistasis and convexity means synergistic epistasis. According to this model epistasis (ε) can be defined as:

$$\varepsilon = W_{ab}W_{AB} - W_{Ab}W_{aB},$$

where W_{Ab} and W_{aB} represent the fitness values of the two single mutants relative to the wild-type (W_{ab}) while W_{AB} represents the fitness of the equivalent double mutant.

This last method was applied to analyze and calculate epistasis on Chapters 2 and 3 of this thesis.

Epistasis and Evolution

In evolutionary terms, the presence of epistasis can constrain the path of evolution. The strength and form of epistasis have been shown to be relevant for several evolutionary issues such as speciation and evolution of sex. Explain the advantage of sex and recombination in evolutionary terms is puzzling. If an organism survives until the reproductive age, this means that its genome went through all the trials successfully. Accordingly, this organism would benefit to pass to its offspring exactly the same information coded on its genome. However, sexual reproduction, underlying shuffling of its own genetic information with the mate genetic information, seems to be widespread in nature. This paradox is called in literature the paradox of sex: if sexual reproduction is detrimental why should it prevail? A body of theories, seeking for a plausible answer for this question, was build. One class of such theories is based on the presence of negative epistasis among mutations affecting fitness (as is the case for instance of the Red Queen Hypothesis (Peters and Lively 1999)). Perhaps the most prominent explanation for the prevalence of sexual reproduction, considering the presence of epistasis, is the mutational deterministic hypothesis (Kondrashov 1988). According to this hypothesis, the advantage of recombination (sex) can be sustained for high deleterious mutation rate, if synergistic epistasis is operating between deleterious mutations turning more efficient the purge of those mutations by the selection process. This theory has, at least, three flaws. The first one is the requirement for deleterious mutation rates per genome per generation to be bigger than one. Although it was seen for some species such as *Caenorhabditis elegans* (Denver et al. 2004) and some RNA viruses such as vesicular stomatitis virus (Elena and Moya 1999), this is not the case for other species like *Arabidopsis thaliana* (Schultz et al. 1999; Shaw et

al. 2000). A priori, one of the prerequisites of this theory is not wide enough to embrace at least a large group of species. The second flaw is that this model applies to populations where the effect of genetic drift is small. For populations where genetic drift has a bigger effect this force, in concert with selection, can play the same role as epistasis in the evolution of sex (the coined Hill-Robertson effect (Hill and Robertson 1966)): it can even override the effects of epistasis (Keightley and Otto 2006; Otto and Barton 2001) which lead one thinking if epistasis plays such a crucial role. The third flaw relates exactly with the role of epistasis. The model assumes a mandatory presence of synergistic epistasis operating among deleterious mutations. The ubiquity of this type of epistasis has been shown hard to prove as will be discussed afterwards.

Despite not being the perfect model, this model from the top of its simplicity postulates a good argument for the maintenance of sex. Improve the understanding of this process involves the joint of various model assumptions. For instance what is exactly the joint role of migration, drift and epistasis in the maintenance of sex, because it is certain that these three factors play a role, but their combined effect is still unknown. In other words, a more realistic model is needed; a model that mimics better what is happening naturally, even at the expense of making it harder to analyze the outcome data.

Still, several studies looked for empirical evidence supporting this theory. Mukai's results were among the first reporting the occurrence of synergistic epistasis (Mukai 1969). In his study, Mukai measured the homozygous viability reduction of 72 lines of *Drosophila melanogaster* carrying mutant polygenes derived from a mutation accumulation experiment. He concluded that there was a synergistic interaction, on average, operating among those mutant polygenes responsible for the control of viability under the studied conditions. However this study has various limitations potentially affecting the estimative of epistasis: the

unknown exact number of mutations accumulated during the mutation accumulation (the number of mutations was estimated assuming that the period of mutation accumulation was proportional to the number of mutations accumulated) and the several assumptions made, like the lack of dominance and the approximately direct proportionality between viability (fitness component usually used in fruit flies studies) and total fitness. This lack of knowledge on the advantage of sex revealed challenging and various studies followed this one. The bulk of the studies were done over the last 15 years. These studies looked for evidences of epistasis on a range of different organisms by means of a variety of methods. The global outcome was a miscellaneous result: some revealed antagonistic epistasis (Bonhoeffer et al. 2004; de Visser et al. 1997a; Jasnos and Korona 2007; Sanjuán et al. 2004) few showed prevailing synergistic epistasis (de Visser et al. 1996; Whitlock and Bourguet 2000; Wloch et al. 2001) and others showed, on average, no predominant type of epistasis (de Visser et al. 1997b; Elena and Lenski 1997). It remains unclear whether these results are due to methodological limitations (such as for instance the lack of statistical power or indirect estimates of mutation number) or just reflect the diversity of genetic systems, since the type of epistasis might depend on the affected biological processes under study.

Some of these studies still base its findings in mutation accumulation experiments (de Visser et al. 1997a; Elena and Moya 1999), and consequently continue to lack which and how many mutations are contributing for the final results. This might probably bias the final outcomes and conduct to lack of knowledge on the real absence of epistasis as well. Adding to this, the fraction of isolated strains usually decreases with the increase in mutation numbers, leading to a bias of the entire data toward antagonistic epistasis (as is the case for de Visser et al. (1997b)). In my opinion, a good example of an improvement on this field is the study conducted by Elena and Lenski (1997). Elena and

Lenski (1997) used a sample of 225 genotypes of *Escherichia coli* with one, two or three mutations generated by mini Tn-10 and measure their fitness in competition with a wild-type competitor. By analyzing the effect of the increasing number of mutations on fitness, like previously done in literature, they did not find deviations to the expected linearity in the absence of genetic interactions. The problem with this type of approach is that the real nature of this lack of interactions is unknown, it could be due to the real absence of epistasis but it could also be due to the existence of both types of epistasis (synergistic and antagonistic), that are canceling each other out, revealing no epistasis on average. The real novelty of this study (besides being more representative of the whole genome), in comparison to previous ones, was the next step. They devised a procedure that allows the knowledge of the exact number and type of insertion mutations and consequently the sign and strength of interactions among those mutations. A sample of 27 recombinant genotypes having pairs of mutations were studied for their separated and combined effects on fitness and by comparing the observed and the expected fitness for each pair they were able to exclude the hypothesis postulating lack of epistasis. Instead, the absence of genetic interactions was due to equal strength and prevalence of both types of epistasis which on average revealed the apparent nullity. Given the asexuality of the organism in question there is not an obvious search for the advantage of sex and recombination, even knowing that the process of transposition is somewhat similar to the process of homologous recombination that usually is involved in sexual reproduction. Indeed, as Elena and Lenski (1997) mentioned "(…) our primary objective is not to evaluate whether sex would be advantageous for *E. coli*. Rather, we seek to determine if there is a general tendency for genetic architectures to exhibit synergistic epistasis among deleterious mutations, (…)" and I think that this was the motivation of most of the studies done in asexual organisms that

searched for evidence of synergistic epistasis. In addition, even the studies carried on recombining organisms didn't have as outcome the prevalence of synergistic epistasis. One recombining organism studied was the retrovirus HIV-1 (Bonhoeffer et al. 2004). The fact that it recombines frequently which can be viewed as a primitive form of sexual replication (Tenim 1991), is seen as a great advantage for the use of this organism in this type of research. In their study, Bonhoeffer et al. (2004) looked for epistasis between mutations (beneficial and deleterious) of 9466 virus samples extracted from HIV-1 patients submitted to drug therapy. Similarly to Elena and Lenski (1997), they also tested the presence of epistasis in two ways. First, they looked for deviations of the expected linearity between the logarithm of fitness and the Hamming distance between virus and after that, they looked for differences between the expected (by considering the single fitness effects) and the observed fitness of double mutants. Both methods revealed the presence of positive epistasis with the second method revealing also that both forms of epistasis were present but the mean value was positive.

A brief glimpse on the entire body of literature on epistasis and evolution is enough to notice the obvious lack of studies seeking for genetic interactions between beneficial mutations. One exception was the work of Sanjuán et al. (2004) that, among other things, analyzed a small sample size of 15 genotypes carrying two beneficial mutations and showed predominant antagonistic average epistasis. This bias to the study of genetic interactions between deleterious mutations is probably due to the rarity of beneficial mutations. Still it is important to study this type of interactions since beneficial mutations are responsible for the success of adaptation and interactions between those may influence the speed of adaptation. Importantly, this lack of knowledge implies indirect inferences on the role of epistasis in adaptation. The current year has been shown fruitful for this issue. Until present, some papers related

with epistatic interactions between beneficial mutations were published as is the case for Chou et al. (2011) and Khan et al. (2011). Both found prevalence of antagonistic interactions between beneficial mutations, corroborating previously obtained theoretical results (Kryazhimskiy et al. 2009). This type of interactions, being prevalent, can be the cause of the commonly observed diminishing rate of fitness increase in microbial populations, as proposed by the authors. Still, these results are preliminary; more studies are needed before the extrapolation of this conclusion to natural populations. The variation of evolutionary parameters such as population size, rate of mutation and rate of recombination may well change it as discussed by Kryazhimskiy et al. (2011).

On a different level, high-throughput studies (both theoretical and experimental), applying new high-throughput techniques, have proven to be efficient in unravel genetic interactions between metabolic networks (for instance Butland et al. (2008), He et al. (2010) and Segrè et al. (2005)). Generally speaking, this kind of studies seeks, primarily, to understand the modular nature of cellular organization and then use this knowledge to determine the unknown functional annotation of some genes and ultimately to deeply understand evolutionary and environmental adaptability. The large scale systematic generation of combinations of allelic variants has shown to be informative of not only the widely studied (but poorly sample sized) gene to gene interactions but also of the gene to pathway interactions which are very useful on the creation of a logical connection between functional networks operating inside a cell. A good example is the work of Segrè et al. (2005). In their study, Segrè et al. (2005) used a mathematical model entitled Flux Balance Analysis to analyze the spectrum of epistatic interactions between metabolic genes in yeast *Saccharomyces cerevisiae*. On average, they found no evidences of epistasis between mutations. Their next goal was to understand the overall organization of

the network. To do so, they searched for interactions between groups of genes that showed an a priori functional annotation. They found, with some few exceptions, what they called a "monochromaticity" of interactions between gene sets. These results showed to have a biological interpretation and the pattern of such interactions varied clearly with environmental perturbations imposed by them.

The metabolic control theory developed by Szathmary (1993) had previously predicted the existence of antagonistic epistasis operating among enzymes from the same pathway, whereas synergistic epistasis was expected for interactions between enzymes in parallel pathways. Large-scale analysis of genetic interactions confirmed and added more knowledge to these statements.

Besides the experimental improvement allowing for the study of many more mutations than the previous works (supposed to increase the power to determine the prevalent type of epistasis operating in the genomes of the organisms), there still exists a lack of ubiquitous positive or negative epistasis. Just like before high-throughput studies showed positive as well as negative epistasis and sometimes the absence of interactions, on average.

From my point of view, these studies are excellent to understand interactions between functional modules of a cell and with this knowledge try to understand the entire operating functional network as a whole. They are also good in unveil unknown gene functions as well as unknown interactions between genes. This type of revelation has some importance for future studies. As mentioned, it will help to understand some features of the cellular metabolism but it might also be useful for other things such as the development of new antibiotics based on the discovery of new potential targets. Definitely these studies are not the best approach to understand minutely epistasis (at the allele level); they could be a good approach to determine the sign of epistasis but never to determine its magnitude. This is due to the fact that they are all mainly

based in deletions known to be strong loss-of-function mutations and minority based in weaker effect mutations caused by point mutations affecting the gene function.

Despite the great devotion of literature to the evolution of sexual reproduction, the importance of epistasis expands beyond that. The above-mentioned high-throughput studies are good examples of that but many more exist. A topic that certainly has a wide influence of epistasis is the appearance and prevalence of multiple-antibiotic-resistances. Epistasis is believed to be one of the factors responsible for the appearance, increase and prevalence of multiple-antibiotic-resistance (a point introduced afterwards and discussed in this document).

The Antibiotics Era

Antibiotic properties were first discovered by Fleming, purely by chance, but still is one great discovery of the century. He found out, when he was returning from summer holidays on September 1928, that one of his plaques cultured with *Staphylococcus aureus* was contaminated with a fungus and most intriguingly that around the mould there was no staphylococci growth (Hare 1970). This observation leaded him to think that maybe the mould had produced a substance, that he called afterwards penicillin, responsible for the growth inhibition (Diggins 2003). In fact, he and his team found the potential of such a substance but they didn't succeed in finding a way to use it, a process only achieved later on: years later, penicillin was developed into the first significant antibiotic by Howard Florey and Ernest Chain (Chain et al. 1940). Even so, Fleming's accidental discovery on that day is a marker of what we can call the start of the antibiotics era (Lerner 2004).

The term "antibiotic" was first applied in 1942 by Selman Abraham Waksman, an American biochemist and microbiologist whose research was important for the discovery of streptomycin amongst other antibiotics. By that time, an antibiotic was any substance produced by a microorganism that prevents the growth of other microorganisms in high dilution (Waksman 1947). This definition excludes of course, a great part of the antibiotics used in clinics and hospitals nowadays, namely, semisynthetic (e.g. beta-lactams and penicillins) and the three classes of man-made synthetic antibiotics in clinical use (e.g. sulfonamides, quinolones and oxazolidinones) (Walsh 2003). In that sense, currently, the term antibiotic applies to a broader range of substances and not only to the naturally produced ones (Andersson 2004).

Shortly after antibiotic discovery and its introduction in clinics, the microbiologists were so optimistic that they actually believed that the evolution of antibiotic resistance was improbable due to the negligible frequency of mutation to resistance in bacteria (Davies 1994). Nevertheless, Fleming, by the time he received the Nobel Prize, was already conscious of how dangerous resistant bacteria could be and on his speech warned that "The time may come when penicillin can be bought by anyone in the shops. Then there is the danger that the ignorant man may easily underdose himself and by exposing his microbes to non-lethal quantities of the drug make them resistant" (Fleming 1945). He was absolutely right and antibiotic resistance became a problem of major concern at the worldwide level. Bacteria acquiring resistance to each and every new antibiotic in a very short period of time seems to be in vogue in bacterial world (Davies 1996). For instance, penicillin resistant strains of *S. aureus* began to be noted only four years after its massive implementation on the London civilian hospitals (Barber and Rozwadowskadowzenko 1948). For many, this was in fact a surprise at that time but it was also the reason why antibiotics have failed and still fail to cure a considerable number of infectious

diseases. Actually, bacteria producing antibiotics as a way to fight back their enemies seems to be as old as bacteria themselves so it wouldn't be odd that they needed an antidote that keep them alive and kill only the opponents. There are mainly three ways used by antibiotic-producing organisms to avoid suicide (reviewed in Cundliffe (1989)). In some cases the resistance mechanisms were simply modifications of the antibiotics (Forsman et al. 1990; Ogawara et al. 1999) and in other cases resistance was due to efflux pumps (Guilfoile and Hutchinson 1991; Piddock 2006). There are also known cases of modification of the sensitive target to render it insensitive and protect bacteria against the antibiotic, as is the case for *Saccharopolyspora erythraea* that produces an enzyme responsible for the attachment of a methyl group on the ribosomal RNA (Skinner et al. 1983).

Generally speaking, resistance can have its origins in intrinsic genetic events, by means of new mutations (McManus 1997) or gene amplification (Brazas and Hancock 2005); or can be the result of a process of transference of resistance genes (Prescott et al. 2005). Both mutations and horizontal gene transfer, as ways to acquire and perpetuate resistance, will be discussed afterwards.

The Problem of Multiple-Antibiotic-Resistance

Presently, antibiotics are undoubtedly of extreme importance to our society since they are crucial for the treatment of bacterial infections in both humans and animals. However, the effectiveness of this type of treatment has been threatened by the gradual emergence of resistant organisms able to fight back antibiotics.

The antibiotic resistance drama gained a whole set of new layers when the first multi-resistant bacteria appeared. This type of bacteria created an entire "new league" that is gaining more adepts every day.

Multiple-antibiotic-resistance is a huge problem for public health since some of the pathogens are becoming resistant to all known antibiotics, rendering us without alternatives to cure some of the most prevalent and sometimes deathly diseases. For instance, since the 1980s, a resurgence of resistant tuberculosis has occurred (Bloom and Murray 1992). This situation has become worse over the years and currently treating *Mycobacterium tuberculosis* infections might imply the use of as many as eight antibiotics. *Pseudomonas aeruginosa* are also reported as multi-resistant strains. The pattern of multi-resistance has been pointed as the reason why it is so hard to eradicate them from healthcare settings (Karlowsky et al. 2005). Actually, the prevalence in these institutions as been claimed as the major cause of opportunistic infections among immunocompromised patients and could ultimately lead to their death if not contained (Levy 1998). Among *E. coli*, identified as a common cause of urinary tract infection, there are strains resistant to members of six classes of antibiotics; a list that is worryingly per se but gets worst by including little-used antibiotics, proving that antibiotic resistance can appear very quickly.

An important factor contributing for this problem and that is the main focus of my thesis is the interactions between genetic material, in this case between mutations conferring antibiotic resistance or between mutations conferring antibiotic resistance and plasmids or even between plasmids.

Evolution of Antibiotic Resistance

Genetic variation and selection of the generated genotypes are the motors for the evolution of antibiotic resistance. The classical view of the acquisition of antibiotic resistance holds that, once bacteria were exposed to antibiotics, only the pre-existing resistant variants would

survive and get fixed in the population (Lederberg and Lederberg 1952; Luria and Delbruck 1943; Newcombe 1949). However, this view had to be extended to lodge the idea that antibiotic resistance can also be transmitted horizontally (actually, horizontal gene transfer is one of the known causes of acquisition of multidrug resistance (Falkow 1975)); or can be a collateral effect of the pressure exerted by the antibiotics: some antibiotics are known to promote the lateral transfer of antibiotic resistance (Beaber et al. 2004), and antibiotics may act as selectors of mutators (Mao et al. 1997; Ren et al. 1999) which in turn will accelerate the process of appearance of antibiotic resistance as explained below.

Horizontal Gene Transfer

DNA transfer among bacteria is critical for the dissemination of resistance (Davies 1994; Rice and Eliopoulos 2000). The transference of genetic material from resistant bacteria to susceptible bacteria can render the cells resistant to several antibiotics, separately or simultaneously (Levy 1998). This type of transference has been shown to happen in a variety of environments. For instance, the horizontal transfer of resistance is known to be common in the human gut, where resistance elements are transferred to known human commensal bacteria (Balis et al. 1996; Salyers et al. 2004) and from those to pathogens (Aarestrup 2005; Sibold et al. 1994; Witte 1998). Generally speaking, it may happen through three genetic mechanisms: conjugation, transformation or transduction (McManus 1997). Conjugation is a common mechanism of transference of antibiotic resistance. Usually, plasmids are the scaffolds on which various antibiotic resistance genes are assembled (Bennett 2008). Accordingly, those genes can easily be replicated and pass between cells, all at the same time. During conjugation a donor strain project a structure called

pilus that allows joining the two organisms, donor and recipient cells, temporarily. Once joined, the donor cell is able to transfer, via *pilus*, genetic material such as plasmid-containing resistance genes (R plasmids) (mechanism reviewed in Tenover (2006)). Once in the recipient cell the genetic material can remain as a plasmid or it can be incorporated in the cell's DNA (Levy and Miller 1989). Transduction happens with the help of bacterial viruses (bacteriophages) that are able to inject genetic material into host cells. This genetic material is usually from the phages but can also have incorporated genetic material from other bacteria such as antibiotic resistance genes. Transformation is the process whereby bacteria can incorporate directly DNA segments that are wandering freely in the immediate environment after being released by some cell lysis or after active excretion. These segments might also comprise some resistance genes (Levy and Miller 1989).

A common trait to these three mechanisms is the possible use of transposons to facilitate the transference and incorporation of the resistance genes from donor strains to host genomes or plasmids (Levy and Marshall 2004). Resistance genes can be segregated within transposons which in turn move from one place of the DNA strain to another carrying with them the resistance properties. Some transposons have integrons incorporated. Bacterial integrons are gene capture systems containing a site for integrating different antibiotic resistance genes and other gene cassettes in tandem, enabling the expression from a single promoter, and that utilize site specific recombination instead of transposition (Hall et al. 1999). Although these systems explain quite well the movement of antibiotic resistance genes between DNA molecules, they are impotent in explaining the clustering of antibiotic resistance genes not only in genomes but also in plasmids. Some years ago, a new recombination system, consisting of ISCR elements, was discovered and pointed as ideal to fill this gap. ISCR elements are predicted to transpose by a mechanism called rolling circle (RC)

transposition, which is the combination of RC replication and recombination (Tavakoli et al. 2000). This system is now known by its capacity of contribution to the assembly of banks of resistance genes on bacterial plasmids (Toleman et al. 2006).

All three mentioned processes (conjugation, transposition and transformation) are not only intraspecific but also interspecific, occurring even between genera (Doucet-Populaire et al. 1992; McConnell et al. 1991; Salyers and Shoemaker 1996). The lack of confinement is worrying in the sense that this means of transport can achieve every living organism. Moreover, cells can easily compensate for the cost of plasmids. Dionisio et al. (2005) showed that, in some cases, evolved conjugative plasmids (plasmids that were evolved within a cell for 420 generations) confer some advantage to their evolved host cells and, more than that, such evolved plasmids could even confer advantage to strains, from the same and different species that did not evolve with the plasmid. Their results suggest that a plasmid could easily remain in the population and be passed among its members as soon as cells get rid of its cost. If such a plasmid has the ability to confer antibiotic resistance, it could persist in the population even in the absence of the antibiotic and pass this resistance to members of the same species or to new species, perpetuating the antibiotic resistance skill. In that sense, the spread of antibiotic resistance is doomed to happen faster and on a large scale.

This rule has a very important exception: clinically significant antibiotic resistance in mycobacteria is usually associated with mutation. So far, there is no evidence for extrachromossomal elements being responsible for multidrug resistance in *M. tuberculosis* although unusual movements of mobile genetic elements, such as the insertion sequence IS*6110*, and the consequent inactivation of critical genes has been associated with the emergence of new resistance (Dale 1995; Gillespie 2002; Lemaitre et al. 1999). Nonetheless horizontal gene transfer is still

responsible for the transference of antibiotic resistance among the majority of bacteria. As previously mentioned, many plasmids, transposons and integrons are known to carry multiple resistance genes conferring resistance to different antibiotics.

Mutators

The occurrence of chromosomal mutations is also an efficient pathway to resistance (Courvalin 2008; Maciá et al. 2005). Some bacteria, coined mutators, have shown a higher than expected mutation rate when compared with the wild type mutation rate (Miller 1996) which has been related to defects in the DNA mismatch repair system (Giraud et al. 2001). This particular behaviour enables mutators to adapt faster than wild type strains to stressful environments (Chao et al. 1983; Gibson et al. 1970; Siegel and Bryson 1967; Tanaka et al. 2003). So, the chances of acquiring antibiotic resistance by mutational events, under antibiotic pressure, are increased by the acquisition of a mutator phenotype (Daurel et al. 2007; Denamur et al. 2005; Henrichfreise et al. 2007; Maciá et al. 2005). Moreover, hypermutability can be co-selected with antibiotic resistance by a process entitled genetic hitchhiking. According to this process, the selective increase in frequency of a beneficial allele can also raise the frequency of all the linked alleles, whether beneficial or detrimental (Sniegowski et al. 2000). Hence, mutator alleles can spread as a result of hitchhiking with beneficial antibiotic resistance mutations that have arisen in the same genome under antibiotic pressure. This phenomenon may lead to an unpleased scenario in which those mutants with one resistance are more likely to develop other resistances to unrelated antibiotics due to mutator phenotype. More than that, Perron et al. (2010) showed that, mutators not only evolve resistance more rapidly than wild-type bacteria, but they

also compensate better for the costs associated with that resistance, in the sense that mutators entirely compensate the cost. The perfect conditions for multiple resistances seem to be settled. Actually, mutators are often present in clinical habitats (Baquero et al. 2004; LeClerc et al. 1996; Oliver et al. 2000), where selection of mutator alleles is particularly likely in some chronic infections, as populations of bacteria face successive antibiotic administrations. For instance, Oliver et al. (2000) showed that *P. aeruginosa* from cystic fibrosis patients had high antibiotic resistance for at least eight antibiotics after years of treatment with antibiotics and that this behaviour was due to a mutator phenotype present in 19.5% of the isolates.

Given the apparent weight that mutator phenotypes have on the adaptation of populations facing changing environments and in particular on the rapid development and spread of antibiotic resistance, it is important to deeply know the dynamics of adaptation of such populations. For example, Trindade et al. (2010) performed a mutation accumulation experiment in a mutator strain of *E. coli* to study the rate and effect of spontaneous mutations that affect fitness during the course of a serious of extreme bottlenecks. As expected, they observed a decrease in the mean fitness, since that the vast majority of mutations are deleterious, and an increase in variance between lines, a common consequence of genetic drift. With these cadences, they estimated a minimum mutation rate to deleterious mutations (0.005) and a maximum mean fitness effect per deleterious mutation (0.03). Even with such severe bottlenecking, beneficial mutations occurred in the mutator lines, as saw previously in other studies carried on different organisms (Dickinson 2008; Joseph and Hall 2004; Shaw et al. 2000). This observation points to a mutation rate to beneficial mutations high enough to be detected, even when natural selection is operating in a vestigial way. This type of information are valuable to try to predict how such populations will adapt but many more studies need to be done to

intertwine properly mutator phenotype with antibiotic resistance and so be able to predict how resistance will develop for a given antibiotic which might be helpful to try to avoid antibiotic resistance.

Epistasis

As mentioned previously, bacteria get resistant to antibiotics due to *de novo* mutations or acquisition of genes from other bacteria. Those changes confer a competitive advantage against their sensitive counterparts during the antibiotic exposure but once it is taken, the resistance phenotypes are generally deleterious (Andersson and Levin 1999; Björkman et al. 1998; Bouma and Lenski 1988; Dahlberg and Chao 2003; Gagneux et al. 2006; Lenski 1997). There are, however, some examples of the so called negative cases in which the resistance phenotype became neutral. The most well known example is the K43R mutation in the *rpsL* gene at *Salmonella typhimurium* and *E. coli* (Kurland et al. 1996; Tubulekas and Hughes 1993). There are also few examples for antibiotic resistance phenotypes conferring an advantage to the bacteria in the absence of the respective antibiotic (Blot et al. 1994; Luo et al. 2005). Results on Chapters 2 and 3 of this document support the idea that mutations are generally costly in the absence of antibiotic. However, Chapter 4 will show some mutations becoming beneficial under certain environmental conditions.

These fitness costs are believed to be key on the dynamics of resistance evolution of the carrier populations (Andersson 2006; Bonhoeffer et al. 1997; Levin et al. 2000). The biological reasons for antibiotic resistance fitness costs can be several: genes with essential functions being the spot for the resistance mutations which will somehow affect its functions; bacteria acquisition of a new gene (or set of genes) responsible for new functions; expense of extra energy

necessary for replication and maintenance of palsmidic genes (Courvalin 2008).

Despite the cost related with antibiotic withdrawal, bacteria seem to be able to persist in populations without loss of resistance. This biological cost of resistance can be partly or fully compensated by the acquisition of second site mutations (Björkman et al. 1998; Björkman et al. 2000; Johanson et al. 1996; Lofmark et al. 2008; Nagaev et al. 2001; Paulander et al. 2007) that substitute the affected functions with alternative ones or, direct or indirectly, restore the efficiency of cellular functions affected by the antibiotic resistance in relatively short periods of time. Hence, bacteria are able to restore its competitive ability without loss of the antibiotic resistance. The process of compensation has been described to be quite omnipresent. It covers mutations as diverse as those that confer rifampicin and isoniazid resistance in mycobacteria (Gillespie 2001) as well as rifampicin resistance in *P. aeruginosa* (MacLean et al., 2010), fucidin resistance in *S. aureus* (Nagaev et al. 2001), and streptomycin resistance in *Enterobacteriaceae* (Lenski 1997; Maisnier-Patin et al. 2002). It also covers, for instance, plasmids burden such as plasmids conferring tetracycline resistance (pACYC184 and pBR322) (Bouma and Lenski 1988) or a derivative of plasmid pBR322 that encodes resistance to ampicillin and tetracycline (Modi and Adams 1991).

By compensating the burden imposed by the antibiotic resistance, bacteria are able to persist in populations and be resistant at the same time. Like that, they can become resistant to a second antibiotic once they face it and so become multiple resistant. This particular type of genetic interactions between resistance and compensatory mutations is only an example of epistasis operating between mutations and its potential influence in evolution of antibiotic resistance; a poorly exploited field.

The study of epistasis has been stretched to comprise the interactions between antibiotics (Yeh et al. 2009). This decision was based on the premise that drugs, just like mutations, can interact synergistically or antagonistically if the combined effect is bigger or smaller than expected by adding their individual activities, respectively, or don't interact at all, having exactly the expected combined effect (also known as additive interaction) (Bliss 1939; Hartman et al. 2001; Keith et al. 2005; Loewe 1953). Yeh et al. (2006) started this journey by showing that antibiotics can be organized according to their interactions with other groups of antibiotics. In other words, antibiotics can form clusters that interact monotonically with other clusters (in analogy with modular epistasis in metabolic networks as pointed by Segrè et al. (2005)). They suggested that this type of knowledge can give some clues on the mechanism of action of a new drug which might be similar for those of the same cluster. Furthermore, recent studies suggest that antagonistic drug pairs can, counterintuitively, slow down and eventually reverse the evolution of drug resistance (Chait et al. 2007; Hegreness et al. 2008; Michel et al. 2008). For example, Chait et al. (2007) found that under some particular concentrations, combinations of suppressive antibiotics (hyper-antagonism), when in competition with each other, can allow the growth of wild type bacteria but not the resistant bacteria. Apparently they found a gap that allows sensitive bacteria to outcompete resistant ones and that consequently might be helpful as the basic knowledge for the implementation of new therapies. It is just a manner of trade-off between immediate efficacy of antibiotic therapy and future hindrance of the evolution of resistance. More recently, Nichols et al. (2011), in their study of the phenotypic landscape of *E. coli*, proposed a way to expand this knowledge. They claimed that their phenomic profiling information can provide a platform to a deeper study of drug interactions, namely study the mechanisms underlying those drug interactions. This study, by means of its drug-gene ontology interactions, might also be useful to

unravel mechanisms of action of both well and poorly described antibiotics as well as the cellular mechanisms underlying antibiotic resistance.

Just like compensation, depending on the epistatic terms, the antibiotic resistance condition may be benefited and perpetuated in the populations or just be expelled. Understanding how mutations interact is a helpful tool not only to understand the patterns of adaptation in general but also to understand how antibiotic resistance evolves in particular.

The Impact of Environmental Diversity on the Cost of Mutations

E. coli, as many microorganisms, is known to face an array of different environments (Savageau 1983). In order to adapt to a given environment, populations evolved a set of mutations that confer them an advantage on such environment. Yet, it is unlikely that any given mutation will be responsible for a high fitness in the entire range of environmental conditions faced by those populations. Variation of the environmental conditions might be responsible for either an increase or decrease of the fitness effect of a given mutation. A deleterious mutation can become more detrimental once facing a harsh environment (unconditionally deleterious) (for an example, see Fry et al. (1996)). Some mutations can even be deleterious under some environmental conditions and neutral under others (conditionally neutral) (Dykhuizen and Hartl 1983; Lewontin and Matsuo 1963; Suzuki et al. 1976). These patterns of environmental dependent phenotypic expression can be extended to a more extreme condition in which

changes in the environment imply a change in the sign of mutations. For instance, a mutation that is beneficial in a given environment might be detrimental in another (conditionally beneficial) (Poon et al. 2005; Silander et al. 2007). This variation in phenotypes in response to environmental conditions is known as genotype-by-environment interaction (Falconer 1981) and can essentially occur in two ways: the phenotypic effect of genotypes may vary across environments but the rank order of genotypes remains the same or, both the rank order and phenotypic effect of genotypes vary (Cockerham 1963).

Knowledge on the extent and form of genotype-by-environment interactions is paramount to the understanding of how populations evolve. The idea that a given genotype cannot be the best fit in all different experienced environments plays a central role in many ecological and evolutionary theories. For instance, Levene (1953) and Gillespie and Turelli (1989) postulated that genotype-by-environment interactions can operate to maintain the levels of genetic variation within natural populations. These considerations triggered a series of studies of genotype-by-environment interactions in natural populations (for instance, Gupta and Lewontin (1982), Marks (1982), Pigliucci et al. (1995), Rawson and Hilbish (1991), Sultan and Bazzaz (1993), Tachida and Mukai (1985) and Wade (1990)). The main goal of these studies was addressing the question: how much potential variation exists in natural populations? To do so, they assayed natural populations for fitness (or its components) in different environments. Most of the cases (Gupta and Lewontin 1982; Pigliucci et al. 1995; Rawson and Hilbish 1991; Sultan and Bazzaz 1993; Tachida and Mukai 1985; Wade 1990) demonstrated that the ranking of genotypes differs among environments which may be predictive of the sustenance of polymorphisms in natural populations and ultimately of the phenotypic variation observed among those natural populations. Despite being important to understand the extent of variability present in natural populations, these studies were

all made considering genotypes from extant natural populations and accordingly pre-existing genetic variation. Given so, most of these genotypes were already subjected to a process of selection and the studied ones where majorly those able to evade that process. In that sense, considering these studies, no conclusions can be made about the patterns of genotype-by-environment interactions for new mutations incoming randomly in a given population.

A recent study carried by Cooper and Lenski (2010) strengthened this notion that environmental heterogeneity can promote evolutionary diversification. In this study they adapted 42 populations of *E. coli* to 7 different environments (comprising one or two carbon resources at the same time or alternated) for 2000 generations. After this process they measured the fitness of all evolved populations and found different extents of adaptation for the different regimes. The among-populations variance revealed to be higher in those fluctuating regimes compared to constant resource regimes supporting the thought that environmental heterogeneity is able to provide divergence among populations.

The evolution of ecological specialization has also been associated with particular forms of genotype-by-environment interactions (reviewed in Futuyma and Moreno (1988), but see also Fry (1996), Kawecki (1994) and Kawecki et al. (1997)). According to this idea, the extent of genotype-by-environment interactions can sculpt how a subdivided population will adapt to spatial variation in natural selection; by evolving either a "generalist" phenotype (suited for life in any environment) or several "specialist" phenotypes (capable of living in a particular type of environment). Generally speaking, ecological specialization occurs if both the rank order and phenotypic effect of genotypes vary and selection favors different genotypes in each environmental state. At the other extreme, generalism occurs when the phenotypic effect of genotypes may vary across environments but the rank order of genotypes remains the same (for an example see Guntrip and Sibly

(1998)). These patterns of genotype-by-environment interactions, when prolonged for several generations, can ultimately be responsible for sympatric speciation due to more or less genetic isolation accompanied by inhabitance of the same geographic region (see for instance Dickinson and Antonovics (1973), Kawecki (1996), Kawecki et al. (1997) and Smith (1966)).

Genotype-by-environment interactions are also important features for the estimation of genomic deleterious mutation rates (U_d) and mean effects of deleterious mutations (s_d); two key parameters in Evolutionary Biology. Experimental studies intending to measure these two parameters are usually carried out in environments that generally supply populations with all the needed resources. However, mean fitness effect of mutations has been showed experimentally to be environmental dependent namely, the fitness effects of deleterious mutations tend to aggravate or remain unchanged under environmental stress (Fernández and López-Fanjul 1997; Fry and Heinsohn 2002; Fry et al. 1996; Kondrashov and Houle 1994; Korona 1999; Shabalina et al. 1997; Szafraniec et al. 2001; Vassilieva et al. 2000). In case of aggravation with environmental stress, and considering that usually the estimates of U_d and s_d parameters are made in benign environments as aforementioned, the obtained values of U_d can be compromised in the sense that they could have been underestimated; on the contrary, the s_d values were clearly overestimated. The conditionally neutral or quasi-neutral mutations (deleterious under some environment and neutral or quasi-neural under the rest) are also biasing downward those estimates. Considering that, despite being neutral (or quasi-neutral) in benign environments (and so, not measurable experimentally), they are deleterious in stressful environments, any estimation of the values of U_d and s_d made in a stressful environment will give different outcomes in comparison to estimates in beneficial environments (notion also supported by Kondrashov and Houle (1994)).

An analysis of the literature about genotype-by-environment interactions clearly reveals that a large part of the experimental studies done in the last years on this subject are mutation accumulation based (Fry et al. 1996; Fernández and López-Fanjul 1997; Wayne et al. 1997; Korona 1999; Szafraniec et al. 2001; Fry and Heinsohn 2002; Chang and Shaw 2003). With this framework, it is hard to address whether stress exposes more mutations or increases their average effect. Also unclear is whether a given genotype-by-environment interaction depends on a single plasticity gene or on numerous alleles that together produce the resulting pattern. Remold and Lenski (2001) used a different experimental approach to overcome these barriers. In their study they intended to analyze the contribution of individual random mutations to genotype-by-environment interactions. To do so, they generated a set of 26 *E. coli* genotypes differing among them by a single random insertion mutation. Then, they measured the fitness effects of each mutation in a set of 4 environments differing in temperature and limiting resource. Through the analyzes of these results they were able to conclude that single random mutations, that never experienced selection in any of the tested environments, can generate genotype-by-environment interactions and extract the patterns of these interactions. They were also able to determine which of the changing ecological factors contribute more, or if they contribute equally, to genotype-by-environment interactions as well as to make some predictions about how common are beneficial mutations.

Adding to the previously mentioned limitations, mutation accumulation studies also raise the possibility of epistasis to be operating in parallel with genotype-by-environment interactions, which is actually suggested as the main explanation for the results obtained in some experimental studies (Jasnos et al. 2008; Kishony and Leibler 2003). This suggestion was supported theoretically (You and Yin 2002). In general terms, empirical studies tended to show that epistasis is

operating as a way to alleviate the fitness effects of mutations in stressful environments. The theoretical results demonstrated the occurrence of this behaviour but only in highly deleterious mutations. The opposite pattern of genetic interactions (aggravation of the fitness effects) was shown to be characteristic of weak deleterious mutations.

By how much evolution is driven by unusual bouts of exposure to harsh environments remains an open question. Nevertheless, it is certain that genetic variation of a given population can experience major impacts with the exposure of that population to environmental changes. Given so, it becomes obvious that the pattern of adaptation of natural populations suffers a tremendous influence of the surrounding environment. With antibiotic resistance mutations it should not be different. Actually, it is known that most of the times mutations that are beneficial under antibiotic selective pressure become detrimental in its absence. The level of resistance can also be influenced by environmental conditions. For instance, bacteria cultured from the marine air-water interface were shown to be more highly resistant to antibiotics than bacteria cultured from the bulk water (Hermansson et al. 1987) which has been suggested to be due to pollution or radiation. Although the mechanism responsible for this phenomenon is still uncertain what is known for sure is that the environment plays a role on the level of resistance. Moreover clinical and community environments lead to different outcomes of antibiotic resistance evolution (reviewed in Andersson and Hughes (2010)). For this and other reasons it is clearly important, and has been frequently suggested by several authors, to study the fitness costs of antibiotic resistance mutations under multiple experimental conditions not only to acquire an obvious more relevant estimate of fitness but also to establish its predictive influence in determining the clinical outcome of infection as well as to reveal any physiological weakness that may be exploited for drug development (Björkman et al. 1998; Björkman et al. 2000; Paulander et al. 2009).

Afterwards, I will show and discuss what I did to add some knowledge to the previously mentioned issues: interactions between mutations, in general, and their influence on antibiotic resistance evolution. Namely, in Chapter 2 I measured the costs of mutations conferring antibiotic resistance and show the presence of positive epistasis as well as sign epistasis between those mutations; in Chapter 3, I studied epistasis between resistance mutations and plasmids (which carry multiple antibiotic resistances) and between plasmids and show the presence of mainly sign epistasis. Finally, in Chapter 4 I addressed the role of the environment on the costs of mutations conferring antibiotic resistance and show the presence of genotype-by-environment interactions.

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CHAPTER II

POSITIVE EPISTASIS DRIVES THE ACQUISITION OF MULTIDRUG RESISTANCE

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Sandra Trindade, Ana Sousa, Karina B. Xavier, Francisco Dionísio, Miguel G. Ferreira and Isabel Gordo

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The evolution of multiple antibiotic resistance is an increasing global problem. Resistance mutations are known to impair fitness and the evolution of resistance to multiple drugs depends both on their costs individually and how they interact - epistasis. Information on the level of epistasis between antibiotic resistance mutations is of key importance to understanding epistasis amongst deleterious alleles, a key theoretical question, and to improve public health measures. Here we show that in an antibiotic free environment the cost of multiple resistance is smaller than expected, a signature of pervasive positive epistasis amongst alleles that confer resistance to antibiotics. Competition assays reveal that the cost of resistance to a given antibiotic is dependent on the presence of resistance alleles for other antibiotics. Surprisingly we find that a significant fraction of resistant mutations can be beneficial in certain resistant genetic backgrounds, that some double resistances entail no measurable cost and that some allelic combinations are hotspots for rapid compensation. These results provide additional insight as to why multi-resistant bacteria are so prevalent and reveal an extra layer of complexity on epistatic patterns previously unrecognized, since it is hidden in genome-wide studies of genetic interactions using gene knockouts.

Introduction

Epistasis occurs when the phenotypic effect of a mutation in a locus depends on which mutations are present at other loci. When the phenotype of interest is fitness, the existence of such genetic interactions can constrain the course of evolution. The strength and form of epistasis is relevant for the evolution of sex, buffering of genetic variation, speciation and the topography of fitness landscapes. While epistasis between gene deletions (Jasnos and Korona 2007; St Onge et al. 2007) has been the focus of recent research, interactions between randomly selected alleles, which are of the greatest evolutionary interest has not (Zeyl 2007). Since mutations that confer antibiotic resistance are known to affect bacterial fitness, levels of epistasis amongst such mutations may determine how multiple-resistance evolves. Such knowledge can be used to understand and predict what type of resistance mutations are likely to be segregating in microbial populations (Gagneux et al. 2006). To understand and predict the evolution of multiple-resistance it is of key importance to know how the fitness of sensitive and resistant bacteria is affected in different environments, particularly both in the presence and in the absence of drugs. Recent studies have shown that interactions exist amongst pairs of antibiotics, i.e. resistance to one drug affects the action of another drug (Chait et al. 2007; Yeh et al. 2006). In particular the combination of pairs of drugs has been studied and the combinations have been characterized as additive, synergistic, antagonistic or suppressive. Importantly it has been found that in certain drug combinations (suppressive) one of the antibiotics may render the treatment more effective against its resistant mutant than against the wild type (Chait et al. 2007). However, we are lacking data on genetic interactions amongst

single nucleotide mutations conferring antibiotic resistance in a drug free environment, i.e. the cost of multiple-resistance. With the occurring increase in frequency of multiple resistant bacteria and the public health problems associated with it, knowledge on the type and strength of epistasis is of most importance in understanding the evolution of multiple-resistance and, ultimately, the planning of new strategies for human intervention.

One can think that a way to halt the spread of resistance to a given antibiotic is to stop the use of that antibiotic. In the absence of the antibiotic for which resistance has been acquired, antibiotic-resistant mutants have a fitness cost when compared to sensitive bacteria (Andersson and Levin 1999; Williams and Heymann 1998). However, when infection is not resolved, a common strategy is to continue treatment with a different antibiotic to which the infecting bacteria are still susceptible. Unfortunately this strategy has led to rapid increase in multiple-drug resistance and not to the loss of resistance to the first treatment, as it would be desired (Bonhoeffer et al. 1997). This raises the possibility that multi-resistant mutations are not independent. If so, when resistance first develops the following question should be asked: if a pathogenic strain is resistant to antibiotic X, which antibiotic should be administered as a second treatment? Clearly, the strategy will depend not only on knowledge about the fitness costs of single resistance mutations but also on the level of genetic interactions – epistasis - between the alleles that underlie those phenotypes. When epistasis exists, it can be positive (antagonistic or alleviating) or negative (synergistic or aggravating). If strong negative epistasis amongst drug resistance alleles is found, then the cost of multiple resistances is high and one can expect multi-drug resistant microbes to be counter selected and disappear very rapidly in the absence of either drug. On the contrary, a much more worrying scenario is the existence of positive epistasis, for it implies that the expected time for elimination of multiple

resistance, even if antibiotic pressure is inexistent, will be much longer and multiple resistant bacteria are expected to accumulate in the population.

Here we quantify the degree of genetic interactions on cellular fitness in an antibiotic free environment for point mutations which confer resistance to commonly used antibiotics of three different classes. We have focused on resistances to: (i) the quinolone nalidixic acid, which inhibits DNA replication by binding to DNA gyrase; (ii) rifampicin, which belongs to the rifamycins class of antibiotics that bind to the β-subunit of RNA polymerase thereby inhibiting transcription; and (iii) streptomycin, an aminoglycoside that binds to the ribosome and inhibits elongation of protein synthesis (Kurland et al. 1996). We find that epistasis is allele specific and that the vast majority of allelic combinations exhibit positive epistasis. The costs of double resistance are therefore smaller than what one would expect if they were independent. Interestingly we found several cases of sign epistasis, which implies that mutations conferring resistance to a new antibiotic are compensatory, i.e. alleviate the cost of resistance present on another locus.

Materials and Methods

Bacterial Strains and Growth Conditions

The strains used were *Escherichia coli* K12 MG1655 and *Escherichia coli* K12 MG1655 Δara. All clones with antibiotic resistant mutations were derived from the ancestral strain *Escherichia coli* K12 MG1655. *Escherichia coli* K12 MG1655 Δara, was used as reference for

the competition fitness assay. The two strains are distinguishable by phenotypic difference due to a deletion in the arabinose operon: ara^+ and Δara give rise to white and red colonies, respectively, in tetrazolium arabinose (TA) indicator agar (Lenski et al. 1991). The clones were grown at 37°C on plates containing Luria-Bertani (LB) supplemented with agar and the respective antibiotics. The antibiotic concentrations were 100μg/ml for rifampicin, 40μg/ml for nalidixic acid and 100μg/ml for streptomycin. To estimate fitness costs competitions were performed during 24 hours in 50 ml screw-cap tubes containing 10 ml of LB medium at 37°C, with aeration (orbital shaker at 230 RPM). To estimate the frequency of each strain, in the beginning and by the end of the competition, Tetrazolium Agar (TA) medium containing 1% peptone, 0.1% yeast extract, 0.5% sodium chloride, 1.7% agar, 1% arabinose and 0.005% Tetrazolium chloride was used. All sets of dilutions were done in $MgSO_4$ at a concentration of 0.01M.

Isolation of P1 Transducted and Spontaneous Resistant Clones

The measure of two-locus epistasis requires the construction of double mutants from single mutants, in order to obtain clones with the same mutations alone and in combination. The antibiotics chosen to isolate the mutants were rifampicin, nalidixic acid and streptomycin. Sets of 40 single clones resistant to each antibiotic were obtained by growing independent cultures of *Escherichia coli* K12 MG1655, plating in Luria-Bertani (LB) agar medium supplemented with each antibiotic and randomly selecting the clones after 24 hours of incubation at 37°C. Each clone was streak plated, and a single colony was grown in a screw-cap tube with 10 ml of LB medium supplemented with the respective antibiotic and stored in 15% glycerol at -80°C. Generalized transduction

of the resistance mutations with bacteriophage P1 was performed as described previously (Silhavy et al. 1984). These mutations were obtained by isolating spontaneous resistant clones and then using these as donor or recipient strains for the construction of the double mutants. Whereas in the great majority of clones the transduction efficiency was high, in five combinations of clones it was extremely low. These clones were termed synthetic sub-lethals and are indicated on Figure 2C. For two combinations of double resistance mutations, three independent clones were assayed for fitness. No significant differences were observed (Kruskal-Wallis test) between these clones.

Three single spontaneous clones, resistant to each antibiotic, were used to put back the resistance on the wild-type sensitive background through P1 transduction. We then measured in 5 fold replicate competitions the fitnesses of each spontaneous mutant and the corresponding single P1 transducted clone. Pairwise comparisons, by Wilcoxon test, revealed no significant differences between the single resistance clones constructed in different ways.

We exposed the single resistant clones to a second antibiotic to select for spontaneous mutants resistant to two antibiotics. These were obtained by culturing each clone carrying resistance independently and plating in Petri-dishes with the two antibiotics. Clones with the double resistance were picked randomly. All spontaneous resistant clones were tested for a mutator phenotype and those few with evidence of high mutation rate were disregarded.

Detection of Mutations

The main target genes for resistance to rifampicin, nalidixic acid and streptomycin are *rpoB*, *gyrA* and *rpsL*, respectively. To know the mutations that confer the obtained resistances, each target was

amplified and then sequenced. The primers used to amplify the portion of the *rpoB* gene encoding the main set of mutations conferring resistance to rifampicin were: 5′-CGTCGTATCCGTTCCGTTGG-3′ and 5′-TTCACCCGGATAACATCTCGTC-3′; for *gyrA* gene, which encodes for the main set of mutations conferring resistance to nalidixic acid, 5′-TACACCGGTCCACATTGAGG-3′ and 5′-TTAATGATTGCCGCCGTCGG-3′; for *rpsL* gene, that encodes for the mutations conferring streptomycin resistance, 5′-ATGATGGCGGGATCGTTG-3′ and 5′-CTTCCAGTTCAGATTTA CC-3′. The same primers were used for sequencing straight from the PCR (Polymerase Chain Reaction) product.

Fitness Assays

To measure fitness cost of the resistance mutations a competition assay was done. The resistant mutants were competed against a reference strain, *Escherichia coli* K12 MG1655 Δara in an antibiotic free environment, in an approximate proportion of 1:1. To do so, we grew both resistant and reference strains in LB liquid medium for 24 hours at 37^{o}C with aeration. Accurate values of each strain initial ratio were estimated by plating a dilution of the mixture in TA Agar plates. Competitions were performed in 50 ml screw-cap tubes containing 10 ml of LB liquid medium by a period of 24 hours at 37^{o}C with aeration. By the end of this competition process, appropriate dilutions were platted onto TA Agar plates to obtain the final ratios of resistants and reference strains. The fitness cost of each mutant strain- i.e the selection coefficient- was estimated as the per generation difference in Malthusian parameters for the resistant strain and the marker strain (Lenski et al. 1991), discounted by the cost of the Δara marker. The fitness cost was estimated as an average of four and five independent competition assays for P1 and spontaneous resistant clones respectively. No

correlation was observed between the cost of the resistance mutations and the frequency at which they arose (Figure S1).

Measure of Epistasis and Statistical Significance

Pairwise epistasis, ε, can be measured assuming a multiplicative model in which case: $\varepsilon = W_{AB}W_{ab} - W_{Ab}W_{aB}$, where W_{ij} is the fitness of the clone carrying alleles *i* and *j* and capital letters represent the wild-type sensitive alleles. Error (σ_ε) of the value of ε is then estimated by the method of error propagation:

$$\sigma_\varepsilon = \sqrt{W_{ab}{}^2\sigma_{WAB}{}^2 + W_{AB}{}^2\sigma_{Wab}{}^2 + W_{aB}{}^2\sigma_{WAb}{}^2 + W_{Ab}{}^2\sigma_{WaB}{}^2}\,.$$

Whenever the value of ε was within the error we considered that alleles *a* and *b* did not show any significant epistasis (we indicate such combinations as white boxes labeled no epistasis in Figure 2C).

From the distribution of values of ε, provided in Figure 2B, we calculated the median value of ε and its 95% CI by bootstrap where we took 1000 samples. We tested normality of the distribution by a Shapiro-Wilk normality test (P=0.024), and a Wilcoxon test for the location in zero resulted in a P value of P=0.0006.

To query about the presence of sign epistasis in the data we made pairwise comparisons between the fitness of each double resistant clone and its corresponding single resistant clones using a Wilcoxon test to assess if the fitness of the double resistant was higher than the fitness of any of the single resistant clones. The P values are indicated in Figure 3 for those combinations that provided significant results, at 5% confidence level, after Bonferroni correction (n=2 comparisons). For some of the double resistant clones created by P1 transduction that

indicated the presence of sign epistasis- see Figure 3- we also obtained spontaneous double resistant mutants, that equally indicated evidence for sign epistasis.

Epistasis is sometimes calculated assuming an additive model (Phillips 2008): $\varepsilon = c_{ab} - (c_a + c_b)$, such that it measures the deviation of the cost of carrying double resistance from the sum of the costs of each resistance. Since the values of the majority of the cost are small, applying the multiplicative or the additive model leads to the same conclusions, i.e. those combinations of alleles that lead to positive (negative) epistasis under the multiplicative model, also lead to positive (negative) epistasis under the additive model. A Kolmogorov-Smirnov comparing the distributions of ε under multiplicative model with ε under the additive model results on P=0.9. The median value in the distribution of epistasis calculated under the additive model is 0.025 with bootstrap 95% CI [0.015; 0.036] and a Wilcoxon test for location strongly supports the presence of positive epistasis: P=0.00002. This shows that the same pattern occurs applying either the multiplicative or the additive models.

To perform the statistical analysis we used the free software R: http://www.r-project.org.

Results

Single Resistant Mutants' Fitness Costs

To study the degree of epistatic interactions amongst alleles that confer antibiotic resistance we started by selecting a series of *Escherichia coli* spontaneous mutants resistant to commonly used

antibiotics of 3 different classes: nalidixic acid, rifampicin, and streptomycin (Methods). From a panel of 120 sequenced clones that carry a single nucleotide change, we obtained 19 different classes of clones (Table S1) with spontaneous mutations in *gyrA*, *rpoB* and *rpsL*, which are the correspondent common target genes of resistance to nalidixic acid, rifampicin, and streptomycin, respectively. Resistant bacteria with mutations in the same amino acids as those collected here are segregating in microbial populations (Gagneux et al. 2006; Siddiqi et al. 2002). We should notice that our procedure for isolating clones carrying antibiotic resistance requires that viable colonies of resistant bacteria can be formed and detected. Any mutations that can arise and cause very high fitness costs cannot therefore be accounted for in this study. Given that highly deleterious mutations are unlikely to segregate in natural populations (Gagneux et al. 2006), genetic interactions amongst such mutations are likely to be of less clinical importance, unless these can be very easily compensated for.

We determined the cost of resistance of each of the clones in the absence of antibiotics. This was performed by measuring relative competitive fitness of the resistant strains against a marked sensitive strain through a competition assay in liquid LB medium without antibiotics (Lenski et al. 1991) (Methods). Figure 1A shows the distribution of fitness costs of each of the mutants. The genotype of each mutation as well as its fitness costs are provided in Table S1. On average the cost of antibiotic resistance was 9%. A Kolmogorov-Smirnov test does not reject the exponential distribution (P=0.85), a commonly used model to describe the distribution of deleterious mutations in several organisms (Eyre-Walker and Keightley 2007). Mutations that confer resistance to nalidixic acid had a lower average fitness cost (3%) than streptomycin or rifampicin (13% and 9%, respectively). Similar levels of fitness cost were found previously in resistant mutants of

Mycobacterium tuberculosis (Gagneux et al. 2006; Maisnier-Patin et al. 2002) and *E. coli* (Levin et al. 2000; Reynolds 2000).

Epistasis Between Deleterious Mutations that Confer Antibiotic Resistance

To study the fitness cost of double resistance and measure epistasis we constructed by P1 transduction all possible pairwise combinations (103) between different resistance alleles. These correspond to 5x11 streptomycin/rifampin, 5x3 streptomycin /nalidixic acid and 11x3 rifampin/nalidixic acid possible combinations. We measured the fitness of the obtained double resistant by competition assays, determined its cost and compared it with the cost that would be predicted if there was no epistasis. Figure 1B shows that the average cost of double resistance is less than twice the average cost of a single resistance (compare with Figure 1A), indicating positive epistasis. Fitness of double resistance was used to calculate pairwise epistasis. Pairwise epistasis, ε, between locus A and B can be measured as follows (Kouyos et al. 2007). 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:

$$\varepsilon = W_{AB}W_{ab} - W_{Ab}W_{aB}.$$

When the value of the costs is small then this measure becomes very similar to the difference between the cost of double resistance and the sum of the costs of each resistance.

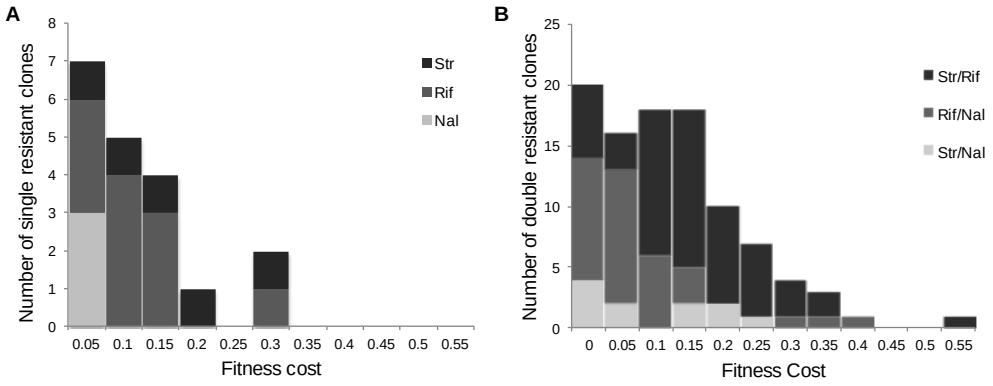

Figure 1. Distribution of fitness costs of single and double mutants indicative of positive epistasis. A Distribution of fitness costs of clones carrying single point mutations that confer resistance to streptomycin (black), rifampicin (dark grey) and nalidixic acid (light grey). **B** Distribution of fitness costs of clones carrying double resistance to streptomycin/rifampicin (black), rifampicin/nalidixic acid (dark grey) and streptomycin/nalidixic acid (light grey). The mean fitness cost of double resistants is less than twice the mean cost of single resistance mutations.

Strikingly, the majority of mutations show positive epistasis. 68% of the points are above the line in Figure 2A and from the clones that show significant epistasis 15% show negative epistasis and 42% show positive epistasis. The later ones correspond to clones where the cost is less than the sum of the costs of each resistance (see also Figure 2C for the specific combinations of alleles showing significant positive epistasis).

It has been suggested from theoretical modeling of RNA secondary structures and from studies in digital organisms – computer programs that mutate and evolve- (Wilke and Adami 2001) that epistasis and the fitness effect of mutations may be correlated, such that mutations with larger effects are more epistatic. In our data we find significant correlations between the strength of epistasis (deviation from zero in absolute value) and the costs of the mutations (Pearson's correlation $r=0.61$, $P<0.001$), which support this theoretical prediction. We also find a marginally significant correlation between the value of epistasis, ε, and the cost of the mutations (Pearson's correlation $r=0.19$, $P=0.06$).

Figure 2B shows the distribution of the ε values, the median is significantly positive (median=0.025, Bootstrap 95% CI [0.016; 0.032]), and its value corresponds to about 1/3 of the average cost of each single mutation. From the allelic combinations for which there is significant epistasis (53%), only 27% give rise to negative epistasis, whereas 73% of these combinations result in positive epistasis. Epistatic interactions were observed more frequently between mutations in *gyrA* and in *rpsL*, with high frequency of positive epistasis but also extreme cases of negative epistasis (synthetic sub-lethal, Figure 2C). Combination of *gyrA* and *rpoB* mutations produced the lowest frequency of negative epistasis (6%), which suggests that the sequential prescription of antibiotics leading to these resistances may easily result in multi-resistance development.

Focusing on the mutations we notice that the interactions are not gene but allele specific. For example *R529H* and *H526L* mutations in *rpoB* notably showed very high negative epistasis frequency, whereas four other mutations (*D516V*, *H526N*, *I572F* and *S512F*) in the same gene showed no negative epistasis regardless of the combination, and *D516V* and *I572F* mutations revealed high frequency of positive epistasis. The latter are potential candidates to segregate in natural populations. The *rpoB H526D* mutation is a specific example where its epistasis is highly dependent on the particular allele of the second mutation: *rpoB H526D* interacts positively with *rpsL K43N*, but negatively with *rpsL K43T*. Interestingly, the *rpoB H526D* mutation has been found in multi-drug resistant *M. tuberculosis* strains and also in this organism its cost was shown to depend on the genetic background (Gagneux et al. 2006). This suggests two important predictions: that we should find resistance alleles with strong positive interaction segregating at higher frequencies (such as *rpoB H526D*/ *rpsL K43N*) in natural populations and that these genetic interactions should also apply to microorganisms other than *E. coli*.

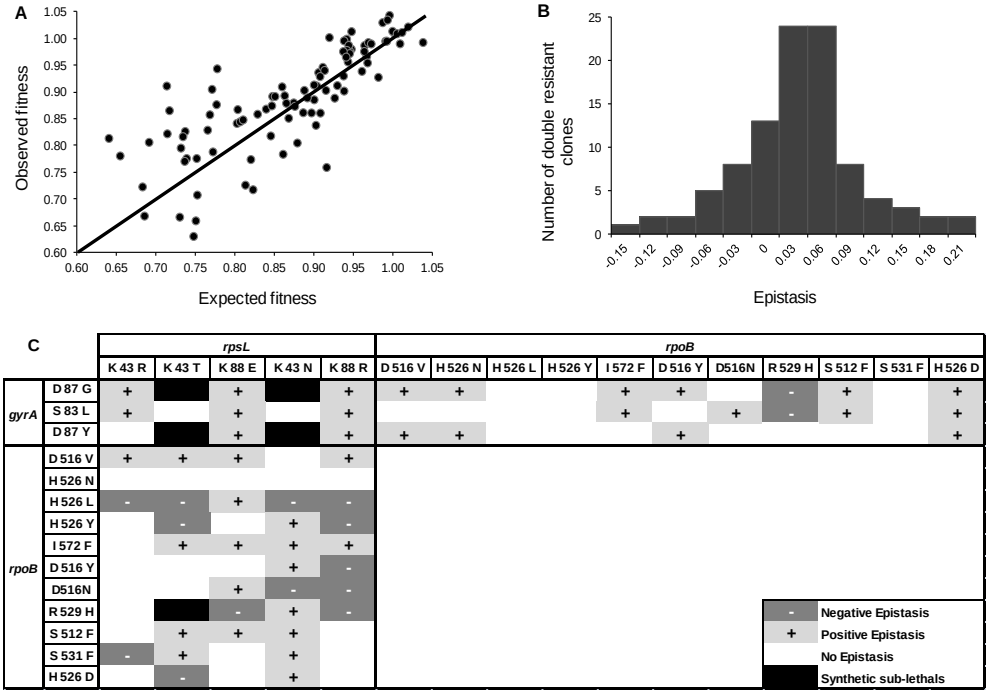

Figure 2. Evidence for positive epistasis. A Relation between the observed fitness of the double resistance genotypes and the expected fitness under the assumption of no epistasis. **B** Distribution of the epistasis level ε, whose median is 0.025 with bootstrap confidence interval [0.016, 0.032], showing positive epistasis. **C** Allelic dependence of epistasis between the *rpsL*, *rpoB* and *gyrA* (positive epistasis in light grey, negative in dark grey and not significant in white. Black indicates combinations of alleles for which there was a low efficiency of transduction - synthetic sub-lethals).

Sign Epistasis in Antibiotic Resistance Mutations

Recently it has been shown that an important type of epistasis, which is known as sign epistasis (Weinreich et al. 2005), may constrain the evolution of resistance to high penicillin concentrations (Weinreich et al. 2006). Sign epistasis happens when the sign of the fitness effect of a mutation (deleterious or beneficial) is itself epistatic, i.e. sign epistasis exists when a mutation is deleterious on some genetic backgrounds but beneficial on others. This form of epistasis, if common, may give rise to

multiple peaks on the fitness landscape. In the context of antibiotic resistance, the implication is simple: if sign epistasis is pervasive then it will be much more difficult to move from a scenario of multiple drug resistance to a scenario where all the bacteria are sensitive, even if antibiotic selection pressure is stopped. Experimental evolution studies have shown that, within hundreds of generations, mutations which compensate for the cost of antibiotic resistance (compensatory) are more likely to occur (Björkman et al. 2000; Levin et al. 2000; Schrag and Perrot 1996) than revertants (Maisnier-Patin et al. 2002). This suggests that sign epistasis might have an important role for the evolution of antibiotic resistance. Within the allelic combinations studied, 12% of our clones showed unexpected sign epistasis between drug resistance alleles. This corresponds to double mutants that have a fitness bigger than the fitness of at least one of the single mutants (Figure 3), and here it means that the mutation conferring resistance to a new antibiotic is beneficial (compensatory) when in a genetic background that contains a mutation conferring resistance to a different antibiotic. This is the worst possible scenario for the host and the best possible for the microbe. Given that a particular mutation was just selected by application of an antibiotic, evolution by natural selection makes it likely that the fixation of a mutation conferring resistance to another antibiotic will occur, even if selective pressure is not applied. Specifically, we find that the same mutation conferring streptomycin resistance (*rpsL K88E*, Figure 3) can be compensated by different mutations that confer rifampicin resistance showing that rifampicin treatment should be avoided in patients infected with *rpsL K88E* streptomycin resistant mutants. Given these results, knowledge of both the clinical history of patient antibiotic use as well as the specific genotypes associated with a given resistance is recommended for predicting the optimal clinical outcomes.

Another worrying class of clones that we found corresponds to combination of double resistant mutations that entail no significant cost. These are 6 out of the 103: *rpsL K43R*/*gyrA D87G*, *rpsL K43R*/*gyrA D87Y*, *rpoB H526N*/*gyrA D87G*, *rpoB H526N*/*gyrA D87Y*, *rpoB D516V*/*gyrA D87Y*, *rpsL K43R*/*rpoB H526N*. These mutants have no disadvantage against the wild-type. Although this constitutes a small percentage, it is nevertheless of extreme importance since for these double mutants little, if any, compensation is required for restoring the competitive ability of the wild-type. Given the known association between the cost of resistance mutations measured in the laboratory and the frequency at which they are found in clinical settings has been demonstrated, at least in *M. tuberculosis* (Gagneux et al. 2006), we predict that those combinations of double mutations are the ones which are more likely to be found. Future studies are planned to test this prediction, although it should be noted that the target for resistance may vary between species and environmental conditions (MacLean and Buckling 2009). In the opposite extreme we found five clones for which the transduction efficiency was very low (combinations shown in black, Figure 2C) which might correspond to combinations of mutations that must entail high fitness cost.

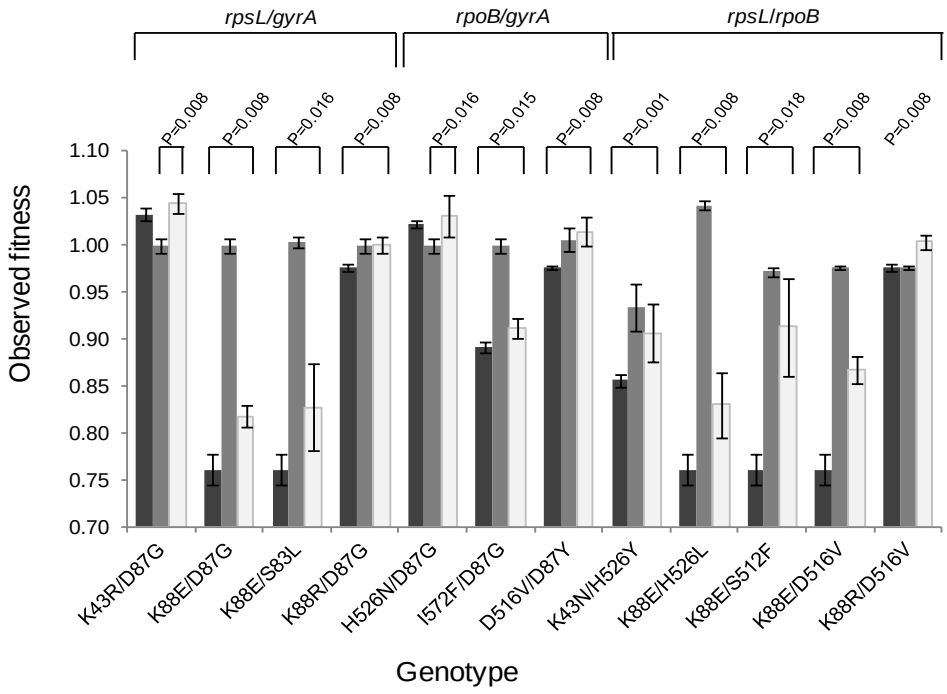

Figure 3. Evidence for sign epistasis amongst alleles conferring resistance. Sign epistasis occurs when the fitness of the double mutant (white bar) is greater than the fitness of at least one single mutant (dark grey and light grey bars). The genotypes of the double mutants where we found sign epistasis are indicated below the bars and the P values of the Wilcoxon test are indicated above the bars.

Level and Pattern of Epistasis in Spontaneous Double Resistant Clones

To make predictions about which resistance alleles are likely to be segregating it is important to study the frequency at which they spontaneously arise. To query which of the P1 transducted mutants are likely to naturally occur and if these also show pervasive epistasis we collected hundreds of spontaneous double resistant mutants. From 289 clones that were sequenced, we obtained 76 different genotypes, whose frequencies are given in Figure 4. We performed a χ^2-square test to investigate the effects of genetic background on the spectrum of

mutations that spontaneously arise. We observe that there are significant differences between the types of new resistance mutations that appear in certain resistant backgrounds and the wild-type sensitive background (Table S2).

We measured the fitness of each of the different spontaneous double resistant clones and compared it with the fitness in the corresponding clones constructed by P1 transduction. 67 out of 72 spontaneous clones were not different from the P1 constructed mutants (Figure S2), but surprisingly, five double resistant clones (*rpoB S531F*/*gyrA D87Y*; *rpsL K43T*/*rpoB H526Y*; *rpsL K88R*/*rpoB H526D*; *rpsL K43R*/*rpoB S531F*; *rpoB I572F*/*rpsL K88R*) had a significantly higher fitness (Wilcoxon test, $P<0.01$). These five spontaneous mutants have a higher fitness because they must have acquired an extra mutation during their isolation which is compensatory. To show that this is in fact the case we measured the fitness of independent clones carrying the same resistance mutations. For three of these haplotypes (*rpsL K43T*/ *rpoB H526Y*, *rpsL K88R*/ *rpoB H526D*, *rpsL K43R*/ *rpoB S531F*) we measured the fitness of spontaneous clones which were obtained applying the reversed antibiotic selection procedure. (i.e. the clones were now isolated by selecting first for rifampicin and secondly for streptomycin). A Wilcoxon-test revealed that the fitness values of these new independent clones were not different from the corresponding P1 clones, showing that the original spontaneous clones carry a compensatory mutation. Given the reduced number of generations in the procedure of generating spontaneous double resistant mutants, observing a compensatory mutation is only probable if such mutation has a very strong effect (s_c). This is because only with a large s_c it will not be stochastically lost (probability of fixation $\sim 2s_c$), and can fix in such short time (time to fixation $\sim 1/s_c$) (Crow and Kimura 1970). Indeed for the five mutants mentioned above, the estimated fitness effect s_c is very large, on average 0.09 (with the corresponding effects of each compensatory mutation 0.07; 0.13; 0.11;

0.07; 0.06). Adaptive mutations of such strong effect emerging and fixing so rapidly in bacterial populations under such small effective population size, is surprising given previous estimates of effects of beneficial mutations (on average 0.01) (Perfeito et al. 2007). A strong compensatory mutation must also have occurred in the spontaneous clones carrying *rpsL K43T*/*gyrA D87G*, *rpsL K43N*/*gyrA D87G*, *rpsL K43T*/*gyrA D87Y* and *rpsL K43N*/*gyrA D87Y* mutations, which were determined as synthetic sub-lethals by P1 transduction.

We calculated the ε values for the 67 spontaneous clones where we do not have evidences for extra compensatory mutations and obtained the same trend as with the double mutants constructed by P1 transduction (58% showed significant epistasis from which 74% showed positive epistasis and 7% that had no significant cost (Figure S3)). These results indicate that positive epistasis is also pervasive in spontaneous mutants supporting the relevance of our results. Additionally, there is evidence from epidemiological studies that in *M. tuberculosis* environmental resistant isolates more than 96% of the strains resistant to rifampicin have at least one mutation in *rpoB*, 52 to 59% of the streptomycin resistant strains have mutations in *rpsL* and 74 to 94% of the strains resistant to ofloxacin or levofloxacin (quinolones like nalidixic acid used here) have mutations in *gyrA* (Sekiguchi et al. 2007). Also, the same type of mutations have been isolated in *E. coli* (Dominguez et al. 2002) and in *Salmonella enterica* where 42% of the isolates showed substitutions in *gyrA* at position S83 and 35% at position D87 (Hopkins et al. 2007), that we show here to exhibit epistasis. Thus we expect the traits observed to be relevant for the evolution of multi-drug resistance acquired during treatment of infectious agents.

				1st Mutation															
				rpsL					*rpoB*										
				K 43 R	K 43 T	K 88 E	K 43 N	K 88 R	D 516 V	H 526 N	H 526 L	H 526 Y	I 572 F	D 516 Y	D516N	R 529 H	S 512 F	S 531 F	H 526 D
2nd Mutation	*gyrA*	80	D 87 G	30	60	30	40	0	30	70	60	50	70	10	50	70	50	50	50
		5	S 83 L	10	0	0	0	10	20	10	20	0	0	20	40	0	10	30	0
		12.5	D 87 Y	20	10	20	30	0	0	10	10	40	10	60	0	30	0	10	10
	rpoB	37.5	D 516 V	0	0	10	10	0											
		12.5	H 526 N	10	0	0	0	0											
		5	H 526 L	0	0	40	0	70											
		15	H 526 Y	30	20	0	10	0											
		5	I 572 F	10	10	20	10	0											
		2.5	D 516 Y	0	0	10	0	0											
		2.5	D516N	0	0	10	0	0											
		2.5	R 529 H	0	0	0		0											
		2.5	S 512 F	0	0	0	10	0											
		5	S 531 F	40	30	0	10	0											
		2.5	H 526 D	10	0	0		10											
	rpsL	2.5	K 43 R						30	20	12.5	0	0	33.3	0	66.7	0	10	0
		52.5	K 43 T						10	40	62.5	50	80	33.3	0	0	33.3	70	60
		2.5	K 88 E						0	0	0	0	0	0	0	0	0	0	0
		32.5	K 43 N						60	30	25	0	10	33.3	33.3	0	66.7	20	20
		5	K 88 R						0	10	0	50	10	0	50	33.3	0	0	20

0%-10%	11%-20%	21%-30%	31%-40%	41%-50%	51%-60%	61%-70%	71%-80%	81%-90%	91%-100%

Figure 4. Mutational spectrum and frequency of spontaneous double resistance mutations. Given the genetic background of the first mutation (column) the frequency of the second mutation (raw) appears as percentage in grey scale gradient. For a given background the percentage of mutations in the second locus adds up to 100%, in most cases, except when the second mutation occurred in a different gene. The percentage of the occurrence of each mutation in a wild-type background is also shown in the third column.

Discussion

Given the importance of epistasis in a variety of biological features such as sex and recombination, buffering of genetic variation, speciation, and the evolution of genetic architecture (Kondrashov 1988), recent studies have focused on measuring genome-wide levels of epistasis. However, typically only deletions or knockout mutations were studied (Elena and Lenski 1997; Jasnos and Korona 2007; Segrè et al. 2005; St Onge et al. 2007). Here, albeit focusing in a small number of genes, we measured epistasis on fitness at the scale of the allele given that these may be the most common type of mutations segregating in natural populations and because it also allows us to study mutations in essential genes. Our results not only show the significance of these interactions for the evolution of antibiotic resistance but also reveal an extra layer of complexity on epistatic patterns previously unpredicted, since it is hidden in genome-wide studies of genetic interactions using gene knockouts.

The data obtained here revealed an average level of positive epistasis, which differs strongly from the results of the degree of epistasis among slightly deleterious mutations caused by random transposon insertions in *E. coli*, where on average no epistasis was found (Elena and Lenski 1997). Positive epistasis has also been found in HIV-1 isolates (Bonhoeffer et al. 2004). Since the data in that study was obtained by sampling mutants from natural populations, comparisons between the results obtained and those found here should be taken carefully. We note nevertheless that some bias towards positive epistasis that may be present in the HIV study is not present in our study, since we constructed all possible pairwise combinations of double mutants. It remains to be explored whether the type of mutations

(single nucleotide changes, deletions or tranpositions) can affect the pattern of genetic interaction which can be observed. We predict that it can since we show that the type of interactions is not gene but allele specific. Positive epistasis was also detected when studying interactions between rifampin and streptomycin resistant mutants in *Pseudomonas aeruginosa* (Ward et al. 2009). The pattern in *E. coli* presented here and the results found in *P. aeruginosa* (even though having a different genetic basis) (Ward et al. 2009) indicate that the presence of positive epistasis amongst antibiotic resistance mutations is not species specific. Furthermore epistatic interactions involving fluoroquinolone resistance mutations in *gyrA* have also been found in *Streptococcus pneumoniae* (Rozen et al. 2007).

Although we have studied different classes of antibiotics that affect different cell targets, our finding of pervasive epistasis can be reflecting the fact that these target genes are part of the fundamental flow from DNA to RNA to protein, and thus can be considered to be working in the same pathway. Because these affect highly conserved cell processes they should be relevant in many different organisms. An interaction between some of these genes has been described for specific traits, namely propagation of bacteriophage T7 (interaction between *rpsL* and *rpoB*) and mitomycin C resistance in *E. coli* (between *rpoB* and *gyrA*) (Chakrabarti and Gorini 1977; Kumaresan and Jayaraman 1988).

Our data, both of spontaneous and P1 transducted double resistant clones, also indicates the presence of sign epistasis in the cost of multi-drug resistance involving rifampin, streptomycin and nalidixic acid, that is, a small fraction of double resistant clones showed a higher fitness than at least one of the corresponding single resistant mutants. Sign epistasis implies that the fixation of one mutation (for example by strong selection pressure of a given antibiotic) may alter the adaptive path in both number and type of subsequent beneficial mutations. Sign epistasis was also previously found in the context of resistance to the

antibiotic cefotaxime (Weinreich et al. 2006). In this system, of the 120 possible mutational paths from the low resistance to high resistance, only 18 can actually occur due to the occurrence of sign epistasis. Although there are not many examples in the literature (Weinreich et al. 2005), this one clearly shows the power of sign epistasis in constraining protein adaptation.

Another interesting example in the context of this work is bacterial adaptation to the cost of resistance through the acquisition of new compensatory mutations. In such an adaptive process it was observed that adaptive mutations which reduce the cost of resistance to streptomycin in *E. coli* (Schrag et al. 1997) and Salmonella (Lenski et al. 1991) are deleterious in the streptomycin-sensitive background and therefore constitute an example of sign epistasis.

In this work we have determined the fitness effect of mutations in the absence of antibiotics. Future studies should focus on epistatic interactions when bacteria grow in the presence of antibiotics, a condition already shown to be relevant to the evolution of resistance (Hegreness et al. 2008; Michel et al. 2008).

Our results highlight the importance of determining the costs of single and multiple resistances and, accordingly ordering allele combinations by the degree of epistasis they exhibit. Given that at the present time, infections are likely to be caused by microbes that carry resistance to at least one drug, the strategy expected to give the best outcome is one in which the next drug is the one leading simultaneously to the resistant mutant with the biggest cost and strongest negative epistasis. Another approach to prevent the evolution of multidrug resistance would be to use drug combinations (at certain concentrations) that select for sensitive bacteria. This is a plausible scenario since it has been shown that when competing in the presence of the two drugs, sensitive

bacteria outgrow resistant (Chait et al. 2007). Our finding of pervasive positive epistasis suggests one possible explanation for the difficulty of eradicating multi-drug resistance in organisms like *M. tuberculosis*, for which current treatments involve combinations of the same drugs as studied here.

Author Contribution

Karina B. Xavier, Francisco Dionísio, Miguel G. Ferreira and Isabel Gordo conceived and designed the experiments. Sandra Trindade and Ana Sousa performed the experiments. Sandra Trindade, Ana Sousa, Karina B. Xavier and Isabel Gordo analyzed the data. Sandra Trindade, Ana Sousa, Karina B. Xavier and Isabel Gordo wrote the paper. Francisco Dionísio and Miguel G. Ferreira contributed to the writing of the paper.

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Supplementary Material

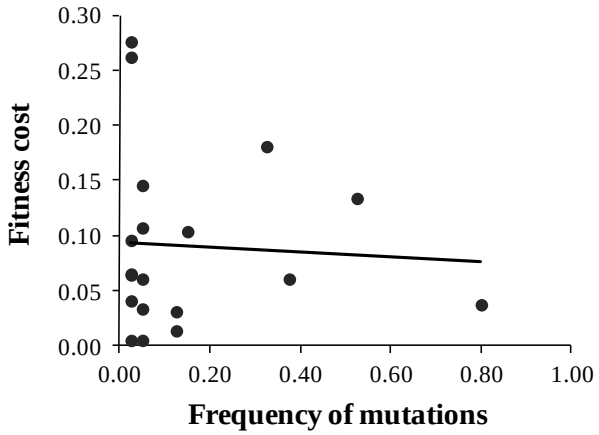

Figure S1. Frequency of appearance of spontaneous mutations as a function of their fitness costs. Linear regression: slope -0.02 ± 0.09(SE).

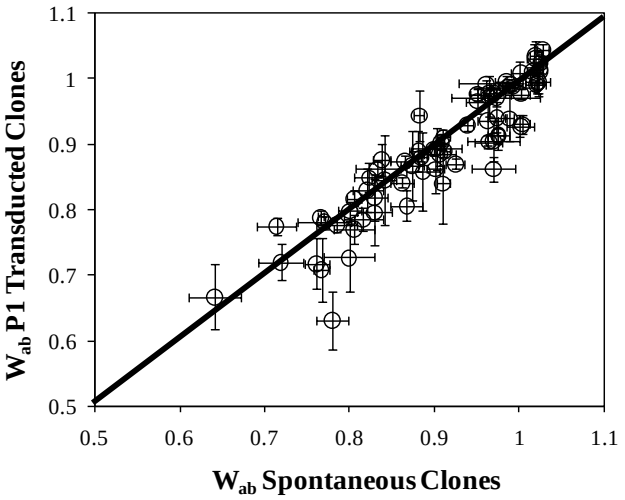

Figure S2. Comparison between double resistant spontaneous clones and the corresponding double mutants constructed by P1 transduction. Wilcoxon signed rank tests were made for assaying fitness differences between every pair of mutants constructed by the two independent methods. At a significance level of α=0.01, 5 spontaneous resistance clones were found to have higher fitness than the corresponding P1 transducted clones, and are therefore candidates to carry compensatory mutations.

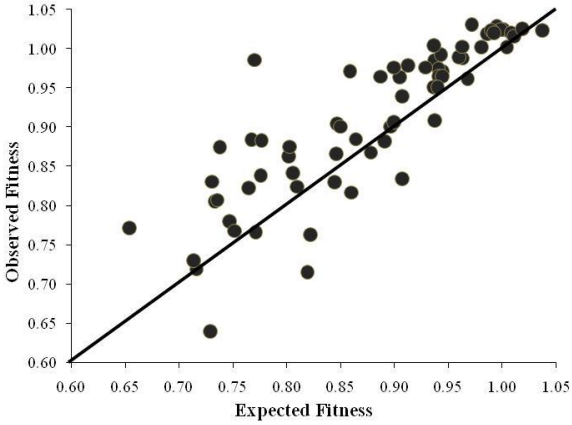

Figure S3. Evidence of positive epistasis in spontaneous double resistant clones. Relation between the observed fitness of the 67 spontaneous double resistance genotypes and the expected fitness under the assumption of no epistasis (compensated and synthetic sub-lethals clones are excluded from the data).

Table S1. Genotypes and fitness costs of single resistance mutations

Gene	Genotype aa change ; nt change	Cost (2SE)%	Resistance
gyrA	D 87 G ; GAC to GGC	3.7 (1.5)	Nal
gyrA	S 83 L; TCG to TTG	3.3 (1.2)	Nal
gyrA	D 87 Y; GAC to TAC	3.1 (1.9)	Nal
rpoB	D 516 V; GAC to GTC	6.0 (1.0)	Rif
rpoB	H 526 N; CAC to AAC	1.4 (1.1)	Rif
rpoB	H 526 L; CAC to CTC	0.4 (1.5)	Rif
rpoB	H 526 Y; CAC to TAC	10.3 (3.2)	Rif
rpoB	I 572 F; ATC to TTC	14.6 (1.2)	Rif
rpoB	D 516 Y; GAC to TAC	6.4 (4.0)	Rif
rpoB	D516N; GAC to AAC	4.1 (2.0)	Rif
rpoB	R 529 H; CGT to CAT	26.2 (4.9)	Rif
rpoB	S 512 F; TCT to TTT	6.5 (1.3)	Rif
rpoB	S 531 F; TCC to TTC	10.7 (1.5)	Rif
rpoB	H 526 D; CAC to GAC	9.6 (1.2)	Rif
rpsL	K 43 R; AAA to AGA	0.5 (1.4)	Str
rpsL	K 43 T; AAA to ACA	13.4 (2.0)	Str
rpsL	K 88 E; AAA to GAA	27.5 (2.8)	Str
rpsL	K 43 N; AAA to AAC	18.0 (1.9)	Str
rpsL	K 88 R; AAA to AGA	6.1 (1.2)	Str

Table S2. Results of χ^2 test on the effects of genetic background (wild type *versus* antibiotic resistant) on the spectrum of mutations that spontaneously arise

			Type of resistance conferred by second mutation		
			NalR	RifR	StrR
Background	*rpsL*	K 43 R	**		
		K 43 T			
		K 88 E	***		
		K 43 N	*		
		K 88 R	***	***	
	rpoB	D 516 V	***		*
		H 526 N			
		H 526 L			
		H 526 Y			**
		I 572 F			
		D 516 Y	**		
		D516N	*		*
		R 529 H			**
		S 512 F	**		
		S 531 F			
		H 526 D	**		

*P<0.05 **P<0.01 ***P<0.001

CHAPTER III

PERVASIVE SIGN EPISTASIS BETWEEN CONJUGATIVE PLASMIDS AND DRUG-RESISTANCE CHROMOSOMAL MUTATIONS

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Rui F. Silva, Sílvia C. M. Mendonça, Luis M. Carvalho, Ana M. Reis, Isabel Gordo, **Sandra Trindade** and Francisco Dionísio

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Multidrug-resistant bacteria arise mostly by the accumulation of plasmids and chromosomal mutations. Typically, these resistant determinants are costly to the bacterial cell. Yet, 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 study the interaction between antibiotic-resistance chromosomal mutations and conjugative (i.e., self-transmissible) plasmids and find many cases of sign epistasis (40%) – including one of reciprocal sign epistasis where the strain carrying both resistance determinants is fitter than the two strains carrying only one of the determinants. 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

Introduction

Multidrug resistance is a major hurdle for modern medicine, putting at risk commonplace medical practices (Martinez et al. 2009) and the treatment of infection by bacterial pathogens (Bertino 2009). Bacteria can become resistant by spontaneous mutation of chromosomal genes or through the acquisition of horizontally mobile genetic elements (Martinez et al. 2009). In the absence of antibiotics, resistance mutations are often deleterious and confer a fitness cost to the cell (Andersson 2006; Andersson and Levin 1999; Lenski 1998; Nilsson et al. 2003). It is logical to expect that, in the absence of antibiotic selective pressure, resistant strains will be outcompeted by the susceptible ones. Thus, a possible procedure to eliminate resistance is to ban the use of an antibiotic. This policy has been applied in different countries with varying results. For example, a deliberate reduction in the prescription of macrolides in Finland, resulted in a 50% decrease in the frequency of macrolide-resistant group A streptococci (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. For example, resistant-bacteria often gain second-site mutations that ameliorate the fitness cost of resistance (Andersson 2006; Andersson and Levin 1999; Bjorkman et al. 2000; Gagneux et al. 2006; Maisnier-Patin et al. 2002; Schrag and Perrot 1996; Schrag et al. 1997). Sometimes, compensatory mutations even increase the level of resistance itself (Orio et al. 2011; Trzcinski et al. 2006).

The exchange of accessory genetic elements, in particular of conjugative plasmids, is frequent (Boucher et al. 2003; Cohen and Pupko 2009; Denamur et al. 2000) and can disseminate genes among

related and phylogenetically distant bacteria (Cohen and Pupko 2009; Denamur et al. 2000; Dionisio et al. 2002). In addition, conjugative plasmids are able to mobilize other plasmids from a donor to a recipient cell (Amábile-Cuevas and Chicurel 1992). Thus, resistance genes can quickly spread among bacterial communities. Plasmid-encoded resistance is generally the result of the activity of efflux pumps, agent-modifying enzymes (Andersson and Levin 1999), or protection of the antibiotic target (Bennett 2008).

Harboring mobile genetic elements generally creates a cost to the host, associated with the replication and maintenance of the genetic element and with the expression of its genes. Such cost has been experimentally demonstrated in a number of resistance-encoding plasmids (Bouma and Lenski 1988; Dahlberg and Chao 2003; Dionisio et al. 2005; McDermott et al. 1993; Smith and Bidochka 1998).

A recent 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 one would expect if they were independent (Trindade et al. 2009). Gene interaction, or epistasis, is generally accepted as being relevant for the understanding of the evolution and dynamics of complex genetic systems (Phillips 2008). Epistasis can vary in strength and form. When epistasis affects fitness, one can expect two possible outcomes. A positive epistatic interaction has an antagonistic effect on deleterious mutations. Thus, the double mutant has a higher fitness than the expected sum of costs. Negative epistasis between deleterious mutations creates a synergistic effect. Here, the double mutant is less fit than the expected sum of costs. 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 and Chao 2004), *Salmonella typhimurium* (Maisnier-Patin et al. 2005) and in the yeast *Saccharomyces cerevisiae* (Jasnos and Korona 2007). Other studies have found no evidence of epistatic interactions within the HIV-1 transcriptional promoter (van Opijnen et al. 2006), in RNA virus (Crotty et al. 2001; Elena 1999) and in *S. cerevisiae* (Jasnos and 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 and Lenski 1997).

Here we focus on the interplay between conjugative plasmids and chromosomal mutations in *E. coli*. In particular, we look at how bacterial fitness is affected by genetic interactions between plasmids and resistance mutations. First, we quantify the degree of epistasis between five conjugative resistance plasmids (R124, R831, R16, R702 and RP4, carrying between one and four resistance genes and belonging to four different incompatibility groups) and 10 mutant alleles of the housekeeping genes *gyrA*, *rpoB* and *rpsL*, conferring resistance to nalidixic acid, rifampicin and streptomycin, respectively. The plasmids were isolated from nature and the resistance mutations are polymorphic in natural populations of different species of bacteria (Gagneux et al. 2006). These genes are involved in different steps of the cell's essential flow of information from DNA to protein. Specifically, *gyrA* codes for DNA gyrase, an enzyme involved in DNA replication. Nalidixic acid and other quinolones inhibit DNA replication by binding to DNA gyrase and resistance to this class of drugs arises from the prevention of this binding. Rifampicin belongs to the rifamycin class of antibiotics which bind to the β-subunit of RNA polymerase, coded by *rpoB*, thereby inhibiting transcription. Mutations in *rpsL*, which codes for ribosomal protein S12, interfere with translation and can produce resistance to streptomycin by blocking the binding of this drug to the ribosome 30S subunit. Secondly, using the same plasmids, we estimate epistasis

between pairwise combinations of conjugative plasmids inside the same cell.

We find pervasive sign epistasis in the interaction between resistance mutations and conjugative plasmids. This implies that the acquisition of an additional resistance plasmid to the existing chromosomal resistance or the appearance of a chromosomal drug-resistance mutation in a bacterial cell already containing a plasmid may ameliorate the initial fitness cost of resistance and therefore complicate resistance reversal. We also observed an overall positive level of epistasis between mutations and plasmids. Both the chromosomal allele and the plasmid seem to contribute to determine the nature of the epistatic interaction, although the host genotype appears to have a more determinant effect. In contrast, the interaction between plasmids exhibit sign epistasis only once, and, despite the occurrence of several cases of somewhat strong epistasis, on average it appears to be null.

Materials and Methods

Bacterial Strains, Plasmids and Growth Conditions

We used five natural conjugative plasmids, R124, R702, R16, R831, and RP4, kindly provided by the Institute for Health, Environment and Safety of the Belgian Nuclear Research Centre. Plasmid characteristics are listed in Table S1. 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 S2). These mutations have

been previously mapped to *gyrA*, *rpoB* and *rpsL* resulting in resistance to nalidixic acid, rifampicin and streptomycin (Trindade et al. 2009).

For the construction of bacterial strains with conjugative plasmids, donors and recipients (either wild-type *E. coli* K12 MG1655 or strains shown in Table S2) were put together for 24 hours. All donor strains are auxotrophic for specific amino-acids and/or unable to use maltose, due to deletions in essential genes/operons, as indicated in chromosomal markers: Mal^-: maltose; Trp^-: tryptophan; Met^-: methionine and Pro^-: proline. Selection of transconjugants 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. If donors are auxotrophic (Table S4) for two amino-acids (*E. coli* CM140 and *E. coli* CM597), transconjugants were selected on minimal medium plates containing glucose and no amino-acids. Otherwise, we used maltose and tryptophan (*E. coli* CM317, *E. coli* CM319, *E. coli* CM312). As a control we confirmed that neither donors (due to auxotrophies or inability to use maltose as carbon source) nor recipient (due to antibiotics selecting for plasmidic resistance genes) grow on these plates.

Transduction was done with P1 bacteriophage, according to the methods described by Trindade et al. (2009).

In 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. TA medium contains 1% peptone, 0.1% yeast extract, 0.5% sodium chloride, 1.5% agar, 1% arabinose and 0.005% tetrazolium chloride.

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 ampicillin.

Dilutions of cultures were done in $MgSO_4$ 0.01 M. All strains were kept frozen in 15% glycerol stocks.

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 or 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. (i) Both strains were grown in 10 ml of liquid LB medium for 24 hours at 37ºC with aeration. (ii) 50 μl of the dilution 10^{-4} of each strain was added to 50 ml screw-cap tubes containing 10 ml of liquid LB medium. (iii) Values of both strain's initial ratio were estimated by plating a dilution of the mixture in TA agar medium. (iv) Competitions proceeded by a period of 24 hours at 37ºC with aeration. (v) At the end of the competition, appropriate dilutions were plated onto TA agar plates to obtain the final ratios of both competitors. These competitions spanned about 19 to 22 bacterial generations. 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 does not allow the growth of the reference strain. The fitness cost of each strain – i.e. the selection coefficient, s, – was estimated as the per generation difference in Malthusian parameters between the mutant and the wild-type (r_m and r_w

respectively): specifically, $s = r_m - r_w = \log\left[\frac{m(T)/m(0)}{w(T)/w(0)}\right]/g$, where T is the final time and g is the total number of generations from t=0 until t=T. Then, we discounted the cost of the ∆ara marker (Lenski et al. 1991). The fitness cost was estimated as an average of three independent competition assays.

Measurement of Epistasis and Statistical Significance

As explained in the main text, epistasis between a mutation and plasmid can be calculated as $\varepsilon = W_{(a;plasmid)} - W_{(A;plasmid)}W_{(a;-)}$, where $W_{(A;plasmid)}$ is the relative fitness of the strain with the wild-type allele (*A*) and carrying the plasmid, $W_{(a;-)}$ is the relative fitness of the mutant strain (with *A* allele replaced by the *a* allele), and $W_{(a;plasmid)}$ is the relative fitness of the strain containing both the mutation (*a* allele) and the plasmid. Similarly, we defined epistasis between plasmids as $\varepsilon = W_{(plasmid1;plasmid2)} - W_{(plasmid1;-)}W_{(-;plasmid2)}$, where $W_{(-;plasmid2)}$ and $W_{(plasmid1;-)}$ are the fitnesses of single-plasmid strains relative to the wild-type plasmid-free strain, and $W_{(plasmid1;plasmid2)}$ is the fitness of the strain carrying two types of plasmids, relative to the same wild-type plasmid-free strain. Then, the error (σ_ε) of the value of ε is estimated by the method of error propagation;

for pairwise combinations of mutation and plasmid:

$$\sigma_\varepsilon = \sqrt{\sigma^2_{(a;plasmid)} + \sigma^2_{w_{(A;plasmid)}} w^2_{(a;-)} + \sigma^2_{(a;-)} w^2_{(A;plasmid)}} \;;$$

for pairwise combinations of plasmids:

$$\sigma_{\varepsilon} = \sqrt{\sigma^2_{(plasmid1;plasmid2)} + \sigma^2_{w_{(plasmid1;-)}} w^2_{(-;plasmid2)} + \sigma^2_{(-;plasmid2)} w^2_{(plasmid1;-)}}\,.$$

If the value of ε was within the calculated error, we considered that the two resistance determinants (mutation and plasmid or plasmid and plasmid) did not show significant epistasis (indicated as white boxes labeled "no epistasis" in Figure 1A and Figure 5A). From the distribution of values of ε, provided in Figure 1B and Figure 5B, we calculated the median value of ε and its 95% CI by bootstrap where we took 10 000 samples.

To test the presence of sign epistasis, we compared the fitness of each strain carrying two resistance determinants (mutation and plasmid or plasmid and plasmid) and its corresponding single resistance-determinant strains. We used a Student t-test to assess if the fitness of the double-resistance-determinants strain was higher than the fitness of any of the single resistance-determinant strains.

Statistical analyses performed using software Statistica 9.0 and MatLab R2009b. Computer simulations performed with Mathematica 7.

Results

Interaction Between Antibiotic Resistance Mutations and Resistance Plasmids

Pairwise epistasis, ε, between loci A and B can be measured as follows. Suppose that the wild-type strain contains alleles A and B. If

W_{Ab} and W_{aB} are the fitnesses of each of the single mutants relative to the wild-type strain, and W_{ab} the relative fitness of the double mutant, then multiplicative epistasis is given by: $\varepsilon = W_{AB}W_{ab} - W_{Ab}W_{aB}$. To estimate epistasis between plasmids and mutations, we defined these quantities in a similar way. If $W_{(A;plasmid)}$ is the relative fitness of the strain with the wild-type allele (*A*) and containing a plasmid, $W_{(a;-)}$ is the relative fitness of the mutant strain (with *A* allele replaced by the *a* allele), and $W_{(a;plasmid)}$ is the relative fitness of the strain containing both the mutation (*a* allele) and the plasmid, then epistasis between a plasmid and a chromosomal mutation becomes:

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

Each conjugative plasmid was introduced in *E. coli* K12 MG1655 cells by conjugation. Then, we determined the fitness cost due to the presence of each plasmid relative to plasmid-free *E. coli* K12 MG1655 cells. This was performed using a competition assay, in the absence of antibiotics (see Materials and Methods). Fitness costs of plasmids span from 2.8% to 8% (Table S1).

The fitness cost imposed by ten different spontaneous antibiotic-resistance mutations was previously determined in Trindade et al. (2009). Table S2 presents the clones chosen from Trindade et al. (2009) and the fitness costs of these mutations. Fitness costs of mutations vary between 0.5% and 27.5% (Table S2) (Trindade et al. 2009).

To screen for epistatic interactions between chromosomal mutations and conjugative plasmids, we further constructed, by conjugation, all possible 50 combinations between these ten mutations and the five plasmids. Then we determined the fitness for each of these 50 combinations.

We found that 52% of the interactions present positive epistasis and only 8% present negative epistasis (Figure 1A). Figure 1A additionally shows that the nature of the epistatic interaction is not gene but allele specific. In fact, the conjugative plasmid influences how a specific allele

interacts with plasmid-borne resistance determinants. This means that, depending on the plasmid, an allele can display no epistasis, positive epistasis or negative epistasis. For example, allele *gyrA D87G* exhibits no epistatic interaction with plasmid R124, however the same allele displays negative epistasis with plasmid R831 and positive epistasis with plasmids R16, R702 and RP4 (Figure 1A). The same pattern (allele specific nature of epistasis) had been observed for epistasis between resistance chromosomal mutations (Trindade et al. 2009). Supporting the pervasive nature of antagonistic interactions between mutations and plasmids, the distribution of the ε values (Figure 1B) has a significant positive median (median=0.037, bootstrap 95% CI [0.021; 0.065]). Figure S1 plots the observed fitness against the fitness expected in the absence of epistasis (ε=0).

To rule out the existence of compensatory mutations, we reconstructed five (double) combinations independently and in the opposite direction from what we did before: *gyrA S83L*(R16), *rpoB I572F*(R831), *rpoB H526N*(R16), *rpoB R529H*(R702), and *rpsL K43N*(RP4). We constructed these five clones by transducting (Trindade et al. 2009) the antibiotic resistance mutation from our mutant *E. coli* strains into the wild-type strain (*E. coli* K12 MG1655) already containing the plasmid. Using this method we decreased the number of generations involving the antibiotic-resistance mutation by a half (because the plasmid was already there). In this way, we decrease the probability of occurrence of compensatory mutations. We measured the fitness of two independent clones corresponding to each of the five (double) combinations. For all five combinations, fitness values are not significantly different from the ones obtained using the previous method (Kruskal-Wallis, P>0.05). Four (*gyrA S83L*(R16), *rpoB I572F*(R831), *rpoB H526N*(R16) and *rpoB R529H*(R702)) of these five combinations correspond therefore to cases of sign epistasis (Figure 1A). One combination (*rpsL K43N*(RP4)) shows no interaction with these new

independent clones as observed before (Figure 1A). The fact that independent clones exhibit the same fitness (and the same type of epistasis) shows that our results are robust. To further strengthen this point, we constructed two new independent clones of *rpoB R529H*(R702), this time using yet a different method: by simultaneous conjugation and transduction. The fitnesses of these clones were again not significantly different from before (Kruskal-Wallis, P>0.05).

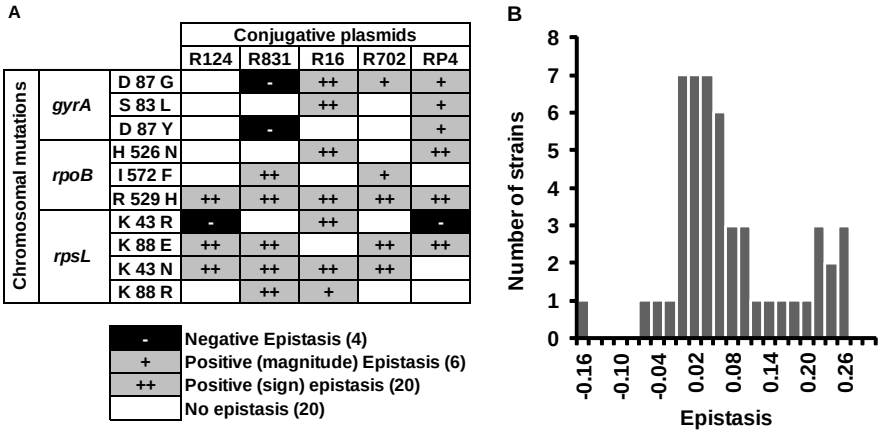

Figure 1. Epistasis between antibiotic resistance mutations and conjugative plasmids. A Epistasis between mutations in genes *gyrA*, *rpoB* and *rpsL* and conjugative resistance plasmids (positive epistasis in grey, negative in black and no epistasis in white). Sign epistasis is indicated with "++". **B** Distribution of the epistasis values. Median is 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 =0.000968).

Focusing on the resistance mutations, we notice that the mean epistatic value significantly varies among them (Kruskal-Wallis P=0.0016). Figure 2A shows that mutations *rpoB R529H*, *rpsL K88E* and *rpsL K43N* exhibit positive and large ε values. The other mutations show lower mean ε values (Mann-Whitney U-Test, P=0.000002).

Figure 2B shows that there is a significant correlation between the fitness cost created by a mutation and its mean epistatic value

(deviation from zero in absolute value) (Spearman P=0.006). In other words, mutations with a more deleterious effect on the cell tend to be more epistatic. This relationship had been initially proposed after *in silico* studies of digital organisms and theoretical modeling of RNA secondary structures (Wilke and Adami 2001). Our results 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).

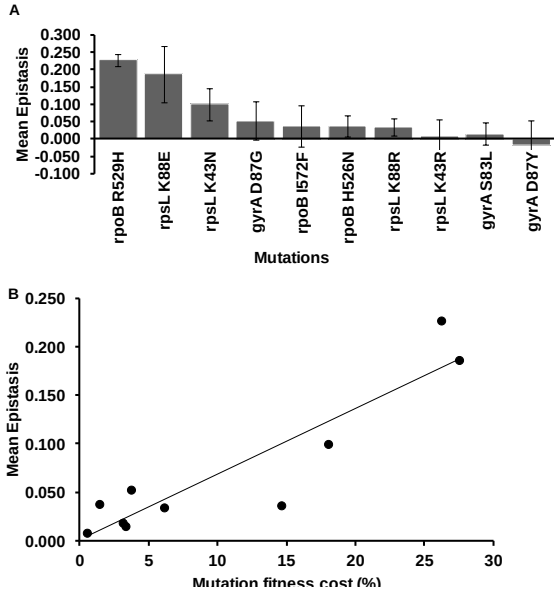

Figure 2. Mutation effect on the mean epistatic value. A Mean epistatic value for each mutation. Error bars indicate twice the standard error. Note how the mean epistatic effect significantly differ between mutations (Kruskal-Wallis P=0.0016) **B** Mutations become increasingly epistatic as their severity increases. Note how the mean absolute epistatic effect correlates with the fitness cost associated with each mutation (Spearman p=0.006).

Focusing on the conjugative plasmids, Figure 3A shows their mean ε values. There are no significant differences in the mean ε values between plasmids (Kruskal-Wallis P=0.676). This means that, on

average, all studied conjugative plasmids tend to interact in the same way with chromosomal mutations. Moreover, comparison between Figures 2A and 3A seems to suggest that the mutation (and not the plasmid) may be the major factor determining the type and the strength of the epistatic interactions we observed. In contrast to what was observed with the effect of mutations on epistasis (Trindade et al. 2009), there is no significant correlation between the fitness cost created by a plasmid and its mean epistatic value (Figure 3B – Spearman P=0.188). This may simply be due to lack of power, as variation in plasmids cost is much smaller than for mutations.

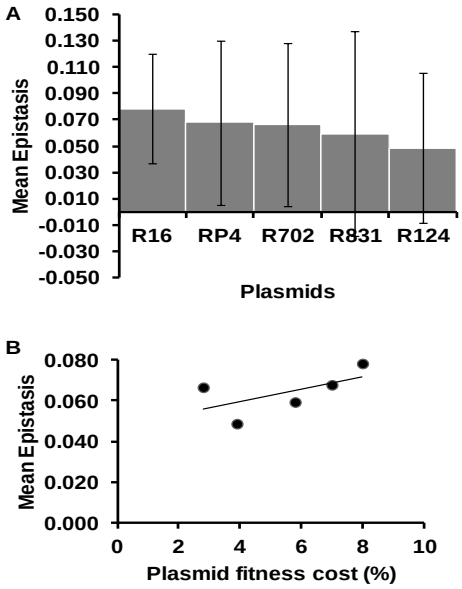

Figure 3. Plasmid effect on the mean epistatic value. A Mean epistatic value for each plasmid. Error bars indicate twice the standard error. Note how the mean epistatic effect does not significantly differ between plasmids (Kruskal-Wallis P=0.6758). **B** Evidence for the lack of correlation between the fitness cost associated with each plasmid and its mean epistatic value (Spearman p=0.188).

Sign Epistasis

A specific mutation can be deleterious on a particular genetic background and beneficial on others – a phenomenon known as sign epistasis. Strikingly, we report that 40% of the combinations between resistance chromosomal mutations and conjugative plasmids present sign epistasis (Figure 1A, where "++" indicates sign epistasis). These are cases where the strain carrying both resistant determinants was fitter than the strain carrying only the mutation or only the plasmid (Figure 4). One of the genotypes (*gyrA D87G*(R16)) presents reciprocal sign epistasis (Carneiro and Hartl 2010), meaning that the mutant *D87G* and harboring the R16 plasmid is fitter than both the plasmid-free mutant and the plasmid-bearing strain without the mutation (hence sensitive to nalidixic acid).

We found examples of sign epistasis in all resistance alleles and all plasmids of this study, i.e. there was no plasmid nor mutation where we did not find, at least one case of sign epistasis. This high prevalence of positive epistasis is not a consequence of plasmid transfer to the reference strain. The proportion of transconjugants was monitored at stationary phase, when bacterial density is higher than 10^9 cells per ml, and was found to be less than 3% (Table S3). Also, computer simulations show that these plasmid transfer events imply an error in the calculation of epistasis that is less than 1%, hence less than the experimental error.

Another interesting aspect of our data is that, three out of the 50 combinations (plasmid+mutation) presented fitness costs not significantly different from zero (t-test, $P>0.05$); these strains are the following: *rpsL K43R*(R16), *rpsL K43R*(R831) and *gyrA D87G*(R16).

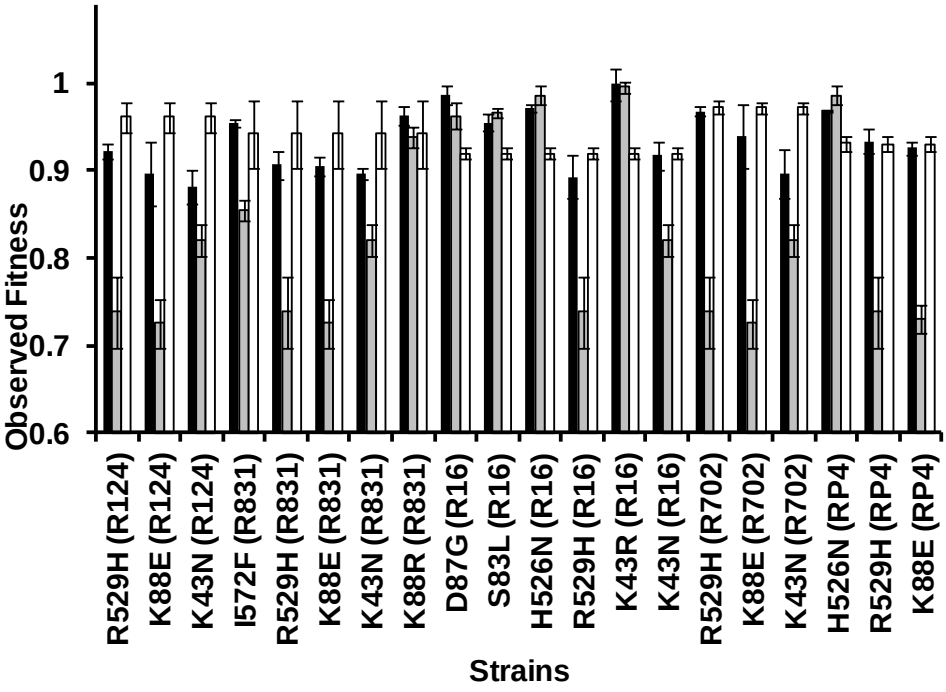

Figure 4. Sign epistasis between chromosomal mutations and conjugative plasmids. Sign epistasis occurs when the fitness of the strain carrying both resistance determinants (black bars) is greater than the fitness of at least one of the strains carrying a single resistance determinant (mutation - grey bars; or plasmid – white bars). The genotype D87G(R16) presents reciprocal sign epistasis (Carneiro and Hartl 2010). Error bars represent twice the standard error.

Interaction Between Conjugative Resistance Plasmids

Finally, we measured epistasis between conjugative plasmids. This is relevant because there have been several reports of bacterial pathogens harboring multiple resistance plasmids (Casjens et al. 2000; San Millan et al. 2009). We constructed nine out of the 10 possible pairwise combinations of the five plasmids (plasmids R702 and RP4 belong to the same incompatibility group, thereby preventing the construction of this

double transconjugant). In this context, epistasis was estimated as: $\varepsilon = W_{(plasmid1;plasmid2)} - W_{(plasmid1;-)}W_{(-;plasmid2)}$.

In this mathematical expression, $W_{(-;plasmid2)}$ and $W_{(plasmid1;-)}$ are the fitnesses of single-plasmid carrying strains relative to the wild-type plasmid-free strain, and $W_{(plasmid1;plasmid2)}$ is the fitness of the strain carrying both plasmids, relative to the same wild-type plasmid-free strain.

Two different plasmids inside the same bacterial cell can interact either antagonistically or synergistically (Figure 5A). Epistatic interaction was found in seven out of nine (78%) strains. Positive epistasis (antagonistic interaction) is nearly as frequent (4/9) as negative epistasis (3/9). One out of nine pairwise combinations of plasmids presented sign epistasis ("++" in Figure 5A). Figure 5B shows the distribution of ε values for all pairwise combinations of plasmids. On average, plasmid pairwise epistasis is close to 0 (median = -0.000830, bootstrap 95% CI [-0.034690; 0.061360]). It is interesting to note that one of the plasmids, R16, interacts antagonistically with all other plasmids, and also with most mutations. This plasmid is also the most costly (Table S1).

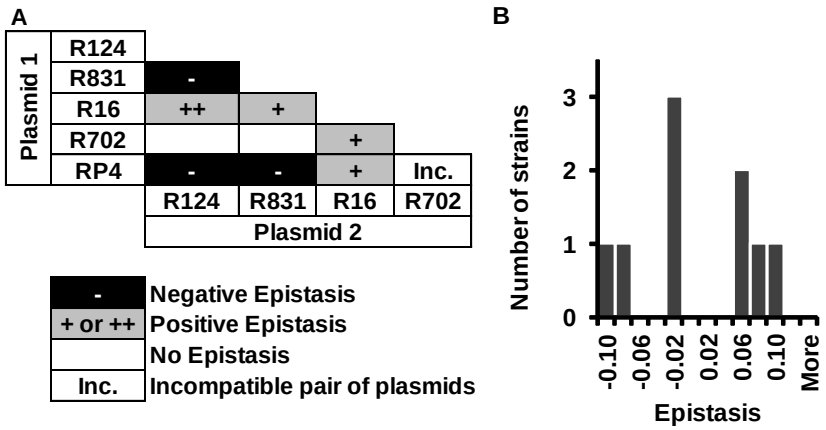

Figure 5. Evidence for epistasis between conjugative plasmids. A Distribution of the types of epistatic interaction found between conjugative plasmids (positive epistasis in grey, negative epistasis in black and no epistasis in white). "Inc" indicates a combination of incompatible plasmids. The single case of sign epistasis is marked with '++'. **B** Distribution of the epistasis level, ε, whose median is -0.00083 with bootstrap confidence interval [-0.035; 0.061], showing that there are several cases of strong positive and negative epistasis despite the near 0 median.

Discussion

Our results 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 determinant. Moreover, 20 out of these 26 antagonistic interactions (77%) exhibit sign epistasis or reciprocal sign epistasis, also an outstanding finding because it means that the fitness cost of harboring both resistance determinants is lower than the fitness cost of bearing one of them. In other words, an initially deleterious antibiotic resistance mutation can become beneficial through the acquisition of a transferable antibiotic resistant plasmid (16 cases); likewise, an initially costly antibiotic resistant plasmid may become

beneficial through the acquisition of a mutation conferring resistance to an additional antibiotic (five cases). This adds up to 20 cases of sign epistasis because of one instance of reciprocal sign epistasis. Last, but not least, three of the plasmid+mutation combinations presented fitnesses not significantly different from the fitness of the wild-type strain.

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 associated with multidrug resistance and may drive its spread. Our study aimed to detect the putative occurrence of epistatic 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.

Our data strikingly suggests the pervasive occurrence of sign epistasis in the interaction between chromosomal antibiotic resistance mutations and conjugative plasmids. Sign epistasis has been shown to have the power to constrain protein adaptation by limiting the number of possible mutational paths and is therefore relevant to the understanding of multidrug resistance emergence (Weinreich et al. 2005). 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 (Schrag et al. 1997). Thus, compensatory mutations are an example of sign epistasis (Andersson and Levin 1999; Maisnier-Patin et al. 2002; Schrag et al. 1997; Weinreich et al. 2005). Our finding of pervasive sign epistasis with conjugative plasmids is one of the worst possible scenarios for the current efforts to eradicate resistance through antibiotic bans. Sign epistasis allows strains carrying a resistance mutation and a plasmid to exhibit higher fitness, thus being able of outcompeting strains carrying

only the mutation or the plasmid (depending on the specific case). These results pinpoint the need for future studies involving other plasmids and other resistances.

Also important in the context of antibiotic resistance is our finding of the ubiquitous occurrence of positive epistasis between resistance plasmids and chromosomal resistance mutations. If such antagonistic interaction is a common phenomenon, then multidrug 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 costs. Hence, such multiresistant strains may be able to persist at significant frequencies in populations where the antibiotic selective pressure has been removed.

Our findings 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 and Schlotterer 2006). Such study found 107 genes exhibiting epistatic effects with the F-plasmid. Although such effect was not found for *gyrA*, *rpoB* and *rpsL*, other host genes involved in information transfer were reported to be affected by the presence of the F-plasmid (Harr and Schlotterer 2006). Under the framework of the complexity hypothesis, these interactions between plasmids and informational genes (*rpoB* (Drlica et al. 1988; Fisher and Yanofsky 1983; Jin and Gross 1989) and *rpsL* (Zengel et al. 1977)) and a topoisomerase (*gyrA* (Jeong et al. 2006)) are expected, given their pleiotropic interactions with other genes. For example, Schmitt et al. (1995) have shown that certain *rpoB*, *rpsL* and *gyrA* alleles affect F-exclusion of bacteriophage T7. In addition, Ozawa et al. (2005) showed that *rpoB* mutations interact with a plasmidic gene (in *Enterococcus faecalis*). Similarly, *gyrB* may also interact with plasmids, eventually leading to their elimination from cells (Wolfson et al. 1982). In conclusion, resistance genes present on plasmids are not necessarily responsible for the epistatic interactions observed.

We also report here the occurrence of significant epistasis between two types of conjugative plasmids within the same host. This finding 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 the conjugative plasmids is close to zero. However, we do not believe that our results suggest a tendency for no epistatic interactions between conjugative plasmids. In fact, our near-zero median level of epistasis between conjugative plasmids is the consequence of having a similar frequency of somewhat strong positive and negative epistatic interaction pairs. Our results may indicate that plasmid interaction follows an all-or-nothing type of response where the net epistatic effect is either strongly negative or strongly positive. However, further studies should use a larger sample of plasmids. Recently, *in silico* studies of *E. coli* and *S. 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 chromosomal mutations and a plasmid than between two types of plasmids.

Our finding of pervasive positive epistasis and, in particular, of sign epistasis, between 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 the original host to a new cell. Many plasmids are even able to move between phylogenetically distant organisms. 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 in prokaryotes it has been suggested that microbes share a common gene pool (Norman et al. 2009). Therefore, we predict that plasmid-borne resistance dissemination control through antibiotic bans is not likely to be successful. We suggest that resistance reversal policies must target plasmids vulnerabilities. Three approaches have been suggested (Williams and Hergenrother 2008): inhibition of plasmid conjugation, inhibition of plasmid replication, and exploitation of plasmid-encoded toxin-antitoxin systems.

Author Contribution

Rui F. Silva and Francisco Dionísio conceived and designed the experiments. Rui F. Silva, Sílvia C. M. Mendonça and Sandra Trindade performed the experiments. Rui F. Silva, Sílvia C. M. Mendonça, Luis M. Carvalho, Ana M. Reis, Isabel Gordo, Sandra Trindade and Francisco Dionísio analyzed the data. Rui F. Silva, Francisco Dionísio and Sílvia C. M. Mendonça wrote the paper. Rui F. Silva, Luis M. Carvalho, Isabel Gordo and Francisco Dionísio did the statistical analyzes. Luis M. Carvalho, Ana M. Reis, Isabel Gordo and Sandra Trindade contributed to the writing of the paper.

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Supplementary Material

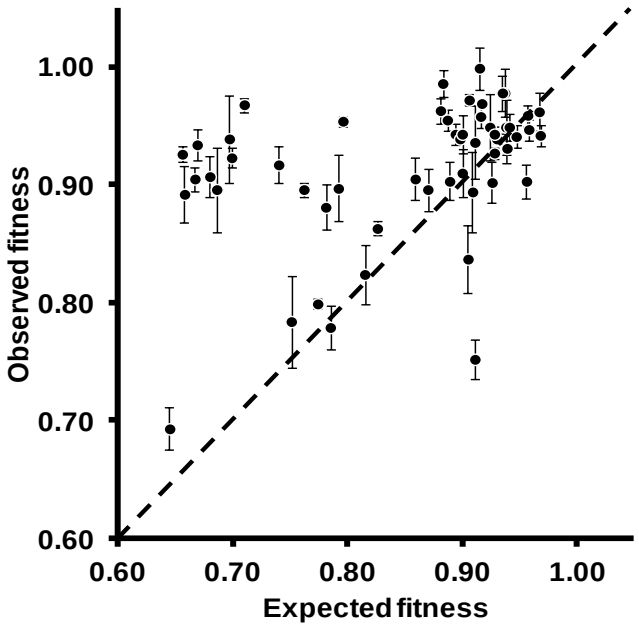

Figure S1. Evidence for positive epistasis between plasmids and mutations. Relation between the observed fitness of the strains carrying a resistance mutation and a conjugative plasmid and the expected fitness under the assumption of no epistasis (represented by the line). Most points (52%) are significantly above the line. Error bars represent twice the standard error.

Table S1. List of the conjugative plasmids used in the present study. We present their incompatibly group, antibiotic resistance markers, size, the fitness cost they create when inserted in *Escherichia coli* K12 MG1655, and origin.

Plasmid	Incompatibility group	Antibiotic Resistance Markers*	Size (kb)	Fitness Cost (%) (2*standard error)	Origin of plasmid	References
R124	IncF IV	Tc	125.7	3.9 (1.7)	*Salmonella enterica* serovar Typhimurium	[1,2]
R831	IncL	Km, Sm	?	5.8 (3.8)	*Serratia marcescens*	[3]
R16	IncB	Ap, Sm, Su, Tc	104	8.0 (0.6)	*E.coli*	[4]
R702	IncP	Km, Sm, Su, Tc	69.7	2.8 (0.7)	*Proteus mirabilis*	[5]
RP4	IncP1	Ap, Km, Tc	60.1	7.0 (0.9)	*Pseudomonas aeruginosa*	[6,7]

*Ap: ampicillin; Km: kanamycin; Sm: streptomycin; Su: sulphonamides; Tc: tetracycline.

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Table S2. List of antibiotic resistant mutants and fitness cost.

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

¶ Nal: nalidixic acid; Rif: rifampicin; Sm: streptomycin

† Data from ref. 27 (main text).

Table S3. Monitoring of conjugative transfer of plasmids to reference strain.

	Total cell density (cfu/mL)*	Transconjugants (% of Δara)#
R124	(1.29± 0.86) x 10^9	1
R702	(1.03± 0.09) x 10^9	2
R16	(1.08± 0.12) x 10^9	<0.5
R831	(1.17± 0.06) x 10^9	<0.5
RP4	(1.30 ± 0.17) x 10^9	1

* Colony forming units per milliliter; # percentage of recipients that received the plasmid (transconjugants) at stationary phase.

Table S4. List of the plasmid donors used for strain construction.

Bacterial strain	Plasmid harbored	Chromosomal markers
E. coli CM317	R124	Mal^- Trp^-
E. coli CM319	R831	Mal^- Trp^-
E. coli CM312	R16	Mal^- Trp^-
E. coli CM597	R702	Met^- Pro^-
E. coli CM140	RP4	Met^- Pro^-

CHAPTER IV

THE BURDEN OF ANTIBIOTIC RESISTANCE MUTATIONS UNDER DIFFERENT STRESSFUL CONDITIONS

Manuscript in Preparation

A mutation may be deleterious in one environment but not necessarily deleterious in a new one. Trying to determine the effects of mutations in various environments is an important approach to understand the pattern of adaptation of natural populations. Antibiotic resistance mutations are known to have different fitness effects depending on the presence or absence of the respective antibiotic. Also clinical and community environments have been shown to lead to different outcomes of antibiotic resistance evolution. In this work, we studied how the costs of antibiotic resistance depend on the environment. To do so, we constructed clones resistant to four antibiotics and measure their fitness by head to head competition assays against the wild type strain in two different stressful antibiotic free environments. We found strong genotype-by-environment interactions for mutations conferring antibiotic resistance. For some rifampicin resistance mutations, extreme cases of total switch of their fitness effects were observed; some mutations became beneficial when subjected to stress.

Introduction

Natural populations are known to face environmental changes. These changes oblige the populations that inhabit a given place to be constantly adapting to new environmental conditions in order to survive. In general, beneficial mutations are thought to be rare (Fisher 1930). According to theory, most of the occurring mutations are neutral or deleterious (Drake et al. 1998; Lynch et al. 1999), a pattern in part confirmed by mutation accumulation experiments (Trindade et al. 2010; Gordo et al. 2011). However deleterious mutations may not always be deleterious. In fact, it is highly unlikely that a given mutation keeps its fitness effect constant across environments. Indeed, it has been demonstrated that adaptation to a novel environment may occur from standing genetic variation, where the preexisting mutations which were neutral or deleterious become beneficial in the novel environment (Dykhuizen and Hartl 1980; Orr and Betancourt 2001; Gibson and Dworkin 2004; Hermisson and Pennings 2005). Environmental changes can therefore play a key role on mutations' fitness effects.

Genotype-by-environment interactions manifest itself every time there is variation in phenotypic effect of a given genotype with a change in the environmental conditions (Falconer 1981). Knowledge on the extent and underlying form of genotype-by-environment interactions is of extreme importance to understand how natural populations evolve. Actually, the presence of this type of interactions has been attributed as responsible for some important evolutionary processes such as the maintenance of genetic variation among natural populations (Anand P. Gupta and Lewontin 1982; Tachida and Mukai 1985; Wade 1990; Rawson and Hilbish 1991; Sultan and Bazzaz 1993; Pigliucci et al. 1995), the process of ecological specialization (Futuyma and Moreno 1988; Kawecki 1994; Fry 1996; Kawecki et al. 1997) and the occurrence

of speciation (Smith 1966; Dickinson and Antonovics 1973; Kawecki 1997).

The vast list of empirical studies related with genotype-by-environment interactions was mainly filled with studies done in extant natural populations (Anand P. Gupta and Lewontin 1982; Pigliucci et al. 1995; Rawson and Hilbish 1991; Sultan and Bazzaz 1993; Tachida and Mukai 1985; Wade 1990) or in laboratory changed populations by means of mutation accumulation experiments (Fernández and López-Fanjul 1997; Fry and Heinsohn 2002; Fry et al. 1996; Kondrashov and Houle 1994; Korona 1999; Shabalina et al. 1997; Szafraniec et al. 2001; Vassilieva et al. 2000). Both approaches are perfectly viable however, they do not allow one to determine if a given genotype-by-environment interaction depends on a single gene or on numerous alleles that together produce the resulting pattern. Since the single effect of mutations was not defined and that mutations can be conditionally neutral or beneficial, depending on the environment, it is also unclear whether stress exposes more mutations or stress increases their average effect every time an alteration of the combined fitness effect was observed. In addition, as more than one mutation might be occurring on a given genetic background, the problem of epistasis should also not be ignored in this type of studies.

The majority of the aforementioned studies typically examined phenotypic variation of genotypes across a set of different environmental conditions. More recent studies, also analyzed the patterns of genotype-by-environment interactions across environments but more than that, they studied the effect of particular stressful conditions on the phenotypic effect of a given genotype (see for instance Remold and Lenski (2001)). Two stressful conditions common to these studies, and also applied in our study, are variation in the amount of available resources and in temperature. One general conclusion is that varying the amount of available resources tends to generate genotype-

by-environment interactions (Remold and Lenski 2001; Ostrowski et al. 2005; Jasnos et al. 2008). However, in respect to temperature, the conclusion is not that straightforward. The magnitude of change seems to be determinant for the presence or absence of interactions between genotype and environment; more extreme changes in temperature give rise to genotype-by-environment interactions not observed for milder changes. Namely, Remold and Lenski (2001) analyzed genotype-by-environment interactions on individual random mutations for a set of 4 environments differing in temperature (37ºC and 28ºC) and limiting resource (maltose and glucose). To do so, they generated 26 *Escherichia coli* genotypes comprising a single random insertion mutation and measured the fitness effects of each mutation in the 4 environments. Authors concluded that single random mutations can generate genotype-by-environment interactions in response to resource but not in response to low temperature. On the contrary, Kishony and Leibler (2003) found that lowering the temperature does have influence on the effects of mutations. They studied random mutations in *E. coli* (most likely one per bacterium), generated by chemical mutagenesis, and by measuring the mutants' growth rates in a set of different environments found that lowering temperature to 17ºC does have influence on the effects of mutations. As previously mentioned, the amplitude of temperature change might be on the cause of these contradicting results; it is possible that genotype-by-environment interactions only exist for extreme temperature conditions capable of implement a higher level of stress. The raise in temperature was also studied in the context of genotype-by-environment interactions. Jasnos et al. (2008) studied the effect of 4 stressful conditions in yeast gene deletions. By estimating the relative growth rate of the mutated strains in those 4 stressful environments and comparing those results to previously assayed maximum growth rates in a favorable environment (Jasnos and Korona 2007; Jasnos et al. 2008), they were able to

conclude that raise the temperature to 37ºC has no or slight influence on the phenotypic effects of the studied genotypes in opposition to the other 3 conditions (Jasnos et al. 2008).

In the applied context of antibiotic resistance mutations, the importance of genotype-by-environment interactions is clear: a given mutation conferring resistance is beneficial in the presence of the drug, but may be costly in its absence (Andersson and Levin 1999; Björkman et al. 1998; Bouma and Lenski 1988; Dahlberg and Chao 2003; Gagneux et al. 2006; Lenski 1997). In addition, the costs of antibiotic resistance can be simply relieved when a resistant population is confronted with other particular environmental conditions or, on the other hand, it can be aggravated depending on the molecular demands exerted by the environment. Given so, it is important to study if those costs vary across environments or if there is little genotype-by-environment interactions for antibiotic resistance mutations. All the above studies have measured fitness effects for antibiotic resistance mutations in different species and environments separately. Still, there is some experimental evidence pointing to the cost of resistance varying as a result of environmental conditions (Björkman et al. 2000; Nagaev et al. 2001; MacLean and Buckling 2009; Hall et al. 2011). However none of these studies measured the fitness effects of the same mutations in different environments in order to define the patterns of genotype-by-environment interactions. For example, MacLean and Buckling (2009) studied the effect of an increased gradient of rifampicin concentrations on the fitness effects of 15 rifampicin resistance mutations in *Pseudomonas aeruginosa*. They did so to understand how the distribution of the fitness effects of beneficial mutations varies with the fitness effect of the ancestral population. In the process to realize this they had to study the fitness effect of each of the 15 mutations in 4 different environmental conditions showing the presence of genotype-by-environment interactions for some of the studied mutations.

Two recent studies analyzed the variation of the phenotypic effects of mutations conferring resistance to the same antibiotics as the ones studied here across a set of different environmental conditions. Paulander et al. (2009) studied the effect of 4 alternative carbon sources (succinate, glucose, glycerol and pyruvate) on the fitness effects of 3 streptomycin resistance mutations for *Salmonella enterica* var. Typhimurium. They found that the fitness effect of all antibiotic resistance mutations varied with the carbon source. More recently, Bataillon et al. (2011) studied the patterns of genotype-by-environment interactions of a bigger set of resistance mutations. They studied nalidixic acid resistance mutations in *Pseudomonas fluorescens* by analyzing the growth rate of those strains across a wide range of 95 environments differing in their carbon source and concluded that the phenotypic effect of those mutations is varying with the environment.

Though genotype-by-environment interactions have been reported previously for mutations conferring resistance to some of the antibiotics studied here (as well as other mutations in general), we present a study that simultaneously comprises mutations conferring resistance to more than one antibiotic and involved in more than one cellular mechanism which might provide a better understanding on the patterns of genotype-by-environment interactions operating among antibiotic resistance mutations and in a broader scale, in natural populations. We studied the effect of stress imposed by environmental conditions on the fitness effects of antibiotic resistance mutations. We measured the fitness effects of single mutations conferring resistance to 4 types of commonly used antibiotics, in 3 different environments. We observed strong genotype-by-environment interactions, meaning that the fitness effects of antibiotic resistance mutations generally differ across environments. This variation in fitness effects took several forms: in some cases mutations got more detrimental with stress, on another, they even become beneficial.

Materials and Methods

Bacterial Strains and Environmental Conditions

The strains used were *Escherichia coli* K12 MG1655 and *Escherichia coli* K12 MG1655 Δara. Rifampicin, streptomycin and nalidixic acid resistant clones were the same used in Trindade et al. (2009). D-cycloserine resistant mutants were derived from the ancestral strain *Escherichia coli* K12 MG1655 by plating the clones at 37^{o}C on plates containing M9 minimal medium (MM) supplemented with agar and the respective antibiotic at the final concentration of 0.04mM. We used MM instead of Luria-Bertani (LB) because D-cycloserine activity is known to be inhibited in the presence of alanine (Bondi et al. 1957; Hoeprich 1965) which is one of the composites of LB medium (Sezonov et al. 2007). All the D-cycloserine spontaneous resistant clones were tested for mutator phenotype and those four that showed evidences of high mutation rate were not considered. Also a knockout strain for *cycA* gene of the Keio collection was tested for D-cycloserine resistance. To study the influence of stressful conditions, we chose three different environments: Luria-Bertani (LB) at 37ºC (LB(37)) and M9 minimal medium at 37ºC (MM(37)) and at 40ºC (MM(40)). All the three environments were antibiotic-free. *Escherichia coli* K12 MG1655 Δara, was used as reference for performing competitive fitness assays. The two strains are distinguishable by phenotypic difference due to a deletion in the arabinose operon: ara^{+} and Δara give rise to white and red colonies, respectively, in tetrazolium arabinose (TA) indicator agar (Lenski et al. 1991). To estimate fitness costs, competitions were performed during 24 hours in 50 ml screw-cap tubes containing 10 ml of each medium at the respective temperature, with aeration (orbital

shaker at 230 RPM). To estimate the frequency of each strain, in the beginning and by the end of the competition, Tetrazolium Agar (TA) medium containing 1% peptone, 0.1% yeast extract, 0.5% sodium chloride, 1.7% agar, 1% arabinose and 0.005% Tetrazolium chloride was used. All sets of dilutions were done in $MgSO_4$ at a concentration of 0.01M.

Detection of Mutations

The known target gene for resistance to D-cyloserine is *cycA* (Fehér et al. 2006). Mutations responsible for resistance were identified by amplifying and sequencing the target gene. The primers used to amplify the *cycA* gene were: 5'-TTTCC AGCCACATGATGAAA-3' and 5'-GTTTATGGGGTCTGGCAAAA-3'. The first primer as well as 5'-AGCGCGAAGACGTAGAACAT-3', 5'-GTCGGCCTGGTCATG GTGGC-3', 5'-TACCAATAAACAGCCAGCCC-3', 5'-CGTGGTGGCGATCACGGC TT-3' and 5'-CGAGGC GACCCAGTCGGAGA-3' were used for sequencing directly from the PCR product.

Fitness Assays

To measure the fitness effect of the resistance mutations in the three different environmental conditions, competition assays were done. The resistant mutants were competed against a reference strain, *Escherichia coli* K12 MG1655 Δara in the three different antibiotic free environments, in an approximate proportion of 1:1. To do so, we grew both resistant and reference strains in LB or MM at 37ºC or MM at 40ºC (all liquid media) for 24 hours with aeration. Accurate values of each strain initial ratio were estimated by plating a dilution of the mixture in

TA Agar plates. Competitions were performed in 50 ml screw-cap tubes containing 10 ml of each medium by a period of 24 hours at the corresponding temperature with aeration. By the end of this 24 hours competition period, appropriate dilutions were platted onto TA Agar plates to obtain the final ratios of resistant and reference strains. The fitness effect of each mutant strain- i.e the selection coefficient- was estimated as the per generation difference in Malthusian parameters for the resistant strain and the marker strain (Lenski et al. 1991), discounted by the cost of the Δara marker, in the case of LB. The marker is neutral in MM at both studied temperatures.

Statistical Analysis

To query about the presence of different fitness effects operating for the same genotype among different environments we made pairwise comparisons between the fitness of each of the 25 resistance mutations in different environmental conditions using a Wilcoxon rank-sum test. To query if the mean fitness effects of the 25 mutations differ across different environmental conditions we used a paired Wilcoxon signed-rank test. For testing the differences in variance of the fitness effects between environments we performed a Pitman's T test. A Bonferroni correction was used to correct for multiple testing (Rice 1989).

To perform the statistical analysis we used the free software R: http://www.r-project.org.

Results

D-cycloserine Resistant Mutants

To study the influence of the environment on the fitness effects of antibiotic resistance mutations we used a sample of 25 different antibiotic resistant mutants. 19 of those mutants accrued from the study of epistasis between antibiotic resistance mutations (Trindade et al. 2009) (Methods). The other set of six clones was composed by D-cycloserine resistant clones. D-cycloserine is an antibiotic known to interfere with cell wall synthesis (Neuhaus 1967). The resistance to this particular antibiotic is conferred by mutations in the *cycA* gene (Fehér et al. 2006). Contrarily to the other studied genes, *cycA* is a non-essential gene since that its knockout renders viable bacteria in opposition to knockout of the genes involved in the other resistances (Baba et al. 2006). From the 30 sequenced clones resistant to D-cycloserine we found that 60% of the genetic alterations corresponded to large insertions, 7% to small insertions, 17% to deletions and 17% to point mutations (Table S1). The large insertions were all detected by analyzing the amplified fragment on agarose gel electrophoresis in comparison to the wild-type template; the large insertions revealed shorter runs on the agarose gel due to its bigger size. On the other extreme, deletions were identified by the total lack of fragment after running on the agarose gel. The small insertions as well as point mutations did not produce any difference in fragment size in comparison to the wild type template. Further sequencing revealed the precise identity of this type of mutations; whether point mutations or small insertions/deletions. Fehér et al. (2006) also found on their study of the characterization of the *cycA* mutants this diversity in type of mutations

conferring antibiotic resistance. Yet, there are no common point mutations between the ones found in their study and the present study. This might be indicative of a vast diversity of genetic alterations that could give rise to D-cycloserine resistance.

More than half of the insertions (55%) analyzed by Fehér et al. (2006) were IS150. This type of insertion is a well known member of the widespread IS3 family of insertions. *E. coli* IS150 is 1433bp long (Schwartz et al. 1988) which is in agreement with the range of values obtained from the analysis of the agarose gel in this study (Table S1). This suggests that our large insertions could be IS150 insertions but, the real identity of the obtained 18 large insertions is beyond the scope of the present study. Also, the amount of obtained insertions is remarkably similar to the percentage of insertions found for growth-dependent mutations (61%) in another *E. coli* gene *ebgR*, when selecting for growth in minimal medium supplemented with lactulose (Hall 1999). Another study in *E. coli* also showed an approximate 60% of insertions occurring among spontaneous mutations in *tonB* gene, when selecting for colicin-B resistants (Rodriguez et al. 1992). This insertion element domination of the mutation spectrum points to the fact that, for most of the reported cases, insertions are the most common when we select for the knockout of a gene. Given that large insertions are a common route to acquire resistance to D-cycloserine, we tested a knockout strain for *cycA* gene of the Keio collection and found it to be resistant.

Genotype-by-Environment Interactions

To test for the presence of genotype-by-environment interactions on antibiotic resistance mutations we studied three different environments generated by two experimental manipulations. We sequentially add environmental factors known to impair fitness and to be common

ecological conditions faced by bacterial natural populations (in this case availability of resources for one environment and availability of resources and temperature for another) (Savageau 1983; Ron and Davis 1971; Poulsen et al. 1995; Bronikowski et al. 2001; Cooper et al. 2001). LB medium was coined as "reference benign environment". All environments were antibiotic-free so, the null expectation is that mutations will be deleterious (Andersson and Levin 1999; Björkman et al. 1998; Bouma and Lenski 1988; Dahlberg and Chao 2003; Gagneux et al. 2006; Lenski 1997), and that their effect will increase with increasing stress, as generally observed (Kondrashov and Houle 1994; Jimenez et al. 1994; Shabalina et al. 1997; Korona 1999; Vassilieva et al. 2000; Szafraniec et al. 2001).

The fitness effect of all the 25 antibiotic resistance mutations was measured, independently, in the three different environments (see Material and Methods). The mean fitness effect of each mutation in the three environments is provided in Table 1. We found that for the majority of the studied mutations (64%) the mean fitness effects varied significantly across environments (Wilcoxon test, $P<0.025$). This variation was not the same for all mutations suggesting the presence of genotype-by-environment interactions. These patterns were also seen in previous studies of either extant natural populations (Anand P. Gupta and Lewontin 1982; Tachida and Mukai 1985; Wade 1990; Rawson and Hilbish 1991; Sultan and Bazzaz 1993; Pigliucci et al. 1995) or laboratory populations (Remold and Lenski 2001).

Table 1. Genotypes and fitness costs of single resistance mutations in different stressful environments

		Fitness Effect (2SE)		
Resistance (Gene)	**Genotype**	**LB(37)**	**MM(37)**	**MM(40)**
Nal (*gyrA*)	D 87 G	-0.037 (0.015)	-0.007 (0.016)	-0.024 (0.016)
Nal (*gyrA*)	S 83 L	-0.034 (0.012)	-0.009 (0.014)	-0.030 (0.037)
Nal (*gyrA*)	D 87 Y	-0.031 (0.019)	0.006 (0.014)	-0.158 (0.009)
Rif (*rpoB*)	D 516 V	-0.061 (0.010)	0.048 (0.031)	0.085 (0.046)
Rif (*rpoB*)	H 526 N	-0.014 (0.011)	0.094 (0.012)	-0.024 (0.049)
Rif (*rpoB*)	H 526 L	0.005 (0.012)	-0.020 (0.011)	-0.062 (0.081)
Rif (*rpoB*)	H 526 Y	-0.104 (0.032)	0.123 (0.008)	-0.471 (0.072)
Rif (*rpoB*)	I 572 F	-0.146 (0.013)	-0.078 (0.019)	-0.293 (0.047)
Rif (*rpoB*)	D 516 Y	-0.064 (0.040)	-0.061 (0.012)	-0.265 (0.182)
Rif (*rpoB*)	D516 N	-0.041 (0.020)	0.088 (0.007)	-0.130 (0.053)
Rif (*rpoB*)	R 529 H	-0.262 (0.049)	-0.238 (0.012)	-0.473 (0.187)
Rif (*rpoB*)	S 512 F	-0.065 (0.013)	0.017 (0.023)	0.150 (0.060)
Rif (*rpoB*)	S 531 F	-0.107 (0.016)	-0.208 (0.009)	-0.388 (0.056)
Rif (*rpoB*)	H 526 D	-0.096 (0.013)	-0.181 (0.027)	-0.391 (0.030)
Str (*rpsL*)	K 43 R	-0.005 (0.014)	-0.063 (0.020)	-0.062 (0.082)
Str (*rpsL*)	K 43 T	-0.134 (0.020)	-0.220 (0.029)	-0.412 (0.109)
Str (*rpsL*)	K 88 E	-0.276 (0.024)	-0.273 (0.047)	-0.871 (0.107)
Str (*rpsL*)	K 43 N	-0.181 (0.013)	-0.245 (0.020)	-0.546 (0.104)
Str (*rpsL*)	K 88 R	-0.061 (0.012)	-0.106 (0.029)	-0.133 (0.058)
D-cyc (*cycA*)	L 432 Stop	-0.027 (0.011)	-0.005 (0.030)	0.008 (0.038)
D-cyc (*cycA*)	3bp Insertion	-0.038 (0.007)	-0.024 (0.036)	0.088 (0.108)
D-cyc (*cycA*)	S 275 Stop	-0.045 (0.013)	-0.014 (0.032)	0.047 (0.059)
D-cyc (*cycA*)	V 284 F	-0.026 (0.012)	-0.003 (0.016)	-0.016 (0.024)
D-cyc (*cycA*)	E 237 K	-0.038 (0.014)	0.012 (0.035)	-0.035 (0.022)
D-cyc (*cycA*)	S 313 R	-0.047 (0.007)	-0.005 (0.020)	-0.003 (0.043)

Figure 1 shows the presence of genotype-by-environment interactions where it is evident the differences both in extent and rank orders of mutation fitness effects across environments. The mean deleterious effect on fitness of the 25 mutations was 0.077±0.017 for LB(37), 0.055±0.021 for MM(37) and 0.176±0.067 for MM(40). Those values did not vary significantly from LB(37) to MM(37) (Wilcoxon test, $P>0.025$), but they did from LB(37) to MM(40) and from MM(37) to MM(40) (Wilcoxon test, $P<0.025$). Regarding the variance, it is 0.005 for LB(37), 0.013 for MM(37) and 0.061 for MM(40). The variances increased from LB(37) to MM(37) and to MM(40) and from MM(37) to MM(40) (from LB(37) to MM(37), Pitman's T test, $P<0.005$ and between LB(37) and MM(40) and MM(37) and MM(40), Pitman's T test, $P<0.0005$) if the conservative Bonferroni correction was not considered. The lack of evidence for an increased mean of the fitness effects between LB(37) and MM(37) added to the knowledge on the fitness effect of the mutations suggests that, between these two environments, genotype-by-environment interactions are mainly due to the rank rather than the amplitude of fitness effects. This evidence is not observed for the fitness effects between LB(37) and MM(40) and MM(37) and MM(40), where there is significant differences in both the rank order and amplitude of fitness effects.

The aforementioned 64% of the cases that showed genotype-by-environment interactions also have some common relevant features. Four mutations (16%), two conferring rifampicin resistance (*rpoB S531F*, *rpoB H526D*) and two conferring streptomycin resistance (*rpsL K43T* and *rpsL K43N*), monotonically increased their deleterious effect. One (4%) rifampicin resistance mutation (*rpoB S512F*) was monotonically becoming positive, meaning that it is detrimental in LB(37), neutral in MM(37) and beneficial in MM(40). Along with rifampicin resistance mutations, there were also evidences (in 12% of the cases) for fitness effects being bigger in MM(37) than in LB(37) and

then decreased again in MM(40): *rpoB H526N* mutation is neutral in LB(37), positive in MM(37) and neutral in MM(40) and *rpoB H526Y* and *rpoB D516N* mutations are both deleterious in LB(37), both are beneficial in MM(37) and then detrimental again in MM(40). Only one (4%) streptomycin resistance mutation (*rpsL K43R*) showed the opposite pattern, a minimum fitness effect at MM(37). This mutation was neutral in LB(37) and MM(40) but deleterious in MM(37). The remaining antibiotic resistance mutations (36%) revealed no significant changes on their fitness effects across environments (Wilcoxon test, $P>0.025$).

By looking separately to the different antibiotic resistance mutations (Figure S1) two things are evident. First, both D-cycloserine and some nalidixic acid antibiotic resistance mutations did not show detectable genotype-by-environment interactions. Second, streptomycin resistance mutations, despite presenting genotype-by-environment interactions, did not show changes in the rank order of their fitness effects across environments. These changes were mostly confined to rifampicin resistance mutations and there was also one case among nalidixic acid mutations.

These observed changes in the rank orders of mutations were previously reported in studies conducted in natural populations (Fry et al. 1996). Actually, they have been pointed as plausible explanations for the maintenance of the high levels of heritable genetic variability operating in natural populations (Gillespie and Turelli 1989).

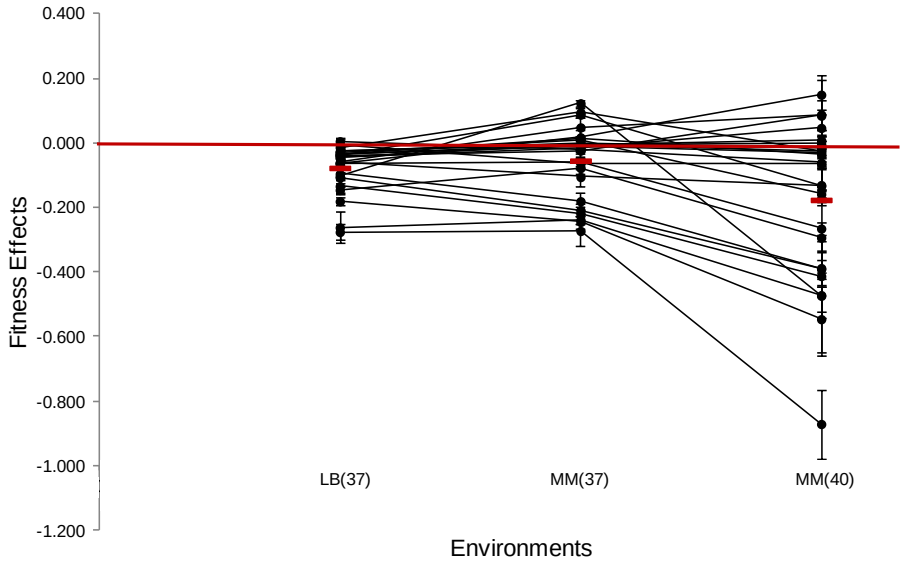

Figure 1: Evidence for genotype-by-environment interactions between mutations conferring antibiotic resistance. Fitness effects of the 25 mutations conferring antibiotic resistance in the three different environments. The majority of mutations contributed to genotype-by-environment interactions through their differential fitness effects across environments. The mean fitness effect for each mutation is represented as a black dot and the mean fitness effect for each environment as a red line. Mean and variance differed between LB(37) and MM(40) and MM(37) and MM(40) but not between LB(37) and MM(37), where only the variance differed.

By analyzing the mutations per gene some patterns can be seen. Figure 2 shows that all mutations that seem to have suffered major fitness effect alterations with stress are related with the *rpoB* and *rpsL* genes that are involved in the transcription process and ribosomal activity, respectively (Kurland et al. 1996). Contrary to our initial expectations, some deleterious mutations on the "reference benign environment" (16%) became beneficial when faced higher levels of stress (*rpoB D516V*, *rpoB H526Y*, *rpoB D516N*, and *rpoB S512F*). Because they did so only under particular environmental conditions, they are conditionally beneficial mutations. They are all mutations from the same gene (*rpoB*), thus likely involved in the same cellular process. Evidences for positive fitness effects operating among mutations in

some of the primary RNA polymerase genes (*rpoB* and *rpoC* genes) were found previously in adapted populations of *E. coli* to M9 minimal medium supplemented with glycerol (Herring et al. 2006; Applebee et al. 2008). This condition was proved to be transversal to other carbon sources, at least for the *rpoC* gene, by Conrad et al. (2010). Given that both genes encode the two major subunits of RNA polymerase they are expected to interfere with the cellular biology in similar ways and so render similar outcomes in terms of fitness effects. Moreover, in *Pseudomonas aeruginosa*, the fitness effects of rifampicin resistance mutations are alleviated on average, under stressful conditions (Hall et al. 2011). All together, these findings point towards a general pattern of alleviation of the fitness effects of rifampicin resistance mutations that, encompasses not only *E. coli*, as shown here, but also other microorganisms. This conditional benefit found for four of the studied mutations is particularly worrying since, for this group, the presence of the respective antibiotic is not a request for their appearance. They are able to be selected in the absence of the drug when combined with stressful environmental conditions known to impair fitness. We found a single case of a neutral mutation (*rpoB H526N*) on the "reference benign environment" that became beneficial under stress. Remold and Lenski (2001) also reported the presence of conditional beneficial mutations for *E. coli* random insertions in MM supplemented with maltose, suggesting the ubiquity of this particular form of genotype-by-environment interactions. Contrarily, Figure 2 also reveals that 16% of the cases showed an increased detrimental effect with stress and that in 12% of the cases the detrimental effect was more pronounced only in MM(40). Moreover, 36% of mutations did not suffer any variation in their fitness effects with the considered environmental changes meaning, once neutral (or slightly detrimental) or detrimental, always neutral (or slightly detrimental) or detrimental, respectively, no matter the level of stress they were exposed to (Figure 2). For the 25 studied mutations, neutrality,

irrespective of the levels of stress seems to be related mostly with mutations on the gene involved in cell wall synthesis (*cycA*) although some cases also happen in the gene associated with the process of DNA replication (*gyrA*), where it is not exclusive.

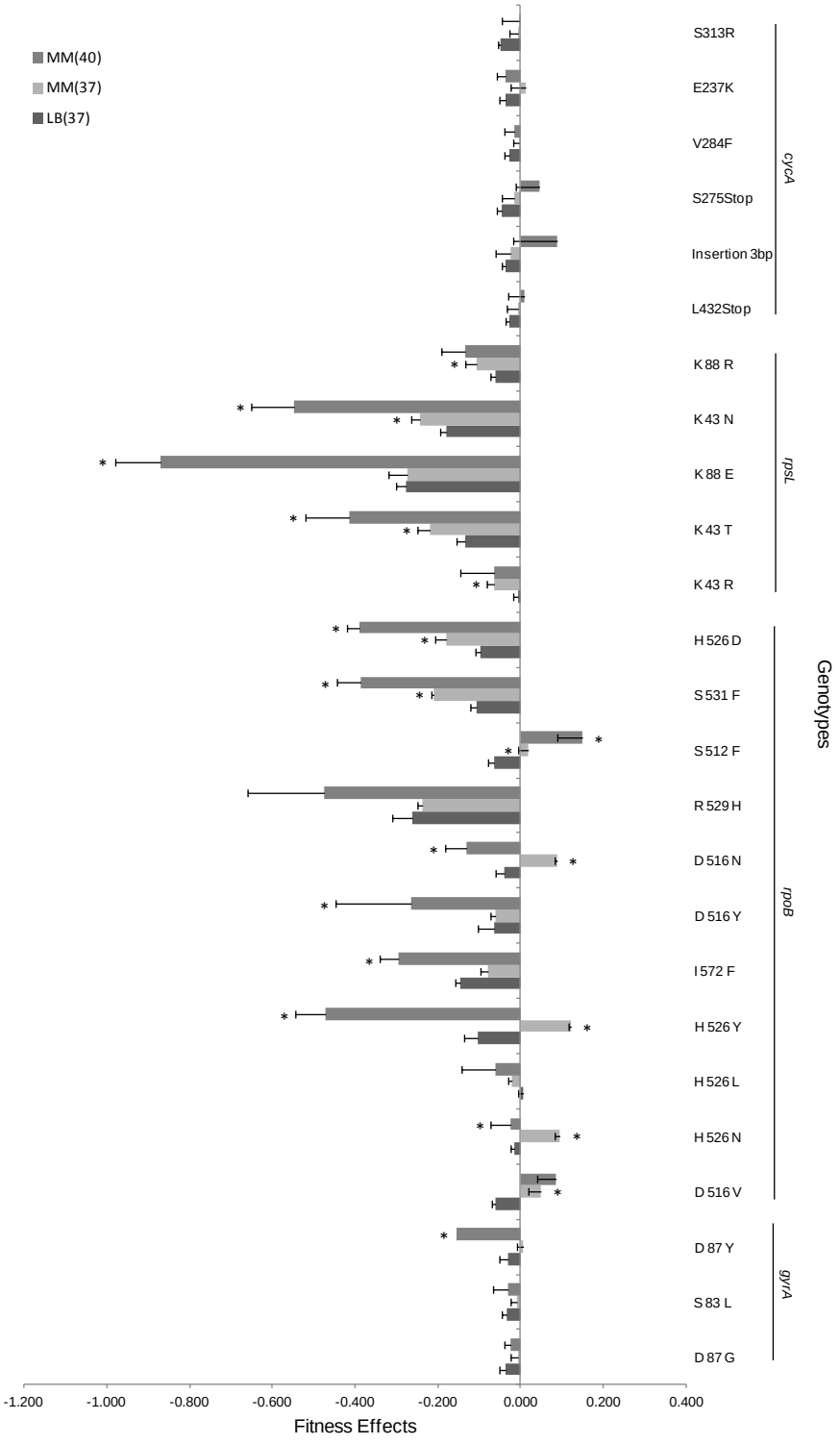
MM(40)
MM(37)
LB(37)
S313R
E237K
V284F
S275Stop
Insertion 3bp
L432Stop
cycA
K 88 R
K 43 N
K 88 E
K 43 T
K 43 R
rpsL
H 526 D
S 531 F
S 512 F
R 529 H
D 516 N
D 516 Y
I 572 F
H 526 Y
H 526 L
H 526 N
D 516 V
rpoB
D 87 Y
S 83 L
D 87 G
gyrA
Genotypes
-1.200
-1.000
-0.800
-0.600
-0.400
-0.200
0.000
0.200
0.400
Fitness Effects

Figure 2: Differences on the fitness effects of the antibiotic resistance mutations between the three environments. Some deleterious mutations (16%) became more deleterious with stress increase. However, 20% of the mutations are beneficial when facing certain levels of stress. 73% of the total neutral mutations remained neutral independently of the level of stress they were exposed to. The vast majority of genotype-by-environment interactions occurred among mutations in genes related with transcription and ribosomal activity. Wilcoxon signed rank test, at 5% confidence level with Bonferroni correction (n=2 comparisons), was performed to access differences in the effects of mutations (as a all) across environments and Wilcoxon rank-sum test, at 5% confidence level with Bonferroni correction (n=2 comparisons), was performed to access if the mutations fitness effects (of each mutation) differed or not with the increase in stress. Asterisks indicate statistically significant differences on the mean fitness effect of each mutation between two consecutive environments.

Discussion

The knowledge on the effects of new mutations in relation to the environment where they emerge is important to understand for instance the evolution of phenotypic plasticity and ecological specialization (Whitlock 1996; Whitlock 2000; Kawecki et al. 1997). Relatively little is known about the individual effects of mutations across environments and their contribution to the evolution of natural populations despite the theoretical predictions. Still, there have been some empirical studies which addressed this important issue (see for instance Remold and Lenski (2001), Kishony and Leibler (2003) and Bataillon et al. (2011)). Here, we focused mainly on point mutations responsible for a single amino acid change. Furthermore, since those changes confer antibiotic resistance we addressed the effect of stress on the cost of resistance. Given that bacteria are known to be often exposed to particular conditions of stress it is imperative to know how these ecological conditions affect resistant bacteria. Some recent experimental studies have shown that the cost of resistance varies with variation of the environmental conditions (Paulander et al. 2009; MacLean and Buckling 2009; Bataillon et al. 2011; Hall et al. 2011). Here, we present a study

that encompasses mutations conferring resistance to more than one antibiotic and consequently involved in more than one cellular mechanism. This analysis might add more knowledge on the patterns of genotype-by-environment interactions in antibiotic resistant populations and in a broader scale, in natural populations.

In our study, the mean fitness effects as well as the variance of the 25 studied mutations were shown to increase under particular conditions of stress. Incrementing the temperature and decreasing the nutritional supplement simultaneously led to a decrease in average fitness effect and an increase in the variance compared to the "reference benign environment". Similar results were observed in relation to the environment where only the nutritional supplement was altered. In general, mutations in different genes seem to react in different ways to different environmental stresses suggesting that these and previous studies results are in agreement for the presence of strong genotype-by-environment interactions operating in a vast range of mutations throughout the genome. However, our results are contrary to previous results obtained for the same microorganism, that pointed to temperature not influencing the effects of mutations (Kishony and Leibler 2003). Nevertheless, there are also some studies showing high influence of nutrients deprivation and no, or slight, influence of temperature on mutations effects (Remold and Lenski 2001; Jasnos et al. 2008).

Even with mutations reacting differently under the same and different stressful conditions, some trends were found for mutations of different genes. Mutations related with DNA replication (*gyrA* that codes for DNA gyrase), are close to neutral. Mutations on the *rpoB* gene, which codes for ß-subunit of the RNA polymerase showed two patterns: more deleterious with the increased level of stress or beneficial under stress (either under nutritional or nutritional and temperature stresses). Recently, Dutta et al. (2011) studied the connections between RNA

polymerase backtracking and genome instability in *E coli*. One of their conclusions was that a particular RNA polymerase mutant (*rpoB H1244Q*) prevents the backtracking process and consequently suppresses the formation of double-strand breaks induced by collisions between the replisome and the elongation complexes. The same results were also obtained in previous studies where the decrease in the probability of collisions was postulated to be due to the fact that this same mutant generates less stable complexes with template DNA (McGlynn and Lloyd 2000; Trautinger and Lloyd 2002). Dutta et al. (2011) add knowledge to previous conclusions by showing that some RNA polymerase mutants have elongation complexes as stable as the wild type. They found that this ability to suppress the formation of double-strand breaks was instead due to failure of the mutant RNA polymerase to pause and backtrack. Following these results, it is expectable that all mutations that underlie more stalls of the RNA polymerase will be deleterious because they decelerate the transcription process and consequently lead to more DNA double breaks due to collisions of those polymerases with the replisomes. Indeed RNA polymerase mutations deleterious in all studied stressful environments were observed. On contrary, mutations that prevent backtracking will be beneficial in the sense that they will avoid such collisions and consequent double-strand breaks. Some of the studied mutations showed to be beneficial however, they were conditionally beneficial because they only do so in stressful conditions. In the "reference benign environment" beneficial mutations were not observed. This could be related with the speed of the cellular machinery that is increased under benign conditions, originating an extra amount of backtracking in comparison to stressful environments. Hence, the prevention of the backtracking generated by certain RNA polymerase mutations might not be enough to overcome the high amount of backtracking due to environmental impositions typical of benign environments.

Also interesting results for the molecular explanation of the observed patterns are those produced by Hall et al. (2011). They determined the contribution of demand for RNA polymerase to the cost of rifampicin resistance and concluded that RNA polymerase demands are responsible for the fitness cost of rifampicin resistance. To do so, they estimated the fitness effects of 53 rifampicin resistant *Pseudomonas aeruginosa* in particular stressful conditions. They started by measuring those effects across a range of sublethal doses of ribosomal inhibitors and concluded that reducing demand for RNA polymerase eliminates the cost of rifampicin resistance. After, they explored the possibility that other factors of stress could also lead to the same patterns observed in the presence of ribosomal inhibitors; they measured the fitness effects of the same 53 rifampicin resistance mutations in the presence of antibiotics that inhibit either DNA supercoiling or cell wall assembly and across a set of 41 different single carbon sources which showed no consistent effect on the elimination of the fitness costs of rifampicin resistance mutations. These results are telling us that the observed patterns of rifampicin resistance, in molecular terms, are due to a particular type of growth inhibitor, namely ribosome inhibitor which consequently led to postulating an interaction between RNA polymerase and the ribosome (discussed in more detail ahead).

On evolutionary terms, the observed abrupt change in the rank order caused by environment-dependent effects suggests the maintenance of polymorphism in fluctuating environments. Environmental heterogeneity as well as stressful conditions are probably common in natural populations which suggests that this type of resistance should be frequent in the sense that it provides an advantage when bacteria divide under stress. Curiously, most of the mutations that were found here to be causing positive fitness effects under stressful conditions (*rpoB H526N*, *rpoB H526L*, *rpoB S512F*, *rpoB D516V* and *rpoB H526Y*) were found in our previous study to interact epistatically with other antibiotic

resistance mutations in the "reference benign environment" (Trindade et al. 2009); clones bearing these five mutations (separately) showed sign epistasis between antibiotic resistance mutations. Once again, the aforementioned study of Dutta et al. (2011) supplied the molecular basis to explain this pattern of sign epistasis. Given so, if a mutation affects the RNA polymerase and concomitantly a second mutation affects the ribosome, the fine tune of both effects on the process of transcription can lead to an increase in fitness conferred by the appearance of the second mutation. Particularly, when a mutation decelerates the RNA polymerase, if the ribosome acquires a mutation that decelerates its mode of action, this mutation, in this context could be beneficial instead of deleterious, giving the observed patterns of sign epistasis. Actually, five of the 12 mutants showing sign epistasis on Trindade et al. (2009) study are precisely rifampicin and streptomycin resistant mutants. In respect to *rpoB H526N* mutation, it showed to produce sign epistasis in combination to *gyrA D87G* mutation. This type of interaction could be molecularly explained if the *rpoB H526N* mutation decelerates the RNA polymerase at the same time that the *gyrA D87G* mutation decelerates the replisome causing fewer collisions. In this case, the gyrase mutation could be beneficial in a background where the RNA polymerase is mutated. Moreover, previous studies also showed that under stress the tendency is for the alleviation of the expected detrimental fitness effect of pairs of mutations (You and Yin 2002; Jasnos et al. 2008). The combined occurrence of these two interactions, both genetic and environmental, confers a potential justification for the high prevalence of those rifampicin resistance mutations. Reinforcing these suggestions is the high level of resistance to rifampicin found in natural isolates of *Mycobacterium tuberculosis* (Telenti et al. 1993).

Mutations in the *rpsL* gene had their detrimental fitness effects increased with stress levels which strikes exactly the suggestion of Kondrashov and Houle (1994). They suggested that the effects of

deleterious mutations may be more severely detrimental in harsh environments. Adding to Kondrashov and Houle (1994) prediction, other experimental studies in *Saccharomyces cerevisiae* showed that the effect of mutations on the growth rate was more deleterious in stressful than in benign environments (Korona 1999; Szafraniec et al. 2001). This situation might imply that if these mutations don't get compensated fast after appearing, they will rapidly be eradicated from the populations.

Finally, the last set of mutations comprises those that being neutral or slightly deleterious in the "reference benign environment" remained neutral in the 2 stressful tested environments. These mutations are mainly related with *cycA* gene (besides those two mutations from *gyrA* reported formerly) which codes for a permease located in the outer membrane (Wargel et al. 1970; Wargel et al. 1971; Cosloy 1973; Robbins and Oxender 1973). This is also an important set of mutations for those no compensation is needed to remain in the populations and they will not be counter-selected either. Mutations being neutral in all the studied environments might indicate that those mutations are able, most of the times, to be polymorphic in natural bacterial populations. Given so, the presence of antibiotic will immediately select for a rapid local selective sweep, which will easily become global. This type of neutrality prevalence irrespective of the levels of stress was also reported by Remold and Lenski (2001). They also found the same percentage of neutrality in their study indicating that this pattern is not confined to this gene in particular but also happens in other genes (as also suggested by the two other mutations in *gyrA* gene that we obtained).

Despite the study of genotype-by-environment interactions have been carried out in point mutations affecting only one or few nucleotides, D-cycloserine mutants revealed to be fertile in all kinds of mutations as previously reported by Fehér et al. (2006). Indeed, the vast majority of the obtained mutants carried large insertions in *cycA*

gene responsible for the observed pattern of resistance. Adding to this we also saw a considerable fraction of deletions. Both types of mutations could be responsible for the complete disruption of the gene and consequent loss of function. This occurrence does not seem to be so worrying for the cell since that the gene in question does not code for an essential function. In fact, the knockout of the entire *cycA* gene renders viable (Baba et al. 2006) and resistant (see Results) *E. coli*. Considering this, our prediction is that the patterns of neutrality across the studied environments would remain the same if large insertions and deletions responsible for the disruption of the gene were taken into account. Goebl and Petes (1986) work supported this notion. In their study they determined the fraction of the yeast *S. cerevisiae* genome required for normal cell growth and division. In this process of determination they found a surprising amount of 80% of random insertions that resulted in no evident phenotypic effects.

The *cycA* gene is a non essential gene that codes for a permease through which the antibiotic crosses the membrane and enters in the cell (Russel 1972; Chopra and Ball 1982). The fact that mutations on this non essential gene were neutral across the three studied environments suggests two things: first that neutrality might occur across several environments with few exceptions for which the gene is essential and second that resistance related with these mechanisms might appear easily and because of that be more frequent.

The present study showed that stress can change the fitness effect of mutations conferring antibiotic resistance in different ways pointing to an obvious genotype-by-environment interaction for that type of mutations. This knowledge is important to understand how populations bearing antibiotic resistance mutations adapt to heterogeneous and constantly changing environments. Additionally, it can help predict the

appearance of resistance under certain conditions and may aid some knowledge in the drawing of strategies to reduce the spread of resistance. One strategy could be as simple as to use the knowledge extracted from the fitness effects of antibiotic resistance mutations in different environments to predict which type of antibiotics are more prone to create successful antibiotic resistance bacteria with no extra cost and avoid their use or find a way to revert their survival strategy.

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Supplementary Material

Table S1. Types and frequency of D-cycloserine resistance mutations

Type of mutation	Description	Fequency	Detection Process
Insertion	≈1100 bp-1600 bp	60%	Agarose Gel
	3bp	7%	Sequencing
Deletion	Unknown	17%	Agarose Gel
Point mutations	Base substitution	17%	Sequencing

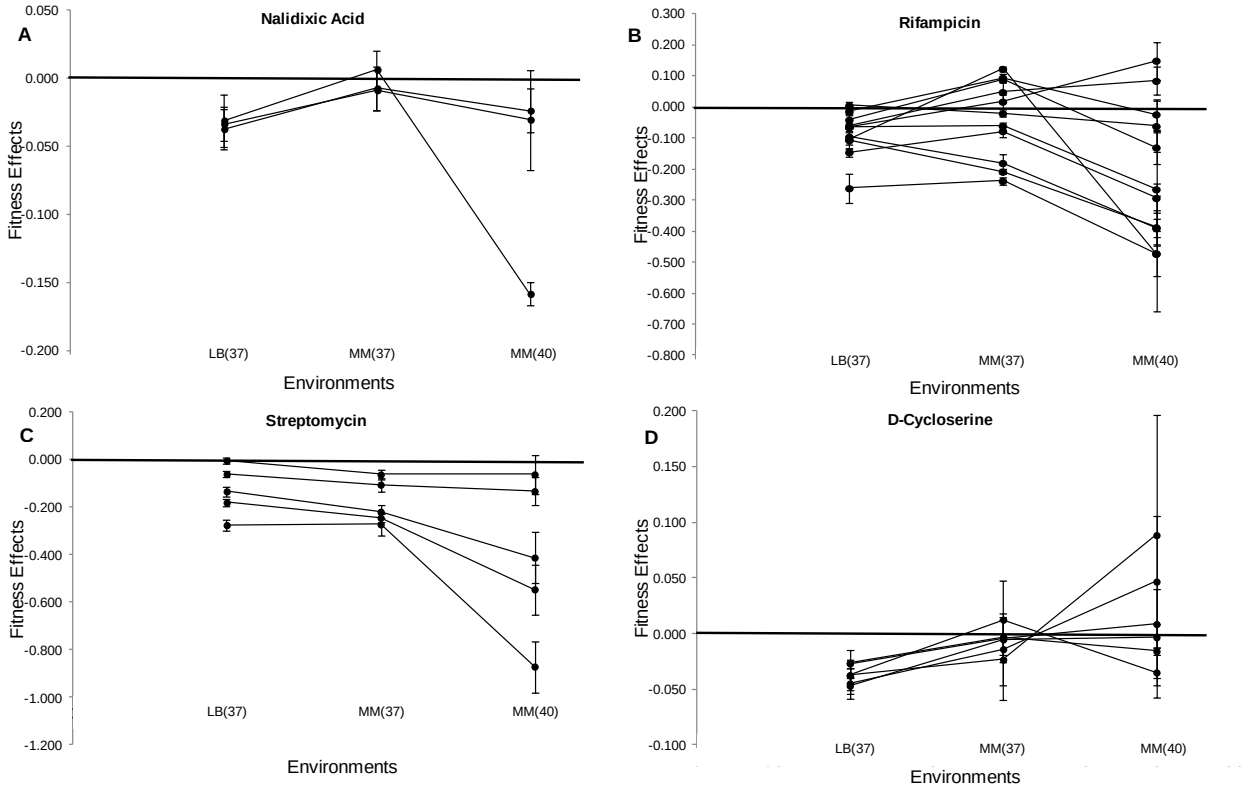

Figure S1. Fitness effects of antibiotic resistance mutations divided by antibiotics. A Nalidixic Acid **B** Rifampicin **C** Sreptomycin **D** D-cycloserine. Nalidixic acid (except for one case) and d-cycloserine antibiotic resistance mutations did not present genotype-by-environment interactions. Streptomycin and rifampicin antibiotic resistance mutations showed strong patterns of genotype-by-environment interactions.

CHAPTER V

DISCUSSION

Facing deathly concentrations of antibiotics is, nowadays, a common scenario for natural populations of bacteria. Yet, bacteria managed to escape by means of acquiring resistance. Actually bacteria found a highly successful manner to deal with antibiotics such that they can even get resistance to more than one antibiotic (multi-resistant bacteria). This occurrence and the resulting prevalence of multi-resistant bacteria are questioning the effectiveness of current treatments of bacterial infections. Epistasis has been suggested to be important in the process of understanding the observed high levels of multi-resistance (see for instance Ward et al. (2009)).

The main aim of the research described in this thesis was to study the fitness effects of mutations conferring resistance to antibiotics and understand how these effects may vary depending on the presence of additional mutations, or accessory genetic elements, and on the environmental conditions. This knowledge might be helpful in disentangle the complex process of evolution of natural populations as well as in designing new therapies to eradicate or decelerate the evolution of antibiotic resistance.

The study described on Chapter 2 revealed the presence of epistatic interactions operating between known mutations on essential genes conferring antibiotic resistance on an antibiotic free environment. These interactions were shown to be majorly of the buffering type: the combined fitness cost of pairs of mutations was smaller than expected. Besides that, a more worrying scenario was also found for 12% of the cases where a second antibiotic resistance mutation was able to compensate for the cost of a previous resistance (sign epistasis). This type of interactions was also reported on Chapter 3 for interactions between a sub-sample of the aforementioned resistance chromosomal

mutations and conjugative plasmids, where they clearly dominated (40%). Positive epistasis was also found on previous studies of antibiotic resistance mutations. Ward et al. (2009), for instance, studied the cost of multiple drug resistance in *Pseudomonas aeruginosa* by analyzing the fitness cost of double mutants carrying rifampicin and streptomycin resistances in antibiotic free rich medium. They found positive epistasis for multiple-drug-resistance. Additionally, Rozen et al. (2007) results on the study about the fitness cost of fluoroquinolones resistance in *Streptococcus pneumonia* also suggest the presence of positive epistasis. Together, these results show a strong tendency for the presence of positive epistasis operating between antibiotic resistance mutations transversal to various microorganisms. The positive pattern of epistasis is not only confined to deleterious mutations. Beneficial mutations were proved to also interact, in a way that two beneficial mutations cause less increase in fitness than the sum of the effects of each (Chou et al. 2011; Khan et al. 2011; Sanjuán et al. 2004). It looks like independently of the type of mutations, whether beneficial or deleterious, the tendency is always to attenuate the fitness effects of those mutations, a pattern that gets stronger with the increase in the fitness effects.

Genetic interactions between two different plasmids were also studied in Chapter 3, where no epistasis was observed, on average. Though, this does not mean the complete absence of epistasis; simply, positive and negative epistases were operating in the same proportion giving a null average. Given the dynamics of antibiotic resistance proliferation, the knowledge on this type of interactions is paramount; it is known that antibiotic resistance is easily passed between individuals of the same species and even between different species by horizontal gene transfer (Doucet-Populaire et al. 1992; McConnell et al. 1991; Salyers and Shoemaker 1996). Importantly, some studies revealed the possibility of

coexistence of more than one resistance plasmid in the same individual (Casjens et al. 2000; San Millan et al. 2009).

The observed dominant buffering behaviour between antibiotic resistant mutations might be sufficient to maintain resistant bacteria in natural populations and so contribute to the patterns of multiple-drug-resistance. Moreover, understanding the aforementioned patterns of genetic interactions and which alleles are contributing to those interactions can shed some light on the working process of the molecular machinery behind this scenario and with this knowledge new therapies can be designed. In a broader context, ultimately, this study adds knowledge to what is previously described for epistasis. Most studies relied on mutation accumulation experiments and those that do not, typically studied deletions or knockout mutations (see for instance Elena and Lenski (1997), Segrè et al. (2005), Jasnos and Korona (2007) and St Onge et al. (2007)). With the work described here it is shown that epistasis is allele specific which adds an extra layer of complexity to the full understanding of the mechanism underlying the genetic interactions. The fact that different alleles were the object of study also allowed us to understand what might be the final outcome of different alterations in essential genes of the cell, something that was not possible with previous studies where gene deletions or knockout mutations were analyzed.

Finally, on Chapter 4, I studied the consequence of external environmental conditions on the fitness effect of mutations conferring antibiotic resistance. I showed that the environment has a strong component in the fitness effects of such mutations in such a way that some rifampicin mutations changed from deleterious to beneficial, given certain levels of stress. Mutations related with essential processes of the cell showed a strong pattern of genotype-by-environment interactions while those in a non essential gene remained neutral or slightly deleterious in all three studied environments. Interestingly, Bataillon et

al. (2011) published their work on the study of the fitness effects of nalidixic acid resistance mutations in the bacterium *Pseudomonas fluorescens* in a wide variety of environments differing on the limiting carbon source. With this work, among other things, they concluded that nalidixic acid resistance mutations (resistance involving essential genes) showed genotype-by-environment interactions. This pattern of interactions seems to be of the same magnitude either considering only beneficial mutations or a wide range of mutations including both beneficial and deleterious mutations (21% for the former and 32% for the latter). As Bataillon et al. (2011) pointed, these magnitude values are on the same order as that achieved by Remold and Lenski (2001) in their study of a small set of random insertions in *Escherichia coli*. Considering that mutations conferring quinolone resistance are usually located in the quinolone resistance-determining region of the gene (Yoshida et al. 1990) which is highly conserved (Weigel et al. 1998) it is expect that, by increasing the sample size for nalidixic acid mutations, it will be observed the same type of mutations as well as the same pattern. This strikes exactly my predictions that mutations in an essential gene will show genotype-by-environment interactions on a wide range of environments contrarily to mutations in a non essential gene that might show genotype-by-environment interactions in few environments.

Usually mutation fitness effects are thought to increase with stress. However this prediction has been shown not to be a general rule (Kishony and Leibler 2003; Remold and Lenski 2001; Jasnos et al. 2008). My results strengthened this lack of consistency by showing that the fitness effects did not always increased with stressful environmental conditions. Instead, it seems to be specific of the gene in question and, more than that on the affected allele and consequently on the cellular processes and on the way those cellular processes are altered by the environment. Optimality implies different environmental conditions

triggering cellular functions in different ways. The explanation for the alleviating or aggravating mutation phenotypic effects of a given mutation seems to depend on the type o stress imposed (see for instance Kishony and Leibler (2003)). In the case of the studied environments it could be as simple as a matter of fine tuning between the affected cellular processes according to each particular environmental demand. For instance, if a change from LB to MM underlies a slower growth, the RNA polymerase will stall less; consistently with this if a mutation appears that will cause even less stalls, this mutation might be beneficial. On the other hand, if the appearing mutation increased the rate of stalling it will most likely be deleterious.

Like individual fitness effects of mutations, the combined fitness effects of mutations, epistasis, are also known to depend on the environment (You and Yin 2002; Pepin and Wichman 2007; Jasnos et al. 2008; Ward et al. 2009; Hall and MacLean 2011). Since environment is clearly an important determinant of the distribution of epistatic effects, studies that test the effects of environmental factors on the variability of epistatic effects operating between antibiotic resistance mutations are crucial for the understanding of the role of epistasis in evolution in general and in evolution of antibiotic resistance in particular. You and Yin (2002) conducted an *in silico* study of bacteriophage T7 to determine the dependence of epistasis on environment and mutation severity. They showed that severe mutations interact positively in poor and rich environments but mildly deleterious mutations interact negatively in poor environments and positively in rich environments. These predictions were tested empirically by Pepin and Wichman (2007) on their study conducted on ΦX174 bacteriophage. They showed that 62%

of the observed variation in epistasis operating among mutations at host recognition sites of these phages was explained by You and Yin (2002) hypothesis. Jasnos et al. (2008) also studied the effects of environmental stress on epistasis. They found that epistasis was operating among gene deletions in yeast, alleviating the expected fitness loss, in a variety of environmental stresses. However, they did not see the aforementioned variation on epistasis. This might be due to the criterion used in selecting deletions: they only studied deletions that generated detectable growth effects in rich medium meaning, large effect mutations. This type of mutations was exactly the one shown to interact positively in poor and rich environments on previous studies (You and Yin 2002). Ward et al. (2009) and Hall and MacLean (2011) saw a similar pattern for antibiotic resistance mutations in *P. aeruginosa*. This type of knowledge should be complemented with more experiments. One possibility is, for instance, perform competitions in sub lethal concentrations of antibiotic, a framework that is not perfect but gets close to the desired conditions. The other alternative is, performing competitions of all resistant strains against a reference resistant strain, in media with lethal concentrations of antibiotic. In the studied sample of resistant clones, for each antibiotic, there is one mutation that is neutral or has a slight fitness effect for at least three different environments (the three environments studied in Chapter 4). These values were obtained by competing resistant clones against a reference wild-type strain indicating that in terms of fitness effects they are alike. So, compete the neutral or quasi neutral strain against the resistant strains should be the same as performing competitions between a wild-type and those same strains, giving similar results. Each of these strains can now be the reference for the competition processes allowing us to measure the relative fitness effects of mutations in the presence of lethal doses of antibiotics. Moreover, in nature resistant strains have to compete with each other quite often in the presence of

lethal concentrations of antibiotics so it would also be interesting to understand the dynamics of such a competition. Another obvious important environment for which epistasis should be tested is *in vivo* for those are the conditions that bacteria have to deal with, when they invade and cause an infection. For instance, Björkman et al. (2000) study in *Salmonella typhimurium* about the effects of the environment on mutations compensating the fitness costs of antibiotic resistance showed that the nature of those compensatory mutations is environment-dependent. They saw that compensation in LB and mice was genetically different. This observation opens the possibility for different types of epistasis occurring *in vivo* and *in vitro*.

Also, a missing piece of data common to all studies that analyzed the patterns of epistasis, including mine, is the lack of knowledge on the mechanistic basis of the reported epistasis. The work presented in this thesis was all based in genetic interactions between antibiotic resistances measured in terms of fitness effects but the underlying molecular mechanisms and the variations on such mechanisms were not studied. Understand how molecular mechanisms change to produce the observed patterns of epistasis is crucial for the complete comprehension of the process. A deeper study focused on this subject should be done. To start with, protein crystallography for instance, might be helpful in unraveling which parts of the enzymes are being affected by the different antibiotic resistance mutations. With this type of knowledge I would be able to see which mutations affect more directly the underlying molecular mechanism and which only affect it indirectly and ultimately associate this knowledge with the observed diversity of fitness costs. Besides that, some published papers pointed to some molecular mechanisms that could explain the obtained results. Bollenbach et al. (2009) studied *E. coli* responses to several drugs related with the inhibition of DNA and protein synthesis. Specifically they showed that the observed suppressive interaction between the referred two types of

antibiotics is due to a nonoptimallity on the rate of ribosome synthesis in bacteria under DNA stress; this equilibrium is restored with the addition of protein synthesis inhibitors to DNA synthesis inhibitors. These interactions are not drug specific since that different pairing of DNA synthesis and translation inhibitors showed this suppressive drug interaction. Instead, it is related with the effect of the drugs on bacterial physiology. On an analogous way, mutations affecting DNA synthesis when combined to mutations affecting translation improved fitness comparatively to the expected outcome. Also, a recent study of Dutta et al. (2011) explored the relationship between DNA replication and transcription and between transcription and ribosomal translation. The rate of replication is known to be much faster than that of transcription turning collisions between replisomes and RNA polymerases unavoidable (Kornberg and Baker 1992). Dutta et al. (2011) studied the molecular mechanisms behind the frequent co-directional collisions between the replisome and the elongation complexes and the mechanisms used by the cell to avoid that and assure the overall genome stability. They showed that, in *E. coli*, collisions between replisomes and backtracked elongation complexes induce double-strand breaks. Having this in mind, I postulate that mutations in DNA gyrase and RNA polymerase decelerate the enzyme and prevent backtrack respectively, and consequently avoid collision. Likely, fitness would be less impaired when compared to the expected combined effect of mutations in DNA gyrase and RNA polymerase. Another important outcome of the study conducted by Dutta et al. (2011) relates to the fact that RNA polymerase mutants as well as translating ribosomes, among other factors, are known to prevent the backtracking process and consequently suppress the formation of double-strand breaks (translating ribosomes evidence was also debated in Proshkin et al. (2010)). This last finding might well explain the presence of positive epistasis in the results presented in Chapter 2. If some of those

mutations belong to the group of mutations that reduce the frequency of stalled RNA polymerases, the concomitant appearance of a mutation that impairs the ribosomal translation activity will not be so detrimental as if it appears on a background where RNA polymerase stalls more frequently. Ultimately, Dutta et al. (2011) conclusions can also shed some light on the explanation for the observed patterns of negative epistasis between streptomycin and rifampicin resistance mutations found here. If the translating process in ribosomes is affected, they can no longer interfere to suppress the RNA polymerase backtracking and consequently double-strand breaks might occur, a process known to be detrimental for bacteria. This situation will be more aggravated if such mutations appear on a background where RNA polymerase mutations that increase the frequency of stalling are present. Of course this is not simple because, as I previously mentioned, other factors also interfere in the process to avoid the detrimental outcome but still it could explain some of the occurrences. In conclusion, it would be interesting to study whether mutations studied in this thesis work provide those types of molecular perturbation in order to explain our patterns of epistasis.

In the preceding Chapters, epistasis was measured between deleterious mutations. In addition, epistasis between beneficial mutations was also reported (Chou et al. 2011; Khan et al. 2011; Sanjuán et al. 2004). The general observation is that the observed epistasis is predominantly of the buffering type, both for deleterious and beneficial mutations. However, there is no study aiming to identify which type of epistasis might be operating between beneficial and deleterious mutations. The results reported on Chapter 4 show that some antibiotic resistant mutations are beneficial under certain conditions. This gives us the tools to study interactions between beneficial and deleterious mutations. Also interesting is the knowledge on which type of epistasis will emerge from the combination of mutations on one non essential and one essential gene. Will the patterns of epistasis be sustained if instead

of two mutations on essential genes we have one mutation on an essential and one on a non essential gene? Double mutants of D-cycloserine and one of the other studied antibiotics are good tools to test this query.

To properly understand and be able to design clinical therapies to avoid the antibiotic resistance problem, more important than study the occurrence of antibiotic resistance is to understand how this resistance prevail in natural populations. Epistasis is believed to be one of the factors responsible for the appearance, increase and prevalence of multiple-antibiotic-resistance (see for instance Hall and MacLean (2011)). Depending on the prevalent type of epistasis, antibiotic resistance can be perpetuated in the populations or simply be expelled. Chapters 2 and 3 provide evidences for both types of epistasis, the prevalent type being positive epistasis which might help in the perpetuation of resistant populations.

Given that at the present time, infections are likely to be caused by microbes that carry resistance to at least one drug, the strategy expected to give the best outcome is one in which the next drug is the one leading simultaneously to the resistant mutant with the biggest cost and strongest negative epistasis. However this approach should be taken carefully since the environmental conditions are also known to alter the costs of resistance (Chapter 4), as well as the type of epistasis operating among mutations. Accordingly, this knowledge must also be taken into account to design the best therapy.

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"What does not kill me makes me stronger."
-Friedrich Nietzsche, *Twilight of the Idols, 1888*-