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ABSTRACT

Antibiotic resistance is, usually, associated with fitness costs for the bacteria. Therefore, it is expected that if one competes a resistant bacterial strain with an otherwise isogenic susceptible strain in an antibiotic free environment, resistant bacteria will be eliminated. Here, I report the occurrence of stabilizing frequency-dependent selection in 43% of a set of antibiotic resistant strains. Stabilizing frequency-dependent selection may promote the maintenance of a stable polymorphism between resistant and susceptible bacteria. If resistant bacteria are rare, their fitness is higher than that of the susceptible strain. If resistant bacteria are the majority in the competition assay, their fitness is smaller than that of the susceptible strain. This enables a stable coexistence between sensitive and resistant strains. Genetic and ecological causes of frequency-dependent selection were investigated. The demonstration of frequency-dependent selection involving drug resistance strains implies that antibiotic resistant bacteria will not be easily eliminated or reversed upon reduction of antibiotic use. This reveals a worrying scenario for the eradication of antibiotic resistance.

Keywords: Antibiotic Resistance, Fitness Cost of Resistance, Bacteria, Competition, Frequency-Dependent Selection, Coexistence.

RESUMO

Durante muito tempo foi admitido que as populações bacterianas seriam mantidas num estado monomórfico devido a dois fenómenos: competição exclusiva e selecção periódica. O principio da exclusão competitiva refere que duas ou mais espécies que competem pelos mesmos recursos não podem coexistir, uma vez que a espécie mais eficiente irá eliminar gradualmente todas as outras (Hardin, 1960). O processo de selecção periódica, em que a evolução de populações bacterianas num recurso limitante consiste numa série temporal de substituições por genótipos cada vez com maior sucesso reprodutivo ou *fitness*, é responsável por eliminar toda a variabilidade genética (Atwood*, et al.*, 1951, Levin, 1981). A existência de polimorfismos seria, então, apenas uma etapa transitória no processo evolutivo.

Contudo, o processo de competição entre espécies é muito mais complexo. O ambiente dos organismos, biótico e físico, é heterogéneo espacial e temporalmente. Portanto, as frequências dos diferentes genótipos numa população estão continuamente a sofrer alterações. Ao mesmo tempo, existem mecanismos responsáveis pela manutenção de polimorfismos nas populações bacterianas. A selecção dependente da frequência é um desses mecanismos.

A selecção dependente da frequência já foi observada em organismos como *Drosophila* (Ayala, 1971), *Escherichia coli* (Levin, 1972, Helling*, et al.*, 1987) e em vírus (Elena*, et al.*, 1997, Yuste*, et al.*, 2002). Num sistema biológico, em que a selecção dependente da frequência actua, o sucesso reprodutivo de um organismo está dependente da sua frequência. Este mecanismo pode resultar em dois cenários diferentes. Assim sendo, a selecção pode favorecer o genótipo mais comum, definindo-se como selecção dependente da frequência disruptiva. Por outro lado, se a selecção favorecer o genótipo mais raro, então estamos perante selecção dependente da frequência estabilizante.

A selecção dependente da frequência disruptiva leva à eliminação de polimorfismos na população. Um genótipo, mesmo possuindo uma qualquer vantagem, apenas será seleccionado face a outro genótipo se a sua frequência se encontrar acima de um certo valor (Levin, 1988). Caso contrário, o genótipo é eliminado.

A selecção dependente da frequência estabilizante, sobre a qual nos iremos focar neste trabalho, resulta num ponto de equilíbrio que permite a coexistência estável entre os genótipos. Se a frequência de um dos genótipos está abaixo do equilíbrio estável, o seu *fitness* relativamente ao outro genótipo será maior, permitindo-lhe aumentar a sua frequência. Se a sua frequência estiver acima do ponto de equilíbrio, o seu *fitness* será menor do que o outro genótipo em competição, e consequentemente irá ocorrer uma

redução do seu número. No ponto de equilíbrio o *fitness* de ambos os genótipos é idêntico, levando a um polimorfismo estável. Vários estudos demonstram a coexistência de genótipos diferentes mesmo em ambientes simples. Algumas sugestões foram sendo dadas na tentativa de explicar os mecanismos ecológicos que permitem a coexistência:

(i) *Trade-off* demográfico, em que cada genótipo é especializado num diferente nicho ecológico (Levin, 1972, Helling*, et al.*, 1987, Turner*, et al.*, 1996, Rozen & Lenski, 2000). Por exemplo, um dos genótipos possui um maior *fitness* quando a glucose no meio é abundante, enquanto o outro genótipo possui um *fitness* mais elevado quando a glucose é escassa (Turner*, et al.*, 1996);

(ii) *Cross-feeding*, em que o genótipo com maior sucesso excreta um metabolito resultante do seu metabolismo ou morte celular para o meio. O segundo genótipo é eficiente a utilizar este metabolito (Turner*, et al.*, 1996, Rozen & Lenski, 2000);

(iii) Interacção antagonista, em que durante a fase estacionária um dos genótipos devido a uma mutação ou a um efeito pleiotrópico de uma mutação é capaz de inibir o genótipo mais frequente, interferindo com o seu crescimento máximo (Lemonnier*, et al.*, 2008).

Os mecanismos ecológicos acima descritos não se excluem entre si. Aliás, a ocorrência de um *trade-off* demográfico está geralmente associado a uma interacção por *cross-feeding* (Rosenzweig*, et al.*, 1994, Turner*, et al.*, 1996).

No presente estudo demonstro a ocorrência de selecção dependente da frequência em bactérias resistentes a antibióticos.

A resistência aos antibióticos pode resultar de mutações genéticas e/ou aquisição de determinantes de resistência por transferência horizontal de genes (revisto emMartinez*, et al.*, 2009). As mutações que conferem resistência podem alterar processos fisiológicos da célula (Andersson & Levin, 1999). De forma similar, quando a resistência está localizada em elementos genéticos móveis (plasmídeos, integrões ou transposões), a replicação e expressão dos seus genes é responsabilidade da bactéria hospedeira. Tal irá interferir no crescimento normal da bactéria (Bjorkman & Andersson, 2000). Assim, um fenótipo resistente acarreta na maioria das vezes, custos para a bactéria, fazendo com que sofra uma diminuição do seu *fitness* (Andersson, 2006). Neste trabalho, utilizei bactérias resistentes a três antibióticos diferentes: ácido nalidíxico, rifampicina e estreptomicina. Para estes antibióticos a resistência é causada por mutações cromossomais em genes envolvidos na replicação (Marcusson*, et al.*, 2009) , transcrição (Reynolds, 2000) e tradução (Kurland*, et al.*, 1996), respectivamente.

Estudos anteriores têm demonstrado que, face às alterações fisiológicas inerentes à resistência, bactérias resistentes em competição com bactérias sensíveis e na ausência de antibióticos, possuem uma desvantagem competitiva (Gillespie, 2001, Trindade*, et al.*,

2009). Deste modo, a simples redução no uso de antibióticos deveria erradicar o grave problema das resistências aos antibióticos. Ou seja, as estirpes resistentes seriam eliminadas. Contudo, o cenário apresenta-se muito mais complexo (Johnsen*, et al.*, 2009). Existem vários processos que permitem manter bactérias resistentes na população ao reduzirem ou eliminarem o custo provocado pelas mesmas: mutações compensatórias (Bjorkman & Andersson, 2000), epistasia entre resistências a antibióticos (Trindade*, et al.*, 2009), mutações sem custo (Ramadhan & Hegedus, 2005, Criswell*, et al.*, 2006) e co-selecção de resistências a antibióticos (Enne*, et al.*, 2004, Andersson & Hughes, 2010). A ocorrência de selecção dependente da frequência aqui apresentada poderá ser um outro fenómeno.

Os resultados aqui apresentados mostram que 13 dos 30 clones espontâneos (43%) resistentes a antibióticos testados apresentam um fenótipo de dependência na frequência quando em competição com um clone sensível. É de notar que, para as mesmas mutações pontuais nos genes envolvidos na resistência ao antibiótico respectivo, os clones resistentes apresentavam um comportamento diferente durante a competição. Isto sugere que o fenótipo para a dependência na frequência seria causado, não pelas mutações para a resistência, mas por outra qualquer mutação a ocorrer no cromossoma. Contudo, tal hipótese não ficou demonstrada.

Relativamente, ao mecanismo ecológico responsável pela dependência na frequência sugeri a ocorrência de um *cross-feeding* ou uma interacção antagonista. Relativamente, ao *cross-feeding* os resultados não foram esclarecedores. No entanto, a hipótese de uma interacção antagonista, apesar de ainda não testada, aponta-se esclarecedora: os clones resistentes seriam portadores de uma mutação capaz de inibir os clones sensíveis através de um contacto célula a célula.

Os resultados mostram uma incidência elevada de selecção dependente da frequência (43%) nos clones resistentes aos antibióticos. Tal incidência pode ser reflexo do método utilizado para selecção dos clones, o qual poderá estar a favorecer a selecção de clones com estas características. Apesar desta hipótese não estar ainda esclarecida, e portanto, desconhecermos se a selecção dependente da frequência tem uma baixa ou elevada incidência nas populações bacterianas resistentes, este estudo vem reforçar que a simples redução ou mesmo eliminação do uso de antibióticos não é uma medida eficaz no combate às resistências aos antibióticos. A ocorrência de selecção dependente da frequência em bactérias resistentes irá possibilitar a sua manutenção nas populações bacterianas, mesmo em baixas frequências. Assim que se reintroduzir novamente o antibiótico a resistência irá emergir em larga escala (De Gelder*, et al.*, 2004).

A selecção dependente da frequência deve ser um mecanismo considerado no estudo das dinâmicas das populações bacterianas e, em particular, nas populações resistentes aos

antibióticos. Deste modo, torna-se necessário o desenvolvimento de novas terapêuticas alternativas aos antibióticos no combate às infecções bacterianas. Tal necessidade está na base do recente desenvolvimento de terapêuticas profiláticas, tais como a interferência bacteriana, vacinas bacterianas e a terapia fágica.

Palavras-Chave: Resistência a Antibióticos, Custo de *Fitness* da Resistência, Bactéria, Competição, Selecção Dependente da Frequência, Coexistência.

INDEX

I – INTRODUCTION

1 COMPETITIVE EXCLUSION PRINCIPLE

Can two or more related species coexist when competing for the same limited resources? The question has been debated for a long time. Gause's principle, or principle of competitive exclusion, states that species using the same resource cannot coexist. The reason is that the more efficient species will gradually increase over the competition and displace the less efficient (Hardin, 1960). Given that bacteria reproduce asexually, it is generally assumed that their evolution in a limiting resource will consist of a temporal series of replacements by ever more fit genotypes, via the process of periodic selection, eliminating all genetic variability (Atwood*, et al.*, 1951, Levin, 1981). Therefore, the existence of polymorphisms would be a process, only transitive, for evolution. So, natural populations should be maintained monomorphic via these two phenomena: periodic selection and competitive exclusion.

However, Gause's principle ignores the complexities of the competition process. In fact, the environment of organisms, physical and biotic, is heterogeneous both spatially and temporally. So, the frequencies of genotypes in a population are continually changing. At the same time, mechanisms responsible for the maintenance of polymorphisms are acting in bacterial populations. Frequency-dependent selection is one of those mechanisms.

2 FREQUENCY-DEPENDENT SELECTION

Frequency-dependent selection phenomena have been demonstrated to occur in very different organisms, such as *Drosophila* (Wright & Dobzhansky, 1946, Ayala, 1971), *Escherichia coli* (Chao*, et al.*, 1977, Helling*, et al.*, 1987), vesicular stomatitis (VSV) and human immunodeficiency type 1 (HIV-1) viruses (Elena*, et al.*, 1997, Yuste*, et al.*, 2002). It has been hypothesized that frequency-dependent selection occurs in natural populations (Ayala & Campbell, 1974, Hori, 1993). However, model organisms with short generation time, large population size and the possibility to control environmental factors, allow studying more precisely the phenomenon of frequency-dependent selection.

In a biological system where there is frequency-dependent selection, the fitness of a given organism depends on its frequency. If selection favors the most common genotype it is called disrupting frequency-dependent selection. If selection favors the rare genotype over a

frequent one it is called stabilizing frequency-dependent selection. The latter will result in the maintenance of genetic variability in a population [containing at least one stable equilibrium].

2.1 DISRUPTING FREQUENCY-DEPENDENT SELECTION

Disrupting frequency-dependent selection implies that there is no internal stable equilibrium in the population and only the most common genotype will be selected.

A good example for disrupting frequency-dependent selection in bacterial populations is the competition between bacteria producing allelopathic molecules and a sensitive one. Allelopathic molecules are capable of killing or inhibiting the growth of competing bacteria, including antibiotics, bacteriocins (Reeves, 1972) and temperate bacteriophages (Levin & Lenski, 1983, Stewart & Levin, 1984). Competitions between bacteriocin sensitive and bacteriocin-producing *E. coli* in liquid media have demonstrated that the latter only invade the population if it is above frequency on the order of 10^{-2} (Chao & Levin, 1981). In other words, only when bacteriocin-producing bacteria are in a high frequency, they will able to invade [where their quantity is sufficient to produce enough bacteriocin to overcome the growth rate disadvantage of its production]. If not, bacteriocin-producing bacteria will be gradually eliminated due to its growth disadvantage (Figure 1).

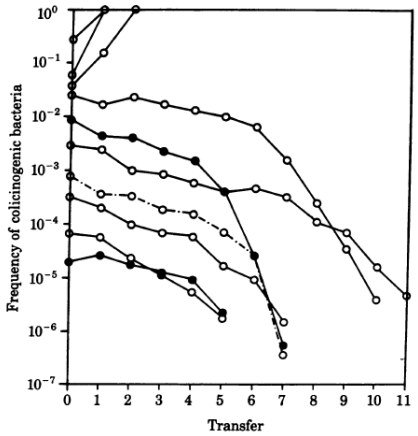

Figure 1: Changes in frequency of colicinogenic bacteria competing with sensitive bacteria in liquid medium cultures (adapted from Chao & Levin, 1981).

Another example for disrupting frequency-dependent selection involves bacterial toxin-antitoxin (TA) gene systems. TA systems code for a stable toxin that kills or inhibits their own cell, and an unstable antitoxin which counteracts the toxin's effect. The toxin is always a protein. The antitoxin can be: (i) an antisense small RNA complementary to the toxin mRNA (type I) inhibiting its translation or (ii) a protein capable of neutralizing the toxin through formation of a proteic complex (type II). These systems are widely distributed in *Bacteria* and *Archaea* (Gerdes, 2000) and are found both in plasmids and in chromosomes (Gerdes*, et al.*,

2005, Pandey & Gerdes, 2005). The presence of TA systems in plasmids, especially those with low-copy number, contributes to plasmid maintenance in growing bacterial populations. Plasmid-free daughter cells are selectively eliminated by post-segregational killing. This elimination occurs after cell division. The antitoxin is unstable and easily degraded by cytoplasmatic proteases. Without its replacement, because the plasmid is lost, the toxin may act (Jaffe*, et al.*, 1985). Conjugative plasmids with TA *loci* (TA^+) have the ability to invade a bacterial population of the same incompatibility group carrying plasmids without TA *loci* (TA^-). This means that, within-cell plasmid competition between TA^+ e TA^- will provide an advantage to TA^+ plasmids (Figure 2) (Cooper & Heinemann, 2000, Cooper & Heinemann, 2005). In liquid cultures where all resources are distributed to all surviving bacteria, TA plasmids would only have an advantage when their initial frequency is high enough to overcome the costs for the host of carrying it. If not, the TA plasmids are unable to invade and tend to extinction (Cooper & Heinemann, 2005).

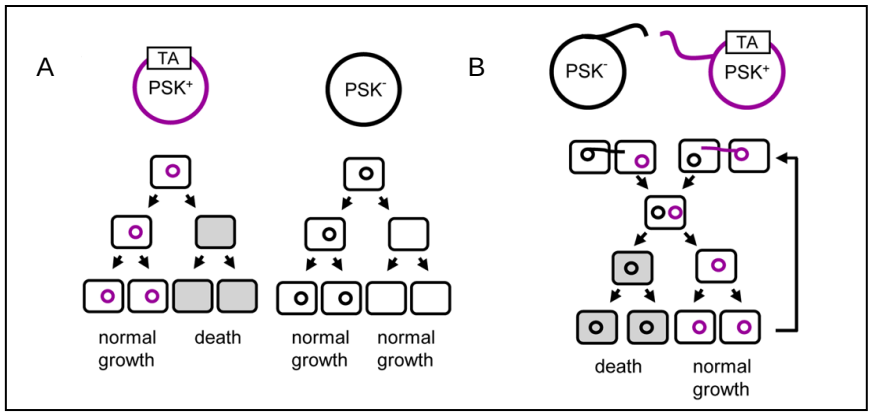

Figure 2: Advantage conferred to bacteria carrying a TA plasmid. (A) Vertical transmission. Prevalence of TA plasmids is increased by post-segregational killing of plasmid-free daughter bacteria. (B) Horizontal transmission. Plasmid-plasmid competition gives an advantage to TA^+ plasmid that outcompete TA^- plasmid. Bacteria that lose TA+ plasmid are killed. On the contrary, bacteria that lose TA- plasmid suffer no damage. Bacterial dead (grey), TA^+ plasmid (purple) and TA^- plasmid (black) (adapted from Van Melderen & Saavedra De Bast, 2009).

2.2 STABILIZING FREQUENCY-DEPENDENT SELECTION

Stabilizing frequency-dependent selection occurs when the fitness of a genotype is higher when that genotype is rare relative to other genotype. This selection results in a stable coexistence. The system is stable because the competitive fitness of one genotype relative to the other is smaller when its frequency is above the equilibrium point and greater when its frequency is below the equilibrium. At the equilibrium frequency, the competitive fitness of the two genotypes is identical.

In natural bacterial populations, one can find some examples of stabilizing frequency dependence selection. One example refers to bacteria capable of neutralizing compounds that kill or inhibit cell growth and cell division (like antibiotics or heavy metals). These bacteria reduce the concentration of those toxic compounds in the environment. Consequently, susceptible bacteria that have disadvantage due to the presence of the toxin are only capable of invading and maintaining in a population where resistant cells are present. When this happens, both bacteria will eventually reach a stable equilibrium. In that equilibrium, the disadvantage of resistant bacteria owing to costs associated to neutralization of the toxin is overcome by its resistance; and the advantage of susceptible bacteria is counterbalanced by dead due to toxin's action. In a theoretical study, Lenski and Hatting showed that *E. coli* strains carrying a chloramphenicol resistant (Cm^r) plasmid and plasmid-free strains: (i) in an environment without chloramphenicol, Cm^r plasmid reduced the fitness of the host bacterium relative to plasmid-free bacteria; (ii) in an environment with chloramphenicol, plasmid-free cells were unable to maintain if resistant ones were not present. However, if plasmid-free bacteria were introduced, at low frequencies, into resistant bacteria cultures with chloramphenicol, plasmid-free bacteria were able to invade and maintain a stable equilibrium with the resistant population (Lenski & Hattingh, 1986). In another theoretical model, presence of beta-lactamase producing bacteria in an environment with the antibiotic (ampicillin) allowed sensitive bacteria to survive at low frequencies. Sensitive bacteria were regarded as cheaters, because they do not harbor a cost from beta-lactamase production, taking advantage from the producing-bacteria (Domingues, 2010).

Besides the biological system above there are several experimental works, mostly done with *E. coli* showing that different genotypes are able to maintain ecologically relevant genetic diversity even in simple environments (Levin, 1972, Helling*, et al.*, 1987, Turner*, et al.*, 1996, Rozen & Lenski, 2000).

In attempting to explain the observation of coexistence in simple environments, some hypotheses have been suggested:

(i) A demographic trade-off. Each genotype is specialized in a different ecological niche (Levin, 1972, Rosenzweig*, et al.*, 1994, Turner*, et al.*, 1996, Rozen & Lenski, 2000). For example, one genotype is competitively superior when glucose is abundant whereas the other is the better competitor for sparse glucose (Turner*, et al.*, 1996). Therefore each genotype will have a growth benefit at a different stage of the cycle and both can stably coexist. So, even though consuming the same resources the two genotypes are not in a direct competition

(ii) A cross-feeding interaction. One genotype is competitively superior for the limiting resource that is exogenously supplied (for example, glucose) and excretes some metabolite into the environment for which the other genotype is efficient to use. The metabolite could be

a result of cell metabolism or a by-product from dead cells. If the amount of the metabolite is proportional to the density of the genotype that is producing it, the rare genotype will benefit. Likewise, the frequent competitor will also benefit when rare because it is more efficient to use the limiting resource, having a higher fitness. Thus, the relative fitnesses of both genotypes will be decreasing functions of their own frequencies, because each has an advantage when rare (Turner*, et al.*, 1996, Rozen & Lenski, 2000);

(iii) An antagonistic interaction. During stationary-phase a genotype carrying a mutation or a pleiotropic effect of a mutation is able to inhibit or kill the other genotype. Such interaction was defined as stationary phase contact-dependent inhibition (SCDI) (Lemonnier*, et al.*, 2008). The inhibition does not occur until bacteria are at (or close to) stationary phase and the total cell density being sufficiently high. Hence, rare SCDI-bacteria are able to increase in frequency by directly decreasing frequency of the other competitor. In that study, the SCDI phenomenon was attributed to mutations in *glgC*, which codes for a regulatory enzyme that catalyzes the first reaction of bacterial glycogen synthesis (Ballicora*, et al.*, 2003).

The above three hypotheses do not exclude each other and stable coexistence could be explained by all of them simultaneously. It is important to emphasize that these stable equilibria will eventually change or disappear. Over generations, environment could change and/or mutations will constantly arise and allow the fixation of beneficial mutations. One genotype will be allowed to win the competition by displacing the other, or both genotypes will coexist in a different stable equilibrium.

In the present study, I attempt to understand if stabilizing frequency-dependent selection is acting in antibiotic resistant bacterial populations.

3 ANTIBIOTIC RESISTANCE

The widespread use of antibiotics in past decades to treat bacterial infectious diseases has greatly assisted the improvement of modern medicine. However, their extensive usage sometimes without rule was responsible for the considerable selection and spread of antibiotic resistant bacteria in both hospital and community settings (Reichler*, et al.*, 1992, Mulvey & Simon, 2009). This scenario imposes a serious problem for public health. Bacterial pathogens are now widely resistant to common antibiotics and new developing drugs became quickly ineffective (Woodford & Livermore, 2009).

3.1 MECHANISMS RESPONSIBLE FOR ANTIBIOTIC RESISTANCE

Antibiotic resistance occurs by genetic mutations and/or acquisition of horizontal gene transfer (HGT) of resistant determinants (for review Martinez*, et al.*, 2009). The rate of formation of resistant bacteria will be determined by the bacteria mutation rate, the biological cost of resistance (Andersson & Levin, 1999) and the rate of antibiotic use (Lipsitch & Levin, 1997). DNA repair systems assure that mutation rate is kept low. However, the mutation rate is greatly determined by the presence of mutator strains. Mutator strains are characterized by increased mutations rates (Oliver*, et al.*, 2000) and recombination frequencies (Matic*, et al.*, 1995) due to a defective methyl-directed mismatch DNA repair (MMR) system (Matic*, et al.*, 1997, Oliver*, et al.*, 2000). The presence of mutators strain in a population will contribute to the emergence of resistant bacteria (Oliver*, et al.*, 2000).

Resistance genes may be found on transferable genetic elements, such as plasmids, transposons or integrons. These elements can spread very fast in bacterial populations, consequently, being fundamental in bacterial genome evolution (Frost*, et al.*, 2005) and in antibiotic resistance dissemination (Whittle*, et al.*, 2002, Bennett, 2008).

The most common mechanisms for antibiotic resistance are the following (Walsh, 2000): (i) alteration of antibiotic target, (ii) enzymatic modification and degradation of the antibiotic or (iii) drug efflux or reduced membrane permeability to prevent antibiotic entry into the cell.

3.2 BIOLOGICAL COSTS OF RESISTANCE

Mutations conferring resistance may disrupt normal physiological processes in the cell. Examples include inhibition of bacterial cell wall synthesis, protein synthesis or nucleic acid replication (Andersson & Levin, 1999). Moreover, if resistance is located in a mobile genetic element, as bacteria are responsible for their replication and gene expression, these elements may interfere with the normal cell growth (Bjorkman & Andersson, 2000). As a consequence, antibiotic resistance is usually associated with fitness costs (Lenski, 1998, Andersson, 2006, Gagneux*, et al.*, 2006, Andersson & Hughes, 2010), which can be reflected in a lower growth rate and virulence.

In the present study, I will focus on chromosomal mutations. Strains harboring resistance mutations in the *rpo*B, *rps*L and *gyr*A genes were used. Mutations in the *rpo*B gene, which codes for RNA polymerase β-subunit, confer resistance to rifampicin in *E. coli*. Those mutations exhibit reduced efficiency of the transcription (Reynolds, 2000). Mutations on *rps*L gene, which codes for the S12 ribosomal protein, are responsible for resistance to streptomycin in *E. coli*. In these resistant bacteria a diminished translational rate is observed

(Kurland, *et al.*, 1996). Mutations in *gyr*A gene, which confer resistance to quinolones, result in alterations in DNA supercoiling. Such alterations give rise to disturbed patterns of transcriptional regulation in *E. coli* (Marcusson, *et al.*, 2009).

In previous studies it has been demonstrated that, in the absence of antibiotic selective pressure, resistant bacteria have a fitness cost, being in disadvantage compared to their counterpart antibiotic-sensitive bacteria (Lenski, 1998, Gillespie, 2001). For this reason it is not clear why resistant bacteria are not eliminated or reverse to a susceptible phenotype upon antibiotic control strategies, i.e. reduction of antibiotic prescription (Johnsen, *et al.*, 2009).

There are four known processes for stability of resistance caused by chromosomal mutation(s) which reduce/eliminate the cost of resistance and make reversion a less probable event: compensatory mutations, epistasic interaction between antibiotic resistances, cost-free mutations and co-selection.

3.3 COMPENSATORY EVOLUTION

In the absence of antibiotic pressure the growth disadvantage of resistant bacteria could be compensated by second-site mutations elsewhere in the chromosome, resulting in an antibiotic resistant bacterial population as fit as susceptible bacteria (Bjorkman & Andersson, 2000) (Figure 3). Compensatory mutation seems to be a more common event than reversion (Schrag, *et al.*, 1997, Kugelberg, *et al.*, 2005), possibly because there is a larger mutation target (Schrag, *et al.*, 1997). Strikingly, the compensation of an antibiotic resistance fitness cost has been shown to occur both in the presence and absence of the antibiotic (Bouma & Lenski, 1988, Schrag & Perrot, 1996, Schrag, *et al.*, 1997, Dahlberg & Chao, 2003, Dionisio, *et al.*, 2005).

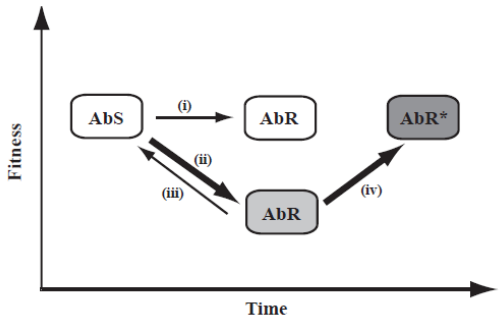

Figure 3: Stability of antibiotic resistance in bacteria. Antibiotic resistant bacteria (AbR) may be as fit (i) or less fit (ii) than their sensitive variants. Fitness may be restored (iii) by true reversion of the resistance conferring mutation or (iv) by acquisition of compensatory mutations that restore fitness to different extent without causing loss of resistance (AbR*) (Bjorkman & Andersson, 2000).

Compensatory mutations can occur by different mechanisms. The most common is the restoration of the structure and function of an altered RNA or protein by intragenic (Hanson*, et al.*, 1993, Reynolds, 2000, Nagaev*, et al.*, 2001) or extragenic (Bjorkman*, et al.*, 1998, Maisnier-Patin*, et al.*, 2007) mutations.

3.4 EPISTASIC INTERACTIONS BETWEEN ANTIBIOTIC RESISTANCES

Epistasis occurs when two or more genes interact with each other resulting in a phenotypically different scenario then if they were acting independently. There are epistasic gene interactions between different antibiotic resistance genes. If different antibiotic mutations enhance each other's costs it is defined as synergistic. If antibiotic mutations mask each other's costs, it is defined antagonistic. In the former case, synergistic interactions will constrain evolution of multiple drug resistance by aggravating its costs. In the latter case antagonistic interactions will promote stability of multidrug-resistant bacteria by reducing its costs. Many studies in this area have found that epistasis tends to be antagonistic (Trindade*, et al.*, 2009, Ward*, et al.*, 2009) and recently a similar epistasis study between antibiotic resistance mutations occurring in the chromosome and in a plasmid has revealed the same trend (Silva, 2010). These findings suggest that epistasis interactions between antibiotic resistances have a key role in the evolution and could explain the difficulties to eliminate multidrug resistant bacteria, as in the case of *Mycobacterium* tuberculosis (Santos*, et al.*, 2010) or methicillin-resistant *Staphylococcus aureus* (MRSA) (Rodriguez-Noriega*, et al.*, 2010).

3.5 COST-FREE MUTATIONS

Cost-free mutations refer to the occurrence of resistance mutations that confer no fitness costs, therefore reducing the driving force for reversibility. Such mutations have been found for several types of antibiotics and bacterial species (Ramadhan & Hegedus, 2005, Criswell*, et al.*, 2006). For instance, substitutions in the 30S ribosomal protein S12, which confer resistance to streptomycin, seems to be a no-cost mutation in *E. coli* and *Salmonella typhimurium* (Kurland*, et al.*, 1996). It should be noted that methods used to measure fitness costs might not detect the lower ones. So, it could appear that certain mutations do not harbor fitness cost. However, those mutations may reduce growth of bacteria in other environments.

3.6 CO-SELECTION OF ANTIBIOTIC RESISTANCE

Co-selection appears mainly in two different scenarios. First, a genetic linkage between a resistance gene and other genetic marker under selection could occur. Therefore, a costly resistance mutation could be maintained in the population (Enne*, et al.*, 2004). Second, resistance to one antibiotic could lead to resistance of antibiotics with a similar structure. If different variants of an antibiotic are used in two different environments [for example, in agriculture and in veterinary] that have different antibiotic control strategies, acquisition of resistance in one environment may develop resistance in the other environment (Andersson & Hughes, 2010).

4 OBJECTIVES

This study aims to demonstrate the occurrence of frequency-dependent selection in antibiotic resistant bacteria. To measure the cost of resistance, competitions experiments are usually performed between resistant and sensitive bacteria in a 1:1 proportion (for example Bjorkman & Andersson, 2000, Trindade*, et al.*, 2009). Nevertheless, only rarely would resistant and sensitive bacteria would be present in a similar proportion in natural environments. Moreover, their proportions are in constant fluctuation. For example, the migration of antibiotic resistant bacteria to antibiotic-free environments, that will result in a lower frequency of the resistant bacteria. Therefore, I performed competitions in antibiotic free environment between spontaneous antibiotic resistant and sensitive clones at different proportions.

I tried to understand if the genetic cause of the frequency-dependent phenotype is a result of a second chromosomal mutation, besides the antibiotic resistance mutation. Then, I tried to determine if a cross-feeding interaction could be the ecological mechanism underlying frequency-dependent selection.

Finally, I developed an experimental protocol to understand the origin of clones with high fitness when rare.

II – MATERIALS AND METHODS

1. BACTERIAL STRAINS AND GROWTH CONDITIONS

The *Escherichia coli* strains used were K12 MG1655 (wild-type), K12 MG1655 Δara, K12 MG1655 srl::Tn10 *mutS*$^{-}$ Str^{R} (mutator strain) (Giraud*, et al.*, 2001) and K12 MG1655 srl::Tn10 Str^{R} (non-mutator strain) (Giraud*, et al.*, 2001). Spontaneous resistant clones to nalidixic acid, rifampicin and streptomycin, were derived from the ancestral strain *E. coli* K12 MG1655. Genes known to be involved in the resistance to the corresponding antibiotics have been sequenced (Dionisio, *et al.*, unpublished data): *gyr*A, *gyr*B, *par*C and *par*E for resistance to nalidixic acid (Nal^{R}) clones; *rpo*B for resistance to rifampicin (Rif^{R}) clones; and *rps*L for resistance to streptomycin (Str^{R}) clones (Tables 1, 2 and 3).

Clones were grown in liquid Luria Bertani (LB) or LB supplemented with agar (LA).

In all experiments, the final antibiotic concentrations in the media were: 40 µg/ml of nalidixic acid, 100 µg/ml of rifampicin and 100 µg/ml of streptomycin.

Competitions assays were performed between *E. coli* K12 MG1655 or *E. coli* K12 MG1655 Δara and spontaneous antibiotic resistant clones. Δara is due to a deletion in the arabinose operon giving rise to red colonies on the tetrazolium arabinose (TA) indicator agar (Lenski, 1988), being distinguished from antibiotic resistant clones (ara^{+}) that produce white colonies. Competitions were performed over a pre-determined time in 50 ml tubes containing 10 ml of LB medium at 37 ºC with constant shaking (170 rpm).

All serial dilutions were done in $MgSO_4$ 10^{-2} M. All clones were stored in 15% (V/V) glycerol at -80 °C for future experiments.

2. SELECTION OF SPONTANEOUS ANTIBIOTIC RESISTANT CLONES

Escherichia coli K12 MG1655 cells were inoculated on several tubes of 10 ml of LB medium for 24 hours, at 37°C and constant shaking. The stationary phase of each culture was platted on LA supplemented with the appropriate antibiotic, followed by incubation at 37°C over 24 hours. Resistant well individualized colonies from each culture were selected and streaked twice onto other selective plates. Then, each clone was grown overnight in LB with the appropriated antibiotic at 37 °C with constant shaking and stored.

Table 1: Sequence of genes involved in resistance to nalidixic acid (Nal; clone number) for wild-type (wt) and resistant clones (Dionisio, *et al*., unpublished data).

Clone	Nalidixic acid resistance genes			
	gyrA gene	*gyrB* gene	*parC* gene	*parE* gene
wt	87th codon: GAC – translates to Aspartic Acid	wt allele	wt allele	wt allele
Nal41	87th codon TAC - translates to Tyrosine	wt allele	wt allele	wt allele
Nal43	87th codon GGC - translates to Glycin	wt allele	wt allele	wt allele
Nal45	87th codon GGC - translates to Glycin	wt allele	wt allele	wt allele
Nal48	87th codon GGC - translates to Glycin	wt allele	wt allele	wt allele
Nal50	87th codon GGC - translates to Glycin	wt allele	wt allele	wt allele
Nal51	87th codon TAC - translates to Tyrosine	wt allele	wt allele	wt allele
Nal54	87th codon TAC - translates to Tyrosine	wt allele	wt allele	wt allele
Nal56	87th codon GGC - translates to Glycin	wt allele	wt allele	wt allele
Nal58	87th codon GGC - translates to Glycin	wt allele	wt allele	wt allele
Nal59	87th codon TAC - translates to Tyrosine	wt allele	wt allele	wt allele

Table 2: Sequence of genes involved in resistance to rifampicin (Rif; clone number) for wild-type (wt) and resistant clones (Dionisio, *et al*., unpublished data).

Clone	Rifampicin resistance genes	
	rpoB1 gene	*rpoB2* gene
wt	511th codon CTG - translates to Leucine 512th codon TCT - translates to Serine 516th codon GAC - translates to Aspartic acid 526th codon CAC - translates to Histidine 529th codon CGT - translates to Arginine 531st codon TCC - translates to Serine	wt allele
Rif1	516th codon AAC - translates to Methionine	wt allele
Rif3	516th codon TAC - translates to Tyrosine	wt allele
Rif4	526th codon TAC - translates to Tyrosine	wt allele
Rif7	526th codon TAC - translates to Tyrosine	wt allele
Rif8	511th codon CCG - translates to Proline	wt allele
Rif12	529th codon CAT - translates to Histidine	wt allele
Rif15	526th codon TAC - translates to Tyrosine	wt allele
Rif17	512th codon TTT - translates to Phenylalanine	wt allele
Rif18	526th codon TAC - translates to Tyrosine	wt allele
Rif19	531st codon TTC - translates to Phenylalanine	wt allele

Table 3: Sequence of genes involved in resistance to streptomycin (Str; clone number) for wild-type (wt) and resistant clones (Dionisio, *et al*., unpublished data).

Clone	Streptomyicin resistance genes
	rpsL gene
wt	43rd codon AAA - translates to Lysine 88th codon CCG - translates to Proline 91st codon CCG - translates to Proline
Str20	43rd codon AAC - translates to Asparagine
Str21	91st codon CAG - translates to Glutamine
Str23	43rd codon AAC - translates to Asparagine
Str24	43rd codon AAC - translates to Asparagine
Str29	43rd codon ACA - translates to Threonine
Str30	88th codon CGG - translates to Arginine
Str31	43rd codon AAC - translates to Asparagine
Str34	43rd codon ACA - translates to Threonine
Str35	43rd codon ACA - translates to Threonine
Str36	43rd codon ACA - translates to Threonine

3. COMPETITION ASSAYS

Competition assays were performed between antibiotic resistant clones and a susceptible clone to determine the ratio of the antibiotic resistant clones [relative to the susceptible clone]. Clones were grown to stationary phase in liquid LB medium. The susceptible clone [wild-type or Δara) were mixed with the antibiotic resistant clones at initial ratios close to: $1:10^{-5}$; 1:1 and $1:10^{2}$, in LB and incubated for 24 hours at 37 °C with constant shaking. The total number of cells at the beginning of the competition was approximately 10^{7} cells/ml, irrespectively of the initial ratio. The number of colony forming units before competition was measured by serial dilutions of each culture, followed by plating on LA and the respective antibiotic (to select for resistant cells) and without antibiotic (for wild-type or Δara cells). Competitions assays were performed over 5 days, unless otherwise noted, through serial competition passages. Every 24 hours, 10 µl of the resulting culture was transferred to 10 ml of fresh LB medium. All experiments were replicated independently three times. The number of antibiotic resistant and wild-type or Δara cells after competition was determined as follows:

(i) Competitions $1:10^{-5}$ of wild-type to antibiotic resistant clones. Cultures were serial diluted and plated on LA or on LA with the respective antibiotic followed by overnight incubation at 37 °C.

(ii) Competitions 1:1 of wild-type and antibiotic resistant clones. Cultures were serial diluted and plated on LA followed by overnight incubation at 37°C, after which 100 random isolated colonies were picked and plated on both LA and LA with corresponding antibiotic, and incubated overnight at 37 °C.

(iii) Competitions 1:1 and $1:10^2$ of Δara and antibiotic resistant clones. Cultures were serial diluted and plated TA indicator agar. An overnight incubation at 37 °C was followed.

4. SCREENING FOR MUTATOR PHENOTYPES

I followed the method of Leclerc et al (1996). Each clone (resistant and wild-type) was grown, in three independently cultures, until stationary-phase in LB for 48 hours at 37°C, with constant shaking. After incubation, 10^8 bacteria from each culture were plated, for resistant (R) count, on LA supplemented with rifampicin, with the exception of Rif^R clones that were plated on LA with nalidixic acid. To count the total number of cells, dilutions were performed and plated on LA. An overnight incubation at 37 ºC was followed for all plates.

As a positive control, I performed the same assay with a mutator strain (*E. coli* K12 MG1655 srl::Tn10 *mutS*$^-$ Str^R (Giraud*, et al.*, 2001)). This strain was used to compare the results of the resistant and wild-type clones mutation frequency.

The mutation frequency for each clone was calculated by the following formula:

$$Mutation \quad frequency \quad = \frac{R\ cells/ml}{Total\ cfu/ml}$$

5. BACTERIAL GROWTH IN SUPERNATANT PREPARATIONS

Bacterial cells were grown to stationary phase in liquid LB medium over one day at 37 °C with constant shaking. Then, cells were removed by centrifugation (10 min at 5000 RPM), followed by decantation. Finally, the supernatant was filtered twice (pore size 0.22 µm). Then, this supernatant was used to grow other bacterial cells over 50 hours at 37 °C with shaking. The number of cells, during incubation, for the first 6 hours was measured hourly, then measurements were done again at 10 hours and 50 hours, by plating in LA or LA with the appropriate antibiotic. After incubation a growth curve was set up.

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6. DEVELOPMENT OF AN EXPERIMENTAL PROTOCOL TO UNDERSTAND THE ORIGIN OF CLONES WITH HIGH FITNESS WHEN RARE

The experiments performed in this thesis have shown an unexpected high number of clones with a greater fitness when rare. I tried to understand if the resistant bacteria selection method favored the selection of these clones. The ancestral strain *E. coli* K12 MG1655 was grown in LB overnight at 37 ºC with constant shaking (170 rpm). A dilution to 10^{-2} was performed and plated on 24 plates with LA. Plates were divided to carry out two independent experiments during incubation: (A) no mixing and (B) manual mixing with glass beads. For experiment (B), 50 µl of $MgSO_4$ 10^{-2} M was added to the plate before mixing, which happened every one and a half hour, for the first 10 hours. Replica plating (Lederberg & Lederberg, 1952) was performed to LA plates with nalidixic acid. After 48 hours of incubation, one clone of each plate was selected randomly and streaked twice to nalidixic acid plates. All clones were stored. Then I checked for an advantage when rare of each of these 24 clones (12 clones under protocol A and 12 clones under protocol B).

7. ESTIMATION OF FITNESS

To measure the fitness of resistant clones relative to the wild-type clones we use the following formula according to Lenski *et al.* (Lenski, 1991), where the fitness of wild-type is normalized to 1:

$$R(t) = R(0) \cdot 2^{r_R \cdot t}$$

$$wt(t) = wt(0) \cdot 2^{r_{wt} \cdot t}$$

$$\frac{R(t)}{R(0)} = 2^{r_R \cdot t} \Rightarrow Log_2\left[\frac{R(t)}{R(0)}\right] = r_R \cdot t$$

$$\frac{wt(t)}{wt(0)} = 2^{r_{wt} \cdot t} \Rightarrow Log_2\left[\frac{wt(t)}{wt(0)}\right] = r_{wt} \cdot t$$

Hence:

$$r_R / r_{wt} = \frac{Log_2\left(\frac{R(t)}{R(0)}\right)}{Log_2\left(\frac{wt(t)}{wt(0)}\right)} \Rightarrow r_R = r_{wt} \cdot \frac{Log_2\left(\frac{R(t)}{R(0)}\right)}{Log_2\left(\frac{wt(t)}{wt(0)}\right)},$$

where R_0, R_t, wt_0 and wt_t stand for colony forming units of antibiotic resistant and wild-type cells in the beginning and in the end of competition assay. This formula assumes that fitness

is not frequency-dependent. Nevertheless, I used it to measured fitness in a 24 hour competition where I will fundamentally be measuring differences in the exponential growth (the principal component of the formula) of both competing strains. Besides, in a 24 hour competition there is not a huge difference in the frequency of the frequency-dependent clones.

8. STATISTICAL AND MATHEMATICAL ANALYSIS

To test if frequency of resistant clones were in equilibrium I performed a linear regression, given as follows: $y = mt + b$, where y is the log-frequency of the clone, m is the slope, t is time in days, and b is the y-intercept. Then an ANOVA was performed to assess if the slope (m) of the regression line of the last three or four days of competition is statistically significant different from zero.

To assess if fitness values of resistant clones were superior to the fitness of the reference strain I performed a *Student's t-test*.

To find differences between mutation rates of clones and those of mutator strains I tested data for homogeneity with Levene's test and I performed a one-way analysis of variances (ANOVA) followed by a Tukey's HSD test. This was performed in *SPSS 17.0*.

All tests were performed with a confidence level of 95%.

III – RESULTS

1 DEMONSTRATION OF FREQUENCY-DEPENDENCY IN SPONTANEOUS ANTIBIOTIC RESISTANT CLONES

The aim of the present study was to understand if populations of antibiotic resistant clones show a frequency-dependent phenotype. I performed competition assays, in the absence of antibiotics, between antibiotic resistant clones and a sensitive clone at different proportions. In these competitions I used thirty clones conferring resistance to commonly used antibiotics belonging to three different classes: 10 clones resistant to the quinolone nalidixic acid (Nal^R); 10 clones resistant to the rifampicin from rifamicyns class (Rif^R); and 10 clones resistant to the aminoglycoside streptomycin (Str^R). Mutations that confer resistance are located in important genes involved in replication (*gyrA*), transcription (*rpoB*) and translation (*rpsL*), respectively. I measured the ratio of the resistant clones in the population along 5 days (unless otherwise noted) of serial dilutions [serial dilutions of 1/1000, implying about 9.966 generations per day]. Each new culture had an initial inoculum of 10^7 cells/ml.

1.1 COMPETITIONS 1:10^5 OF RESISTANT TO WILD-TYPE CELLS

I performed competitions in an approximate proportion of 1:10^5 of resistant to wild-type clones. Figures 4, 5 and 6 show the resistant clones frequencies over competition, for the three different antibiotics.

As observed in Figures 4, 5 and 6 some clones showed an advantage when rare and were maintained in the population in a certain frequency (without replacement of the wild-type strain). At this point it was necessary to establish a criterion to distinguish advantageous resistant clones from disadvantageous. The criterion was: the equilibrium point is not stable if the slope (*m*) of the regression line of the last three or four days of competition is not statistically significant different from zero.

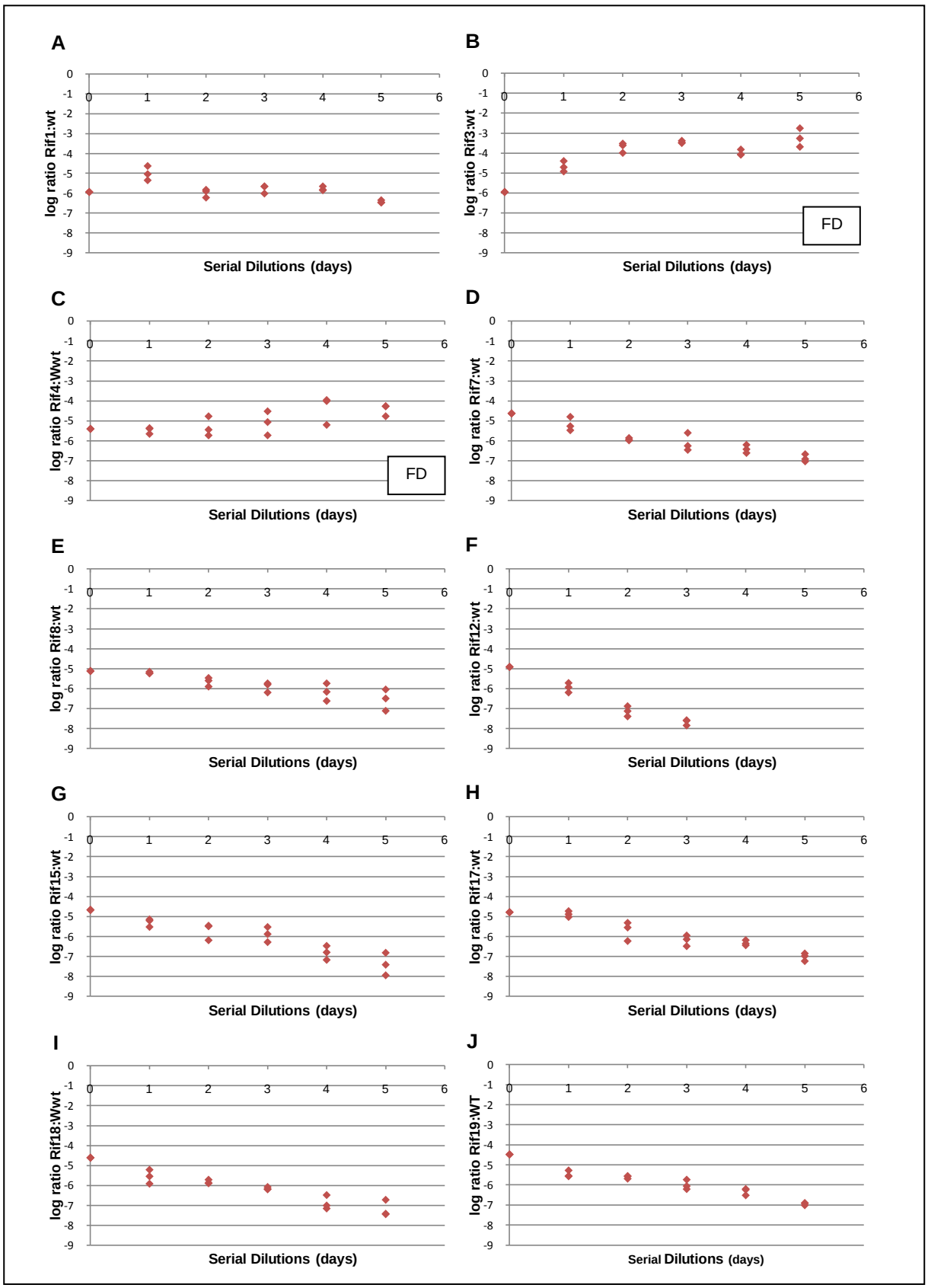

Figure 4: Frequency of rifampicin resistant clones over time. Competitions were performed with initial frequencies of resistant clones close to 10^{-5} over 5 days (about 10 generations per day) against wild-type (wt). The ratio of resistant (R) cells over the wild-type was calculated as follows: $ratio_R = \log_{10}(R/R+wt)$. Frequency-dependent (FD) clones are indicated.

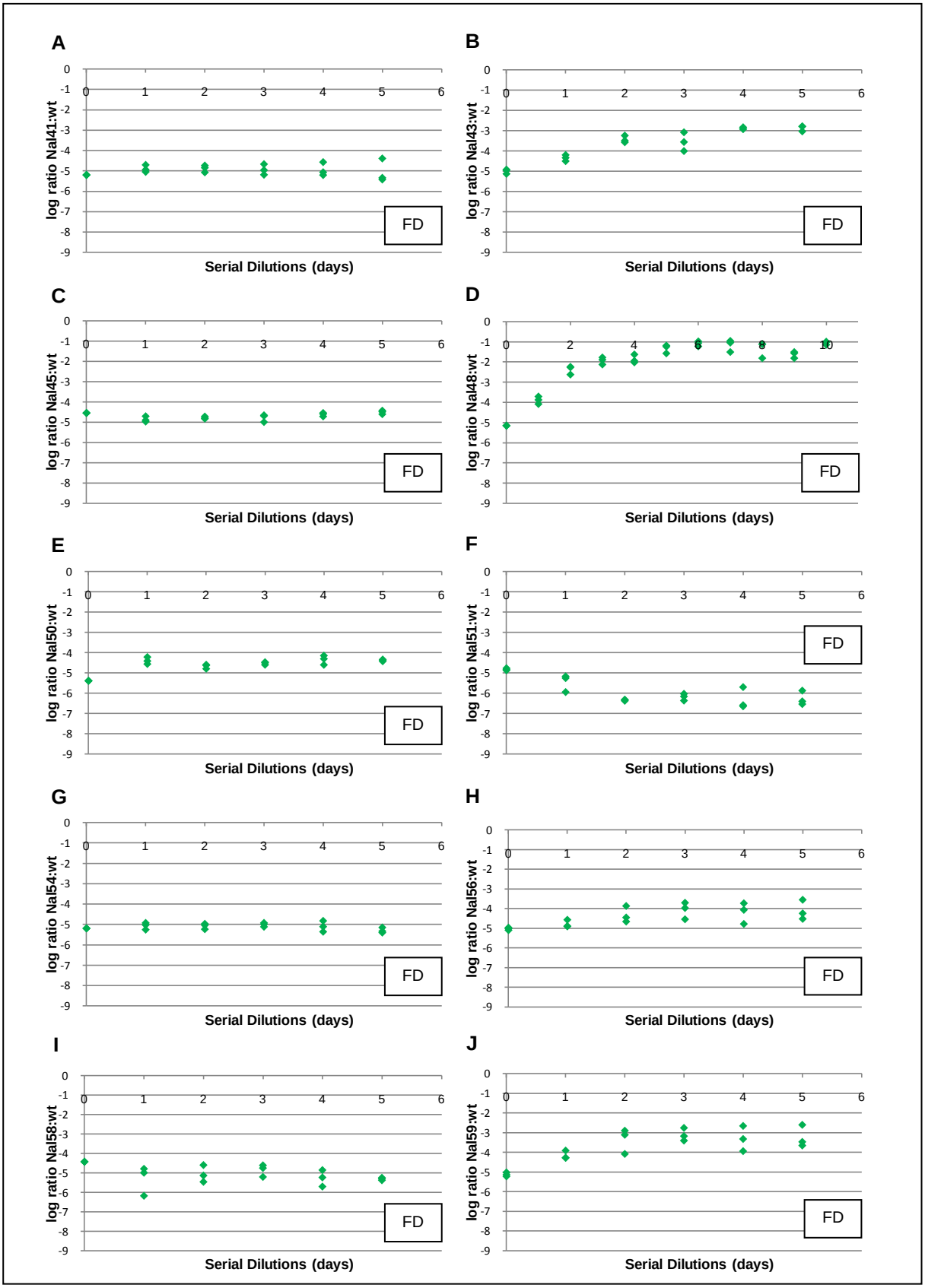

Figure 5: Frequency of nalidixic acid resistant clones over time. Competitions were performed with initial frequencies of resistant clones close to 10^{-5} over 5 days (about 10 generations per day) against wild-type (wt). The ratio of resistant (R) cells over the wild-type was calculated as follows: $ratio_R = \log_{10}(R/R+wt)$. Frequency-dependent (FD) clones are indicated.

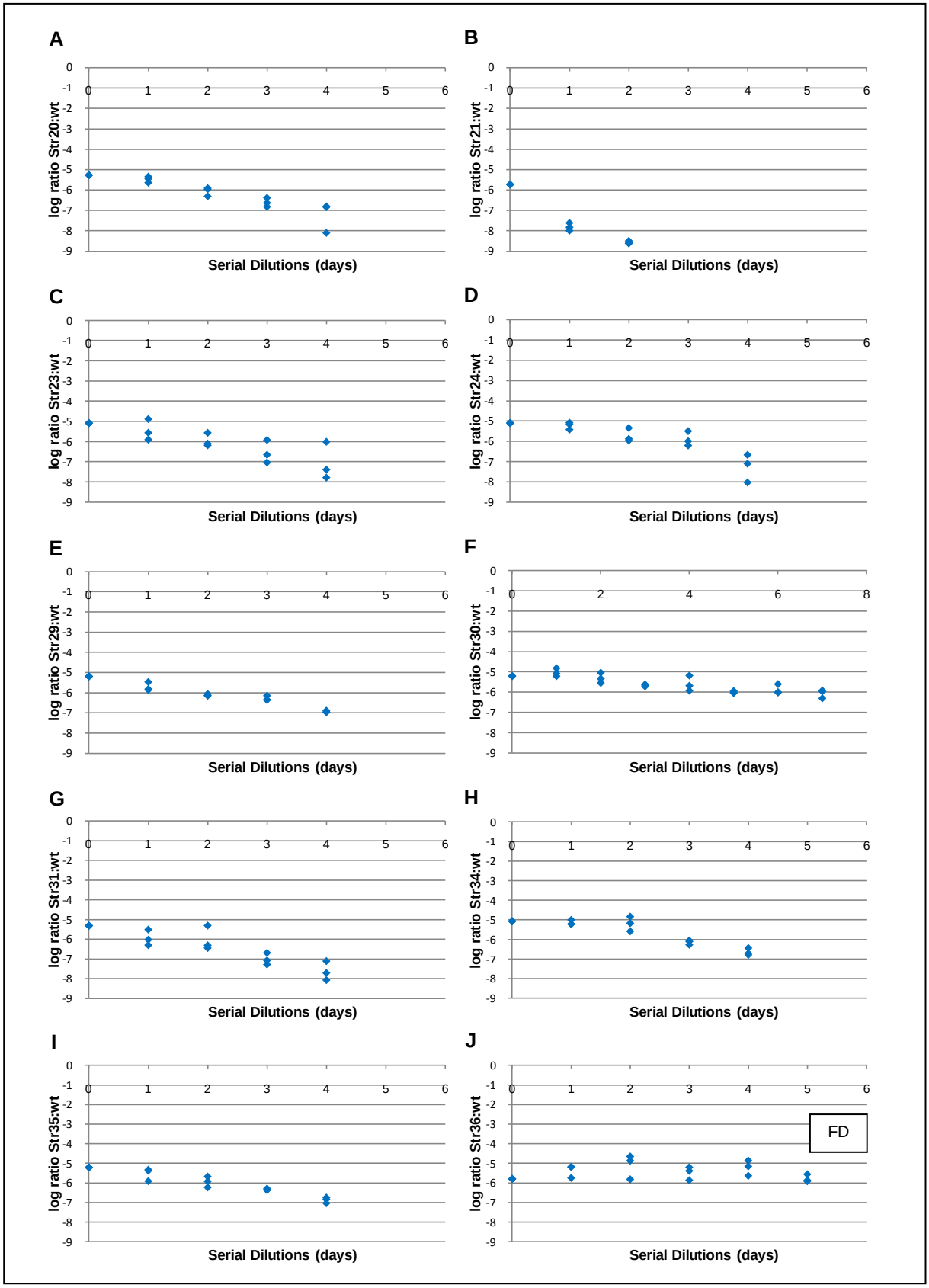

Figure 6: Frequency of streptomycin resistant clones over time. Competitions were performed with initial frequencies of resistant clones close to 10^{-5} over 5 days (about 10 generations per day) against wild-type. The ratio of resistant (R) cells over the wild-type was calculated as follows: $ratio_R = \log_{10}(R/R+wt)$. Frequency-dependent (FD) clones are indicated.

Table 4a: Values of the stable equilibrium of frequency-dependent clones obtained from a linear regression analysis.

Clone	Intersection (*b*)	Slope (*m*)	*P* (ANOVA) †
Nal41	-4.832	-0.036	0.515
Nal43	-2.962	0.017	0.850
Nal45	-4.881	0.040	0.474
Nal48	-1.184	-0.011	0.785
Nal50	-4.571	0.036	0.269
Nal51	-6.086	-0.043	0.776
Nal54	-4.962	-0.047	0.150
Nal56	-4.058	-0.017	0.932
Nal58	-4.237	-0.226	0,108
Nal59	-2.960	-0.065	0.757
Str36	-4.902	-0.148	0.359
Rif3	-3.985	0.107	0.564
Rif4	-4.202	-0.045	0.923

† Last column refers to ANOVA for regression analysis testing if slope is significantly different from zero.

Table 4b: Clones without frequency-dependency assessed from a linear regression analysis.

Clone	Slope (*m*)	*P* (ANOVA) †
Str20	-0.589	9.99×10^{-5}
Str21	-1.425	3.30×10^{-5}
Str23	-0.541	0.004
Str24	-0.630	5.86×10^{-4}
Str29	-0.383	3.036×10^{-6}
Str30	-0.107	0.021
Str31	-0.606	5.54×10^{-4}
Str34	-0.544	2.41×10^{-5}
Str35	-0.440	8.96×10^{-6}
Rif1	-0.327	7.98×10^{-3}
Rif7	-0.387	0.012
Rif8	-0.295	8.06×10^{-3}
Rif12	-0.864	9.18×10^{-5}
Rif15	-0.748	3.26×10^{-3}
Rif17	-0.413	4.14×10^{-3}
Rif18	-0.522	4.58×10^{-3}
Rif19	-0.473	3.74×10^{-4}

† Last column refers to ANOVA for regression analysis testing if slope is significantly different from zero.

Thirteen of the thirty (43%) spontaneous resistant clones (Table 4a) have reached a stable equilibrium ($P > 0.05$): 10 Nal^R clones, two Rif^R clones (Rif3 and Rif4) and one Str^R clone (Str36). These results suggest that their fitness is high when rare having an ability to maintain in the population by stable coexisting with the wild-type. This coexistence occurs without selective pressure (i.e. presence of antibiotic). These thirteen clones were denoted 'frequency-dependent mutants'. For clone Nal43 I only considered two days for the stable equilibrium, because the frequencies at day 3 showed a large variance (Figure 5B).

For the remaining clones (Table 4b) I did not find a stable equilibrium, because their frequency was gradually decreased over the competition period. In one case - Str21 - the resistant clone was eliminated on day 3 of the competition experiment. The clone has a relative fitness of 0.32 (± 0.078). Therefore, these clones did not present a frequency-dependent phenotype.

Each frequency-dependent clone seems to present a characteristic stable equilibrium at low frequencies (Table 4a) and regardless of its low starting frequency relative to the wild-type, each clone reached its point of balance. For example, the clone Nal48, that showed the largest increase of its frequency over the competition period, when the starting frequency was 10^{-5} it rose rapidly to 0.1 (b = -1.184). After reaching this frequency Nal48 showed a stable equilibrium with the wild-type; when it started with a frequency close to 10^{-1} it was observed the stable equilibrium since the first day of competition (Dionisio *et al*., unpublished results). The clone Nal51 presents a stable equilibrium beneath its starting frequency (b = -6.086). So, its frequency in the first two days decreased until it reaches its equilibrium point. The clone Str36 reached the equilibrium on the first day of competition, because its stable point is very similar to the initial frequency of 10^{-5} (b = -4.902) and there it keeps along the competition period.

1.2 COMPETITIONS 1:1 OF RESISTANT AND WILD-TYPE CELLS

Previous authors have performed competition experiments between sensitive and resistant clones in environments free of antibiotics. With no reasons to do otherwise, most authors have performed 1:1 competitions, not $1:10^{-5}$ or any other frequency. Their results show that resistant clones, either have a cost or an equivalent fitness to the wild-type (Tubulekas & Hughes, 1993, Lenski, 1998, Gillespie, 2001). So, I performed 1:1 competitions to test if our clones are not different from common clones tested in previous works. I performed competitions between clones of the Table 4a and the wild-type along 7 days. I measured the resistant clones' frequencies in the first and seventh days. Results are shown in Figure 7.

I follow the same criterion defined in page 16, section 1.1 to assess for a statistically significant frequency decrease. The clones Nal51, Str36, Rif3 and Rif4 presented a statistically significant disadvantage over the wild-type along the competition (for all $P < 0.001$). Hence, they present a cost. The remaining 8 Nal^R frequency-dependent clones did not show a statistically significant disadvantage ($P > 0.05$), even though neither showed any advantage. These clones did not show a growth cost. I also performed a competition with one clone without a frequency-dependent phenotype (Rif18, see table 4b) which confirmed that these clones still present a disadvantage against the wild-type. This 1:1 competition assay was not performed for clone Nal41.

1.3 COMPETITIONS 1:1 OF RESISTANT AND ΔARA CELLS

I performed competitions 1:1 between Δara and resistant clones to evaluate if using a Δara strain, instead of the wild-type strain would give similar results (Figure 8). I performed competitions for the 8 Nal^R clones without a statistically significant frequency decrease, for Rif3 and Str36. I observed identical outcomes to the Figure 7. The cost of carrying a Δara marker was measured by performing a competition assay against the wild-type. I observed a cost of 0.6% not statistically different from zero (*Student's t-test*, $t = -0.509$, d.f. = 2, $P = 0.661$). For that reason I disregard the cost.

1.4 COMPETITIONS 1:10^2 OF ΔARA TO RESISTANT CELLS

As frequency-dependent clones have an advantage when rare, I attempt to demonstrate that such advantage is somehow compensated by a disadvantage when resistant clones are the majority. When in majority, discrimination between resistant clones and the wild-type it is not possible. Therefore, I performed competitions in a proportion 1:10^2 of Δara to resistant clones. I used the Δara strain, which showed similar results to the wild-type (Section 1.3), to overcome technical difficulties in discriminating between resistant and sensitive clones. For these competitions I used three frequency-dependent clones and one clone without frequency-dependency. All four clones tested presented a clear disadvantage over the Δara along the competition experiment, here shown by the increase in frequency of Δara cells. Since resistant clones are at a higher proportion in the competition, in Figure 9 I calculated the ratio of Δara to the total number of cells.

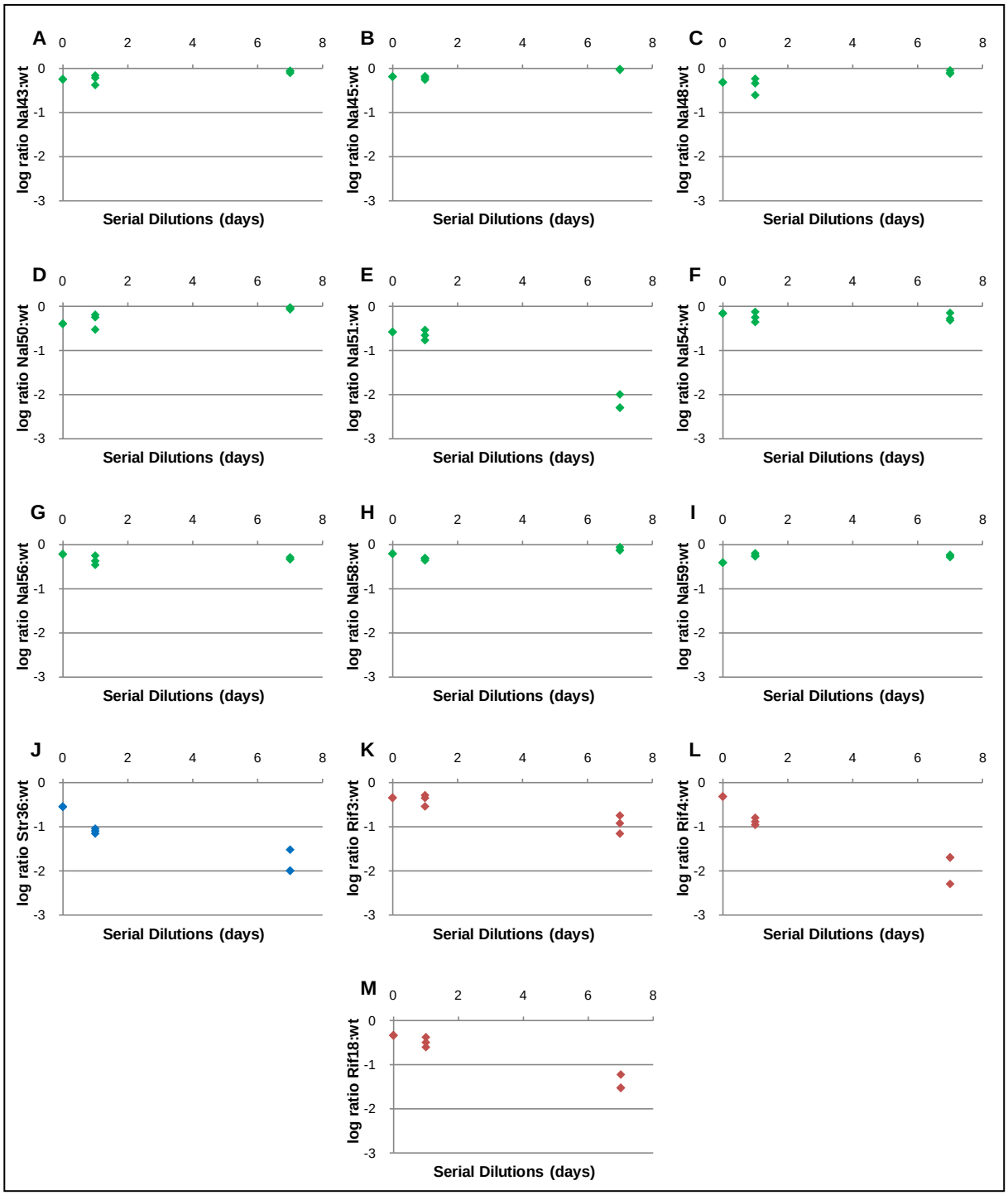

Figure 7: One to one competitions of each clone of Table 4a and Rif18 against the wild-type strain. Competitions were performed along 7 days. The ratio of resistant (R) cells over the wild-type was calculated as follows: $ratio_R = \log_{10}(R/R+wt)$. To assess statistically significant decrease an ANOVA of the regression line was performed. Clones Rif3, Rif4, Rif18, Str36 and Nal51 decrease over time in competition with wild-type strain ($P < 0.05$). Clones Nal43, Nal45, Nal48, Nal50, Nal54 and Nal56 present no cost ($P > 0.05$).

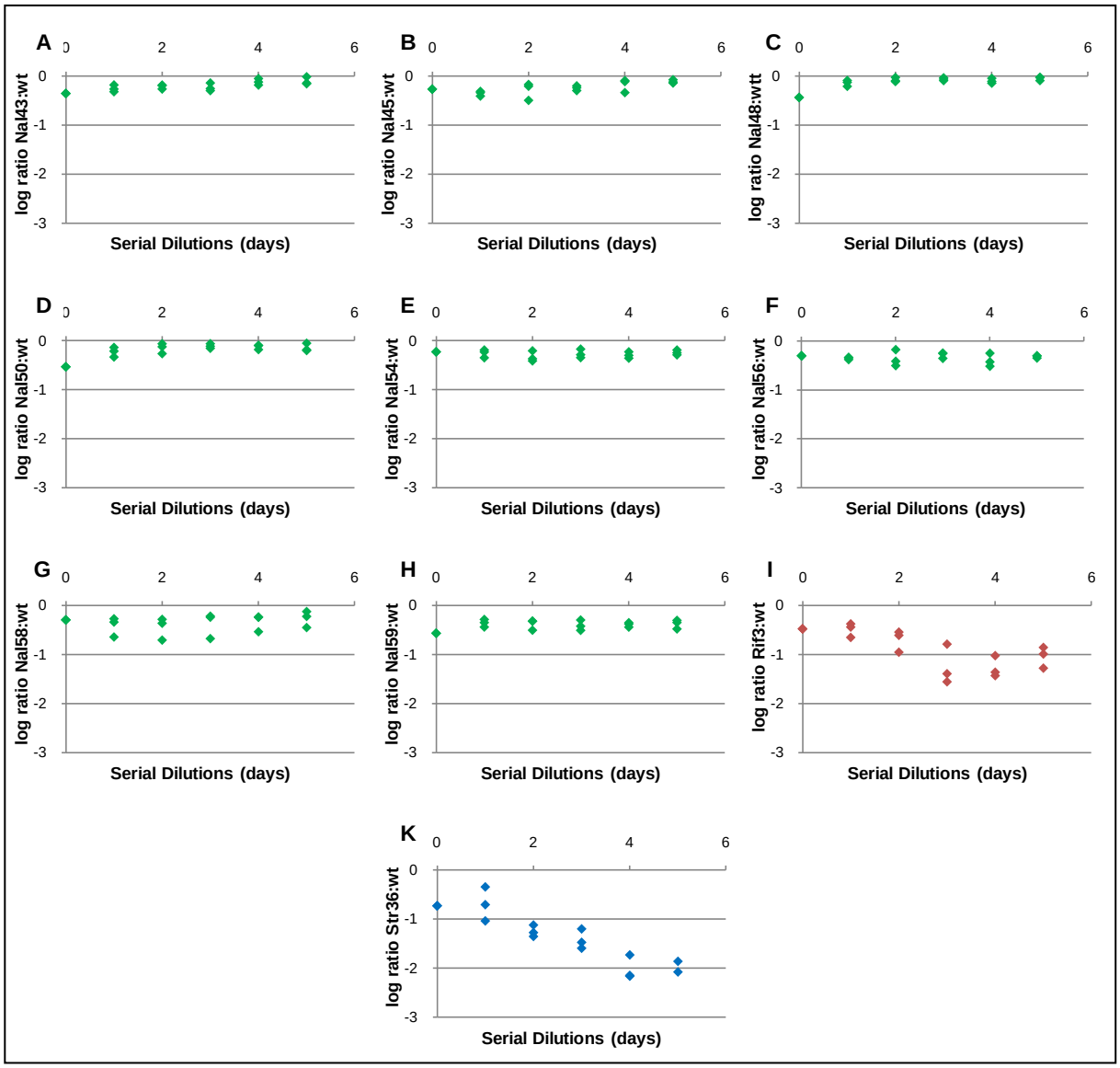

Figure 8: One to one competitions between each clone of table 4a (with the exception of Rif4 and Nal51) and Δara strain. Competitions were performed along 5 days. The ratio of resistant (R) cells over the wild-type was calculated as follows: $ratio_R = \log_{10}(R/R+wt)$. To assess statistically significant decrease an ANOVA of the regression line was performed. Clones Rif3 and Str36 decrease over time in competition with Δara strain ($P < 0.05$). Clones Nal43, Nal45, Nal48, Nal50, Nal54 and Nal56 present no cost ($P > 0.05$). The outcomes of these competitions are similar to those of Figure 7.

1.5 FREQUENCY-DEPENDENT FITNESS OF NAL48 CLONE

Figure 10 shows the fitness of the Nal48 clone plotted against its frequency in the population. Fitness values were calculated using data shown in Figures 5, 7 and 9.

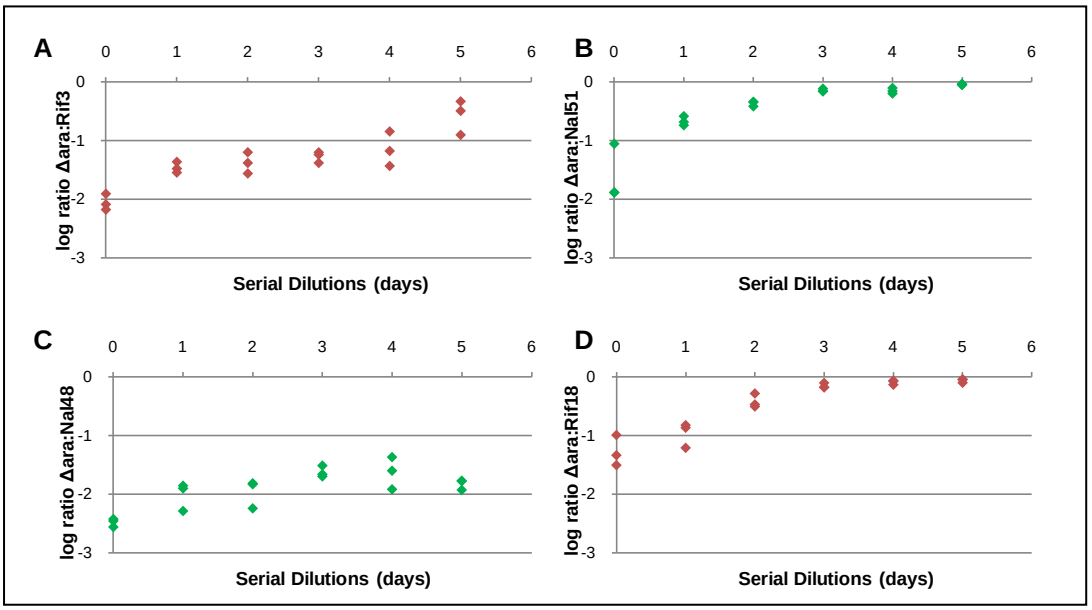

Figure 9: Frequency of Δara strain over time when competing 1:10^2 against antibiotic resistant clones. Frequency increased in the population over 5 days (about 10 generations per day) of competition, meaning that frequency of resistant clones decreased over the over time. The ratio of the Δara over resistant (R) cells was calculated as follows: $ratio_{\Delta ara} = \log_{10}(\Delta ara/\Delta ara+R)$. To assess statistically significant increase an ANOVA of the regression line was performed. (A) Δara:Rif3 (d.f. = 17, *P* = 0.000001); (B) Δara:Nal51 (d.f. = 17, *P* = 0.000016); (C) Δara:Nal48 (d.f. = 17, *P* = 0.001); and (D) Δara:Rif18 (d.f. = 17, *P* = 0.0000002).

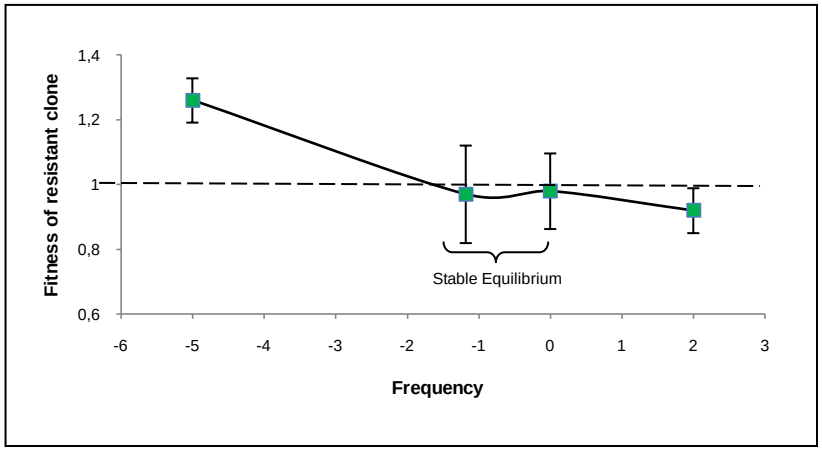

Figure 10: Relative fitness of Nal48 at different frequencies in the population. Competitions were performed against wild-type. The stable equilibrium of Nal48 is located in the indicated area. The broken line represents the fitness of the wild-type normalized. Error bars represent twofold standard error.

1.6 EVIDENCE FOR ANOTHER MUTATION OCCURRING IN THE CHROMOSOME

As already mentioned, all spontaneous resistant clones were sequenced (Table 1, 2 and 3) for genes involved in antibiotic resistance. Some of the isolated clones carry the same

mutation conferring antibiotic resistance, but present different behavior in competition with the wild-type clone. For example, the fitness of Rif4 depends on its frequency, while the clone Rif7 does not, but both presented a substitution of the amino-acid 526 from histidine to tyrosine (Figure 4C and 4D, respectively). Both Str36 and Str29 have a substitution of the amino-acid 43 from lysine to threonine. However, Str36 presented a frequency-dependent phenotype, unlike the clone Str29 (Figure 6J and 6E, respectively). Clones Nal59 and Nal51 have a substitution of the amino-acid 87 from aspartic acid to tyrosine. Both clones had an advantage when rare, but presented a different phenotype: Nal59 clone had a stable equilibrium of -2.960, increasing its frequency before reaching the stable equilibrium, whereas Nal51 has a stable equilibrium of -6.086, decreasing its frequency to reach the point of balance (Figure 5J and 5F, respectively).

2 SCREENING FOR MUTATORS

It could be argued that, if other mutations occur besides antibiotic resistance, some of these mutations can be advantageous. Hence, if these clones were mutators they could provide a short-term advantage. To seek if mutators are responsible to increase the frequency-dependent phenotype, I screened frequent-dependent and wild-type clones for mutator phenotypes. A mutator strain is defined by a mutation rate higher than the average of the species being usually caused by a defect in the methyl-directed mismatch repair system (LeClerc*, et al.*, 1996, Matic*, et al.*, 1997, Picard*, et al.*, 2001). In *E. coli* this system is very well described, being MutS, MutL MutH and UvrD the most important enzymes. MutS recognizes and binds mismatch, MutL binds to MutS and together activate MutH. The latter recognizes the “correct” strand (the oldest and methylated one) and introduces a nick in the non-methylated strand. In the next phase, ssDNA-binding proteins and *uvrD* gene product bind to the region around the nick allowing nucleases to degrade the “inaccuracy”. Finally, DNA polymerase III resynthesizes new strand to replace the mismatch region. Defective MMR system, in most natural isolates, is due to mutations in the *mutS* or *mutL* genes (LeClerc*, et al.*, 1996, Oliver*, et al.*, 2000). For the screening I used *mutS*$^{-}$ strains.

Frequency-dependent clones and the wild-type show mutation rates for rifampicin significant different from those of the mutator strain (ANOVA: $F_{11,24}$ = 16,267, P < 0.001; Tukey HSD: P < 0.001). The same was assessed for mutation rates for nalidixic acid (ANOVA: $F_{2,6}$ = 58.843, P < 0.001; Tukey HSD: P < 0.001). Hence, frequency-dependent clones and wild-type did not show a mutator phenotype.

3 TESTING IF ANOTHER MUTATION IS OCCURRING ELSEWHERE IN THE CHROMOSOME

As mentioned in 1.6 Section, clones presenting the same antibiotic resistance mutations, nevertheless present different phenotypes. This suggests that other mutation occurred while the mutants were isolated. I used a mutator strain (*E. coli* K12 MG1655 srl::Tn10 *mutS*$^{-}$ Str^{R}) and generated spontaneous rifampicin resistant mutants. The same procedure using a non-mutator strain was performed. I isolated ten clones of each strain to initiate competition against the wild-type. If another mutation, responsible for the frequency-dependent phenotype, is occurring in the chromosome besides the mutation causing the antibiotic resistance, then a mutator strain would probably generate more often frequency-dependent mutants [relatively to the non-mutator one].

The results observed after competition showed no difference among clones generated by the two different strains. Both generated five clones [mutator clones - 3, 5, 7, 8 and 10; non-mutator clones - 1, 2, 3, 5 and 10] with a frequency-dependency phenotype. All those frequency-dependent clones were characterized by a decrease in their frequency, followed by a stable equilibrium (Figures 11 and 12). Hence, I did not find a correlation between increased mutational rate and appearance of a frequency-dependent phenotype.

Table 5: Mutation frequencies of wild-type (wt), mutator strain (*mutS*$^{-}$) and frequency-dependent clones for rifampicin or nalidixic acid resistance.

Clone	Resistant cfu/ml	Total cfu/ml	Mutation Frequency †
Mutator	7800.00; 8118.33*	1.17 x 109	6.67 x 10-6; 6.94 x 10-6*
Rif3	28.33*	1.12 x 109	2.42 x 10-8*
Rif4	33.33*	1.25 x 109	2.85 x 10-8*
Str36	6.67	1.31 x 109	5.09 x 10-9
Nal43	30.00	1.09 x 109	2.75 x 10-8
Nal45	26.67	1.47 x 109	1.81 x 10-8
Nal48	43.33	1.39 x 109	3.12 x 10-8
Nal50	38.33	1.52 x 109	2.52 x 10-8
Nal51	23.33	1.22 x 109	1.91 x 10-8
Nal54	43.33	1.65 x 109	2.63 x 10-8
Nal56	18.33	1.39 x 109	1.32 x 10-8
Nal58	30.00	1.46 x 109	2.05 x 10-8
Nal59	43.33	1.76 x 109	2.46 x 10-8
wt	42.50	1.38 x 109	3.08 x 10-8

† Mutation frequency is the ratio of resistant bacteria divided by the total number of bacteria in the population.

(*) Nalidixic acid resistance.

4 TESTING THE OCCURRENCE OF CROSS-FEEDING INTERACTION

Why would 10 out of 10 Nal^R clones, two out of 10 Rif^R and one out of 10 Str^R present an advantage when rare in competition with the wild-type strain? Is wild-type strain producing or excreting some metabolite useful to these antibiotic resistant strains? To answer these questions I seek to explore if stable coexistence between the rare frequency-dependent clones and the wild-type is due to a cross-feeding interaction. I tested this idea with clone Nal48. For that, I grew Nal48 frequency-dependent clone, independently, in the supernatant of wild-type (Swt) and supernatant of Nal48 (SNal) (Figure 13). I expected to observe a higher growth rate of Nal48 growing in Swt than in SNal. I performed a *Student's t-test* to assess differences between exponential growth in both supernatants. However, I did not find differences (*Student's t-test*, $t = 2.477$, d.f. = 3, $P = 0.089$). The end-point of both was similar (*Student's t-test*, $t = 0.931$, d.f. = 4, $P = 0.404$).

5 EVALUATION OF RESISTANT CLONES SELECTION METHOD

Such a high incidence of resistant clones with fitness frequency-dependent raised the question of whether the resistant bacteria selection method favored the selection of clones with advantage when rare. I isolated new spontaneous nalidixic acid mutants by a different method. Besides growth of wild-type in LB medium I added another stage for growth [structured environment] before selecting the resistant clones. I obtained 12 Nal^R clones from each method (A and B). Mixture was performed, in method B, only for the first 10 hours. This period of time refers to the exponential growth phase (when cells are replicating the emergence of mutants is more likely). I measured the fitness of 24 Nal^R clones by a 1:1 competition assay against the wild-type.

I expected that clones generated by method B would show a higher incidence rate of frequency-dependency than clones generated by method A. Clones from method B were allowed to compete (by mixing) in a heterogeneous environment. If one frequency-dependent clone appears, it will be in rarity [so, it will have a higher fitness] and will divide more frequently. Nevertheless, when I mix the environment those 'daughter-clones' will spread. Those clones will possibly be again in rarity and will divide more frequently. Hence, every mixture will result on the spread of the clones. At the time I select clones the probability to find frequency-dependent clones will be higher.

Results do not support the hypothesis, indicating the opposite. For method A, four of the 12 clones showed a fitness superior to the wild-type (*Student's t-test,* $P < 0.05$). Clones A11

(1.576 ± 0.379, *P* = 0.268) and A12 (1.371 ± 0.188, *P* = 0.187) present a fitness marginally superior to the wild-type (Figure 14). For method B, B8 clone present a fitness indistinguishable from the wild-type (1.045 ± 0.037, *P* = 0.350) (Figure 15).

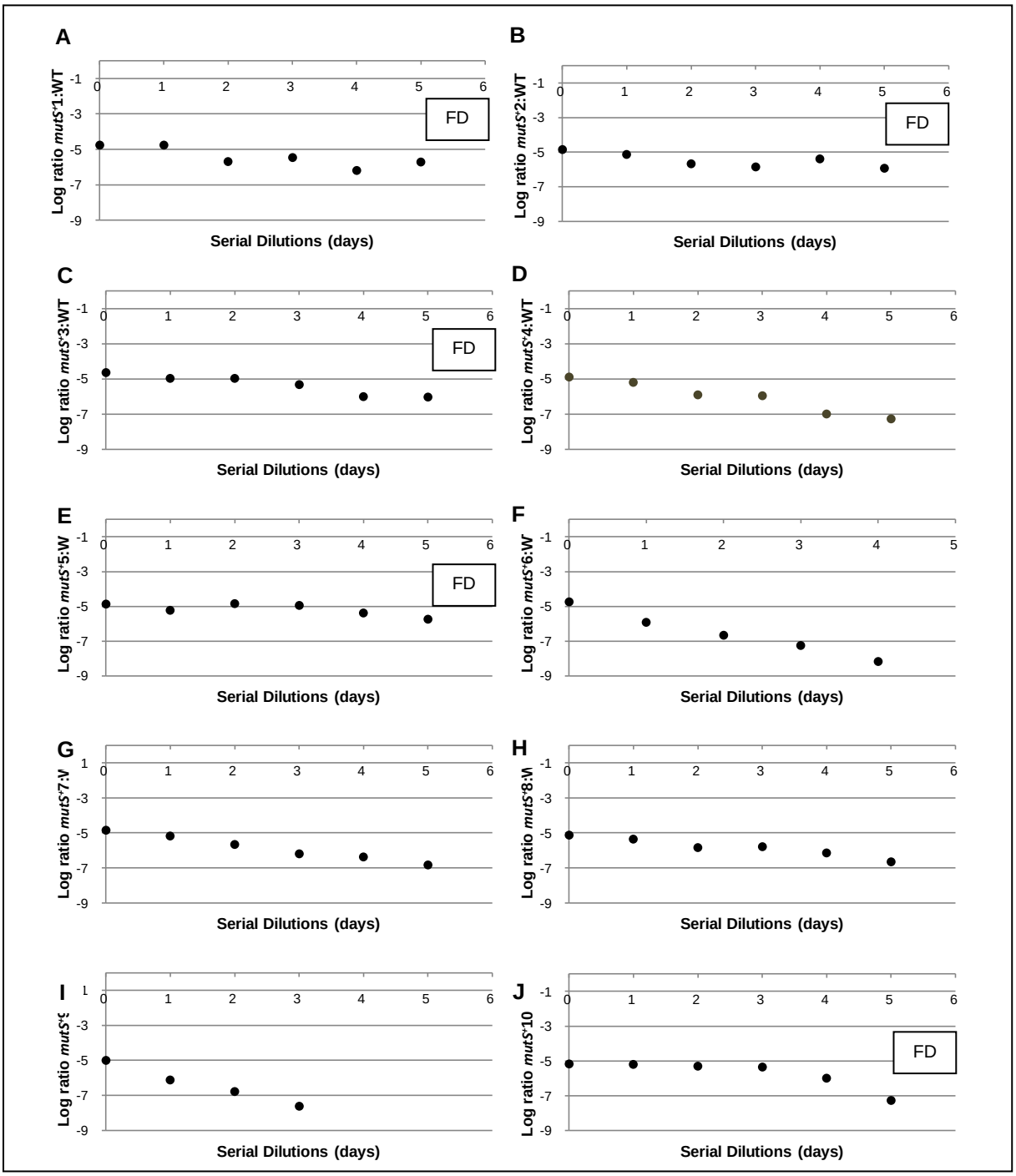

Figure 11: Frequency of rifampicin resistant clones generated from a non-mutator (*mutS*$^+$) strain over time. Competitions were performed over 5 days (about 10 generations per day) in a proportion 1:10^5 of resistant clones to wild-type. The ratio of resistant (R) cells over the wild-type was calculated as follows: $ratio_R = \log_{10}(R/R+wt)$. To assess for a stable equilibrium an ANOVA of the regression line was performed. Frequency-dependent (FD) clones (*P* > 0.05) are indicated.

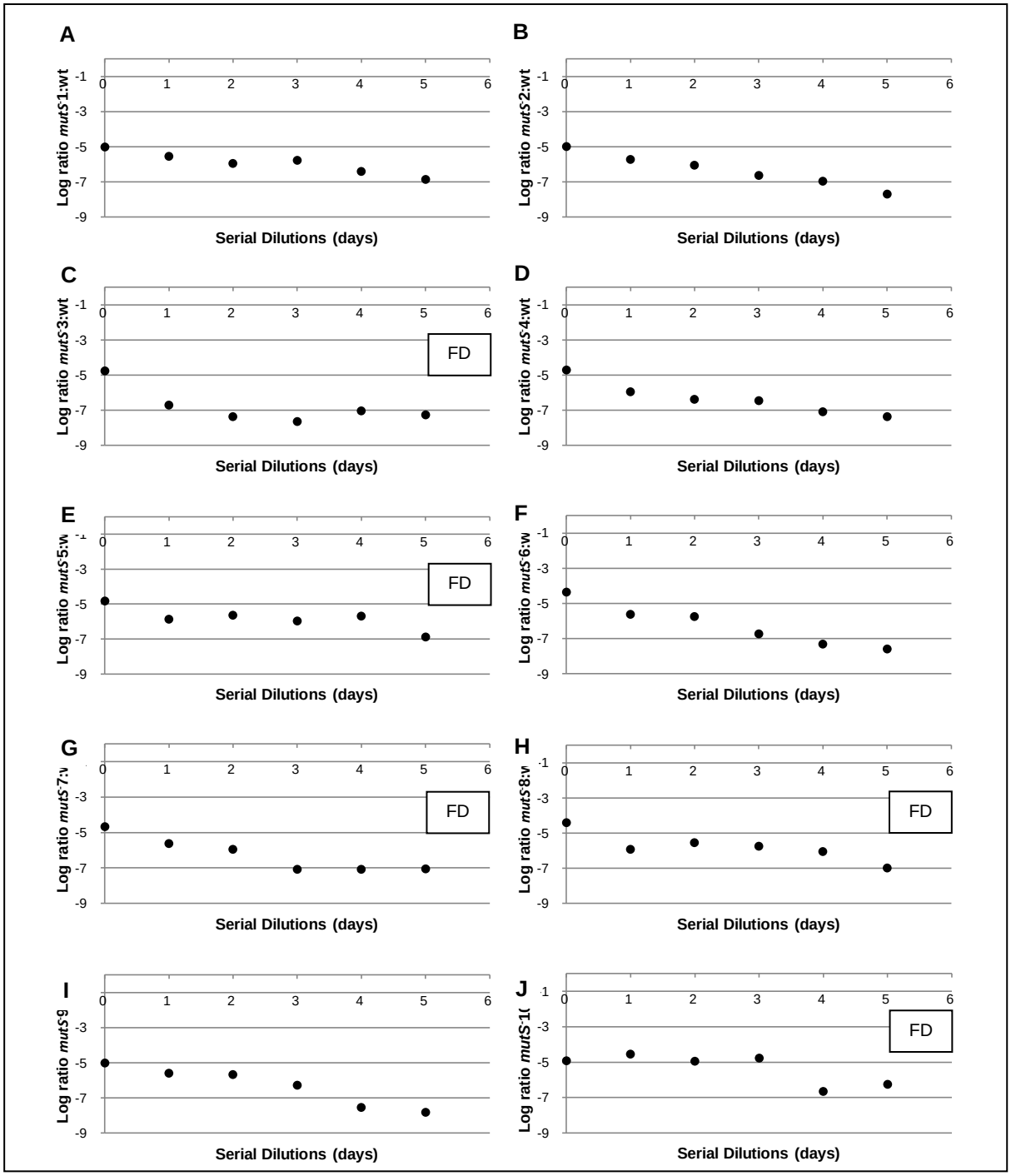

Figure 12: Frequency of rifampicin resistant clones generated from a mutator strain (*mutS*$^-$) over time. Competitions were performed over 5 days (about 10 generations per day) in a proportion 1:10^5 of resistant clones to wild-type. The ratio of resistant (R) cells over the wild-type was calculated as follows: $ratio_R = \log_{10}(R/R+wt)$. To assess for a stable equilibrium an ANOVA of the regression line was performed. Frequency-dependent (FD) clones ($P > 0.05$) are indicated.

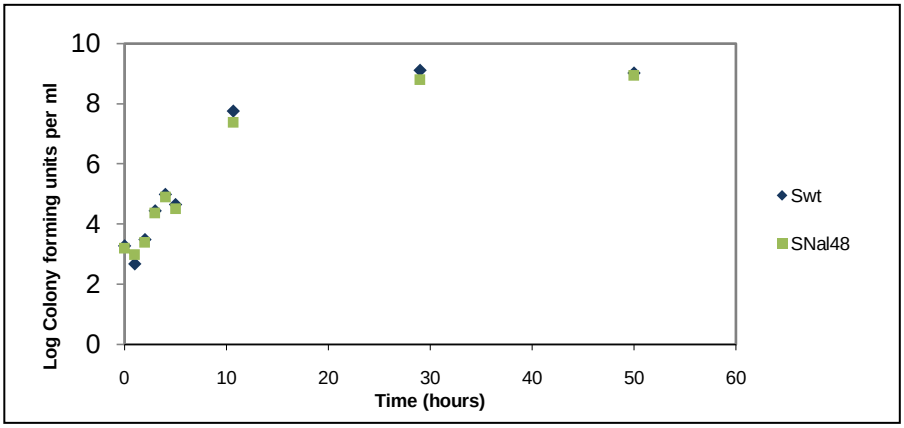

Figure 13: Growth of Nal48 in both wild-type (Swt) and Nal48 (SNal48) supernatant preparations. Clones were grown over 50 hours. To assess differences between growth in both supernatants a *Student's t-test* was performed. No differences were observed (t = 2.477, d.f. = 3, P = 0.089).

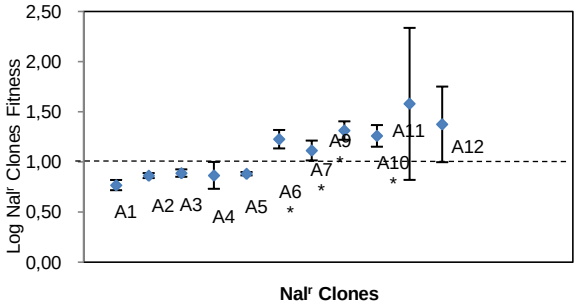

Figure 14: Fitness of nalidixic acid resistant clones obtained by method A. Four clones have a fitness higher than one. The broken line represents the fitness of wild-type normalized. Statistically significance of fitness superior to the wild-type was assessed by performing a *Student's t-test* for each clone. P-values are indicated (*: P < 0.05). Error bars represent twofold standard error.

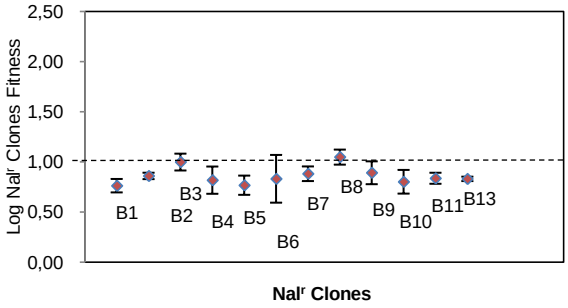

Figure 15: Fitness of nalidixic acid resistant clones obtained by method B. None clones were find with fitness higher than one. B8 clone present a fitness not distinguishable from one (1.045 ± 0.037, P = 0.350). The broken line represents the fitness of wild-type normalized. Statistically significance of fitness superior to the wild-type was assessed by performing a Student's t-test for each clone. Error bars represent twofold standard error.

IV – DISCUSSION

This thesis shows that the fitness of 13 out of 30 (43%) spontaneous antibiotic resistant strains is dependent on their frequency. This means that, if one competes the wild-type strain with one of these 13 strains, their fitness changes with their frequency in the population. In particular, when in rarity (competitions $1:10^{-5}$), the fitness of our antibiotic resistant clones is higher than the fitness of the wild-type strain. When common (competition 1:100) the fitness of antibiotic resistant clones is smaller than the fitness of the wild-type strain. At the stable equilibrium their fitness equals the wild-type fitness, that is, the fitness of both strains is equal to one (Figure 10). Such a scenario, where a strain varies its fitness until reaching a stable coexistence with another one, is defined as "stabilizing frequency-dependent selection" (Levin, 1988).

The 17 clones of this study that did not show a behavior suggestive of frequency-dependent selection present a constant disadvantage. In other words, irrespective of their initial frequency in the competition, their fitness is always smaller than that of the wild-type clone. In fact, such behavior is not surprising (for example Andersson, 2006, Trindade*, et al.*, 2009) and would be expected given that mutations to antibiotic resistance are coupled to cellular physiologic changes. One would expect that these physiological changes impose a growth cost (Kurland*, et al.*, 1996, Reynolds, 2000, Marcusson*, et al.*, 2009) at all frequencies.

The set of 13 strains with frequency-dependent fitness is composed of: 10 out of 10 Nal^R clones, two out of 10 Rif^R clones (Rif3 and Rif4) and one out of 10 Str^R clone (Str36).

The mutators screening of frequent-dependent clones allows me to demonstrate that the advantage in rarity is not due to advantageous mutations emerging as the result of a mutator phenotype.

For competitions 1:1 I expected to observe the loss of the fitness advantage for frequency-dependent clones. This was confirmed, indeed, as none of those clones showed an increase in their frequency. Four clones (Rif3, Rif4, Str36 and Nal51) present a fitness cost shown by a decrease in their frequency. For 8 Nal^R clones I did not observe a drop of their frequency over 7 days of competition (~70 generations). In others words, these eight resistant clones do not present a growth cost. Recently, another study demonstrated that mutations in *gyrA* gene conferring resistance to fluoroquinolones did not impose a growth disadvantage for the bacteria in the absence of antibiotic (Luo*, et al.*, 2005). Therefore, the same is apparently happening with these 8 clones resistant to the quinolone nalidixic acid.

One to one competitions were performed in parallel in two ways: either the sensitive strain was a wild-type strain, or the sensitive strain was unable to use arabinose as carbon source. The two methods gave similar results. Therefore, one may conclude that the two

methods are equivalent. In fact, the Δara strain has been used as reference for competition assays in several works (see for example Lenski*, et al.*, 1991, Trindade*, et al.*, 2009).

This fact enabled us to use the Δara strain to perform 1:100 competitions. This time the sensitive strain is the rare strain. By performing competitions between the wild-type and resistant cells (in which resistant cells are the majority) I would be unable to discriminate the two clones. Hence, using the marked strain Δara the discrimination was possible.

I tried to understand why I obtained such a high number of antibiotic resistant clones having a frequency-dependent fitness. At first, one could think that the phenotype would be associated or caused by the antibiotic resistance point mutations. Yet, when analyzing the sequences of genes involved in each antibiotic resistance one can see that similar mutations result, in most cases, in different behaviors. For example, for the same mutation, one clone showing a frequency-dependent phenotype, but the other not. Consequently, other mutations may have occurred elsewhere on the chromosome. This other mutation should explain and be responsible for the frequency-dependent phenotype. During bacteria replication there are functions in the cell responsible for keeping mutation rates low [for example, proofreading by DNA polymerases and DNA repair systems]. Even so, the probability for an error to occur and consequently a mutation, is in the order of 10^{-8} in natural isolates (Matic*, et al.*, 1997). Since frequency-dependent clones already had a mutation responsible for resistance, the probability that another error in the replication system has occurred is extremely low. The appearance of another mutation could be explained if bacteria enter in a “transitory mutator period”, when inhibited by a given antibiotic [when I isolated the resistant clones]. Bacteria under antibiotic stress are able to produce increased genetic variation (Foster, 1995). In that period mutations will eventually emerge, for example an antibiotic resistance mutation along with one responsible for the frequency-dependent phenotype.

Of the three antibiotics used to select resistant clones, one can see that nalidixic acid resistant clones show the higher incidence of frequency-dependence – all ten clones tested. It is known that pleiotropic interactions occur between *gyrA* gene and many other genes (Jeong*, et al.*, 2006, Drlica*, et al.*, 2009). Hence, if another mutation responsible for the frequency-dependent phenotype is occurring elsewhere in the chromosome, its effect could be enhanced by mutations in the *gyrA* gene.

A chromosomal mutation, besides the one for antibiotic resistance, could be the genetic cause of occurrence of frequency-dependent phenotype in resistant clones. When I generated resistant clones, from a mutator strain, I was expecting clones with a higher number of mutations. Hence, it would be more likely to be frequency-dependent than the clones generated from the non-mutator strain. Nevertheless, such hypothesis was not confirmed. Results showed the same number of frequency-dependent clones generated from both strains (see in this thesis Figures 11 and 12). One possible explanation is the fact that

mutator organisms, having a higher mutation rate (Matic*, et al.*, 1997), will generate a high proportion of deleterious mutations (Giraud*, et al.*, 2001). Hence, a mutation for frequency-dependent phenotype may indeed arise in the population. However, it could be masked due to deleterious mutations that cause a cost to the bacterial cell.

Frequency-dependent selection in asexual populations was first reported by Levin (1972). He observed a stable equilibrium between *E. coli* B and K12 genotypes in a glucose minimal medium. Nevertheless, the ecological mechanisms underlying the coexistence were not clarified (Levin, 1972). In this study, I used a rich and density-limiting culture medium.

The role played by a mutation responsible for the frequency-dependent phenotype was investigated. I analyzed two hypotheses: a cross-feeding interaction and an antagonistic interaction.

The extra mutation may enable the rare resistant cells to be efficient in consuming some extracellular compound released by the wild-type cells. Such extracellular compound could be a result of cell metabolism or dead of wild-type cells. Hence, when wild-type cells are the majority in the population there is a large density of that putative compound in the environment. Resistant clones at a low frequency are all able to exploit it, accelerating their growth. With the increase in the resistant cells' frequency [consequently, wild-type cells decrease] the extracellular compound will be shared by a higher number of cells. Hence, it will not be available for all, which is reflected in a reduction in the resistant cells growth and frequency. Several studies suggest the existence of such a cross-feeding interaction in the maintenance of coexistence in populations (Turner*, et al.*, 1996, Rozen & Lenski, 2000). In most of these cases, cross-feeding interaction is coupled with a demographic trade-off, where the genotypes are more efficient to use different compounds from the environment. Rosenzweig and colleagues demonstrated that in a long-term experiment, stable polymorphisms emerge as a result of bacterial evolution of an acetate cross-feeding interaction. One genotype secretes acetate coupled with a high rate of glucose uptake and the other harbors a mutation that causes semiconstitutive overexpression of acetyl CoA synthetase (Rosenzweig*, et al.*, 1994). However, in the present work I did not find differences between Nal48 growth in both wild-type and Nal48 supernatants.

The occurrence of a demographic trade-off was not considered. A rich medium is composed by several sugars for bacterial growth. Resistant and susceptible bacteria could be using different sugars, therefore not being in a direct competition. However, if the competitions were to be performed in minimal medium, which is composed only of one sugar [for example, glucose], the stable coexistence should disappear. Nevertheless, competitions between frequency-dependent clones and wild-type in minimal medium were performed and stable coexistence was still observed (Dionisio, *et al.*, unpublished results).

Secondly, the mutation could lead to expression of some membrane protein that somehow inhibits the maximum growth rate of common wild-type cells. This inhibition would be possible only through cell-to-cell contact. As a result, rare resistant clones would be able to increase in frequency. A previous study found such an antagonistic interaction, defined as "stationary phase contact-dependent inhibition", between ancestral and inhibitory evolved bacteria (Lemonnier*, et al.*, 2008). This study suggests that the inhibition is caused by mutations in *glgC* gene, involved in the glycogen synthesis pathway. However, the mechanism underlying the contact inhibition was not clarified. They also refer that bacteria harboring such mutations may engender some fitness costs that will not enable them to increase in frequency when in rarity (Chao & Levin, 1981). Yet, Lemonier *et al.* (2008) assume that it is possible that rare bacteria increase in frequency if their costs were compensated by a higher rate of mortality of inhibited bacteria. This hypothesis deserves further research. For future studies, I propose the "U-tubes" experimental protocol carried out by Lemonnier *et al.* (2008) to find if a cell-cell contact for inhibition takes place. If rare, frequency-dependent clones have a higher probability to form cellular aggregates (contact) with sensitive bacteria. Therefore, I would observe an advantage for resistant clones. When I compete the clones in similar proportions (1:1), resistant clones will have a smaller probability to form aggregates with susceptible ones. Resistant cells will probably form aggregates more easily with "sister-cells" than before ($1:10^{-5}$ competition). Thus, resistant clones will not gain enough advantage and will decrease its frequency as a result of the fitness cost of harboring such a mutation. I believe that cell-cell contact takes place only at (or close to) stationary-phase, when there is a high cell density.

I do not think the scenario where frequency-dependent clones have an advantage by releasing a toxin into the environment. If this happened, when I performed competitions in equal proportions (1:1) of resistant and susceptible clones I should still observe an advantage for resistant clones, and consequently, a frequency decrease of susceptible one. Yet, in those competitions resistant clones did not show an advantage. Moreover, in competitions $1:10^{-5}$, one would have to postulate an extremely high amount of toxin produced by the resistant clones (see also Chao & Levin, 1981).

Some studies assume that stable coexistence may be also influenced by extrinsic factors (Ayala, 1969, Icenhour*, et al.*, 2006). Ayala (1969) showed that the resultant stable equilibrium between two species of *Drosophila* was temperature dependent by mediating the fitness of the larval and adult forms. Frequency-dependency and stable coexistence of bacterial populations may be under laboratory dependence. Laboratory dependence occurs when organisms cultured in different locations behave in a different way. In another study, a stable equilibrium between two strains of *E. coli* was modified depending on which laboratory the strains were cultured. In this study, O'Keefe and colleagues found that differences in the

stable equilibrium were not due to bacterial changes. Instead, differences were owing to differences in the growth culture medium, in particular the composition of deionized water (O'Keefe*, et al.*, 2006).

Upon the observation of such a high incidence of frequency-dependency, a question arose. Frequency-dependent clones appear naturally so often in LB medium or, on the contrary, they appear more scarcely but, due to their growth advantage in rarity, the clones show up more frequently? If the latter happens, frequency-dependent clones will have higher probability of being selected when resistant clones are isolated. I tested this hypothesis. However, the outcomes of the experiments did not corroborate the hypothesis. Indeed, results were the opposite of what I was expecting. Perhaps I did not undergo an efficient mixture for frequency-dependent clones competition. In addition, I performed the mixture (method B) only during exponential phase (first 10 hours). However, it has been described that in stationary-phase and under stress [caused by starvation] bacteria increase mutagenesis as a strategy for bacterial adaptive evolution (Bjedov, et al., 2003). The experimental protocols could be further improved along with a larger sample to allow comparisons.

Let's hypothesize that this phenomenon was not confined only to resistant bacteria, but could exist in other bacteria that establish a stable equilibrium with others. For example, regarding an infectious bacterial disease, a genotype that is at a low frequency would better escape from host response. The explanation will be that the immune system will respond only to the most common genotype. Indeed, in a very recent study regarding the dynamics of commensal bacteria that typically colonize humans, it was observed that different strains of some species seem to stable coexist. If one of these strains is pathogenic and is at low frequency, it could trigger an infectious disease without opposition from the immune system. Indeed, some species are able to colonize and increase their infectious load when other species pre-colonize (Margolis*, et al.*, 2010).

V – CONCLUSION

I reported here the pervasive occurrence of stabilizing frequency-dependent selection in bacterial populations. Such phenomenon contributes to maintenance of genetic polymorphisms and is one of several other mechanisms driving the genetic diversity of bacterial populations. Furthermore, I reported here that frequency-dependent selection occurs in antibiotic resistant bacteria. This phenomenon could counteract antibiotic resistance eradication in the absence of antibiotic selective pressure, in addition to other well studied phenomena: compensatory mutations, epistasis, no cost-mutations and co-selection.

This thesis reinforces the idea that the avoidance of antibiotics for a certain period of time will not reverse the antibiotic resistance clinical problem (Levin, 2001). The reintroduction of the antibiotic to treat the infectious disease will allow rare resistant bacteria to have a selective advantage over the sensitive ones, reaching high frequencies at a much faster rate (De Gelder*, et al.*, 2004). Highlighting the above, in United Kingdom between 1991 and 1999 consumption of cotrimoxazole [a combination of trimethoprim and sulfamethoxazole] was reduced by 97%, but even that did not result in a reduction in sulfamethoxazole resistance. An update in 2004 showed that sulfamethoxazole resistance in *E. coli* had remained stable (Bean*, et al.*, 2005), perhaps by frequency-dependent selection or one of the others mechanisms maintaining resistance in the environment.

Further investigation is required concerning the role of frequency-dependent selection in the dynamics of bacterial populations and more importantly in antibiotic resistant bacteria. Thus, it is necessary to develop new therapeutics that replace antibiotics in treating bacterial infections. This need is the basis of the recent development of prophylactic therapeutics, such as bacterial interference, bacterial vaccines and phage therapy.

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