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ECOLOGY AND EVOLUTION OF BACTERIOPHAGES

João Pedro Alves Gama

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Abstract

Viruses that infect bacteria (phages) can directly drive host death required for their multiplication or alternatively remain in a dormant state (prophage) inside the host, being vertically transmitted to host offspring. Due to host exploitation, phages are usually seen as parasites. However, as a symbiont, the virus can confer some advantages to the host, such as immunity to further infections caused by related virus. Some authors proposed that lysogens (hosts harbouring prophages) can use their pathogens to harm susceptible conspecifics. This behaviour, in which the actor (lysogenic individual) experiences a cost (death) to cause damage to conspecifics is called spite. In a further extent this behaviour can be seen as indirect altruism, because the actor by displacing competitors allows its relatives to have access to more resources, thus benefiting them.

Here we show that when competing with susceptible conspecifics in unstructured habitats, there is an optimum initial frequency of lysogens leading to benefit. Above this optimum, the cost of phage production is higher than the benefit associated to the displacement of susceptibles, while under the optimum frequency the viral production is not sufficient to efficiently outcompete susceptibles. However, when competing in structured habitats, spiteful advantage is always seen at short term. At long term, conversion of susceptibles into lysogens predominates. After conversion, the frequency of lysogens decreases if initially rare, or maintains at a certain level if initially common.

We also show that after evolution in conditions, phages become more virulent while bacteria become resistant to the phage. Thus, the evolved strain becomes more competitive.

In conclusion, we that indeed there is spiteful behaviour at short term. However, the effect of spite in often cancelled because susceptible individuals became phage-immune by lysogenization.

 Keywords: bacteria, bacteriophage, competition, spite, virulence, evolution.

Resumo

Os vírus que infectam bactérias (bacteriófagos, ou simplesmente fagos) podem classificados como virulentos ou temperados. Os fagos virulentos seguem, obrigatoriamente, um ciclo lítico caracterizado por multiplicação viral no hospedeiro e subsequente libertação dos seus descendentes recorrendo à morte do hospedeiro. Os fagos temperados, por sua vez, podem seguir um ciclo lítico lítico semelhante ao dos fagos líticos ou seguir um ciclo lisogénico. Neste ciclo os fagos passam a um estado inactivo, ficando integrados no genoma do hospedeiro (profago). Este profago é então multiplicado aquando da multiplicação do hospedeiro, sendo portanto transmitido por transmissão vertical à descendência do hospedeiro. Contudo, os profagos podem ser induzidos a replicar-se. Se tal acontecer, o ciclo lítico é seguido (Campbell, 1996).

Devido ao facto de explorarem a maquinaria celular do hospedeiro de modo a conseguirem reproduzir-se, os fagos são geralmente considerados parasitas. Contudo, eles podem conferir alguns tipos de vantagem ao hospedeiro, como por exemplo:

- Imunidade à superinfecção: quando os fagos estão no estado de profago, se outros fagos semelhantes invadirem o hospedeiro, a sua replicação é impedida devido à capacidade de o profago conferir imunidade ao hospedeiro.
- Servem como meio de transmissão horizontal de genes benéficos: os fagos podem ser utilizados como vectores para transmitir genes horizontalmente entre hospedeiros Este processo é denominado por transdução e implica que alguns genes bacterianos sejam por engano adicionados ao genoma do fago e consequentemente quando o fago infecta um novo hospedeiro e nele se integra, o hospedeiro toma proveito desses. Entre estes genes, encontram-se genes que codificam factores de virulência e de resistência a antibióticos.

Existe um outro modo através do qual os fagos poderão beneficiar o hospedeiro. Esta hipótese propõe que as bactérias lisogénicas (bactérias que contêm o profago integrado) produzem fagos de modo a prejudicar membros da mesma espécie (Dionisio, 2007). Este tipo de comportamento é designado por malícia e implica que o alvo desse comportamento seja prejudicado. Pressupõe também que o actor sofra um custo por produzir tal comportamento (West et al, 2006). Neste caso específico, o actor, indivíduo lisogénico, sofre um custo. Este custo é a própria morte, necessária para a produção de vírus. Por sua vez os vírus irão prejudicar o alvo do comportamento, infectando-o. Devido a esta infecção o alvo pode também morrer. Denote-se que ao produzir vários fagos, o actor tem a capacidade de afectar vários alvos simultaneamente. Assim o custo de um

actor é ultrapassado pela soma do custo individual causado a cada alvo, razão pela qual este comportamento pode ser seleccionado evolutivamente.

A diminuição na frequência de competidores susceptíveis na população total deixa livres mais recursos (nutrientes) que poderão ser utilizados por parentes do actor, que são à partida, imunes à infecção e por isso não são penalizados com posterior contágio resultante do comportamento do actor. Deste modo, os parentes do actor recebem um benefício do seu comportamento. Por esta razão, o comportamento malicioso pode também ser designado por altruísmo em segundo grau, visto que ao prejudicar competidores menos aparentados, acaba por beneficiar os seus parentes (Lehmann et al, 2006).

Dos exemplos de malícia reconhecidos, um dos mais referidos em microrganismos é a produção de toxinas que eliminam membros da mesma espécie (Gardner & West, 2004a; Gardner & West, 2004b; Riley et al, 2003; West et al, 2007). As bactérias que produzem estas toxinas, designadas bacteriocinas e, no caso específico de *Escherichia coli* por colinicas, são imunes ao seu efeito lethal, contudo a sua produção comporta um custo, uma vez que para a libertação das toxinas a morte da bactéria produtora é necessária. Assim, o indivíduo produtor sofre um custo (morte) e em contrapartida beneficia os seus parentes porque elimina vários competidores susceptíveis ao efeito lethal das colicinas. Contudo, a vantagem adquirida pelos parentes do actor varia consoante as condições em que as competições se efectuam (Chao & Levin, 1981; Kerr et al, 2002).

Quando num habitat não estruturado (meio líquido), o benefício conferido pela produção de colicinas depende da frequência inicial de bactérias produtoras. Se esta frequência for abaixo de um dado limite, não há benefícios, porque a produção de toxinas não é eficaz para eliminar um número suficiente de competidores. Tal acontece porque neste tipo de habitat, os recursos libertados são divididos de igual modo por todos os indivíduos, portanto os parentes do actor têm de ser inicialmente suficientes para tirarem vantagem destes recursos. Assim há vantagem para os parentes do actor apenas se estiverem inicialmente numa frequência em que o benefício de eliminar competidores ultrapasse o custo de produzir toxinas. Contudo num meio estruturado, a vizinhança e só os susceptíveis próximos do actor sofrem os danos provocados pelas toxinas. Consequentemente, os parentes do actor, que se encontram na sua proximidade beneficiam dos recursos deixados pelas bactérias mortas. Por essa razão, uma dependência na frequência semelhante à que se observa em habitats não estruturados não se verifica neste caso (Chao & Levin, 1981).

Neste trabalho é demonstrado que, quando em competição em habitats não estruturados, a população lisogénica beneficia do comportamento malicioso quando a frequência inicial das duas estirpes é semelhante. Contudo, se a frequência da estirpe lisogénica inicialmente é superior à da susceptível, a interacção dos fagos com os indivíduos susceptíveis é fraca devido ao facto de estes indivíduos serem raros na população. Por outro lado, se inicialmente os indivíduos lisogénicos forem inicialmente raros, a sua produção de fagos não é suficiente para lhes conferir vantagem. Parece então existir uma frequência inicial óptima para a estirpe lisogénica adquirir vantagem; acima ou abaixo desta, o custo inerente ao comportamento malicioso não é compensado pela vantagem adquirida.

Quando as competições são efectuadas em habitats estruturados, os resultados são diferentes. É observada sempre vantagem a curto prazo, quer os indivíduos lisogénicos sejam raros ou frequentes. Assim, o custo do actor é compensado pela vantagem recebida pelos seus parentes quando beneficiam dos nutrientes libertados pelos competidores que foram eliminados na sua proximidade. Contudo, a longo prazo há conversão dos indivíduos susceptíveis em lisogénicos. Este factor é determinante para o resultado final da competição. Se a frequência dos indivíduos lisogénicos for idêntica ou superior à dos inicialmente susceptíveis durante a sua conversão, a frequência da estirpe lisogénica estabiliza. Pelo contrário, se os indivíduos inicialmente susceptíveis ultrapassam os lisogénicos durante o processo de conversão, então a frequência de indivíduos lisogénicos baixa na população. Isto é explicado pelo facto de o uso do fago se tornar obsoleto, uma vez que a estirpe competidora se tornar imune à sua acção infecciosa.

A estirpe lisogénica foi evoluída em laboratório em diferentes condições experimentais: em habitats estruturados e não estruturados, com e sem competição. Independentemente das condições experimentais a que foram submetidas, as estirpes lisogénicas evoluem no mesmo sentido: o fago torna-se mais virulento enquanto bactérias resistentes (mutantes) por sua vez são seleccionadas.

Com os resultados obtidos nesta tese, podemos concluir que de facto existe comportamento malicioso, a curto prazo. O efeito do comportamento é, no entanto, anulado a longo prazo, porque os indivíduos susceptíveis ao serem lisogenizados se tornam imunes ao fago.

Palavras-chave: bactérias, bacteriófagos, competição, malícia, virulência, evolução.

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INTRODUCTION

In 1910's Frederick W. Twort and Felix d'Hérelle, independently, discovered "*a microbe that was "antagonistic" to bacteria and that resulted in their lysis*" (d'Herelle, 1917; Duckworth, 1976; Twort, 1915). This kind of microorganism was later termed bacteriophage, which literally means *bacteria eater (d'Herelle, 1917)*. In 1940, with the advent of the electron microscope, the nature of this microorganism was revealed: it was a virus (Summers, 2006).

Bacteriophages (phages for short) are thus viruses that infect bacteria. More than 30 genera are known. They are naturally diverse in virion symmetry and genome content (Summers, 2006). Usually, phages are classified as virulent or as temperate (for a review on bacteriophages, see (Campbell, 1996)). When a bacterium is infected by a virulent phage, it undergoes phage replication and host death to release the viral progeny. However, if the phage is temperate, it can be integrated in the bacterium's genome and be kept in a dormant state (prophage), being vertically transmitted to bacterial progeny during bacterial replication.

For both phage types the infection cycle always begins with interaction between the virus and the host cell, resulting in irreversible adsorption. This is followed by the injection of the genomic content of the phage into the bacterial cell. A phage is lytic if it simply replicates and lysis the cell.

The infection cycle of a temperate phage can be more complex than that of lytic phages. After the entrance of a temperate phage's genome in the host cell two outcomes are possible: lysis or lysogeny. In one hand, there's production of viral progeny and host cell lysis similarly to what occurs for lytic virus. This is called lytic cycle. In the other hand, the phage genome is stably integrated in the host genome. By this way the host becomes lysogenic. This allows the phage genome to be replicated along with host genome and consequently transmitted vertically to daughter cells. However some conditions lead to induction of the prophage. Induction will allow production of viral progeny and the host cell lysates as a consequence.

Typically phages might be seen as parasites because they exploit their host for reproduction leading to host cell death. Nonetheless, the presence of a prophage can also retrieve some benefits. A way of doing so is through transduction. Transduction is a form of lateral gene transfer where phages act as vectors for transmission of bacterial genes between bacterial strains (Zinder & Lederberg, 1952). Among those genes, there are

several that code for virulence factors such as toxins or even antibiotic resistance determinants (Bossi et al, 2001; Boyd & Brussow, 2002; Colomer-Lluch et al, 2011; Musser et al, 2002; Weiss et al, 2003).

Another benefit of harbouring prophages, observed in *Escherichia coli*, is the fact that during aerobic growth under conditions of continuous carbon source limitation lysogenic strains have higher metabolic rate than non-lysogenic, thus reproducing faster than non-lysogenic strains (Edlin et al, 1977; Edlin et al, 1975; Lin et al, 1977).

Another kind of advantage of harbouring a prophage was suggested. The hypothesis (mathematically demonstrated) states that hosts could use their parasites to harm unrelated conspecifics (Dionisio, 2007; Rozsa, 1999; Rozsa, 2000). Correlating the hypothesis to the case presented in this work, lysogenic strains could use phages to kill non-lysogenic susceptible strains, thus possessing a fitness advantage over them. This kind of act is classified as a spiteful behaviour. To understand it better, some concepts of social behaviour will be presented.

Behaviours that affect the fitness of several individuals are considered social (Gardner & West, 2004a; West et al, 2006). Assuming this principle as the basis of sociality, one should ask if it is possible that microorganisms could present social lives. Traditionally microorganisms were seen as lonely beings obeying only to their own genetic instructions. However this idea is surpassed for there are already enough examples of social behaviours in microorganisms (Gardner & Kummerli, 2008). Microbes do cooperate, but also communicate and these traits ensure the performance of a varied array of activities (Crespi, 2001; West et al, 2007).

There are several types of social behaviours (Hamilton, 1964a; Hamilton, 1964b), here we will follow the classification presented by (West et al, 2006) which is based on the effect the behaviour produces on the fitness of the actor (individual performing the behaviour) and of the recipient (individual who suffers the consequences resulting from the actor's behaviour). This classification is illustrated in Table.1. If a behaviour has a positive effect on both the recipient's and actor's fitness it is called mutual benefit but if only the actor experiences a positive effect while the recipient suffers a negative effect on its fitness it is called selfishness. However the actor must not be always favoured. If the behaviour has a negative effect on the actor's fitness but a positive effect on the recipient's fitness it is called altruism. Yet there is the possibility of both actor and recipient having a negative effect on fitness, in this case the behaviour is called spite.

Table.1: Classification of Social Behaviours			
		Effect on Recipient's Fitness	
		Positive	Negative
Effect on Actor's Fitness	Positive	Mutual Benefit	Selfishness
	Negative	Altruism	Spite

According to the previous paragraph, individuals may decrease their own fitness, both in altruistic and spiteful behaviours. Thus, there is an important question emerging from this: "Why would an organism harm himself?" (i.e. what reason would drive an actor to perform a behaviour that has a negative effect on its own fitness?). Darwin already asked himself how come there is altruism, for example, among social insects. Indeed, in the 8th chapter of his book "On the origin of Species", Darwin writes that altruism among social insects is "*one special difficulty, which at first appeared to me insuperable, and actually fatal to the whole theory* [Natural Selection]". Already in the XX century, Fisher (1930) and (Haldane, 1952) discussed the "problem of altruism", but they left to Hamilton, in 1964, the mission to show the necessary conditions for altruism to be selected in biological populations (Fisher, 1930; Haldane, 1955; Hamilton, 1963; Hamilton, 1964a; Hamilton, 1964b).

A few years later, Hamilton discussed the existence of spiteful behaviour using the same mathematical models used to discuss the existence of altruism. According to these models, an individual is willing to pay a cost C to be altruist to another individual (giving him a benefit B) if RB − C > 0, where R is the relatedness between the altruist and the recipient individuals.

Relatedness is measured as a regression coefficient, so it may also assume negative or positive values as well as 0. For this to be so, it is important to understand that relatedness between two individuals depends upon the composition of the total population. When two individuals are equally related between them and with the average population, then, the relatedness of the two individuals is R = 0.

If two individuals are less related between them than they are related to the average population, R<0 (Gardner & West, 2004b). In this case, spiteful behaviour between the two individuals may be selected. That is, instead of a benefit B, the actor imposes a cost D to the recipient individual. Such behaviour is selected if R.D − C > 0.

If the cost is *negative*, that is, if the actor has a benefit in interacting with the recipient, then the two individuals are either competing (selfish behaviour, in which B<0) or there is mutual benefit (B>0).

Table.2: Social Behaviours and its Parameters				
		Effect on Recipient's Fitness		
		Positive	Negative	Parameters
Effect on Actor's Fitness	Positive	Mutual Benefit	Selfishness	C<0
	Negative	Altruism	Spite	C>0
	Parameters	B>0	B<0	

Applying the parameters to Hamilton's rule as shown in Table.2, one will find that for altruism to occur relatedness must be positive and for spite to occur relatedness must be negative.

Earlier it was questioned why would the actor perform a behaviour that has a negative effect on its own fitness? To answer this question one must have in mind the concept of relatedness. In altruism the actor behaves in a way that reduces his fitness but increases the fitness of the recipient. However for altruism to happen the recipient and the actor must be highly related. Although the actor is directly decreasing its own fitness it will increase it through an indirect way, because it is increasing the frequency of its genes in the population by improving the fitness of a highly related organism. Spite however is the dark side of altruism, by which the recipient have its fitness reduced (Gardner & West, 2004a; Gardner & West, 2004b). Acting so, the actor manages to decrease the frequency of genes of the recipient (less related organism) in the population.

At a greater extent spite can function as indirect altruism because this behaviour gives some advantage to related recipients by reducing the fitness of unrelated competing organisms (Lehmann et al, 2006). However it is important to state that harming unrelated organisms doesn't directly imply helping related ones because benefits are often spread among related and unrelated individuals (Rozsa, 2000). Examples of spite are not easy to find in nature (Hamilton, 1970; Rozsa, 2000) explained some reasons for this difficult observation. Gardner and West (2004a) interpreted those reasons and summarized two essential conditions for spite to evolve: strong competition for resources and a mechanism for kin recognition.

Bacteriocin production constitutes a good example of spite (Gardner & West, 2004a; Gardner et al, 2004; West et al, 2007). Bacteriocins are antimicrobial compounds which have activity restricted to members of the same or close species (for reviews on bacteriocins see (Baba & Schneewind, 1998) and on colicins see (Cascales et al, 2007)). These molecules play an important role in competition between conspecifics (Riley et al, 2003). Production of bacteriocins is costly to the cell that produces it. For example, in *E. coli* colicin (type of bacteriocin produced by this species) production requires cell death (Chao & Levin, 1981). Additionally, colicinogenic bacteria must carry the genes for colicin production and immunity (often in plasmids) and this carriage results in a reduced growth rate compared to the growth of sensitive bacteria. Since bacteriocins cause target cell's death it also represents a cost to the recipient. But clone mates of the producer organism are protected from its killing effect because they have the gene that provides immunity to the toxin. Therefore, bacteriocin activity is only directed to non-relatives and production of these toxins reduces the level of competition felt by related organisms in complex bacterial communities (Chao & Levin, 1981; Kerr et al, 2002; West et al, 2007).

The effect of bacteriocin activity during competition depends on the habitat. It has been shown that in unstructured habitats (where individuals affect equally the environment of every other individual), the advantage of colicinogenic over sensitive bacteria is frequency dependent. Thus, if the frequency of colicinogenic bacteria is above a certain threshold there is advantage. Otherwise bacteriocin activity is disadvantageous. However in structured habitats (where an individual have a stronger effect on neighbours) colicinogenic bacteria are in advantage even if initially rare. In structured habitats the killing of sensitive bacteria increases resources on the proximity of the colicinogenic colony due to the formation of an inhibitory zone around it. This is different to what happens in unstructured habitats, where the extra resources are randomly distributed. Therefore, while in structured habitats only colicinogenic bacteria are favoured by this behaviour, in unstructured habitats this benefit is diluted among the whole population of bacteria whether they are colicinogenic or not (Chao & Levin, 1981). Thus in structured habitat the effect of spite is more similar to indirect altruism than in unstructured (also known as mass) habitat since the greatest part of the benefits are enjoyed by related individuals instead of being equally spread between both parts.

Dionisio (2007) and Rozsa (2000) have meanwhile proposed that hosts may use parasites to decrease the fitness of susceptible competitors. This would be advantageous if spiteful individuals are able to direct harm to non-relatives rather than to related individuals (Rozsa, 2000). A specific example of a spiteful infected individual proposed by (Dionisio, 2007) is that of *E. coli* and its lambda phage.

Phage lambda is a temperate phage comprising a genome of linear double stranded DNA that infects *E. coli* (Campbell, 1996). It is one of the most studied bacteriophages. Being a temperate phage, λ enters the host and then it can follow one of two different paths: replication and lysis of the host or stable integration into the host genome (Oppenheim et al, 2005). This lysis-lysogeny decision is under influence of factors such as cell physiology and the number of infecting phages. It is known that lysogeny is favoured by high multiplicity of infection (Kourilsk.P, 1973; Kourilsky & Knapp, 1974). If the cell is growing in a rich media lysis is favoured but if it is in carbon starvation lysogeny tends to occur (Kourilsky & Knapp, 1974). There are also *E. coli* mutants where lysogeny is favoured (Belfort & Wulff, 1971; Gautsch & Wulff, 1974). Prophage induction is also under influence of cell physiology. When the cell initiates an SOS response, caused by UV radiation or other agent capable of damaging the host DNA, the phage is induced thus initiating the lytic cycle (Oppenheim et al, 2005).

Phage lambda confers immunity to superinfection. This means that when a lambda phage enters into a lysogenic cell, its capacity to initiate the lytic cycle is impaired by the resident prophage (Oppenheim et al, 2005).

The lysis-lysogeny decision and superinfection immunity are of course dependent on the regulatory network of the phage. The product of gene cII is responsible for the start of the lysis-lysogeny decision. It is responsible for the inhibition of expression of late genes encoding proteins for phage morphogenesis and host cell lysis. However, if the levels of CII are not sufficient to inhibit the late genes, the lytic cycle is followed. CII also stimulates the integration of the phage on the host genome and stimulates the expression of the regulator CI. The CII levels depend on CIII which regulates its proteolytic degradation (Oppenheim et al, 2005).

For the maintenance of the prophage state, CI is the general regulator, thus inhibiting the expression of almost all the phage genes. It also inhibits replication of the phage genome. CI also blocks expression of other related phages that entered the cell, thus conferring immunity to superinfection. However, if CI is inactived the lytic cycle is taken. This is what happens during prophage induction (Oppenheim et al, 2005).

Several mutants of lambda are known. Some are unable to follow the lysogenic cycle, among them there are mutants defective for CI, CII or the oR operator to which CI binds. However, there are also mutants that cannot follow the lytic pathway (Oppenheim et al, 2005).

In the model proposed by Dionisio (2007) phage lambda represents a weapon that lysogenic *E. coli* can use to cause harm on competing non-lysogenic conspecifics. One of the conditions referred above for spite to happen is strong competition. This condition is verified because in a mixed population (lysogens and non-lysogens) there will be competition for resources necessary for individual growth and reproduction. The other condition required is a mechanism of kin recognition. This condition is also verified since relatives (other lysogens) are immune to lambda superinfection, hence only non-relatives recipients (individuals susceptible to the phage) will experience a negative benefit.

As mentioned above, spiteful individuals incur a cost to themselves. To use the phage against competing conspecifics the actor must release the virus into the medium. This release constitutes the cost, because release requires lysis of the cell performing this behaviour. Therefore this model is consistent with the predictions of the spiteful behaviour.

The final frequencies of the competing strains seem to be only dependent on the phage virulence (here defined as probability of dying upon phage infection) and the initial strains ratio (Joo et al, 2006). The phage pathology however depends on culture conditions (Ptashne, 1992).

Bossi et al (2003) tested the prophage contribution to the dynamics of bacteria populations. Competitions took place in unstructured habitats with an initial ratio of 1:10 (lysogenic:non-lysogenic). The results showed that there were three steps during competition. First the ratio suffered no significant changes. Then the phage population increased and susceptible bacteria were reduced due to multiplication of phage inside them which lead to their lysis. Hence, the ratio lysogen:susceptible increased. At last competitive neutrality was reached because remaining susceptible individuals became lysogenic.

Brown et al (2006) shown that whether lysogenic strains were invading or invaded by the susceptible strains, the former always displayed advantage. In the end, the susceptible strain always became lysogenic. However, the final outcome was dependent upon the strains initial frequency. This is explained by the amplification effect. Each infected susceptible not only dies as it is also responsible for the multiplication of the virus, hence the amplification effect. It is now clear that, when invading a susceptible population, the amplification effect will be strong, leading to a fast decrease of the susceptible frequency. On the other hand, if the susceptible strain is present at a low frequency, phage amplification will be weak. As a consequence the phage can only act by direct killing of the infected susceptible individual.

This key aspect of amplification is suspect of being a factor that allows lysogenic strains to invade, even when rare, a population dominated by susceptible strains in unstructured habitats (Brown et al, 2006). Therefore leading to a different outcome to what has been seen when similar conditions are present during colicinogenic strains invasion.

Lysogenic individuals can harbour more than one phage type. Then susceptible individuals will require separate phage integration events to become immune. When susceptible harbour all the different prophages, lysogens won't retain their advantage. Multiple integrations are time consuming and extra time allows lysogens to harm more susceptibles. Then, strains releasing more than one phage type have extra advantage. (Figueroa-Bossi et al, 2003).

As it was stated before there are two essential conditions for spite to evolve: kin recognition and strong competition for resources. The first is an innate character of the phage which prevents further infections by similar phages. Thus, the regular competition for resources would lead to selection of spite. Given these conditions, one could expect that coevolution of bacteria and temperate phages would improve spiteful behaviour. As it was referred earlier, some mutants of phage lambda were already described in nature. λCII^- is one of them. This mutant is unable to integrate in a new host, so the logic fate of the bacterial host is death with production of phage progeny. Moreover this mutant is unable to superinfect lysogenic strains. Mutant like λCII^- would therefore improve spiteful behaviour (Dionisio, 2007).

The first objective of this work is to study the capacity of lysogens to harm non-lysogenic bacteria in both unstructured and structured habitats. Later, the outcomes of these competitions will be compared to those obtained by Chao and Levin (1981) that competed colicinogenic with non-colicinogenic bacteria. However, in their experiment, the production and immunity for the colicin were encoded in a non-conjugative plasmid. Therefore there is no conversion of susceptible individuals into colicinogenic ones. Thus, the ratio colicinogenic/non-colicinogenic could only increase by decreasing the frequency of non-colicinogenic bacteria through colicin-mediated killing. On the other hand, if competition occurs between lysogenic and non-lysogenic bacteria the ratio lysogenic/non-lysogenic can increase in two ways: (i) non-lysogenic phage-mediated killing, as in the case of competition involving colicins; (ii) conversion due to prophage integration in non-lysogenic.

Because lysogens force susceptible individuals to harbour the prophage in order to become immune, this effect can be considered as enforcement behaviour. Enforcement will increase the level of relatedness between both strains. Consequently enforcement represses competition, suppressing spiteful behaviour. To prove that both spite and

enforcement take place during these competitions the sub-population of previously susceptible bacteria will be screened for lysogeny and resistance to the phage. This is the second objective of the work.

The selective pressure of competition will be tested, as a third objective, by evolving the lysogenic strain for several generations. At last, spiteful behaviour and conversion will be tested on evolved strains.

MATERIAL AND METHODS

Bacteriophages and Bacterial Strains

Two phages were used. The wild-type λ phage and a virulent mutant, λ_{vir}, capable of infecting susceptible and lysogenic strains (thus able to produce super-infection) but not resistant strains.

Three strains derived from the same ancestral strain were used in this work. Mutants of *E. coli* K12 MG1655 resistant to streptomycin and rifampicin were obtained by spontaneous mutation. A streptomycin resistant (str^R) mutant was infected with phage λ, thus creating a lysogenic strain (later confirmed not to be a spontaneous resistant mutant). This lysogenic str^R strain will be henceforth denoted Lys. Rifampicin resistant (rif^R) mutants susceptible and resistant to λ were selected from exposure to λ and λ_{vir} Sus. The Susceptible rif^R strain will be denoted Sus while the resistant rif^R strain will be denoted Res.

Growth rates determination

Since the strains are no longer isogenic, their growth rates were measured by the optical density method. 10 μl taken from a pre-culture grown overnight at 37ºC with agitation (170 rpm), were added to 10 ml of Luria Broth (LB) medium and incubated at 37ºC with agitation (170 rpm). 3 replicates of each strain were used. Every 30 minutes, a sample of 500 μl was taken to measure the optical density at 670 nm.

Bacterial Competitions

Competitions between lysogenic and non-lysogenic strains were performed. Four different initial ratios Lys:C (C stands for Competitor, either Sus or Res) were used: 10^4:1, 1:1, 1:10^2, and 1:10^4. Competitions for each initial ratio were made in triplicate.

For each competition in structured habitats 5 μl of the desired dilution of each strain were added to 3 ml of Luria Broth top agar ($_{top}$LA) (the final concentration of agar was 0,375% m/v). After agitation, the mixture was poured on a Petri dish containing an agar (1,5% m/v) basal layer. Followed incubation during 24 hours at 37ºC, the upper layer was scraped into 12 ml of MgSO4 (10^{-2} M) and this mixture was agitated on vortex for 3 minutes. A decimal

dilution of the mixture was then made in MgSO4 (10^{-2} M). 200 µl of the dilution were added to 3 ml of $_{top}$LA. The methods described in this paragraph were repeated six times.

For each competition in unstructured habitats 5 µl of the desired dilution of each strain were added to 10 ml of LB. Followed incubation during 24 hours at 37ºC with agitation (170 rpm), 5 µl were taken from the tube containing the competing bacteria. These 5 µl were added to another tube containing 10 ml of LB. Incubation followed and the procedure was repeated for the next competition cycles.

The next two methods took place every two days. The procedures are similar to competitions in both habitats. For determination on day zero, the dilutions were made from the pre-culture tubes.

Colony forming units (cfu) determination

The desired dilutions (containing 10^2-10^3 cfu/ml) were made from the tube containing the competing bacteria after incubation. 100 µl of these dilutions were then plated in Luria Broth with agar (LA) (1,5% m/v) with Streptomycin (100 µg/ml) and LA with Rifampicin (100 µg/ml). Then the plates were incubated at 37ºC overnight.

Determination of the fate of the initial susceptible subpopulation:

A continuous line of phage λ was inoculated on LA and a line of phage $λ_{vir}$ onto another Petri dish. Then each colony to be screened was cross-streaked across the lines of each phage. The plates were incubated at 37ºC for 24 h. A total of 50 colonies were screened for each replicate. The colonies were classified as lysogenic if they showed continuous growth when crossing the line of λ but not $λ_{vir}$, resistant if they showed continuous growth when crossing both lines and susceptible if they did not show continuous growth when crossing both lines.

Phage-Bacteria Co-evolution

Evolution procedures were performed under four conditions: in structured habitats with and without competition and in unstructured habitats with and without competition. Ten replicates were done under each condition. When in competition, 5 µl of susceptible and

lysogenic strains (from pre-cultures) were used, however in the other case, without competition, only 5 µl of the lysogenic strain were used.

Evolution in structured and unstructured habitats followed the methods described for competitions, with some exceptions. The incubation period was of 12 h instead of 24 h. After incubation, an additional step for selection of lysogenic bacteria was added. This step consisted in adding 200 µl of the culture to 10 ml of LB with streptomycin (100 µg/ml). After incubation for 10 h at 37ºC with agitation (180 rpm), 1ml was centrifuged (5400 rpm during 10min) and the pellet resuspended in 1 ml of MgSO4 (10^{-2} M). Then a new cycle of evolution was initiated, however the Lys strain used came from the resuspended mixture instead of a pre-culture, while the Sus strain (when necessary) came from a new pre-culture.

The evolution cycles were performed until the lysogenic strain reached 200 generations. After isolation of the Lys strain, 200 µl of the culture were added to 10 ml of LB and grown for 24 h at 37ºC with agitation (170 rpm). Then 1 ml of each culture was filtered (0.22 µm) and diluted in MgSO4 (10^{-2} M). 10 µl of each supernatant dilution (10^{0}-10^{-6}) were applied to plates consisting on a basal layer of agar and $_{top}$LA layer inoculated with the Sus strain. The same was done in plates inoculated separately with ancestral Lys strain and Res strain. Then the plates were incubated at 37ºC overnight. To confirm that the ancestral strain did not produce evolved phages, the supernatant was also analysed in 20 replicates.

Each population where phage mutants emerged was also screened for resistance. 100 µl of the population pre-culture was inoculated in 3 ml $_{top}$LA and then plated in Petri dishes with a basal layer of agar. After the $_{top}$LA solidified, a drop of $λ_{vir}$ was placed on the centre of the plate. Overnight incubation followed at 37ºC.

Bacterial Competitions with Evolved Strains

The methods are identical to those described before in the Bacterial Competitions section. However the only initial ratio Lys:C performed was 10^{2}:1. Additionally, the phage titter of pre-cultures was measured by the same method described for evolved cultures. The plates used for this quantification were either inoculated with Sus or Lys strains, never Res strain.

Statistical Parameters and Statistical Analysis

For almost competitions, twice the standard error was used to express value dispersion from the mean. However, in some cases, results were adjusted through use of functions. In these cases, instead twice the standard error, the method of error propagation was performed, according to the formula:

$$\varepsilon_{f(x,y)} = \left| \frac{\partial f}{\partial x} \right| \cdot \varepsilon_x + \left| \frac{\partial f}{\partial y} \right| \cdot \varepsilon_y$$

For comparisons between the growth rates of the different strains an One-Way ANOVA analysis was performed. To analyse the results taken from the evolution experiments Qui-square test was performed to compare the effect between strains evolved under each condition with the ancestral strain and between the effects of each condition.

Statistical analyses performed using Microsoft Excel 2010.

RESULTS

Growth rates

Our main objective is to access if lysogenic strains produce phages as a weapon to displace susceptible conspecific cells (but not the resistant cells) when both occur in the same habitat. Before testing this hypothesis it is necessary to ensure that a strain do not have, *a priori*, a fitness advantage over the other, since they are not isogenic. To do so, we measured the growth rates of the Sus, Lys and Res strains grown separately (Fig.1), each with three replicates. Performing a One-Way ANOVA analysis, we confirmed that the growth rate of the three strains were not significantly different (d.g. =8; F=1,46; p=0,30).
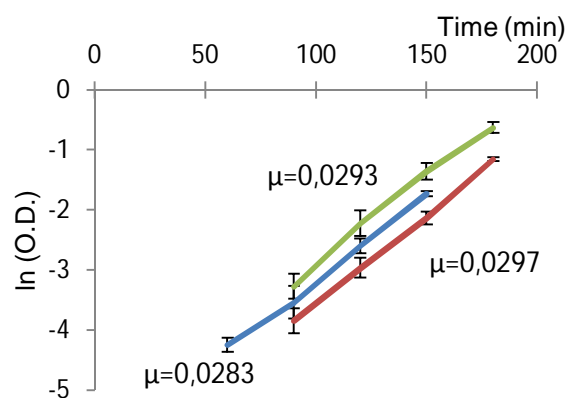
Fig.1. Growth rates (μ) of the three strains during exponential phase. Lines represent the average value of three replicates of the Lys (red), Res (green) and Sus strains (blue). Error bars denote twice the standard error.

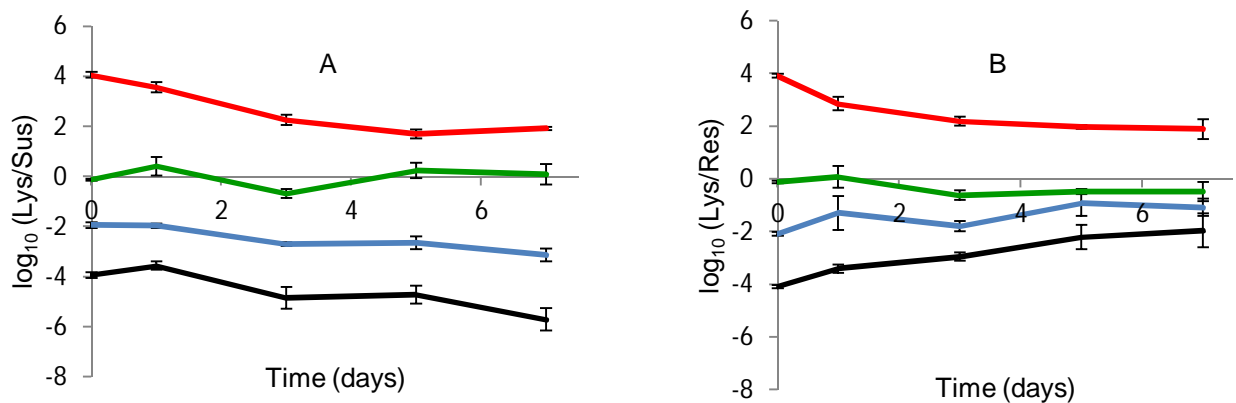## Competitions in unstructured habitats

In Fig.2, we present the results of the competition in unstructured (liquid) habitat. When competing with susceptible conspecifics (Fig.2A), if the lysogens were initially rare, their frequency decreased over the time. When lysogens were initially common, their frequency decreased but, near the end, it seems to fix in the population in a frequency of approximately 100 lysogen per susceptible. When lysogens and susceptibles started at identical frequencies, the proportion was maintained throughout time, despite some fluctuations. If lysogens were competing with resistants (Fig.2B), their frequency increased when they were rare. When lysogens initially outnumbered the resistant conspecifics, they behaved in a similar fashion as they did against susceptibles. However, if both strains started in the same proportion, the frequency of lysogens initially decreased but was later

maintained (near the initial proportion). Since we confirmed, in Fig.1, that no strains have a higher growth rate than the others, the results in Fig.2B seem to indicate intrinsic (not phage mediated) frequency-dependent fitness.

To reveal the actual advantage or disadvantage of the lysogens over the susceptibles, the intrinsic frequency-depedent fitness must be eliminated. The data was transformed as follows:

$$W_{Lys/Sus} = log_{10}\left[\frac{Lys}{Sus}\right] - log_{10}\left[\frac{Lys}{Res}\right]$$

where $W_{Lys/Sus}$ is the relative fitness of Lys when competing with Sus. If $W_{Lys/Sus} > 0$ there is advantage.This is shown in Fig.3. When they started at low frequencies, the lysogens presented themselves less fit to compete with susceptibles than with resistants. The oposite was seen, when the lysogens and the competitors started at equal frequencies. However, if lysogens were initially the majority, they behaved in the same way either competing against susceptibles or resistants.


Fig.2. Temporal dynamics of lysogens to competitors ratio in unstructured habitat.
(A) Lysogens to susceptibles ratio. (B) Lysogens to resistants ratio. Lines represent the initial ratios: lysogens/competitors, 10^4:1(red), 1:1 (green), 10^{-2}:1 (blue) and 10^{-4}:1 (black). Each line is the average value of three replicates. Error bars denote twice the standard error.

We then screened the susceptible subpopulation for lysogens and resistant individuals. Independently of the initial ratio, the susceptible subpopulation remained susceptible throughout the time.
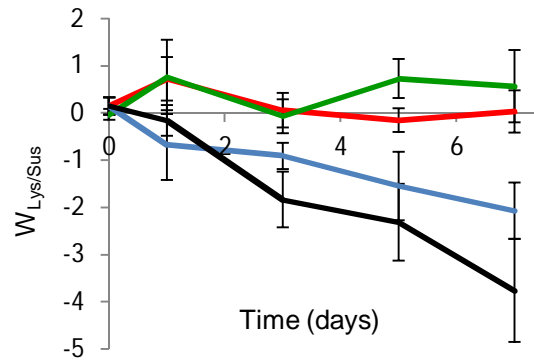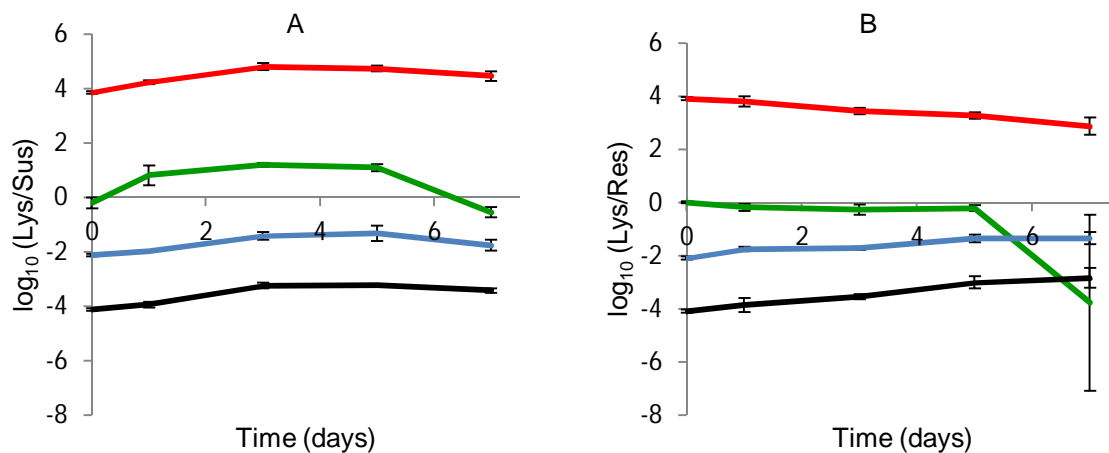
Fig.3. Fitness of lysogens in unstructured habitat. Lines represent the initial ratios: lysogenic/competitor, 10^4:1(red), 1:1 (green), 10^{-2}:1 (blue) and 10^{-4}:1 (black). Each line is the average value of three replicates. Error bars denote twice the standard error.

Competitions in structured habitats

When competing in structured (solid) habitat against susceptibles (Fig.4A), lysogens showed always an early advantage independently of the initial ratio. However, that advantage was lost at later times, when their frequency stabilized or decreased in the population. The frequency of lysogens always increased if they started competing with resistants at lower frequencies, but decreased along the time if they started at higher frequencies (Fig.4B). Until the fifth day, the frequency of the lysogens was maintained when the resistants started at equal frequencies. This is similar to what happened in unstructured habitats when lysogens competed with resistants, suggesting frequency-depend fitness.


Fig.4. Temporal dynamics of lysogens to competitors ratio in structured habitat. (A) Lysogens to susceptibles ratio. (B) Lysogens to resistants ratio. Lines represent the initial ratios: lysogens/competitors, 10^4:1(red), 1:1 (green), 10^{-2}:1 (blue) and 10^{-4}:1 (black). Each line is the average value of three replicates. Error bars denote twice the standard error.

Competitions starting at 1:1 revealed a decrease in the seventh day (both when lysogens compete with susceptibles or with resistants). Thus, the pattern established almost from the beginning was interrupted.

As we will show later, the cause of this decline in the frequencies of the lysogens is the emergence of phage mutants capable of infecting both susceptibles and lysogens but not resistants. This also accounts for the unusual size of the error bars at day seven: in the competition between lysogens and resistants, mutants appear in two of the three replicates, while mutants appeared in all three replicates when lysogens competed with susceptibles.

Similarly to Fig.3, Fig.5 shows the actual advantage/disadvantage of the lysogenic over the susceptible strain. When lysogens were initially equal or superior in frequency to their competitors, they had advantage during the first three days. From this day, their frequency tended to stabilize (except in the case discussed before where phage mutants arose). However, when lysogens were initially rare, instead of stabilizing their frequency on the third day, they experienced disadvantage. (To make sure, that lysogens when rare had advantage, we performed a t-Student test. Black (p=0,03) blue (p=0.001)) points at day three are significantly different than 0.)
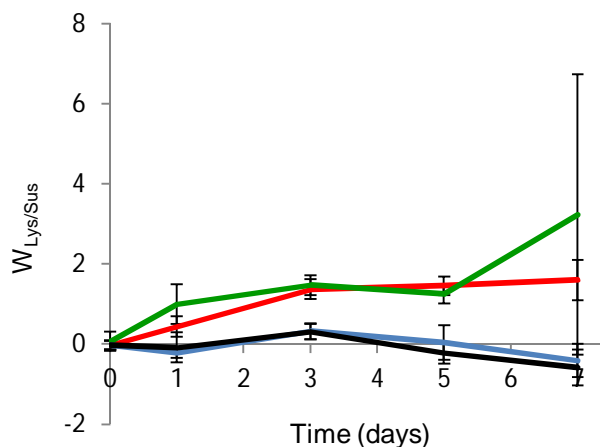
Fig.5. Fitness of lysogens in structured habitat. Lines represent the initial ratios: lysogenic/competitor, 10^4:1(red), 1:1 (green), 10^{-2}:1 (blue) and 10^{-4}:1 (black). Each line is the average value of three replicates. Error bars denote twice the standard error.

To understand this difference between the various competitions we screened the susceptible subpopulation for lysogenic and resistant individuals (Fig.6). From day 0 to day

3 the susceptible subpopulation is 100% constituted by susceptible individuals, independently of the initial frequencies of the strains (Fig.6A). From the third day susceptibles started to be converted into lysogens, so that the initially susceptible population became constituted by susceptible and lysogenic individuals (Fig. 6A and B). The decrease of the susceptible individuals (and the increase of lysogenics) in the subpopulation is greater if the competitor lysogenic strain started at lower frequencies in the global population. However, for all competitions, at day seven there are very few or no susceptible individuals in the subpopulation (Fig.6A). In the end the subpopulation is mainly constituted by lysogenic individuals for all initial ratios, except when both strains started at equal frequencies (Fig.6B). This exception is due to the emergence of phage mutants that could infect both susceptible and lysogenic individuals. Because of this, there was selective pressure for resistant individuals to arise. In fact, Fig.6C shows that at the seventh day, the subpopulation is mainly constituted by resistant individuals. Note that at the same time, the subpopulations of susceptible and lysogenic individuals were reduced to few or no organisms.
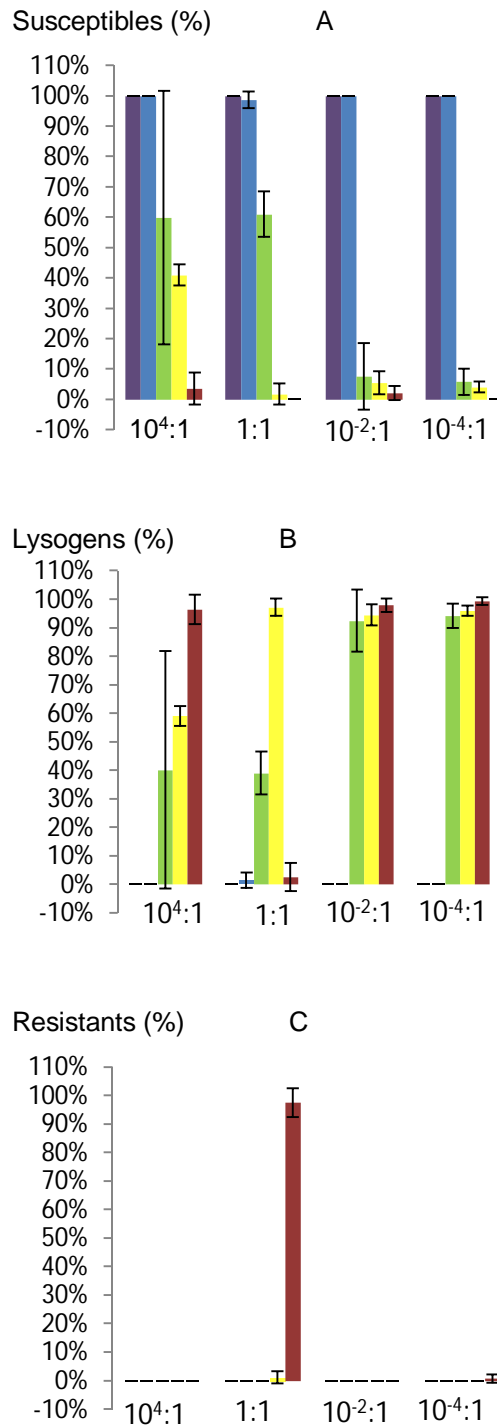
Fig.6. Percentage of lysogenic and resistant individuals in the susceptible subpopulation during competition against lysogens in structured habitat. (A) Percentage of susceptible individuals. (B) Percentage of lysogenic individuals. (C) Percentage of resistant individuals. Bars represent the percentage for each day: 0 (purple), 1 (blue), 3 (green), 5 (yellow) and 7 (red). Each bar is the average value of three replicates. The initial ratios are shown below each set of bars. Error bars denote twice the standard error.

Phage-Bacteria coevolution

Since mutant phages occur easily on these populations, we have decided to test what would happen during evolution for longer periods of time. The lysogenic strain was evolved for 200 generations under four different conditions regarding the habitat and the effect of competition. We confirmed that the ancestral strain did not produce phage mutants. We also tested the host range of the mutants. Mutants described here are capable to superinfect lysogenic strains, yet are unable to infect resistant strains.

We performed Chi squared (χ^2) tests to compare each evolutionary condition with the ancestral, in terms of phage mutants productions. Each condition is significantly different from the ancestral. We also tested the effects of the habitat and competition. The habitat did not significantly influence the course of evolution. However the effect of competition is only significant on unstructured habitats. Results of the statistical testes are shown in Table.3.

| Table.3. Analysis of evolutionary conditions	
Ancestral vs. Evolution in unstructured habitat without competition	χ^2; d.f.=1; p=5,32x10^{-4}
Ancestral vs. Evolution in structured habitat without competition	χ^2; d.f.=1; p=1,93x10^{-5}
Ancestral vs. Evolution in unstructured habitat with competition	χ^2; d.f.=1; p=4,32x10^{-8}
Ancestral vs. Evolution in structured habitat with competition	χ^2; d.f.=1; p=4,32x10^{-8}
Structured habitat vs. Unstructured habitat	χ^2; d.f.=1; p=4,29x10^{-1}
Competition vs. No competition	χ^2; d.f.=1; p=1,57x10^{-3}
Structured habitat with competition vs. Structured habitat without competition	χ^2; d.f.=1; p=6,03x10^{-2}
Unstructured habitat with competition vs. Unstructured habitat without competition	χ^2; d.f.=1; p=9,82x10^{-3}

All bacterial populations where phage mutants emerged were tested for resistance. All, except two, showed resistance. However, although the majority of the population was resistant some phage plaques were detected, suggesting that not all cells of the population are resistant.

After evolution, we selected populations to compete with the ancestral susceptible and resistant clones in the same conditions that produced Figs.5-8. We chose two populations: one evolved in structured habitat with competition and the other evolved in the same habitat without competition. The chosen population in each case was the one that most closely resembled the average of the ten in terms of total phage, total mutant and fraction of mutants produced.

Note that we did not isolate a mutant from evolved populations. In the competitions that follow, the lysogenic population is, in fact, a composite of ancestral and evolved individuals. Since, we don't know if the evolved individuals harbour mutant prophages or are just lytically infected with the mutant (due to phage epidemics), henceforth they are termed carriers (Car) instead of lysogens. The population evolved with competition will be termed A1 and the one evolved without competition will be termed B2.

Competitions in unstructured habitats after evolution

Evolved strains have higher fitness than the ancestral when competing in unstructured habitats against the resistant strain (Fig.7A). However, this advantage is suspected to be just innate frequency-dependent fitness (not phage mediated) as the advantage stops when both strains are at the same frequency in the population. Unlike the ancestral, while competing against the susceptible strain, the evolved strains had advantage instead of disadvantage (Fig.7B).
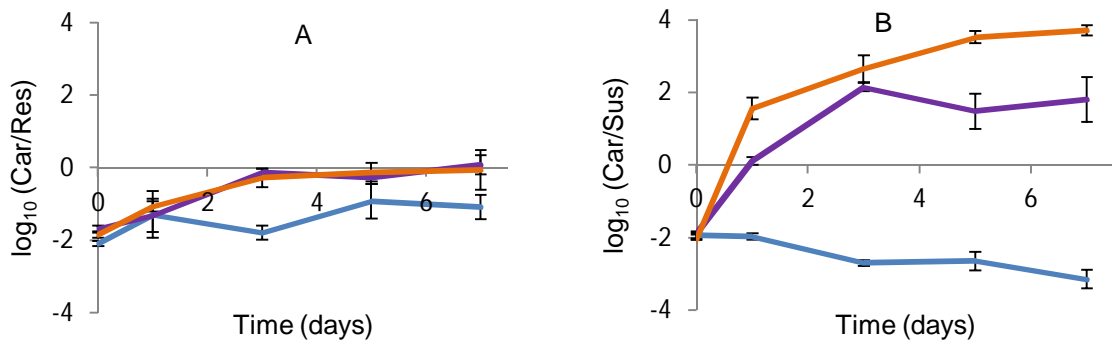

Fig.7. Temporal dynamics of evolved carriers to competitors ratio in unstructured habitat.
(A) Carriers to resistants ratio. (B) Carriers to susceptibles ratio. Each competition started with an initial ratio carrier/competitor of $10^{-2}:1$. Lines represent the carrier strains, ancestral (blue), A1 (purple) and B2 (orange). Each line is the average value of three replicates. Error bars denote twice the standard error.

Indeed, the frequency of this strain seemed to stabilize since the fifth day. Strain A1 had continuous advantage until the third day. From this day, the frequency of the strain seemed to decrease and later stabilize. Strain B2 showed greater advantage than strain A1 in the first 24 hours. The frequency of strain B2 stabilized during the next days. Strain B2 never had disadvantage. Similar conclusions are taken when the carrier population

fitness relative to the resistant strain is subtracted to the relative fitness to the susceptible (Fig.8).

The difference between the evolved strains can be due to production of different levels of phage mutants. We determined the titter of the wild type and mutant phage present in the supernatant of the pre-cultures of strains A1 and B2. While A1 produced 9,28% of mutants, B2 produced 25,68%. This seems to be consistent with the previous results. Higher levels of mutants confer more advantage to carriers.
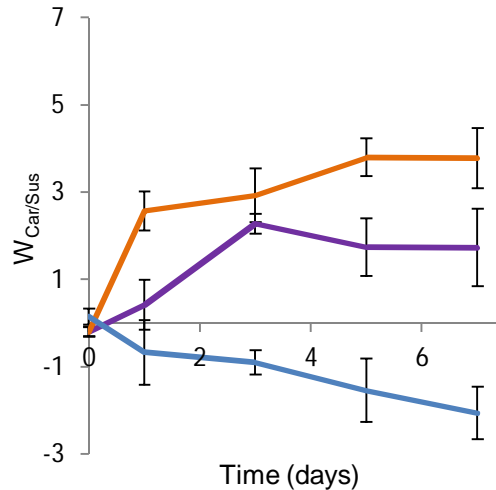
Fig.8. Fitness of evolved carriers in unstructured habitat. Each competition started with an initial ratio carrier/competitor of 10^{-2}:1. Lines represent the carrier strains, ancestral (blue), A1 (purple) and B2 (orange). Each line is the average value of three replicates. Error bars denote twice the standard error.

Next, were screened the initially susceptible population for lysogenic and resistant individuals (Fig.9). No conversion into lysogens occurred. Selection for resistance is dependent on the carrier strain. Competition against strain B2 led to emergence of resistants since the first day. However, after competition with strain A1, resistants start to emerge on day seven. These results are consistent with the previous (Fig.7 and 8), since a higher initial dose of phage mutants exert a stronger selective pressure for resistant individuals to emerge.
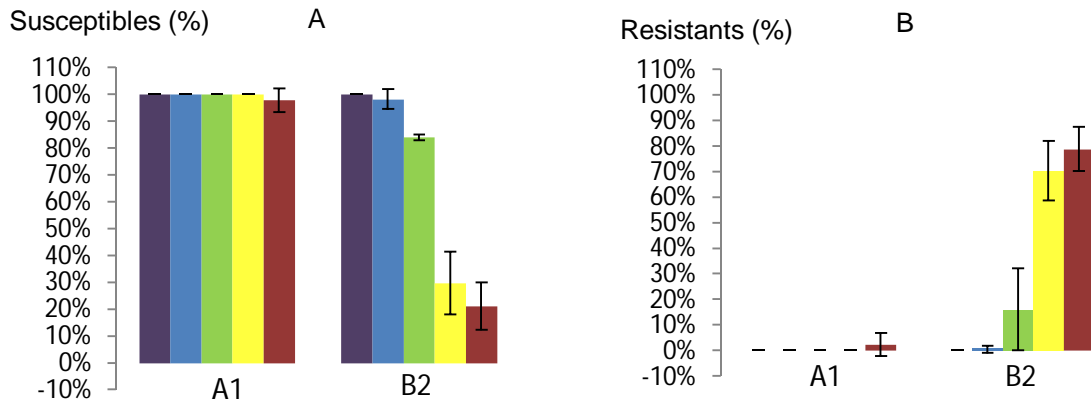
Fig.9. Percentage of lysogenic and resistant individuals in the susceptible subpopulation during competition against evolved carriers in unstructured habitat. (A) Percentage of susceptible individuals. (B) Percentage of resistant individuals. Bars represent the percentage for each day: 0 (purple), 1 (blue), 3 (green), 5 (yellow) and 7 (red). Each bar is the average value of three replicates. The competitor carrier strains are shown below each set of bars. Error bars denote twice the standard error.

Competitions in structured habitats after evolution

While competing in structured habitats against the resistant strain, again the fitness of the evolved strains was higher than the ancestral (Fig.10A). As it happened previously, this advantage seems to be frequency-dependent fitness (not phage mediated). While competing against the susceptible strain, the evolved strains showed higher advantage when compared to the ancestral (Fig.10B). When the intrinsic frequency-depedent fitness is eliminated we can confirm that evolved populations have identical advantages (Fig.11).

If strains have similar advantages, then in this habitat the difference between the proportion of mutant phages produced is not as determinant as in unstructured habitats. In structured habitat the neighbourhood (as an inhibition zone) affected is similar, the killing effect should not be directly proportional to the dose of pathogens. In this case, it only matters if the dose is sufficient (then higher than a fixed threshold) or not to displace susceptible neighbours. Whereas in unstructured habitats there is a direct proportionality, due to interactions between all individuals, the higher the dose of pathogens the higher the number of susceptible neighbours that die.
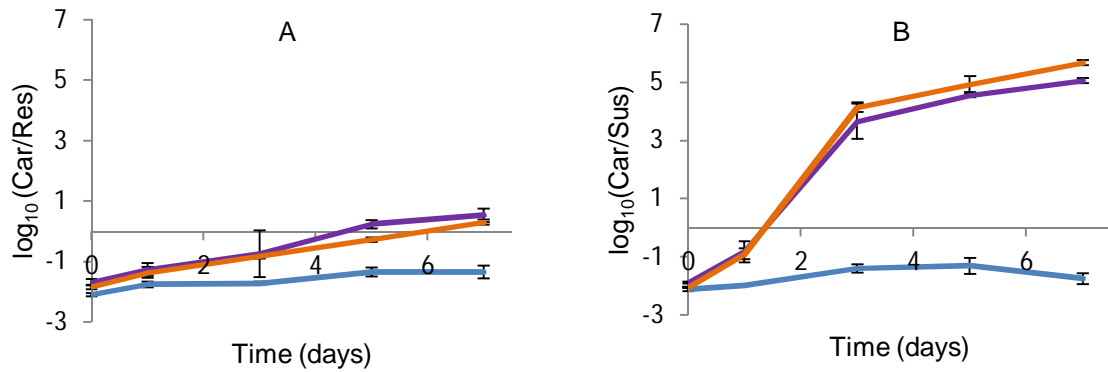
Fig.10. Temporal dynamics of evolved carriers to competitors ratio in unstructured habitat.
(A) Carriers to resistants ratio. (B) Carriers to susceptibles ratio. Each competition started with an initial ratio carrier/competitor of 10^{-2}:1. Lines represent the carrier strains, ancestral (blue), A1 (purple) and B2 (orange). Each line is the average value of three replicates. Error bars denote twice the standard error.
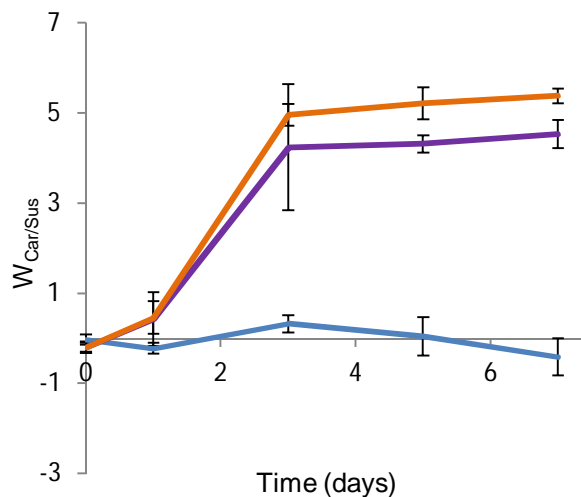
Fig.11. Fitness of evolved carriers in structured habitat. Each competition started with an initial ratio carrier/competitor of 10^{-2}:1. Lines represent the carrier strains, ancestral (blue), A1 (purple) and B2 (orange). Each line is the average value of three replicates. The error bars denote twice the standard error.

At last, in Fig.12 we show that the behaviour of susceptibles is identical when competing with strains A1 or B2. Emergence of resistance starts at day three in both cases.
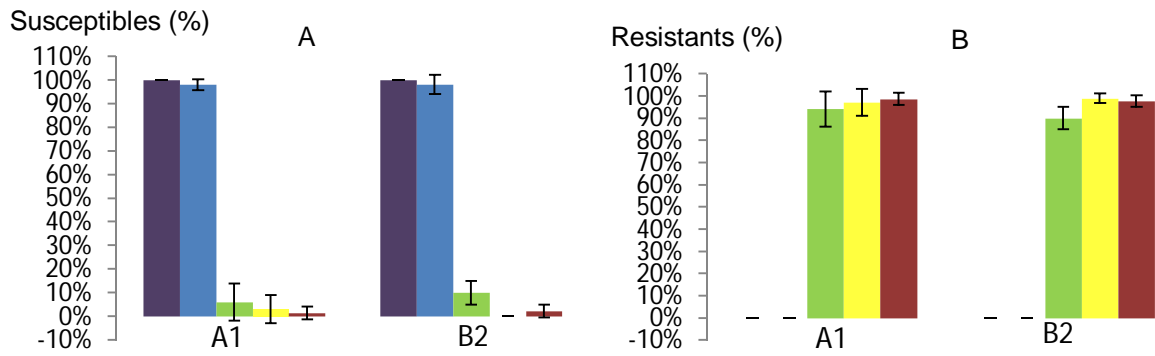
Fig.16. Percentage of lysogenic and resistant individuals in the susceptible subpopulation during competition against evolved carriers in structured habitat. (A) Percentage of susceptible individuals. (B) Percentage of resistant individuals. Bars represent the percentage for each day: 0 (purple), 1 (blue), 3 (green), 5 (yellow) and 7 (red). Each bar is the average value of three replicates. The competitor carrier strains are shown below each set of bars. Error bars denote twice the standard error.

DISCUSSION

The aim of this thesis was to analyze the selective conditions for lysogenic bacterial cells to use temperate bacteriophages as biological weapons to compete with other bacterial strains. Previous authors studied those conditions using mathematical models based on population genetics (Dionisio, 2007; Rozsa, 1999) and found that such conditions are easily met (Dionisio, 2007). Given that phage-producing bacterial cells have a cost (they are killed) when they kill their victims, such interactions between lysogens and bacteriophage-free cells constitute a spiteful behavior. This is in line to what has been shown experimentally for colicins (Chao & Levin, 1981). In fact, previous authors have shown that production of colicins to displace susceptible conspecifics constitutes a spiteful behaviour. That is, production of colicins is a behavior that harms both actor and receptor (Hamilton, 1970). The outcome of this behavior depends on the environment where colicinogenic compete with non-colicinogenic bacterial cells. In structured habitats, colicinogenic bacteria efficiently displace their competitors, even when they are initially present in low frequencies. There is advantage to producers of colicins because the displacement of competitors freed resources that allowed further growth of the colicinogenic individuals. However, in unstructured habitats, toxin producers only gain advantage when they are initially common. This happened because the extra resources were divided by colicinogenic and competitor strains.

It was suggested that lysogenic bacteria could act spitefully as well, not only because phages produced by a few cells could harm susceptible conspecifics, but also because phages can replicate themselves inside victim cells, thus amplifying their destructive action. That is, the spiteful strength of bacteriophages would be much higher than that of colicins because, contrary to colicins, phages can amplify inside victims (Brown et al, 2006). Therefore, in unstructured habitats, lysogenic strains would be advantageous even when initially rare. However, phages also have the capacity to convert susceptible individuals into producers through lysogenization, a phenomenon that was not considered in the mathematical models (Dionisio, 2007) and that could mask the power of temperate phages as biological weapons.

Our results show that in unstructured habitats, the advantage of lysogenic individuals depends on its initial frequency in the bacterial population. When initially rare, lysogens were displaced by susceptibles (Fig. 3). However, when initially common, lysogens also decreased in proportion (Fig 3). Thus, lysogens experienced benefits only when both strains started at identical frequencies: in this case, although lysogen frequency started to

increase slightly, it was only for a short time because soon the frequencies of both strains stabilized. This suggests that there is a maximum and a minimum threshold where advantage is conferred. Under that threshold, the amount of phage produced is not enough to cause sufficient lethal effect on susceptible individuals: few competitors die and the extra resources are divided by the whole population, not conferring advantage to lysogens. On the other hand, when the frequency of lysogens is above the maximum threshold, the interaction between phage and susceptible cells is modest, most probably because susceptible individuals are very rare to be found by phages. Consequently, they are not displaced. No conversion was shown in any case.

Our results seem to be consistent to the ones obtained by Chao and Levin (1981) with the colicin model: the advantage of producers depends on its frequency when competing in a mass habitat. In another study where lysogens competed with non-lysogens in mass habitats, it was shown that, when there is phage amplification inside victim cells advantage does not depend on the frequency of lysogens (Brown et al, 2006) . Therefore, our results are not consistent with this study by Brown et al (2006). Such difference between our results and those of Brown et al (2006) can be explained by higher virulence of the phage used in their works. In the near future, we should study this difference.

In structured habitat lysogens initially have an advantage (Fig. 5). If lysogens were initially common (frequencies 1:1 and 10^4:1), their frequency stabilized after the initial advantage. However, when they were initially rare (frequencies 1:10^2 and 1:10^4), there was a slight increase in frequency, followed by a decrease (Fig. 5). This decrease in lysogen frequency is simultaneous to the conversion of susceptible individuals into lysogenic ones (Fig. 6). Thus, in structured habitats, when lysogenic individuals display a superior fitness similarly to what happens with colicinogenic strains, the advantage is for a short time due to the conversion of susceptible cells into lysogens. Here, there is also a threshold. If the frequency of lysogens is inferior to the frequency of susceptibles when conversion starts, lysogens display disadvantage. On the contrary, lysogens have an advantage if the frequency of lysogens is superior to that of susceptibles when conversion started. The effect of conversion depends on this threshold, because higher conversion rate occurs when lysogens are rare. Competitions in structured habitats are thus constituted by two distinct phases: spite (early) and conversion (late).

The evolved lysogenic populations produce phage mutants. These mutant phages are able to infect lysogenic strains, thus capable of overcoming the immunity conferred by the resident prophage. This indicates that these mutant phages are insensitive to the action of the repressor protein CI coded by the prophage, hence suggesting mutations on the oR

operator . A consequence of such mutations is the incapability of the phage to lysogenize (Oppenheim et al, 2005). Then, these mutants behave as virulent phages, that is, they are no longer temperate phages. Since immunity to superinfection is no longer effective, kin discrimination during competition is not determinant. Then, when such mutants emerge, the overall population is susceptible to infection, which leads to epidemic viral proliferation. Then, phage amplification by the whole population allows persistence of mutants independently of the habitat.

We did not determine if mutants can be kept in the prophage state in the original host before induction. Perhaps lysogens maintain mutant phages in the prophage state (for example, through some compensatory chromosomal mutation). In this case, lysogens would be capable of producing them upon induction. However, we cannot exclude the hypothesis that, during lysogen selection through evolution cycles, virulent phages were not taken with the bacteria from cycle to cycle. This could occur if some phages were present in the pellet after centrifugation (experimental error) or if infected bacteria transmitted to the next cycle contained replicating intracellular phages that would be released during the next cycle, in this sense propagating the epidemics. Considering that no errors occurred, the two most likely situations are: (i) a certain proportion of cells carry the mutant phage as a prophage (and some of these cells enter induce the replication of the phage) or (ii) the mutant phage is unable to integrate in the chromosome, and so a certain proportion of cells carry phages already engaged in lytic phase. In both alternative situations, a certain portion of bacterial cells are engaged in phage production and release them into the bacterial community. This phage production is costly to producers (they die), but, on the other hand, they can be useful to the population as a whole when competing with other strains (Figs. 8 and 11). This "farming" behavior of phages by a small portion of bacterial cells remind a recent work on "primitive farming" by the social amoeba *Dictyostelium discoideum* (Brock et al, 2011).

D. discoideum is a social slime mould that grazes on bacteria. Upon starvation, *D. discoideum* cells aggregate and form a motile multicellular slug that subsequently produces a fruiting body containing spores. A recent work has shown that, during slug formation, *D. discoideum* cells save some bacteria for transmission in their spores (Brock et al, 2011). That is, instead of eating these bacteria, *D. discoideum* cells carry them, despite being in starvation. Carriage of bacteria allows inoculation of new habitats with bacteria on which the population will feed. Bacteria carriage is only performed by a fraction on individuals of the total population. The population is thus constituted by farmer and non-farmer individuals. Farmers pay a fitness cost when compared with non-farmers (one of the factors on which cost depends is the lower capacity to travel).

If we compare our model to the *D. discoideum model*, we find some similarities. There are two types of individuals: carriers (infected) and non-carriers (resistant). As observed with *D. discoideum* carrying bacterial spores, carriage (in our case, carriers of phages) is costly: it leads to death. Carriage of phages is advantageous when bacterial cells migrate to new habitats, another similarity with *D. discoideumi*. Then, one intriguing hypothesis is that the populations could carry their pathogens with them, to displace susceptible conspecifics and consequently colonize new niches.

Despite the mechanism of virulent phage origin and maintenance on these populations, the fact is that ecologically, an adequate bacterial response would be selected. Since epidemics lead to susceptible individual's death, resistant individuals would arise due to this selective pressure. Resistant cells were, indeed, detected in almost all the evolved populations, thus corroborating our expectations. We speculate that in cases where resistants were not detected, the emergence of mutants occurred recently, and then, we did not give enough time for the rising of resistance. Despite the overall resistance, there are individuals in the population that are not resistant: these are the carriers.

Our results showed that after 200 generations, phage mutants able to superinfect lysogens emerged on both structured and unstructured habitats, and that these mutants show up irrespectively of whether there was competition with a naïve (susceptible) strain or not. Consider the random emergence of a mutant that can freely replicate in whatever individual it interacts with. This would lead to its maintenance in that environment until a counter defense was settled. This initial amplification is then enough for their maintenance in short term and independent of competition because both lysogens and susceptibles are permissive to the mutant phages that appeared in our experiments. Thus any random interaction with a bacterium would lead to more amplification. However at long term, competition becomes a decisive factor to phage maintenance.

In unstructured habitats, all individuals have the same probability of interacting with the phage, thus the same probability of dying and the same selective pressure leading to resistance. At some point, resistant bacteria are selected and phage amplification is reduced. This is consistent with the prediction that notwithstanding the majority of the population becoming resistant, some subpopulations are still susceptible allowing phage persistence amongst the bacterial population (Levin et al, 1977). However, if a new susceptible strain is constantly added, phage amplification can continue indefinitely. On the other hand, in structured habitats, individuals near the infection focus are more affected than distant ones. Thus the probability of death and selective pressure for resistance depend on the distance between individuals and the infection focus. Then,

sanctuaries, i.e. zones where the phages cannot reach the bacteria due to a distant location from the infection focus, select individuals unaffected by the epidemics thus preserving susceptibility among the population.

When lysogens evolve in structured habitats without competition, bacterial cells susceptible to mutant phages are protected in sanctuaries, whereas selective pressure near the focus of infection leads to the emergence of resistant individuals. However, when a new cycle of evolution starts, every individual's position is random. Then individuals once located distantly to the infection focus can be repositioned near the focus and consequently protection is no longer conferred. Resistant individuals are also repositioned. If resistant individuals are placed further away from the focus of infection, selective pressure for resistance is attenuated. When evolving in structured habitats with competition, the same idea can be applied, however the frequency of susceptibles is higher than when there is no competitor strain, thus enforcing the maintenance of the epidemics. Moreover, the external addition of susceptible individuals decreases the selective pressure for resistance imposed on the lysogenic susceptibles to the mutants. By contrast, when no susceptible individuals are added, all selective pressure is directed upon lysogenic susceptibles.

These aspects can explain the fact that the appearance of mutant phages occurred in all four conditions. They may also explain why there are no differences of the number of cases in terms of presence of the mutants between structured and unstructured habitats but there is difference in unstructured habitats between the situations of presence and absence of competition.

After evolution, the competitiveness of the population is higher than that of the ancestral strain (Figs 8 and 11). In mass habitats, advantage was observed in early times, but later the frequencies of the carrier strains seemed to stabilize. Now that phage virulence is superior with evolved strains than it was with the ancestral strain, the results are more closely related to those regarding phage amplification. The strain B2, which produces more mutant phages than strain A1, has a higher fitness than the A1 strain (Fig.8). Then the advantage of each strain depends on the number of mutant phages produced. The production of mutant phages (in this thesis referred as virulence) also influences the response of the susceptible cells. If virulence is higher, then the probability that susceptibles are converted into resistants is expected to be high (Fig.9). When the evolved strains competed in structured habitats, we have seen an advantage in the first days. The frequency of carriers rapidly increased in the population, but at later times it seemed to stabilize (Fig.11). The efficiency of susceptible displacement is superior for the evolved

strains than for the ancestral, since phage virulence increased. Moreover, the susceptible strain becomes resistant instead of lysogenic (Fig.16), as it happened with the ancestral (Fig.6), presumably because evolved phages cannot establish lysogeny. In structured habitats, the two evolved strains have similar behaviours suggesting that the difference in phage virulence between populations A1 and B2 do not affect the outcome of the competitions. Consider a dose X of pathogens that kills all susceptible individuals in a certain limited area. A dose superior to X is limited to the same area, then all the susceptibles in the area dies. Therefore, higher doses of phages around the area of the phage producer do not influence the outcome of the competition.

Spiteful behavior, as described in the last two paragraphs, is advantageous, after co-evolution of the dyad phage-bacterium. The evolved phage can infect lysogens, then, if lysogens are no longer immune, there is no kin discrimination and spiteful behavior would fail. The solution for spite to me maintained relies on the fact that the majority of the evolved lysogens became phage resistance. This resistance emerged during co-evolution to counter the lethal effects of the phage mutants. Therefore, resistant individuals are not infected, implying that kin discrimination is preserved. The fraction of the evolved population (carriers) that remains non-resistant includes the cost of phage production (with consequent death). Despite their cost, carriers are responsible for the advantage conferred to the population. Because carriers keep phages in the population, phages can be used to harm other strains.

Evolution of lysogens leads to a higher phage virulence. Phage mutants are unable to follow the lysogenic cycle: when infecting susceptibles, no lysogens are produced. The probability of survival of susceptible individuals is then decreased, because they cannot become lysogens, as they could with the wild type phage. Even if susceptibles could become lysogens, they would die upon infection because the prophage cannot confer immunity against the evolved virus.

Evolution of lysogens led to a higher phage pathology. The phage mutants are unable to follow the lysogenic cycle, the when infecting susceptibles, no lysogens are produced. The probability of survival of susceptible individuals is then decreased, inversely, the killing rate increase, conferring advantage to individuals of the carrier population. However, when the killing effect reaches its maximum, the frequency of carriers stabilizes. This maximum is defined by the habitat. In structured habitats it is limited by the phage dispersion, since different doses (above the sufficient dose to sweep that area) of viruses in the same area cannot exceed the number of susceptible individuals to be infected. In unstructured habitat

the killing effect is dependent on the probability of interaction between host and pathogen. Thus if hosts are rare, pathogens barely have access to them.

CONCLUSIONS AND FUTURE PERSPECTIVES

We studied the parameters affecting the ability of lysogenic bacteria to produce phages to outcompete susceptible conspecifics. We conclude that this ability depends on the habitat and on strain frequency. At short term, lysogens are favoured in structured habitats independently of their initial frequency. In unstructured habitats, lysogens increase in frequency only if both lysogens and susceptibles start at identical frequencies. Phage activity in this work resembles colicin activity (Chao & Levin, 1981), not being consistent with the work of (Brown et al, 2006). Differences in phage virulence could account for the inconsistency between both works.

Furthermore, lysogenization of susceptibles, as a mechanism to disrupt competition, plays a significant effect only in structured habitats. Indeed, lysogenization of susceptibles has a stronger effect than spiteful behaviour alone. However, when evolved strains compete with susceptibles, the lysogeny mechanism is no longer significant. During the evolution of lysogenic strains, phage virulence increases, thus leading to a more efficient spiteful behaviour.

Phage-bacteria coevolution increases phage virulence and bacterial resistance. This is the last conclusion of the present work. Further studies on this respect are required. As a future work, we consider the possibility of analysing the mutations driving coevolution.

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