Evolutionary ecology of social bacterial populations under antibiotic and bacteriophage pressure

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Evolutionary ecology of social bacterial populations under antibiotic and bacteriophage pressure

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A Simon
A mes parents et Camille


Calvin and Hobbes
by Watterson
WOW, IT REALLY SNOWED LAST NIGHT! ISN'T IT WONDERFUL?
EVERYTHING FAMILIAR HAS DISAPPEARED! THE WORLD LOOKS BRAND-NEW!
A NEW YEAR... A FRESH, CLEAN START!
IT'S LIKE HAVING A BIG WHITE SHEET OF PAPER TO DRAW ON!
A DAY FULL OF POSSIBILITIES!
IT'S A MAGICAL WORLD, HOBBES, OL' BUDDY...
-LET'S GO EXPLORING!

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Abstract

Evolutionary ecology of social bacterial populations under antibiotic and bacteriophage pressure

Bacteria are the basis of virtually all ecosystems and examining their dynamics in the face of biotic and abiotic perturbations is essential to understanding their persistence, evolution and diversification. This thesis is directed towards a better understanding of the impact of phage and antibiotic pressure on the evolutionary ecology of bacterial populations and, in particular, on the evolution of bacterial social behaviours. First, using a combination of mathematical modelling and experimental evolution, we studied how antagonisms in the form of antibiotics (Chapters 1 and 2) and phages (Chapter 3) affect the dynamics of public goods production and strategies, and the evolution of resistance in populations of the bacterium *Pseudomonas aeruginosa*. We found that both phages and antibiotics favour cheats over cooperators in well-mixed environments. While the advantage to cheats led to population growth and even increased resistance frequency in the short-term (Chapter 1), the cheat-dominated populations eventually declined in the presence of phage predators, arguably due to the combination of antagonist pressure and cheating load (Chapter 3). Second, based on the evolutionary prediction that multiple control agents will be more efficient at controlling bacterial populations and reducing the evolution of resistance, we investigated *in vitro* the complex interactions between phages and antibiotics in the context of combined therapies. We showed that the combination of phages and antibiotics decreased population survival and resistance evolution significantly more than either alone. While this main result may be mitigated by several factors such as antibiotic dose (Chapters 4 and 5), the timing of inoculation (Chapter 4), and antibiotic mode of action (Chapter 5), it is also obtained in longer-term assays (Chapter 5). Our results highlight the complexity of the interplay between the negative effects exerted by antibiotics and phages and the evolutionary ecology of bacterial populations, and bring new insights both to the understanding of social evolution and to the potential therapeutic use of phages and antibiotics.

Keywords: social evolution, *Pseudomonas aeruginosa*, experimental evolution, antibiotics, bacteriophages, resistance

Résumé

Ecologie évolutive des populations bactériennes sociales sous la pression de bactériophages et d'antibiotiques

Les bactéries constituent le socle de presque tous les écosystèmes et l'étude de leurs dynamiques face aux perturbations biotiques et abiotiques est essentielle à la compréhension de leur maintien, de leur évolution et de leur diversification. Cette thèse vise à une meilleure appréhension de l'impact des bactériophages et des antibiotiques sur l'écologie évolutive des populations bactériennes et, plus particulièrement, sur l'évolution de leurs comportements sociaux. Dans une première partie, nous avons étudié comment les antibiotiques (Chapitres 1 et 2) et les phages (Chapitre 3) affectent les interactions fondées sur la production de biens publics ainsi que l'évolution de la résistance dans les populations de *Pseudomonas aeruginosa*, en combinant modélisation mathématique et évolution expérimentale. Nous avons montré que les phages et les antibiotiques favorisent les tricheurs face aux coopérateurs dans les environnements homogènes. Alors que l'avantage des tricheurs permet la croissance de la population et augmente la fréquence de résistance à court terme (Chapitre 1), les populations dominées par les tricheurs finissent par décliner en présence de phages, vraisemblablement suite aux pressions combinées des phages et des tricheurs (Chapitre 3). Dans une seconde partie, nous avons exploré *in vitro* les interactions complexes entre les phages et les antibiotiques dans le contexte des thérapies combinées. Conformément à la prédiction de la théorie de l'évolution selon laquelle plusieurs moyens de contrôle combinés sont plus efficaces que chacun séparément, nous avons montré que l'usage simultané de phages et d'antibiotiques réduit davantage la survie et la résistance des populations. Si ce résultat principal peut être modulé par différents facteurs tels que la dose d'antibiotiques (Chapitres 4 et 5), le moment d'inoculation (Chapitre 4), et le mode d'action des antibiotiques (Chapitre 5), il persiste sur le long terme (Chapitre 5). Nos résultats soulignent la complexité des interactions entre les effets négatifs des phages et des antibiotiques et l'écologie évolutive des populations bactériennes et apportent de nouveaux éléments à la fois à la compréhension de l'évolution de la socialité et à l'usage thérapeutique potentiel des phages et des antibiotiques.

Mots clés : évolution sociale, *Pseudomonas aeruginosa*, évolution expérimentale, antibiotiques, bactériophages, résistance

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Glossary

Actor: individual performing a focal behaviour.

Altruism: behaviour that involves a fitness cost for the actor and a fitness benefit to the recipient (Table 1).

Antagonism: external constraint that reduces the fitness of individuals by decreasing their survival and/or reproduction.

Cheat: individual whose fitness increases by directly or indirectly exploiting the costly behaviours of others, but participates less in those behaviours at a reduced cost.

Collective behaviour: group-level emergence of aligned behaviours resulting from local interactions between individuals.

Cooperation: direct or indirect behaviour performed by an actor (a 'cooperator') that provides a fitness benefit to a recipient and which is selected for, at least partially, because of its beneficial effect on the fitness of the recipient. It can be beneficial (mutual benefit) or costly (e.g., altruism) to the actor (Table 1).

Direct fitness: component of fitness gained through personal survival and reproduction.

Gene: basic physical unit of heredity, coding for a variation in a focal trait or behaviour.

Inclusive fitness: sum of direct and indirect fitness effects of a focal trait.

Indirect fitness: component of fitness gained from helping the survival and/or reproduction of individuals sharing traits under selection.

Kin selection: selective process by which an allele is favoured because each copy of the allele increases not only the fitness of its bearer, but also the fitness of other individuals carrying the same allele and associated phenotypic expression. These individuals may be genetically related to the bearer only for the focal locus.

Mutual benefit: behaviour that involves a fitness benefit to both the actor and the recipient (Table 1).

Natural selection: process by which relative fitness is increased through differential reproduction between genotypes.

Pathogenicity: capacity of an organism to harm its host.

Public good: good (e.g., resource) that is costly to produce and provides a potential benefit to individuals in the local group or population.

Relatedness: statistical measure of genetic similarity between two individuals relative to the population average, at a gene influencing one or more (social) traits.

Selfishness: behaviour that involves a fitness benefit for the actor and a fitness cost to the recipient (Table 1).

Stressor: factor that induces a challenge to an organism's internal state (the 'stress') and may trigger a cascade of physiological, behavioural and/or phenotypic changes (the 'stress response').

Virulence: degree of pathogenicity, measured as case fatality rate and/or the degree of disease severity.

General introduction

Bacteria are among the oldest forms of life on Earth, form amazingly diverse and complex phyla, and live in virtually all environments. They are not only the functional backbone of many ecosystems, playing important roles in several processes including the decomposition of organic matter, nitrogen fixation, and possibly even the hydrological cycle and climate (DeLeon-Rodriguez *et al.* 2013), but are also involved in a multitude of interactions with other organisms, ranging from mutualistic symbiosis, to commensalism and parasitism. In humans, as well as other mammals, bacteria live, for example, on skin, in nasal passages and lungs, and in the digestive tract. Despite their success in diverse ecosystems, bacteria are confronted with a diverse array of ecological antagonisms. Antagonisms potentially reduce bacterial fitness through sublethal or lethal effects. Historically, humans have taken advantage of some of these antagonisms with the aim of controlling bacterial pathogens and pests. While single cell responses have been extensively studied, the ecological and evolutionary consequences of antagonisms at the population level remain largely unexplored. Yet, recent studies report that bacterial populations can respond to environmental challenges through collective behaviours (Lee *et al.* 2010, Meredith *et al.* 2015). Collective behaviours are widespread in bacteria and may play a fundamental role in responses to and interactions with antagonisms. Investigating the ecological and evolutionary consequences of antagonisms will likely bring key insights into the stability, evolution and diversification of bacterial populations. This will increase our fundamental understanding of social evolution in harsh environments, and present the opportunity to apply these insights to the control of bacterial pathogens and pests.

Living in harsh environments: phages and antibiotics

There are several non-mutually exclusive ways to define and classify ecological antagonisms: they can be of biotic or abiotic origin; physical, chemical or biological; and they may be natural environmental factors or result from human activities (Bijlsma & Loeschcke 2005, Schimel *et al.* 2007, Steinberg 2012, Poole 2012). At the individual organism level, antagonisms may be lethal in the very short-term or induce a series of behavioural, physiological and phenotypic changes in the recipient (Bijlsma & Loeschcke 2005). Beyond the initial effects of harsh environments, a short time scale stress may result in mortality in the longer term if the individual fails to respond to (or tolerate) the stressor (Dwyer *et al.* 2014). The impacts of ecological antagonisms (harshness) are thus defined along a continuum of short and long-term responses. Examples of potentially lethal antagonisms include predation, toxins, and abiotic environmental extremes (e.g., temperature). Ecological antagonisms require a relative definition, as the impact of an environmental component may be specific to a particular organism in a given context (Bijlsma & Loeschcke 2005). For example, a temperature of 37 $^{\circ}$C is stressful for *Pseudomonas fluorescens* which usually grows at lower temperatures, whereas is it the optimal growth temperature for *Pseudomonas aeruginosa* (Farhangi & Safari Sinegani 2014). In addition to ecological antagonisms, stress may arise from internal factors including the accumulation of metabolic by-products (Lasko *et al.* 1997) and reactive oxygen species (Cabiscol *et al.* 2010).

In this thesis, I take a population-level approach and focus on the consequences of antagonisms on the ecology and evolution of bacterial populations. I use an ecological definition of antagonisms as external constraints that decrease the fitness of individuals by decreasing their survival and/or reproduction, and challenge the maintenance of a population or a community in its environment (Koehn & Bayne 1989). I focus on two broad types of antagonism: bacteriophages (or phages) as reproducing, dynamic, and potentially evolving bacterial predators, and antibiotics as stressors acting through time, possibly resulting in cell death.

Phages are the most abundant organisms on Earth and display an extraordinary diversity (Rohwer 2003, Labrie *et al.* 2010, Grose & Casjens 2014). They occur in virtually all environments where their bacterial hosts can survive, and constitute a major ecological and selective force on bacterial populations (Vos *et al.* 2009, Gómez & Buckling 2011, Weitz *et al.* 2013, Haerter *et al.* 2014). Phages may show rapid

coevolution with bacteria, selecting for increased ('arms race') or fluctuating (e.g., 'fluctuating selection dynamics') resistance to phage (e.g., Buckling & Rainey 2002, Hall *et al.* 2011a, Buckling & Brockhurst 2012, Brockhurst & Koskella 2013, Betts *et al.* 2014). Predation by phages can modify the interactions in multispecies communities and result in either increased diversity or in environmentally-contingent extinction for some species (Harcombe & Bull 2005, Rodriguez-Valera *et al.* 2009). In addition to lytic phages that kill their host through membrane lysis following adsorption and replication, temperate phages use either lytic or lysogenic cycles to replicate (Echols 1972, Fig. 1). During the lysogenic cycle, the phage's genetic material integrates into the host genome as prophage. It is transmitted to daughter cells with little or no harm to the host bacterium until the initiation of the lytic cycle in response to environmental cues (Ghosh *et al.* 2009). Prophages may affect their bacterial host's biology in several ways and lead to the expression of phage-derived traits, the best documented being the induction of toxin production that may result in pathogenesis (Hargreaves *et al.* 2014b, Bobay *et al.* 2014). For instance, the secretion of endotoxins responsible for the virulence of *Vibrio cholerae* is encoded by the prophage CTX_{φ} (Plunkett *et al.* 1999).

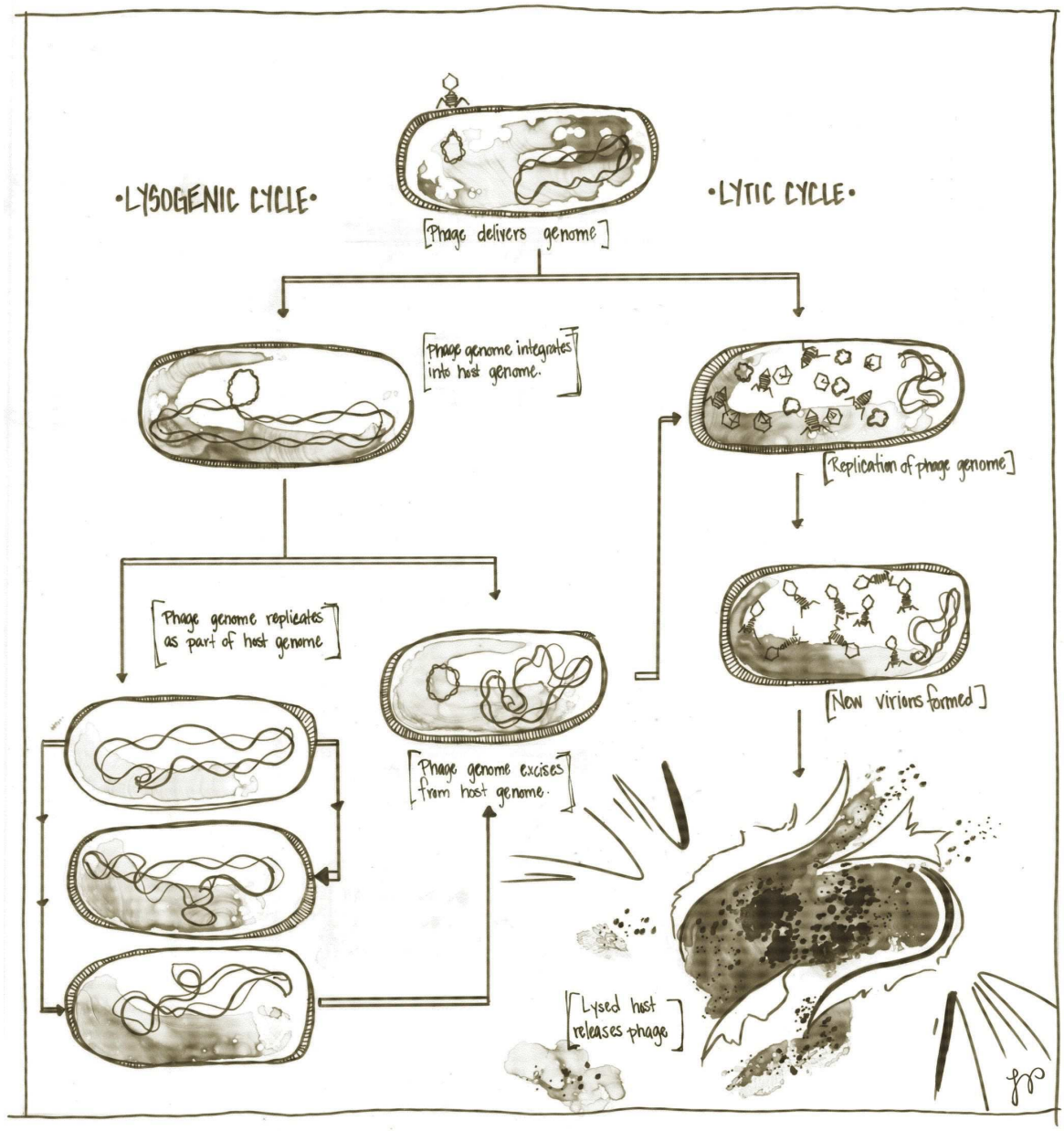


Figure 1: Illustration of phage lytic and lysogenic cycles (Rohwer *et al.* 2014).

In natural environments, antimicrobial compounds are secreted within or between microbial species and may mediate interactions such as competition and spite (Gardner *et al.* 2004, Tyc *et al.* 2014). In addition to sometimes being ecological weapons, antibiotic molecules can play a role in intra- and interspecific signalling and result in the induction of quorum sensing (QS) or biofilm formation, for instance, (Kaufmann *et al.* 2005, Nalca *et al.* 2006, Ghosh *et al.* 2009, Andersson & Hughes 2014). In contrast to the often localised effects in natural situations, the anthropogenic use of antibiotics for human health management, aquaculture and farming has led to the pervasive spread

of resistance and to the massive release of antibiotics into the environment with consequences on genetic variability and bacterial behaviours (Andersson & Hughes 2014, Meek *et al.* 2015). The ecological and evolutionary implications of such large-scale antibiotic exposure on bacterial dynamics and population biology remain largely unknown (Andersson & Hughes 2014, Berendonk *et al.* 2015).

As alluded to above, phages and antibiotics may induce bacterial responses and/or cause mortality. Sufficiently high antibiotic concentrations or quantities of phage able to complete their lytic cycle should cause bacterial death. In contrast, sublethal antibiotic or phage doses or phages unable to complete their lytic cycle may constitute stressors and trigger bacterial responses such as avoidance and phenotypic tolerance (e.g., Poisot *et al.* 2012, Bernier & Surette 2013, Andersson & Hughes 2014). Beyond individual-level stress responses, bacterial populations exhibit a wide range of collective survival mechanisms, including the production of shared antibiotic-degrading enzymes, altruistic cell death, swarming motility and biofilm formation (Meredith *et al.* 2015). Such group- or population-level phenomena may reduce the impact of stressors on bacterial populations and result in higher tolerance and/or avoidance, but also in increased bacterial virulence. For instance, biofilms may be induced under phage or antibiotic pressure (phages: Hosseinidoust *et al.* 2013a, Tan *et al.* 2015; antibiotics: Kaplan 2011, Bleich *et al.* 2015) and beyond increased resistance, may be associated with increased bacterial pathogenicity (e.g., Boyle *et al.* 2013, Marguerettaz *et al.* 2014).

Living in groups: social behaviours in bacteria

A striking characteristic of bacterial populations is the variety and complexity of their life-styles. Bacteria may be planktonic or live clustered in mucoid microcolonies, biofilms or fruiting bodies (Ma *et al.* 2012, Swan *et al.* 2013, Claessen *et al.* 2014). These group-living life-styles may result from local reproduction and non-dispersal or colonisation of suitable habitats, but also from the pressure of antagonisms (Hosseinidoust *et al.* 2013a, Claessen *et al.* 2014, Aka & Haji 2015). Living in groups not only leads to competitive interactions, but also often involves social behaviours, mediated by cell-cell contact, communication or changes in microenvironments (e.g., Crespi 2001, West *et al.* 2007a). Social interactions potentially affect a population's dynamics and evolution. Population growth may rely, for instance, on collective foraging or on the sharing of secreted compounds to scavenge essential metals (e.g., Neilands 1995).

Swarming behaviours enable 'long-range' dispersal and colonisation of novel environments (Velicer & Yu 2003). Furthermore, intraspecific sociality can influence interactions with other strains as exemplified by the spiteful release of targeted antimicrobials (Gardner & West 2006, West & Gardner 2010, Hawlena *et al.* 2010). Social traits in bacterial populations may be environmentally dependent, as in experimental adaptive divergence in *Pseudomonas fluorescens* (e.g., Bantinaki *et al.* 2007). In well-mixed environments, cells largely adopt a planktonic life-style, whereas static environments lead to rapid diversification and ecological stratification. The bottom of the microcosms is occupied by large colonies called 'fuzzy spreaders' due to their aspect on agar plates, 'smooth genotypes' dominate the liquid broth, and 'wrinkly spreaders' colonise the air-liquid interface (Rainey & Travisano 1998). This interface colonisation relies on the collective secretion of an extracellular matrix that 'sticks' the cells together and forms a biofilm. Such biofilms can enable component cells to gain better access to oxygen, but they have also been shown in other systems to enhance niche exploitation, and may constitute protective habitats to evade predation or harsh environmental conditions (Velicer 2003).

Interactions between ecological antagonisms and bacterial evolutionary ecology: unresolved issues

This thesis is composed of two main parts, each addressing a scientific problem related to interactions between harsh environments and the evolutionary ecology of bacterial populations.

Problem 1. Given the ubiquity of social behaviours in bacterial populations and their importance for population dynamics, it is very likely they are strongly impacted by ecological antagonisms such as dynamic predators (phages) and compounds of microbial or human origin (antibiotics). Understanding the consequences of this interplay on population biology requires addressing both the impact of antagonisms on cooperative behaviours within populations, and the consequences of these social interactions on the population response to antagonisms.

Problem 2. Bacterial responses to antagonisms are also a central issue for human health. The evolution of antibiotic resistance has progressively become a serious con-

cern and studying the consequences of alternative therapies is critical for treatment success. This requires identifying the conditions under which a single or combined therapeutic measure reduces bacterial fitness, population size, and evolutionary potential.

The following sections give a brief overview of our current knowledge and specific questions related to the main themes of this thesis. The first part presents my main research on the interplay between the selective pressure exerted by antibiotics or phages and social behaviours in populations of the bacterium *Pseudomonas aeruginosa*. The second part is collaborative work, directed towards a better understanding of the effects of antibiotics and phages on populations of the opportunistic pathogen *P. aeruginosa* in the context of single vs combined therapies.

Interplay between harsh environments and public goods cooperation

Social evolution theory in bacteria

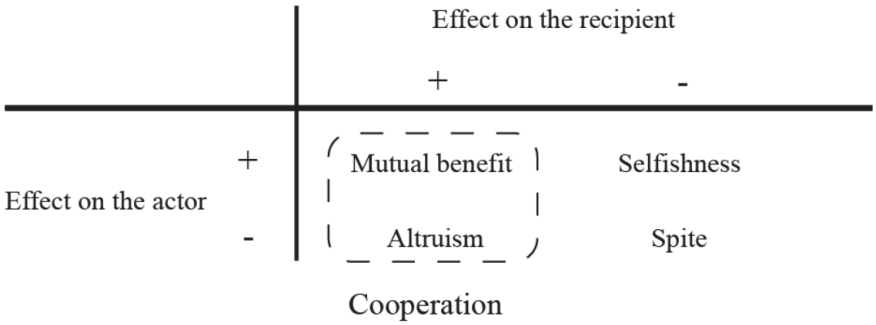
Social traits in bacteria: the importance of public goods

An individual's behaviour is social if it affects its fitness and the fitness of one or more other individuals in a population (Hamilton 1964a;b, West *et al.* 2007c, Table 1). Cooperative behaviours are beneficial to recipients, and can be anywhere from very costly (altruism) to highly beneficial (mutualism) for the actor (West *et al.* 2007c). Honeybees provide a classic example of altruism, where *Apis mellifera* workers forgo reproduction and are dedicated to the welfare of the queen and colony (e.g., Ratnieks & Wenseleers 2008, Ratnieks & Helanterä 2009). Examples of cooperative mutualism include ant-plant interactions, where the plant provides shelter and food resources to the ants and receives protection against herbivores and pathogens or food in return (e.g., Heil & McKey 2003, Bonhomme *et al.* 2011, Mayer *et al.* 2014), or nitrogen-fixation mutualisms between leguminous plants and rhizobial bacteria (e.g., Kiers *et al.* 2003, Denison & Kiers 2004). Ecological conditions, however, may modify the costs and benefits of mutualistic interactions thus leading to conditional outcomes (reviewed by Bronstein 1994).

While insects, mammals and birds have historically received considerable attention in the study of social evolution, recent research has explored the ubiquity and great variety of social behaviours in microbes, challenging the classic view of bacteria as non-interacting cells (Crespi 2001, Parsek & Greenberg 2005, West *et al.* 2007a, Xavier *et al.* 2011). Microbes may interact in diverse ways, including collective protection, dispersal, reproduction, foraging, and competition with other species through chemical warfare (Crespi 2001). Many of these behaviours rely on the release of extracellular compounds that are potentially available to surrounding cells. Under certain environmental conditions, the benefits of these compounds extend beyond the producing individuals, functioning as public goods (West *et al.* 2007a).

Public good molecules may modify the environment in several ways (reviewed by Crespi 2001, Velicer 2003, Keller & Surette 2006, Kolter & Greenberg 2006, West *et al.*

Table 1: Classification of social behaviours. Based on Hamilton (1964a;b) and West *et al.* (2007c).



2006, Brown & Buckling 2008, West *et al.* 2007a, Xavier *et al.* 2011, Celiker & Gore 2013) including: scavenging food (siderophores: West & Buckling 2003, invertase: Travisano & Velicer 2004, lytic secondary metabolites: Gerth *et al.* 2003), surviving antibiotic pressure (Ciofu *et al.* 2000) or predation (Cosson *et al.* 2002), exploiting hosts (Brown 1999, O'Loughlin & Robins-Browne 2001), or dispersing (biosurfactants: Daniels *et al.* 2004, Velicer & Yu 2003) and colonising new habitats (adhesive polymers: Rainey & Rainey 2003). Moreover, public goods behaviours play an important role in bacterial population dynamics, shaping intrapopulation interactions and diversification, but also the structure and stability of the entire community (Kerényi *et al.* 2013, McClean *et al.* 2015). A recent study demonstrated that variation in a social trait (biofilm formation) drove changes in interspecies interactions, resulting in a profound modification of community structure. Suppressing biofilm formation in one of the species in the community generated drastic changes in community composition and the relative abundances of all species (McClean *et al.* 2015).

Exploitation by non-cooperative individuals

The 'public' nature of these cooperative interactions makes them vulnerable to cheating, and considerable variability in individual investment has been reported in several natural and experimental systems (Strassmann *et al.* 2000, Crespi 2001, Fiegna & Velicer 2003, Velicer & Yu 2003, Fiegna & Velicer 2005, Diggle *et al.* 2007b, Kearns 2010, Cordero *et al.* 2012, Jiricny *et al.* 2014, Andersen *et al.* 2015). Cheating occurs

when individuals increase their own fitness by exploiting the cooperative behaviours of others (Ghoul *et al.* 2014a), while participating less or not at all in goods production. Cheating may occur by (i) using available public goods or (ii) manipulating cooperators to produce more and/or use less public goods (Ghoul *et al.* 2014a). In a well-mixed environment, or when the public good is highly diffusible and durable (Kümmerli & Brown 2010), cheats are selected for and may outcompete cooperators. In more elaborate cooperative systems where the good is directed towards an 'intended recipient' based, for example, on inclusive fitness, cheating may emerge to disrupt this link and invade the system (e.g., Driscoll & Pepper 2010, Ghoul *et al.* 2014a).

Cheats can be classified in two broad categories (Travisano & Velicer 2004, Santorelli *et al.* 2008): obligate cheats that do not modulate their behaviour and facultative cheats in which behaviour is conditioned by social and environmental factors (Santorelli *et al.* 2008). In both cases, whether or not a trait or behaviour is 'cheating' will be environment-dependent. Determining this requires accounting for the costs and benefits of social interactions in a given environment, rather than the absolute quantitative production of a public good (Ghoul *et al.* 2014a;b). For instance, lower production of a public good in an environment where it is not needed is not cheating in a functional sense (Ghoul *et al.* 2014a, Kümmerli & Ross-Gillespie 2014).[1]

Cheating is often presented as the major hurdle to the evolution and maintenance of cooperation (Sachs *et al.* 2004, West *et al.* 2007b, Bourke 2011). Nonetheless, cooperation is found across all levels of biological organisation (Bourke 2011, Levin 2014). This raises the central question of how cooperation evolves and is maintained in the face of cheating.

Explanations for the evolution and maintenance of cooperation in bacteria

A basic prediction of evolutionary game theory is that costly cooperative behaviours are subject to the invasion of less costly cheating strategies and, depending on conditions, cooperators and cheats will either coexist, or the cooperators will go extinct (e.g., Doebeli & Hauert 2005, West *et al.* 2007b, Allen *et al.* 2013a). Several mechanisms may explain the persistence of cooperation. Below, I first describe cases where

[1]In addition to cooperators and cheats, loners represent a third strategy that consists of not partaking in social exchanges (e.g., Szabó & Hauert 2002a). Loners can modify their behaviour, however, and defect or contribute to cooperation through volunteering (Doebeli & Hauert 2005, Szabó & Hauert 2002b).

a cheating strategy fails to establish. Then, I explain the main framework for understanding the evolution of cooperation in general and more specifically in bacteria, and the underlying mechanisms. Finally, I examine the impact of environmental factors on the maintenance and evolution of cooperative behaviours. Fig. 2 provides a summary of these different mechanisms in the particular case of public goods cooperation.

Cheats arise in a population by migration or by mutation on a gene involved in the cooperative behaviour. When the mutation results in decreased or no investment in a cooperative behaviour, the mutant is a potential cheat. However, this mutant may fail to establish in the population if the mutation brings no net benefit (Fig. 2). For example, when siderophore-producing populations attain high densities, iron-scavenging molecules are not limiting and production ceases. As a consequence, cooperators do not pay the cost of actual production and (in the absence of a baseline cost of being a cooperator) potential cheats have no fitness advantage (Ghoul 2014). This process also applies to plastic changes in gene expression of facultative cheats. Moreover, the mutation may have negative indirect effects on other traits via antagonistic pleiotropy or epistasis (Foster *et al.* 2004, Friman *et al.* 2013; Fig. 2). For instance, in light-producing colonies of *Vibrio fischeri* symbiotically associated with the bobtail squid *Euprymna scolopes*, the gene for cooperative luminescence is pleiotropically involved in bacterial growth (Visick *et al.* 2000, Sachs *et al.* 2004). In such cases, mutants do not actually cheat and will not be selected to increase in frequency in the population.

The main framework for understanding the maintenance and evolution of cooperation is inclusive fitness theory. Inclusive fitness theory predicts that the maintenance of a given trait/behaviour relies not only on its direct effects on fitness, but also on its indirect effects (Hamilton 1964a;b). These indirect effects result from the genetic correlation at the loci of interest between the actor and the recipient of the social behaviour (kin selection, Hamilton 1964a;b). The simple, formalised version of this theory known as 'Hamilton's rule' states that cooperation is favoured when $rb - c > 0$ where b is the marginal benefit of increased cooperation to the recipient, r the relatedness at the loci for a social act among actors and recipients, and c the marginal cost of cooperation to the actor. The inclusive fitness of an individual is then the sum of its own reproductive success (direct fitness, $-c$ in Hamilton's rule) and its success through helping relatives to reproduce (indirect fitness, rb in Hamilton's rule). Generalisations of Hamilton's model have been formulated and analysed using a regression framework (Queller 1992, Gardner *et al.* 2011, Rousset 2015) that accounts for non-additive or

synergistic interactions between b and c.

As described above, numerous mechanisms that are consistent with inclusive fitness theory have been elucidated and tested or observed in microbial populations (reviewed by e.g., Sachs *et al.* 2004, Lehmann & Keller 2006, Gardner & Foster 2008).

A cooperative trait has a *direct* positive effect on fitness when its benefit outweighs its cost either (i) by leading to higher survival and reproduction and benefitting others as a by-product, (ii) due to enforcement and control mechanisms (Table 2), or (iii) in mutualistic interactions. First, 'by-product' benefits to others occur when cooperation relies on a 'leaky' function such as the production of catalase-peroxidase enzymes: the degradation of the toxic peroxide is beneficial for the enzyme producer but also for surrounding cells (Sachs & Hollowell 2012, Morris *et al.* 2012). Second, enforcement mechanisms coerce the partner to cooperate and include prudent regulation, partner choice and indirect or reputation-based reciprocity (e.g., Sachs *et al.* 2004, Travisano & Velicer 2004, Table 2). Enforcement mechanisms differ from control mechanisms where cheats are punished by cooperators, or by cheats themselves for not partaking in cooperation (Table 2). Both enforcement and control mechanisms limit cheating behaviour in partners, thus increasing the benefit of cooperation. Third, several examples come from microbial mutualistic interactions (i.e., where there is a net positive fitness benefit of the interaction between individuals of two or more independent populations, either strains or species). For instance, *Serratia ficaria* produces quorum sensing molecules that can induce biosurfactant production in *Serratia liquefaciens*, and both strains can swarm together (Andersen *et al.* 2001). Multispecies biofilms represent another example of mutualistic interactions, where individual strains produce their own matrix, but together form an interdependent, complex biofilm with increased protection against antibiotics and other antagonisms (Allison & Matthews 1992, Moons *et al.* 2009).

Mechanisms providing *indirect* benefits rely on the relatedness between the actor and the recipients of cooperation and include population structuring and cheat discrimination. First, structuring or population viscosity, whereby genetically close individuals are clustered, may emerge from limited or budding dispersal (Taylor 1992, Queller 1992, Gardner & West 2006, Kümmerli *et al.* 2009a). This mechanism increases the average relatedness within the group without requiring any higher-order (complex) mechanisms such as cognitive ability. Nonetheless, assortment also increases local competition

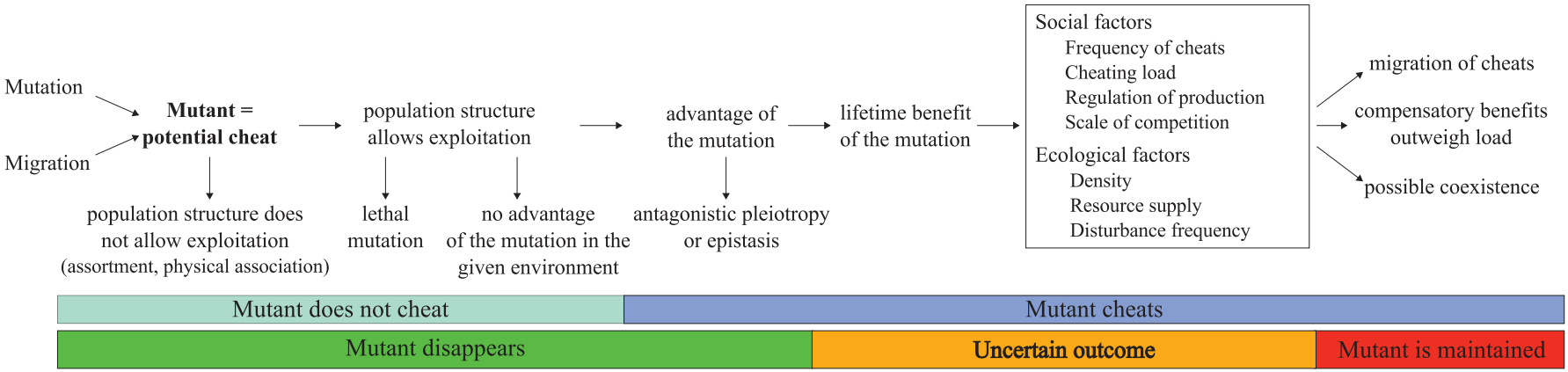


Figure 2: Outcome of a potential mutant for public goods cooperation in a given social and environmental context.

and reduces the benefit of cooperation through negative fitness effects between relatives (e.g., Frank 1998, Griffin *et al.* 2004). Second, increased relatedness between actors and recipients may result from discrimination (West *et al.* 2006, Stefanic *et al.* 2015, Rendueles *et al.* 2015). Discrimination relies on the capacity of individuals to 'recognise' their kin based on environmental and/or genetic cues. Environmental cues lead to cooperation directed towards genealogical and non-genealogical relatives and involve prior association or shared environments (Grafen 1990). When genetic cues imply phenotype matching and fairly high relatedness over most of the genome (i.e., kin recognition), cooperation occurs with genealogical relatives (Crozier 1986, Giron & Strand 2004). When cooperators direct their social behaviour towards non-genealogical relatives, genetic cues entail a pleiotropic or physical linkage between the social gene and some advertisement function that signals to other individuals the presence of the gene (i.e., a 'greenbeard'; Dawkins 1976). Greenbeard recognition has been documented in the slime mold *Dictyostelium discoideum* (Queller *et al.* 2003) and in the yeast *Saccharomyces cerevisiae* (Smukalla *et al.* 2008), but is expected to be relatively rare due to its own vulnerability to cheating ('false' greenbeards, Gardner & West 2010).

The above mechanisms are by no means mutually exclusive and cooperation often results from both direct and indirect fitness benefits (West *et al.* 2007b, West & Gardner 2013). In addition, if cheats are successful in the short-term, they may lead populations to self-extinction and only the populations dominated by cooperators would be maintained (Velicer 2003, Fiegna & Velicer 2003, Travisano & Velicer 2004). Such negative effects of cheating at the group-level ('cheating load') have been shown, for example, in *Pseudomonas fluorescens* where the cheats disrupt biofilm stability (e.g., Rainey & Rainey 2003, Brockhurst *et al.* 2006) and in *Myxococcus xanthus* where fruiting body formation and sporulation may be impaired by high cheat frequencies (Velicer *et al.* 2000).

Moreover, cooperation may be embedded in complex systems and numerous empirical studies have shown that environmental and social factors influence the ecological and evolutionary dynamics of cooperation (Fig. 2). For instance, higher resource supply favours cooperative behaviours by lowering the associated overall fitness cost (Brockhurst *et al.* 2008, Nadell *et al.* 2010). Moreover, increased resource supply enables cooperators to persist in the face of higher frequencies of disturbance by boosting growth following a major perturbation (Brockhurst *et al.* 2010). The social environment and biotic interactions with other species also affect the dynamics of cooperation.

For instance, the presence of non-cooperative individuals may lead to the breakdown of cooperation in well-mixed environments (Kümmerli *et al.* 2009b), whereas interspecific competition may enhance or reduce cooperation depending on its impact on cooperator fitness (Harrison *et al.* 2008, Korb & Foster 2010, Mitri *et al.* 2011, Celiker & Gore 2012). Indeed, ecological factors affect social evolution through changes in the costs and benefits of the focal social behaviour (the c and b of Hamilton's rule), and in the genetic similarity between actors and recipients at loci involved in the behaviour (the relatedness r).

Here I focus on how biotic and abiotic antagonisms interact with social behaviours in the form of public goods. Below I present different mechanisms underlying these interactions.

Effect of antagonisms on the evolutionary ecology of public goods in bacteria

There is no consensus on the effects of antagonisms on cooperative behaviours and their evolution. While it has long been recognised that individuals reduce their investment in cooperative behaviours under pressure to favour short-term benefits (e.g., Mazur 1987, Stephens *et al.* 2002), both theoretical (Andras *et al.* 2003, Beckerman *et al.* 2011) and empirical studies (Callaway *et al.* 2002, de Bono *et al.* 2002, Spieler 2003) have reported positive correlations between the level of cooperation and harsh conditions. Other studies have shown that ecological antagonisms can even foster cooperation (Jousset *et al.* 2009, Krams *et al.* 2010, Quigley *et al.* 2012, Morgan *et al.* 2012, Friman *et al.* 2013). As discussed below, antagonisms may affect cooperation through three non-mutually exclusive processes: demography, plasticity and selection (Fig. 3).

Demography

An obvious effect of antagonisms is to decrease population densities by enhancing mortality (lethal antagonisms) and/or reducing reproduction (non-lethal antagonisms). Lower density may have contrasting effects on cooperation. On the one hand, it may *increase* cooperation by relaxing local competition (Taylor 1992, West *et al.* 2002, Griffin *et al.* 2004), and result in reduced opportunity to exploit cooperators (Ross-

Table 2: Examples of enforcement and control mechanisms involved in the evolution of cooperation (QS = quorum sensing).

Enforcement mechanisms		**Description**	**References**
Intrinsic defector inferiority		Inherently costly loss of social genes	Travisano & Velicer 2004
Physical association		Shared fate (to fitness)	Queller & Strassmann 2009
Autoinducing cooperation (QS)		Cooperation under favourable social conditions	Travisano & Velicer 2004
Trading		Reduced investment in each interaction and increased number of interactions	Friedman & Hammerstein 1991
Suspiciousness		Assessment of the partner's potential before cooperating	Friedman & Hammerstein 1991
Gossiping		Observation of interactions between other individuals	Alexander 1987, Paine 1967, Trivers 1971
Rewards to cooperators		Encouragement of cooperation	Sachs *et al.* 2004
Prudent regulation		Cooperation only when cost-free (minimal costs)	Xavier *et al.* 2011
Partner choice		Cooperation with the individual that offers greater fitness returns	Visick *et al.* 2000
Partner fidelity feedback		Repeated interactions between partners, leading to coupled fitness	Sachs *et al.* 2004
Coercion		Forced interaction and cooperation	Tebbich *et al.* 1996
Control mechanisms			
Punishment by cooperators	Policing	Active punishment directed specifically towards cheats	Manhes & Velicer 2011
	Xenophobia	Generalised exclusion of any "stranger"	Riley & Wertz 2002
Punishment by cheats		Punishment of cheats by other cheats	Eldakar *et al.* 2013
Sanction		End of an interaction with a cheat that harms the cheat as a byproduct	Raihani *et al.* 2012

Gillespie *et al.* 2009). Moreover, lower density results in higher resource supply per cell, which has been shown to favour cooperative behaviours as it decreases the overall fitness cost to cooperators (Brockhurst *et al.* 2008). On the other hand, low densities may *decrease* cooperation in cases where public goods production is triggered by high densities (Darch *et al.* 2012), such as in quorum-sensing dependent cooperation.

It has been shown, however, that population densities may increase under harsh conditions as the result of higher investment in reproduction ('terminal investment'; Poisot *et al.* 2012). While such increases appear to be transient, they may affect cooperation dynamics through demographic effects (e.g., increased competition), but also by generating costs whereby investment in reproduction may curb energy allocation to cooperative behaviours.

Moreover, non-lethal antagonisms may affect population demography via increased emigration. Emigration will modify the density of the resident populations with the above described consequences on cooperation, but also may induce other cooperative behaviours linked to bacterial movement, such as collective swarming (Shen *et al.* 2008, Park *et al.* 2008, Butler *et al.* 2010, Breidenstein *et al.* 2011, Roth *et al.* 2013, Meredith *et al.* 2015).

Plasticity

Plastic changes are phenotypic modifications that occur in response to an environmental stimulus. They may result from direct impacts of the antagonisms through physical contact (Chen *et al.* 2005, Poisot *et al.* 2012), or from indirect impacts when the bacteria respond to environmental cues (Justice *et al.* 2008) such as, for example, by-products of protist predation inducing filamentation as a defence mechanism in *Flectobacillus spp* (Pernthaler 2005).

Recent work has identified several phage- or antibiotic-induced plastic changes associated with cooperative traits in bacterial populations. Specifically, both phages and antibiotics may be associated with enhanced biofilm formation (phages: Hosseinidoust *et al.* 2013a, Tan *et al.* 2015; antibiotics: Kaplan 2011, Bleich *et al.* 2015) and up-regulation of virulence factors (phages: Hosseinidoust *et al.* 2013b;c; antibiotics: Shen *et al.* 2008, Vasse *et al.* 2015). *Escherichia coli* was shown to use quorum sensing to reduce the number of phage receptors, thus decreasing adsorption rate by two-fold and

increasing the frequency of uninfected cells in the population (Høyland-Kroghsbo *et al.* 2013). Indeed, quorum sensing likely plays an important role in plastic responses to stressors and may be either up-regulated (Paulander *et al.* 2012) or down-regulated (Olivares *et al.* 2012) under pressure. Antagonisms may also alter intra- and inter-specific interactions by inducing plastic social behaviours such as avoidance through transient associations with resistant 'cargo' bacteria (Finkelshtein *et al.* 2015). Another illustration comes from collective antibiotic tolerance mechanisms, including cooperative secretions of antibiotic-degrading enzymes, which are often under the control of quorum sensing (Meredith *et al.* 2015).

Selection

In addition to inducing plastic responses, antagonisms may select for certain genotypes that escape, tolerate, or resist antagonisms with potential consequences on cooperative behaviours. Here, antagonisms may favour or impede cooperators though differential effects on cheats, through frequency-dependent selection and/or by affecting population diversity.

First, antagonisms may differentially impact cooperators and cheats. This may occur when cooperators have a direct advantage over cheats in the presence of antagonisms. For instance, the cooperative Type Three Secretion System 1(*ttss-1*) is a virulence trait in *Salmonella enterica* serovar Typhimurium triggering the invasion of gut tissue in mice. The ensuing inflammation benefits both virulent and avirulent (i.e. mutants that do not express *ttss-1*) *S. enterica* serovar Typhimurium as it reverses the outcome of competition between this pathogen and the protective microbiotia in favour of the former (Stecher *et al.* 2007). Crucially, while the avirulent strain outgrows cooperators by avoiding the cost of *ttss-1* expression in the absence of external antagonisms, antibiotics can tip the balance in favour of cooperators (Diard *et al.* 2014). This is because the secretion system allows the cooperative virulent strain to invade the host's tissue thereby evading antibiotic pressure, whereas the avirulent cells cannot leave the lumen and are purged by the treatment. Other studies report pleiotropic or epistatic relationships between cooperation and resistance (Jousset 2012, Friman *et al.* 2013, Yurtsev *et al.* 2013). This is illustrated by QS cooperative *P. fluorescens* being less affected by protist predation than defective mutants, as the former produce more resistant biofilms (Friman *et al.* 2013).

Second, selection from antagonisms may be positively frequency dependent, favouring the most numerous cooperators from the spread of rare mutants. This can be explained by, all else being equal, larger population sizes associated with higher standing variation and increased chances for resistant mutation to occur. Cooperative genes will hitchhike with the resistant mutation, providing that the mutant's fitness outweighs that of the non-resistant cheat (Santos & Szathmáry 2008, Morgan *et al.* 2012, Waite & Shou 2012, Connelly *et al.* 2015, Hammarlund *et al.* 2015). This idea has been experimentally explored in microbes using a novel growth environment (Waite & Shou 2012), and the presence of phages (Morgan *et al.* 2012) as selective pressures. In contrast, should both cooperator and cheat subpopulations harbour resistant mutants, phage populations could be expected to adapt to and differentially impact the more frequent social type (the 'killing the winner' hypothesis; Escobar-Páramo *et al.* 2009, Winter *et al.* 2010). It is nonetheless important to note that the 'winner' type is likely to change over time and rare cheats may well be the final winners in certain microbial social systems (Fiegna & Velicer 2003).

Third, antagonisms may result in increased or decreased genetic diversity in the population, thereby affecting relatedness. Killing the fittest phenotype can help maintain or even increase population diversity, as has been observed in phage predators (Rodriguez-Valera *et al.* 2009). Increased bacterial diversity may also emerge from selection exerted by phages when several resistance mechanisms coexist (Brockhurst *et al.* 2005), or from phages selecting for higher mutation rates in certain bacterial populations (Pal *et al.* 2007, Jousset 2012 but see Gómez & Buckling 2013). Using a rock-paper-scissor like model, Czárán and colleagues (2002) showed that the secretion of diverse antibiotics fosters the maintenance of diversity in bacterial communities. All these mechanisms leading to higher diversity may result in lower relatedness at cooperative loci and favour the emergence of intermediate phenotypes with varying levels of investment in cooperation (Harrison & Buckling 2005). Conversely, the selection of resistant phenotypes (together with decreases in density under predation or antibiotic pressure) may cause clonal sweeps (Hahn *et al.* 2002, Flanagan *et al.* 2007, Miller *et al.* 2011), thereby reducing population diversity and increasing relatedness. With phage pressure, several factors determine whether selection leads to increased or decreased bacterial diversity including resource supply and the degree of spatial heterogeneity (Rodriguez-Valera *et al.* 2009).

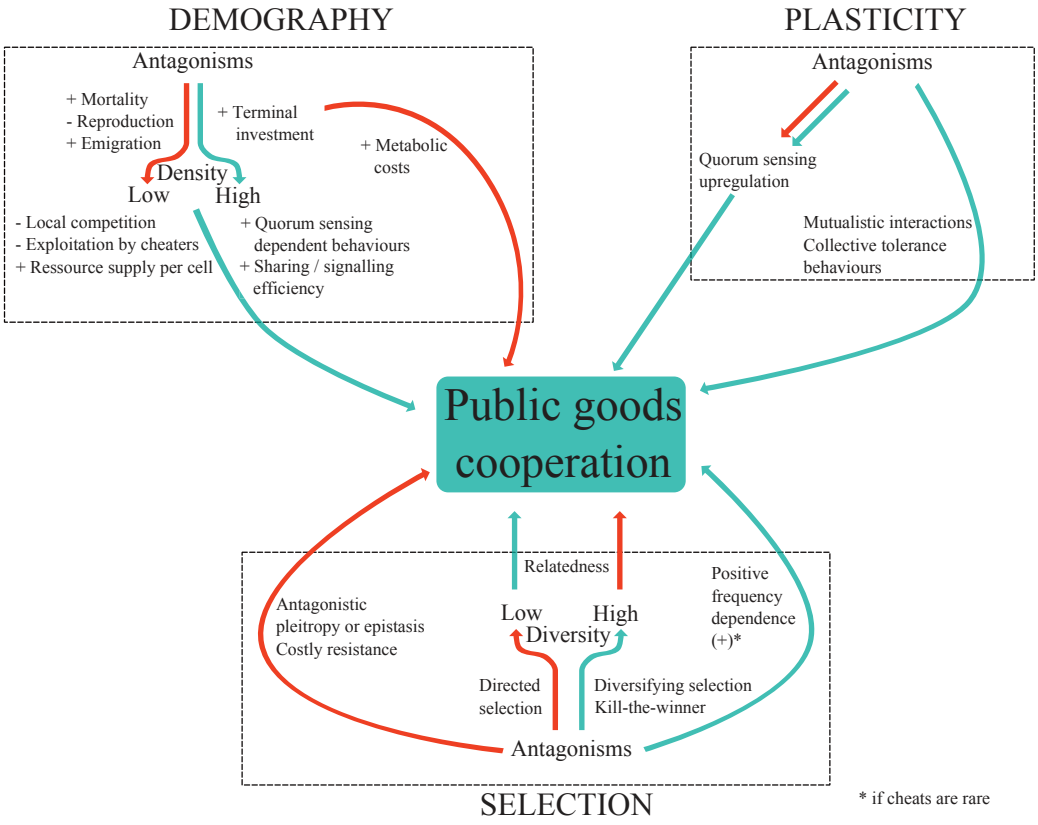


Figure 3: Some effects of antagonisms on public goods cooperation through demography, plasticity and selection. Red arrows represent negative effects and blue arrows positive effects. + (resp. -) indicate an increase (resp. decrease) of the mentioned mechanism.

Research questions and model system

Considering that social behaviours are a critical feature of many bacterial populations emphasises the need to explore how they interact with environments, and more specifically with antagonisms that may impact population biology. Experimental evolution is a powerful approach to identify patterns and test explanatory hypotheses under controlled conditions. The ecology and evolution of social systems in harsh environments is likely to be complex, requiring the identification of the drivers of social evolution and resistance, understanding their interactions in relation with the environment, and dissecting the emerging patterns.

The aim of the first part of my thesis is to examine how ecological antagonisms interact with social behaviours to shape bacterial dynamics and evolution. Specifically, I address the following central questions:

How do antagonisms affect the social dynamics of public goods?

How does the social state of a population affect its response to antagonisms?

We explore these questions using a combination of mathematical modelling (Chapter 2) and experimental evolution (Chapters 1 and 3). We investigate the effect of antagonisms on social behaviours by following the relative frequencies of cooperators and cheats and the production of public goods under different environmental conditions. The impact of social interactions on a population's response to antagonisms is evaluated through population survival and resistance evolution in microcosms with different initial frequencies of cheats.

Biological model: the bacterium *Pseudomonas aeruginosa*

All the experiments presented here were conducted on the gram-negative bacterium *Pseudomonas aeruginosa* PAO1. *P. aeruginosa* is highly versatile, living in a wide range of ecological niches including soil, water, plants, animals and most man-made habitats (Lister *et al.* 2009). It is known to engage in several social behaviours, some of which rely on the collective secretion of molecules or public goods in a shared environment. *P. aeruginosa* has become a textbook example of bacterial social evolution and a wide body of empirical studies have documented the production of public goods such as biofilms, signalling molecules, bacteriocins and nutrient-scavenging compounds (Rainey & Rainey 2003, Griffin *et al.* 2004, Diggle *et al.* 2007b, Xavier *et al.* 2011, Ghoul *et al.* 2015).

In **the first part of this thesis**, I address the interplay between harsh environments due to either an antibiotic or a phage, and public goods cooperation in the form of siderophore production in *P. aeruginosa*. Siderophores are extracellular iron-scavenging molecules, facultatively produced by certain bacteria when soluble iron is limiting (Guerinot & Yi 1994). They are secreted into the environment and, as long as conditions allow surrounding cells to benefit from the secretion, siderophores function as public goods (West & Buckling 2003). This biological model is appropriate for addressing the impacts of antagonisms on cooperative behaviours for several reasons. First, siderophore production is facultative and depends on the characteristics of the environment. The impact of ecological factors on siderophore production by *P. aeruginosa* has been well studied, particularly with relevance to theory on cooperation (Table 3). Second, the production of siderophores is costly; there is thus a selective advantage to cheating (Griffin *et al.* 2004) and cheats spontaneously appear in siderophore-producing

Table 3: Effect of biotic and abiotic factors on siderophore production in *Pseudomonas aeruginosa*. '+' ('-') indicates that the fitness of cooperators (i.e. pyoverdin producers) or pyoverdin production increases (decreases) when the biotic or abiotic factor increases.

	Effect on		
Biotic and abiotic factors	**Fitness of cooperators**	**Pyoverdin production**	**References**
Resource supply	+		Brockhurst *et al.* 2008
Soluble iron availability	+	-	Kümmerli *et al.* 2009b
Low oxygen concentration		+	Cox 1986
Pyoverdin durability	+		Kümmerli & Brown 2010
Cell density		-	Kümmerli *et al.* 2009b
	-		Ross-Gillespie *et al.* 2009
Budding dispersal	+		Kümmerli *et al.* 2009a
Hypermutability	-	-	Harrison & Buckling 2005; 2007
Presence of cheats	- (*)	+	Kümmerli *et al.* 2009b, Harrison 2013
High relatedness	+		Griffin *et al.* 2004
Repression of competition	+ (*)		Kümmerli *et al.* 2010
Global scale competition	+ (*)		Griffin *et al.* 2004
Interspecific competition	- (*)	+	Harrison *et al.* 2008
Phage predation		+	Hosseinidoust *et al.* 2013b

(*) in low-iron medium

populations *in vitro* (Jiricny *et al.* 2010) and *in vivo* (Andersen *et al.* 2015). From a more practical perspective, the genetic basis and the metabolic pathway of siderophore production are both well studied (e.g., Crosa 1989, Meyer *et al.* 1996, Visca *et al.* 2007), and mutant collections are available. Finally, siderophore production has consequences for *P. aeruginosa* virulence. Siderophores may be considered as 'non-specific virulence factors' (Kreibich & Hardt 2015), as they favour nutrient acquisition thereby promoting bacterial growth (Buckling *et al.* 2007, Cornelis & Dingemans 2013), and the major siderophore pyoverdin is also a signalling molecule involved in the regulation of other virulence factors (exotoxin A and protease PrpL, Lamont *et al.* 2002). Moreover, it has recently been shown that pyoverdin may act as a toxin that damages mitochondria and modifies iron homeostasis in *Caenorhabditis elegans* and mammalian cells (Kirienko *et al.* 2015).

Moreover, as an opportunistic human pathogen, *P. aeruginosa* is a major cause of

nosocomial diseases including pneumonia, urinary tract infections and bloodstream infections (Aloush *et al.* 2006, Lister *et al.* 2009). It induces medical complications in immunocompromised patients and those with burn wounds, and is particularly dangerous for cystic fibrosis patients (Schurek *et al.* 2012). The design of appropriate and efficient therapies is very challenging as *P. aeruginosa* presents high levels of resistance to several antibiotics including some penicillins, fluoroquinolones and glucopeptides as well as an impressive capacity to evolve resistance over the course of treatments (Lister *et al.* 2009). The use of bacteriophages is a promising alternative for pathogen control, in particular in combination with antibiotics (Verma *et al.* 2009, Torres-Barceló *et al.* 2014). Combined therapies are supported by the evolutionary rationale of higher efficiency of two different selective pressures compared to either in isolation. While several studies have tested the use of phage and antibiotics *in vivo* and *in vitro* with encouraging results (e.g., Zhang & Buckling 2012, Chhibber *et al.* 2013, Torres-Barceló *et al.* 2014, Kamal & Dennis 2015), the underlying mechanisms remain mostly unknown. Additional studies are needed to explore critical features of the treatments including doses and timing of inoculation, and long-term effects of the drug combination on the evolution of resistance and virulence. I address some of these issues **in the second part of this thesis**. The following section aims at contextualising this work and associated research questions.

Effects of phages and antibiotics as therapeutic agents against bacterial pathogens

We previously highlighted the ubiquity and diversity of bacterial life on Earth. Among all the habitats colonised by bacteria, one of particular interest is our own bodies (Turnbaugh *et al.* 2007, Peterson *et al.* 2009, Qin *et al.* 2010). About 10^{14} microbial cells, at least twofold the number of human cells, live in and on us: our microbiota (Whitman *et al.* 1998, Turnbaugh *et al.* 2007). Several studies, mainly focussing on bacteria, report the considerable positive and negative impacts the microbiota have on human health and physiology (Clemente *et al.* 2012, Cho & Blaser 2012, Lee & Hase 2014, El Aidy *et al.* 2015). Positive effects include the development and functioning of the immune system supported by the gut microbiota (Chow *et al.* 2010), and the facilitation of digestion (Bäckhed *et al.* 2005). In contrast, at the human population level, bacteria may also be the cause of death and widespread epidemics such as tuberculosis, typhoid, cholera and pneumonia (Brachman & Abrutyn 2009), and be the source of certain nosocomial infections in hospitals (Peleg & Hooper 2010). The design of efficient antibacterial treatments requires identifying the evolutionary and ecological factors driving pathogenesis and understanding the mechanisms involved in population-level responses and the evolution of resistance.

The need for new therapies

Antibiotics are our main weapons against bacterial infections (Magill *et al.* 2014). Initiated with the discovery of penicillin and streptomycin in the first half of the 20^{th} century, the wide use of antibiotics, along with better hygiene, have considerably increased life expectancy (Davies & Davies 2010). However, antibiotic treatments are prone to the rapid evolution of resistance through mutation or the horizontal transfer of resistance-conferring genes (Palmer & Kishony 2013). A bacterial infection may involve large numbers of replicating cells resulting in high evolutionary potential within the time frame of a treatment. The overuse of antibiotics and their release into the environment, combined with the lack of care in their management has resulted in pervasive resistance, such that several antibiotics that previously eradicated infections are now virtually useless (Leclercq *et al.* 1988, Reardon 2014; 2015).

Moreover, the discovery of new antibiotics will not counteract the problem of their overuse (Reardon 2014). First, the rate of traditional and synthetic antibiotic discovery may eventually decrease (Projan 2003, Davies 2006, Tomaras & Dunman 2015). Second, the hope of discovering the 'magic' resistance-proof antibiotic seems illusory, especially in light of past experiences and what evolutionary biology would predict (Reardon 2014, Read & Woods 2014, Smith *et al.* 2015).

The consequence is that bacterial infections are still a major cause of global mortality (Reardon 2014; 2015) and we need to consider new therapeutic agents and novel treatment strategies. The use of bacterial viruses (phages) has considerable untapped potential as alternative to antibiotics (Nobrega *et al.* 2015). In this context, combined therapies involving antibiotics and phages appear particularly promising. An increasing number of studies indicate that antibiotic and phages in combination are more effective in controlling pathogenic bacteria than either alone. Below, I present some advantages and possible drawbacks of combined therapies.

Combined therapies: advantages and possible drawbacks

Evolutionary theory supports the use of combined therapies

Evolutionary theory informs our understanding of resistance (Hendry *et al.* 2011, Kouyos *et al.* 2014) and provides a powerful framework for the design of new treatments. In particular, it predicts that the use of multiple agents will likely be more efficient at reducing bacterial load and at limiting the evolution of resistance than single treatments (Cottarel & Wierzbowski 2007, Fischbach 2011). At least three non-mutually exclusive mechanisms may explain a lower probability of resistance. First, the evolution of resistance to several agents may generate high fitness costs, likely resulting in trade-offs with life-history traits such as growth rates (Zhang & Buckling 2012). Second, resistance to one agent may interfere with resistance to the second, as could be the case of phages of the bacterium *Salmonella* attaching to efflux pump receptors that are also employed to rid the cells of antibiotics (Ricci & Piddock 2010, Chaturongakul & Ounjai 2014). Third, a reduction in population size by the agent is expected to concomitantly decrease standing variation and the probability of resistance mutations to other agents (Barrett & Schluter 2008, Bourguet *et al.* 2013).

The promise of phages as adjuvants to antibiotics

The combination of multiple agents is likely to be promoted by agents with sufficiently different targets and involving different genetic and plastic resistance mechanisms. Little is known about interactions between phage and antibiotic resistance (Zhang & Buckling 2012, Tazzyman & Hall 2015); what information exists largely comes from studies of each employed alone. Antibiotic resistance can involve the modification of bacterial permeability, the alteration of the antibiotic target pathway or receptor, the activation of efflux pumps and/or the transformation of the antibiotic molecule (e.g., Poole 2002, Tenover 2006). Phage resistance may occur at different steps of the phage cycle (in the case of lytic phages) and either (i) stops phage adsorption or injection of genetic material, or (ii) kills the phage before it completes its reproductive cycle (Hyman & Abedon 2010, Labrie *et al.* 2010). The inhibition of phage adsorption or injection may be achieved through the loss or masking of phage receptors, by the production of competitive inhibitors or of an extracellular matrix (Hyman & Abedon 2010, Labrie *et al.* 2010). Several mechanisms may result in phage killing within the bacterial cell and include abortive infection, toxin-antitoxin and restriction-modification systems, and an 'adaptive immune response' in the form of clustered regularly interspaced short palindromic repeats (CRISPRs) and CRISPR-associated (Cas) proteins (Dy *et al.* 2014).

Phages also exhibit a number of defining characteristics as therapeutic agents. As previously mentioned, phages are present in most if not all bacterial environments and exhibit remarkable diversity (Clokie *et al.* 2011), meaning that they constitute a massive (and potentially infinite) source of therapeutic variants. The use of temperate phages has been suggested to control bacteria through the transfer of lethal genetic material (e.g., phage-encoded proteins specifically targetting essential bacterial metabolic pathways; Yosef *et al.* 2014). However, this is of limited potential due to superinfection immunity (Hyman & Abedon 2010), and to the risk of encoding bacterial virulence factors (Abedon & LeJeune 2007). In contrast, lytic phages rapidly kill their bacterial hosts and therefore do not suffer from these constraints (Kutter *et al.* 2010). Moreover, lytic phages may coevolve with their bacterial hosts and as such can both overcome bacterial resistance and be 'trained' to improve their impacts on bacterial strains (Pirnay *et al.* 2011, Betts *et al.* 2013) and/or increase the range of strains infected (Poullain *et al.* 2008). Because lytic phages replicate before killing their bacterial

hosts, phage density may increase during the course of a treatment. Phages can be chosen for therapies based on their narrow host ranges (Yosef *et al.* 2014), which limit direct impacts on commensal or mutualistic bacteria. Limited host range requires, however, careful identification of pathogenic bacteria and may be overcome by the employment of phage cocktails (e.g., Zhang *et al.* 2010, Kelly *et al.* 2011), or the use of phages in combination with broader spectrum antibiotics.

Advantages of phage-antibiotic combined therapies

Combining phages and antibiotics increases the spectrum of treatable bacterial pathogens compared to phages alone. This is particularly relevant to multibacterial species infections where single agent treatments may result in the prevalence of one species over others, with unpredictable consequences on bacterial virulence and resistance (McVicker *et al.* 2014, Birger *et al.* 2015). In addition to limiting the evolution of bacterial resistance, phage-antibiotic combined therapy presents a promising opportunity for the control of multidrug resistant bacteria (e.g., Chhibber *et al.* 2013, Kamal & Dennis 2015). Indeed, phages and antibiotics can act synergistically to decrease bacterial densities, both in susceptible and in antibiotic resistant bacteria, and this has been shown both *in vitro* and *in vivo* (Krueger *et al.* 1948, Comeau *et al.* 2007, Ryan *et al.* 2012, Chhibber *et al.* 2013, Torres-Barceló *et al.* 2014). In PAS (Phage Antibiotic Synergy) systems, antibiotics enhance the production of phages by bacterial hosts (Kaur *et al.* 2012, Kamal & Dennis 2015). Moreover, phage-antibiotic combinations may enable the control of bacteria with resistant subpopulations, as in cases of biofilms that are disrupted by phage (Ryan *et al.* 2012, Lehman & Donlan 2015). Synergistic effects may be obtained even when using sublethal antibiotic doses, with positive repercussions on the patient, since sublethal doses impact commensal and beneficial bacteria less strongly, and reduce the risk of resistance evolution in large bacterial populations (Pena-Miller *et al.* 2013, Kouyos *et al.* 2014 but see Goneau *et al.* 2015).

Whereas the impacts of phage-antibiotic combined treatments on bacterial population size and resistance are increasingly understood, effects on the virulence of any bacteria that may survive a treatment is largely unexplored. What information exists again comes from single agents, where both antibiotics and phages have been shown to affect virulence, but with contrasting outcomes (Shen *et al.* 2008, Skindersoe *et al.* 2008, Kaplan 2011, Hosseinidoust *et al.* 2013b;c). As highlighted in the first part of

this introduction (page 33), antibiotics and phages may alter the ecology and evolution of bacterial populations through (i) demography, (ii) selection and (iii) plasticity, each potentially affecting virulence. First, demographic effects leading to decreased densities should result in lower virulence by both reducing the bacterial load in the host and by limiting the expression of quorum-dependent virulence factors (Rutherford & Bassler 2012). Second, in contrast to demographic effects, selection by both antibiotics and phages has been shown to increase virulence by tipping the competitive balance in favour of virulent cooperators (Jousset *et al.* 2009, Diard *et al.* 2014). Moreover, resistance may carry a fitness cost, which potentially leads to reduced virulence by decreasing the growth and the expression of virulence factors (Fernández-Cuenca *et al.* 2011, Laanto *et al.* 2012, Seed *et al.* 2014). Third, bacteria may plastically modulate virulence-related cooperative behaviours such as quorum sensing and biofilms (Skindersoe *et al.* 2008, Kaplan 2011, Hosseinidoust *et al.* 2013a;c, Tan *et al.* 2015, Bleich *et al.* 2015). Quorum sensing, in particular, controls the expression of several virulence factors including proteases, elastase, phospholipase C, pyocyanin or exotoxins (Shen *et al.* 2008, Bjarnsholt *et al.* 2010, Strateva & Mitov 2011, Pollitt *et al.* 2014). Predicting the overall effect of combined therapies on bacterial virulence is challenging and more experimental work is needed to elucidate the interplay between the different factors involved. Nonetheless, as phage-antibiotic combinations may reduce both bacterial densities and the evolution of resistance more than do single treatments, we expect that the former will usually result in lower virulence than the latter. Moreover, an understanding of the underlying virulence mechanisms can inform the design of treatments, for instance, favouring antibiotics known to interfere with quorum sensing (Skindersoe *et al.* 2008), or combinations specifically targetting bacterial virulence factors.

Possible drawbacks of combined therapies

Combined therapies do have risks, some of which are the same as those associated with the single agent components, but others emerging from the specific combinations of phages and antibiotics. In addition to the potential increase in bacterial virulence described above, two main issues may arise: null or negative effects due to inappropriate combinations, and the selection for resistance.

First, one of the agents (phages or antibiotics) may be inappropriate under some

treatment conditions such that a combined therapy will be equivalent to a single treatment (negative antagonist interaction, Piggott *et al.* 2015). For example, the efficiency of some phages may be constrained by the host immune system through neutralisation by antibodies and sequestration in the spleen or liver (Yosef *et al.* 2014). Moreover, combinations may be less effective than single treatments when the agents interfere, but to the best of our knowledge, this has not been described in combined therapies, whereas positive synergistic and additive effects are commonly reported (Krueger *et al.* 1948, Comeau *et al.* 2007, Ryan *et al.* 2012, Kaur *et al.* 2012, Kamal & Dennis 2015).

Second, even if less likely than either agent independently, combined therapy could theoretically select for resistant cells, for example by activating SOS responses (MacLean *et al.* 2013, Baharoglu & Mazel 2014) or selecting for hypermutators (Zhang & Buckling 2012, Tazzyman & Hall 2015). These mutators, should they arise, may not persist in the long-term due to the accumulation of deleterious mutations (Zhang & Buckling 2012). Moreover, SOS-induced mutations do not lead to long-term increased evolvability or resistance but only improve survival in the short-term (Torres-Barceló *et al.* 2015). Another issue may arise if phages select for antibiotic resistant cells. Although no experiment supports this hypothesis in phage-antibiotic combinations, this has been reported in antibiotic cocktails (Pena-Miller *et al.* 2013). Such an effect could arise should phages differentially kill antibiotic susceptible cells, either because they are more metabolically active or more susceptible (e.g., receptor number on outer cell membrane, or receptor conformation; Labrie *et al.* 2010). Careful choice of phages and antibiotics can minimise such effects, such as phages capable of disrupting biofilms when using a biofilm-inducing antibiotic.

Experimental phage-antibiotic combinations and open questions

In the last decade, phage-antibiotic combinations have been tested *in vitro* and *in vivo* in the laboratory with encouraging results (e.g., Verma *et al.* 2009, Escobar-Páramo *et al.* 2009, Zhang & Buckling 2012, Kamal & Dennis 2015). For instance, a *Myoviridae* phage combined with linezolid successfully cured multiresistant *Staphylococcus aureus* hindpaw infections in diabetic mice (Chhibber *et al.* 2013), and the combination of enrofloxacin and a lytic phage led to complete protection against *E. coli* in birds (Huff *et al.* 2004). Crucially, when resistance was measured, the combined therapies led to

limited resistance compared to single treatments (Verma *et al.* 2009, Zhang & Buckling 2012).

However, the mechanistic basis of these promising results is not understood and further research is required to determine the conditions increasing or maximising the effectiveness (e.g., density and virulence reduction, minimising resurgence due to resistance) and safety (e.g., preventing impacts on the microbiota, decreasing virulence and minimising spread to other hosts) of combined treatments. In particular, the effects of antibiotic doses and phage concentrations as well as the impact of timing and sequences of inoculation remain largely unknown. Experimental evolution and molecular analyses should inform our understanding of antibiotic-phage-bacterial interactions in terms of impacts of combined therapies on population biology and behaviours.

The second part of my thesis aims at exploring the ecological and evolutionary effects of phages and antibiotics as therapeutic agents against populations of bacterial pathogens. In particular, I address the following questions:

How is the efficiency of the combined therapy affected by the modalities of the treatment (doses, modes of action, inoculation sequence)?

Do phages modify the evolution of bacterial resistance to antibiotics (and *vice versa*)?

How do the treatments affect bacterial virulence *in vivo*?

We investigate these questions using experimental evolution in *P. aeruginosa* with a panel of antibiotics and lytic phages. We assess the efficiency of single and combined treatments by measuring their effects on bacterial survival, evolution of resistance (Chapter 4 and 5) and virulence *in vivo* (Chapter 5).

Thesis outline

The first part of this thesis addresses the interplay between antagonisms and public goods cooperation. It is composed of the three following chapters.

In Chapter 1, we experimentally examine the impact of different doses of antibiotics on competition between siderophore producers and non-producers in an environment requiring siderophore-based iron acquisition. We also investigate the impact of these resulting intraspecific interactions on the population's response to antibiotic pressure, in particular resistance evolution.

Chapter 2 further explores the interaction between antibiotic pressure and siderophore cooperation using a mathematical model. Linking the empirical results of the competition experiment (Chapter 1) to theory enables us to generate hypotheses about how antibiotics influence public goods production, and to gain insight into the microevolutionary mechanisms that underpin the observed patterns.

In Chapter 3, we extend the study of public goods cooperation under harsh conditions by analysing more complex interactions between bacteria and a biotic antagonism in the form of phages both at ecological and evolutionary timescales. We analyse the interactions between the two strains (producers and non-producers) as well as the ensuing population dynamics under two experimental conditions: a 'social-inducing' environment in which the access to iron relies essentially on siderophore production and a 'social-repressing' environment where iron is freely available.

The second part of the thesis addresses the effects of phage and antibiotic combinations on populations of pathogenic bacteria in an evolutionary framework.

In Chapter 4, we investigate, *in vitro*, the impact of the timing of antibiotic inoculation in a combined therapy with a lytic phage on the reduction of *P. aeruginosa* density and on resistance to both control agents.

In Chapter 5, we study the effect of antibiotic doses and modes of action on the long-term dynamics of bacterial populations in the presence of phage, and assess the adaptation rate of the bacteria and the evolution of resistance to both antibiotics and phages. We further test the consequences of each treatment on bacterial virulence *in vivo*.

Part I

Ecological and evolutionary interplay between harsh environments and public good cooperation

1

Cooperation increases antibiotic resistance in social cheats

Marie Vasse, Clara Torres-Barceló, James Gurney, Oliver Kaltz and Michael E. Hochberg. (*in preparation*)

Abstract

Ecological antagonisms play a key role in shaping the dynamics and evolution of bacterial populations, in particular by inducing stress responses and selecting for resistant or tolerant phenotypes. Little is known, however, about their impact on the social lives of bacteria, and in particular the production of public goods. We investigated how the effect of an abiotic stress in the form of antibiotics interacts with siderophore production to influence resistance evolution and social dynamics in the pathogen *Pseudomonas aeruginosa*. We assessed the impact of antibiotics on (i) the interactions between siderophore producers and non-producers, and (ii) how these interactions feed back to the population's response to antibiotics, particularly for resistance evolution. We found that non-producers were favoured over producers in mixed cultures under antibiotic stress, reaching higher densities and higher frequencies of resistance. We hypothesise that this results from non-producers benefitting from the presence of producers and thus bearing lower overall fitness costs. While the dominance of non-producers led the mixed cultures to higher survival and resistance to antibiotics than either monoculture, we found that the few remaining producers also displayed higher resistance compared to monocultures. Our results highlight the complex interactions between social traits and antibiotic stress and their consequences for social evolution and resistance.

Introduction

Public goods production is a characteristic of a diverse range of taxa, from microbes to humans (West *et al.* 2006, Rankin *et al.* 2007, Cordero *et al.* 2012, Escobedo *et al.* 2014). Explaining the persistence of this costly behaviour is challenging, since it can be exploited by cheats that to not contribute to the common good, yet reap the benefits. Kin selection theory is a successful framework for understanding the evolutionary dynamics of public goods behaviours, with the central prediction that cooperation is favoured by sufficient positive assortment between and benefits to cooperators (Hamilton 1964a;b, West *et al.* 2002, Bourke 2011). A large body of theoretical work (e.g., West *et al.* 2007b, Bijma & Aanen 2010, Gardner *et al.* 2011, Marshall 2011, Débarre *et al.* 2014, Liao *et al.* 2015, Misevic *et al.* 2015) and a growing number of empirical studies (e.g., Harrison & Buckling 2005, Velicer & Vos 2009, Kümmerli *et al.* 2009a, Brockhurst *et al.* 2010, Dumas & Kümmerli 2012, Dobata & Tsuji 2013) have investigated a range of mechanisms that may underlie this central insight.

Microbial populations are an increasing focus for understanding public goods dynamics (Crespi 2001, West *et al.* 2007a, Xavier 2011), because of their relatively simple behaviours, rapid ecological and evolutionary responses, and the ease of controlled experimentation (West *et al.* 2007a, Strassmann *et al.* 2011). Microbes and in particular bacteria, show a variety of behaviours consistent with basic social interactions under controlled laboratory conditions. These frequently rely on the coordinated secretion of beneficial metabolites (i.e. public goods) and include between-individual communication, formation of multicellular-like structures, and collective motility and resource acquisition (reviewed by e.g., Crespi 2001, Velicer 2003, West *et al.* 2007a, Celiker & Gore 2013). Recent study in experimental bacterial populations has elucidated some of the mechanisms consistent with kin selection fostering cooperative behaviours (e.g., West *et al.* 2002, Griffin *et al.* 2004), such as assortment emerging from limited or budding dispersal (Gardner & West 2006, Kümmerli *et al.* 2009a), and kin discrimination (Mehdiabadi *et al.* 2006, Rendueles *et al.* 2015). Despite this accumulating consensus, little is known about how social populations respond to differences and variation in abiotic and biotic components of their environment, and in particular, ecological antagonisms. Such antagonisms could affect both their population ecology and evolution and, should stress responses entail costs, could, in principle, interact with the ecology and evolution of social behaviours.

Bacteria are confronted with a variety of antagonisms, including predation (e.g., other bacteria, phages, metazoans), parasitism (lysogenic phages, plasmids), antimicrobials from other organisms (antibiotics, antimicrobial peptides, toxins), and abiotic environments (extreme temperatures, pH, salinity) that can result in reduced fitness through decreases in survival and/or reproduction. Stress may impact cooperation directly through differential selection on cooperative phenotypes (Friman *et al.* 2013, Diard *et al.* 2014) or by inducing specific plastic behaviours (Hoffman *et al.* 2005, Kaplan *et al.* 2012, Bleich *et al.* 2015, Finkelshtein *et al.* 2015, Vasse *et al.* 2015), and indirectly by affecting population dynamics and structure (Ben-Jacob *et al.* 2000) or selecting for genetically-based resistance. The latter includes the hitchhiking of cooperative genes with resistance mutations (Waite & Shou 2012, Morgan *et al.* 2012, Quigley *et al.* 2012), and potential epistasy or pleiotropy with cooperative genes (Yurtsev *et al.* 2013).

Here, we investigated how antibiotics and public goods behaviours interact to shape resistance evolution and social dynamics in microbial populations. Our hypothesis is that public goods production favours the evolution of resistance by enabling large, growing populations. This is because, all else being equal, population size is associated with the likelihood of the presence or subsequent emergence of resistance or tolerance mutations (Morgan *et al.* 2012, Ramsayer *et al.* 2013). However, the ecological and evolutionary outcomes of interactions involving social behaviours and stress responses are likely to be complex. Exploitation by cheats should limit producer growth, the emergence of novel variants and therefore evolutionary potential. Since antibiotics are expected to increase the overall fitness costs to producers and to non-producers, we expect their numbers to decline under antibiotic pressure. However, should a producer harbour a resistance mutation to a stressful environment, this mutant will increase in frequency provided its absolute fitness is greater than zero, i.e., the producing trait effectively hitchhikes (Santos & Szathmáry 2008, Morgan *et al.* 2012, Waite & Shou 2012). The evolution of traits conferring stress resistance may, in turn, affect ecological interactions and drive changes in population composition through trade-offs with social or other life-history traits. Cooperative interactions highly depend on the densities and relative frequency of cheats and cooperators and, as the composition of the population changes, its dynamics and the social environment will also be affected. This makes predicting changes in the relative frequencies of social traits challenging. To understand the complex ecological and evolutionary dynamics of public goods beha-

viours under stressful conditions, we need to consider the interactions and feedbacks involving resistance and cheating behaviours.

Specifically, we examine how a public goods trait in the form of siderophore production interacts with resistance evolution to the antibiotic gentamicin in the opportunistic pathogen *Pseudomonas aeruginosa*. Siderophores are small secreted molecules that chelate poorly soluble iron in the environment and make it available to bacteria via specific outer-membrane receptors (Hannauer *et al.* 2012). As any cell carrying these receptors can use siderophores, they are a public good in a well-mixed environment and, as such, are vulnerable to 'cheating' by individuals that do not invest in production, but possess receptors and reap the benefit (West & Buckling 2003, Griffin *et al.* 2004). Two major resistance mechanisms to gentamicin have been described in *P. aeruginosa* and both potentially affect siderophore dynamics. First, reduced transport and reduced accumulation of gentamicin within the cell by, respectively, decreased cell permeability and increased efflux-pump activation (Mayer 1986, Livermore 2002) may interfere with siderophore uptake and release through the membrane. Second, expression of modifying enzymes that inactivate the antibiotic (Mingeot-Leclercq *et al.* 1999) likely comes at a cost, with potentially negative consequences for the relative fitness of siderophore producers. Moreover, ecological effects may mediate public good dynamics, for example Ross-Gillespie and colleagues (2009) have suggested that low densities (due to antibiotic pressure in our case) could favour siderophore cooperation.

We grew two genotypes of *P. aeruginosa*, a siderophore-producing strain and a non-producing strain, under iron-limited conditions with different doses of the antibiotic gentamicin. We assessed (i) the impact of antibiotic pressure on the interactions between the two producting genotypes and (ii) the effect of these interactions on the population's response to antibiotics, in particular the evolution of resistance. We found that antibiotic stress favours non-producers over the producers in mixed cultures and leads to decreased relative frequencies of producers. Moreover, the non-producers from mixed cultures reached higher frequencies of resistance than either non-producer or producer monocultures, arguably because of the former's enhanced growth in the presence of producers and lower overall environmental fitness costs (costs of resistance and costs of siderophore production). Surprisingly, the few producers that remained in these mixed cultures also showed higher proportions of resistance than either monoculture, suggesting the role of plastic or genetic 'compensatory' mechanisms.

Materials and Methods

Bacterial strains

We used *Pseudomonas aeruginosa* PAO1 (ATCC 15692) as the pyoverdin-producing strain (hereafter called 'producers') and the mutant PAO1Δ*pvdD* (Ghysels *et al.* 2004), derived from the same genetic background, as the non-producing strain (hereafter called 'non-producers'). The knockout of the non-ribosomal peptide synthetase gene *pvdD* prevents pyoverdin production.

We initiated 5 populations of producers and 5 of non-producers, each from a single arbitrarily chosen colony, in 6 mL of casamino acids medium (CAA; 5 g Casamino acids, 1.18 g $K_2HPO_43H_20$, 0.25 g $MgSO_47H_20$, per litre; Sigma-Aldrich) contained in 30 mL Thermo-Fisher microcosms. Populations were incubated overnight at 37 ° C under constant orbital shaking (200 rpm) before being used as inoculum for the experiment.

Experimental conditions

The experiment was carried out at 37 ° C under constant orbital shaking (350 rpm, 8mm stroke) to ensure well-mixed conditions. We used the inner wells of 48-well plates as microcosms, each containing 800 μL of iron-limited CAA medium. To create the iron-limited conditions, we supplemented the CAA medium with 100 μg/mL of a strong iron chelator, human apotransferrin (Sigma-Aldrich), and 20 mM of sodium bicarbonate. We used millipore water to prepare all solutions to limit the amount of exogenous iron.

We employed the aminoglycoside antibiotic gentamicin (Sigma-Aldrich) at final concentrations of 2, 4 or 8 μg/mL to assess the impact of increasing antibiotic pressure. In a preliminary experiment, we estimated that 10 μg/mL is the minimum gentamicin concentration that prevents *P. aeruginosa* growth under our experimental conditions. Briefly, we initiated 8 replicate populations of producers and non-producers in 800 μL of iron-limited CAA supplemented with gentamicin in 48-well plates with an initial density of 10^7 bacteria per mL. Plates were incubated at 37 ° C under constant orbital shaking at 350 rpm. After 24 hours, we read optical density (OD) of each population using a spectrophotometer (ClarioSTAR microplate reader, BMG Lab Technologies) and scored growth inhibition if OD < 0.1 (corresponding to the minimal value in the

accurate detection range of the spectrophotometer).

Experimental protocol

We inoculated bacteria as either monocultures or mixed cultures of producers and non-producers to a final density of *c.* 10^7 bacteria per mL into fresh medium with either a low (2 μg/mL), intermediate (4 μg/mL), or high (8 μg/mL) dose of gentamicin, or in antibiotic-free medium. Mixed populations were initiated with either 15%, 45% or 75% of non-producers. Each treatment was replicated 5 times for a total of 100 populations (4 antibiotic conditions $\times$ 5 types of cultures $\times$ 5 replicates) that were arbitrarily distributed in the 48-well plates. The experiment was run for 48 hours.

Population densities and relative frequencies of producers and non-producers

We mixed individual populations by pipetting and sampled 20 μL of each at the beginning of the experiment (T_0) and after 10 (T_{10}), 24 (T_{24}), 34 (T_{34}) and 48 (T_{48}) hours. To estimate total densities and relative frequencies, we plated serial dilutions of each sample onto King's B medium (KB) agar plates. Plates were then incubated 24 hours at 37 $^\circ$C for subsequent counting of colony forming units (CFUs). Numbers of bacteria were estimated by averaging the counts from at least 3 different plates. We distinguished colonies of producers from non-producers by their different colours (yellow-green and white, respectively).

Antibiotic resistance

We estimated the proportion of resistant cells at T_0 and T_{48} by plating serially diluted samples of each population onto antibiotic-free KB agar plates and onto KB agar with 10 μg/mL gentamicin, simultaneously. We calculated the proportion of resistant cells as the ratio of the number of colonies able to grow on gentamicin-agar to the total number of colonies, with a detection threshold of 40 resistant cells (i.e., at least 1 CFU in the undiluted 20 μL sample from the 800 μL culture). We distinguished producers from non-producers based on the colour difference as described above and we further checked

the phenotype of the resistant colonies by inoculating a sample of each individual colony into iron-limited CAA overnight and measuring fluorescence: producer cultures showed significantly higher fluorescence than non-producers ($p < 0.001$).

To investigate the observed effects in more detail, we repeated the experiment for a subset of treatments. Using the highest dose of gentamicin (8 μg/mL), we tested monocultures of producers and non-producers, as well as mixed cultures with initially 15% of non-producers. We estimated the densities, relative frequencies and proportion of resistant cells by plating at T_0 and T_{48}, as described above.

Statistical analysis

First, we employed logistic regression (binomial error structure, logit link) to analyse variation in the frequency of non-producer cells over the course of the experiment. Models contained antibiotic treatment (0, 2, 4, 8 μg/ml gentamicin) and initial non-producer frequency (0, 15, 45, 75 and 100%) as explanatory factors, time (hours) as a covariate, and replicate population as a random factor. All possible interactions were statistically fitted. Where necessary in this and other analyses below, models were corrected for overdispersion by dividing the χ^2 values for each factor by the mean residual deviance (χ^2 value of residuals divided by the degrees of freedom of residuals).

Second, we used standard least-square methods (ANOVA) to analyse variation in final (log-transformed) bacterial density. In addition to antibiotic treatment and initial non-producer frequency, we also fitted initial density in each replicate population as a covariate. Analyses were carried out separately for producers and non-producers, as well as for the total population.

Third, we analysed variation in the frequency of antibiotic-resistant cells as a function of antibiotic treatment and initial non-producer frequency. Attempts using logistic regression, with the number of resistant cells as response variable and total cell number as binomial denominator (as above for non-producer frequency, with logit link) led to unsatisfactory model fits, namely a strong deviation from a normal distribution of residuals. This problem was solved by switching to a Poisson error structure (with log link) and using total cell number as a covariate in the model. This way of fitting the model is also more appropriate, given that numbers of resistant and non-resistant cells were determined independently (by plating independent samples on different media,

see above). Analyses were carried out separately for producers and non-producers, and for the total population (producers and non-producers combined).

Results

Changes in non-producer frequency

In all replicate populations and all three initial frequencies, non-producer frequency increased over the course of the experiment (Fig. 1.1). Non-producer frequencies were substantially higher in the presence of the antibiotic than in the antibiotic-free controls. At higher doses (4 and 8 μg/mL), non-producers often reached near-fixation ($> 90\%$) after 48 hours.

Antibiotic dose further affected the timing of the frequency changes, as indicated by the significant time $\times$ gentamicin interaction ($\chi^2_3 = 123.39, p < 0.0001$). Namely, at the two lower doses (2 and 4 μg/mL), non-producer frequencies increased considerably during the first 24 hours and then reached a peak (Fig. 1.1). At the highest antibiotic dose (8 μg/mL), the frequency increase was delayed by *c.* 24 hours.

These patterns were similar for all initial frequencies of non-producers, and the significant triple interaction (time $\times$ gentamicin $\times$ initial frequency, $\chi^2_6 = 26.69$, $p < 0.001$) likely reflects the boundary effect, as frequencies cannot exceed 1.

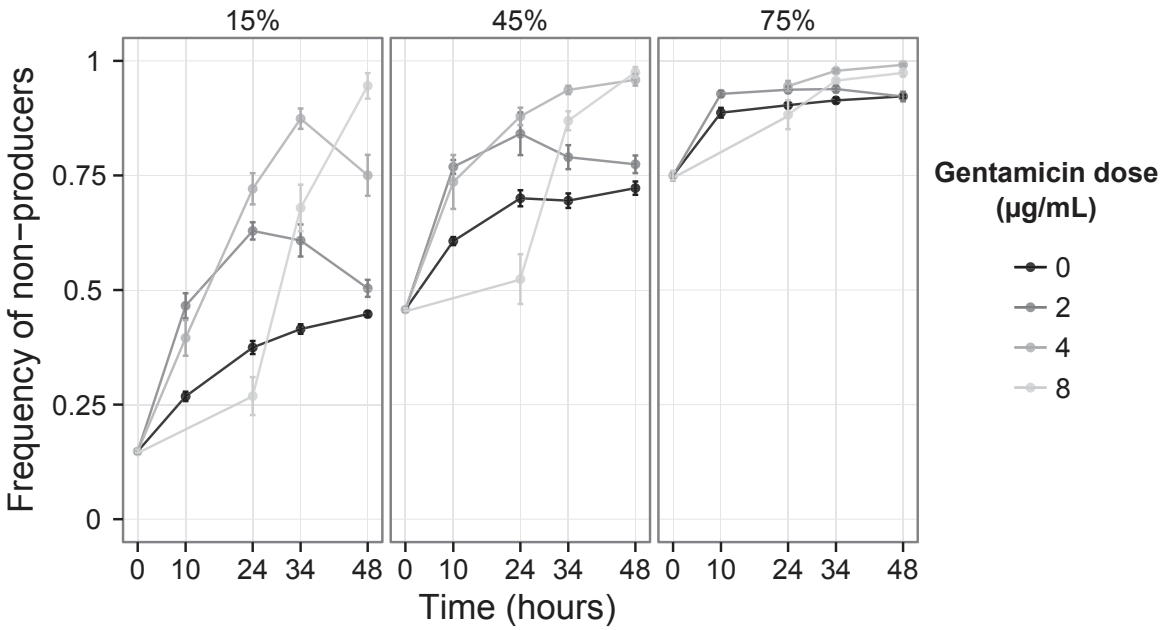


Figure 1.1: Change in non-producer frequencies between T_0 and T_{48}. The three panels correspond to the different initial frequencies of non-producers. Different gentamicin doses (in μg/mL) are represented by the grey scale and the black line is the gentamicin-free control. Bars are standard errors of the mean.

Effects of the antibiotic and initial non-producer frequency on bacterial densities

The addition of the antibiotic slowed down bacterial growth and resulted in lower final densities in all populations compared to the control, with higher doses leading to lower density (effect of gentamicin: $F_{3,79} = 282.24, p < 0.0001$; Fig. 1.2). There was no statistically significant effect of initial densities on final densities ($F_{1,79} = 0.63, p = 0.43$).

In the presence of non-producers in mixed culture, producers grew to lower densities than in monoculture (effect of initial frequency $F_{3,63} = 77.51, p < 0.0001$, contrast mixed vs mono: $p < 0.0001$, Fig. 1.2A). The addition of the antibiotic enhanced this effect, in particular for the highest gentamicin dose (8 μg/mL), where the mean final density of producers in mixed culture was about 20 times lower than in monoculture (antibiotic dose $\times$ initial frequency interaction, $F_{9,63} = 10.84, p < 0.0001$). Indeed, at this dose, producers in mixed culture not only had lower densities than in monoculture, but also decreased in total numbers during the experiment (Fig. 1.4).

Conversely, non-producers exploited producers and thus reached higher final densities in mixed culture than in monoculture, both in the presence and absence of the antibiotic (effect of initial frequency: $F_{3,63} = 18.28, p < 0.0001$, contrast mixed vs. mono: $p < 0.05$, Fig. 1.2B). The significant interaction between gentamicin dose and initial non-producer frequency ($F_{9,63} = 4.21, p < 0.001$) indicates that this mixed culture advantage was influenced by the experimental treatments. Thus there was no consistent mixed culture advantage at the lowest gentamicin dose (2 μg/mL) or at low initial frequency of non-producers (15%). However, when we repeated the experiment for 15% mixes at 8 μg/mL gentamicin, non-producers did grow to higher densities than in monoculture (Fig. 1.6B), similar to the 45% and 75% mixes in the main experiment.

When examining the combined final density of producers and non-producers in the mixes, we found a significant interaction between antibiotic dose $\times$ effect of initial frequency ($F_{12,79} = 5.64, p < 0.0001$). Specifically, at the two high doses (4 μg/mL and 8 μg/mL) the total number of producers and non-producers in the mixed cultures was significantly higher than in either monoculture (contrasts mixed vs. mono: $p < 0.01$; Fig. 1.2C). At low dose or without the antibiotic, there were no consistent overall differences between mixed and monocultures (Fig. 1.2C).

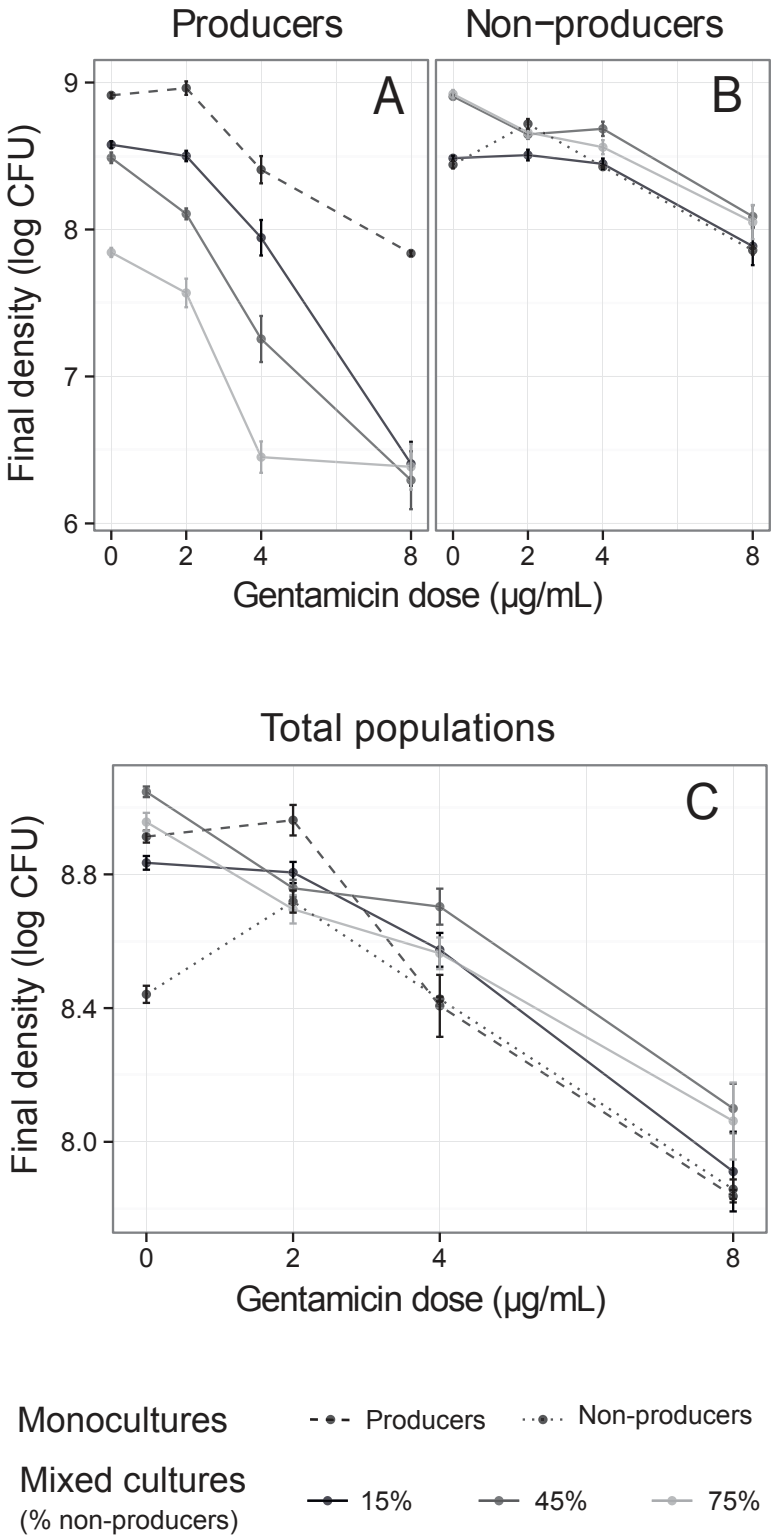


Figure 1.2: Final density of producers (A), non-producers (B), and total populations (C) depending on gentamicin dose and the initial non-producer frequency. The first two panels (A and B) present the final density of each clone in monocultures (dashed line=producers, dotted line=non-producers) and in mixed cultures (grey scale). Panel C shows the final density of the total populations of monocultures and mixed cultures. Data are log-transformed CFUs and bars are standard errors of the mean.

Effects of the antibiotic and initial non-producer frequency on bacterial antibiotic resistance

Increasing the dose of gentamicin led to higher frequencies of resistant cells, with up to a five-order-of-magnitude difference between the highest dose (8 μg/mL) and the control (Fig. 1.3). Nonetheless, the resistant proportion always remained below 10%.

Producer monocultures generally showed higher frequencies of resistance than non-producer monocultures at all three gentamicin doses (producer vs. non-producer: $\chi^2_1 = 9.14, p < 0.01$; Fig. 1.3). Furthermore, when looking at producers and non-producers individually, we found a significant interaction between antibiotic dose and initial non-producer frequency for resistance, both for producers ($\chi^2_9 = 22.89, p < 0.0001$) and non-producers ($\chi^2_9 = 34.84, p < 0.0001$). The frequency of producer resistance in mixed culture was either lower than or not different from that in monoculture at low antibiotic doses, but higher at highest antibiotic dose (Fig. 1.3A). Non-producer resistance showed a similar pattern (Fig. 1.3B), although there was also a more general overall trend of higher resistance in mixed culture compared to monoculture (Fig. 1.3C).

Because of their growth advantage, non-producers represented the majority of resistant cells in mixed cultures. Hence, patterns of resistance at the total population level (Fig. 1.3C) were similar to that for non-producers only (Fig. 1.3B; antibiotic dose $\times$ initial non-producer frequency interaction: $\chi^2_{12} = 18.56, p < 0.05$). The supplementary replicate experiment confirmed these main results (Fig. 1.7).

Taken together, higher antibiotic concentrations resulted in more pronounced differences in density and resistance between mixed cultures and monocultures. At the total population level (producers and non-producers combined), mixed populations showed consistently higher density and resistance than monocultures, when facing high antibiotic doses.

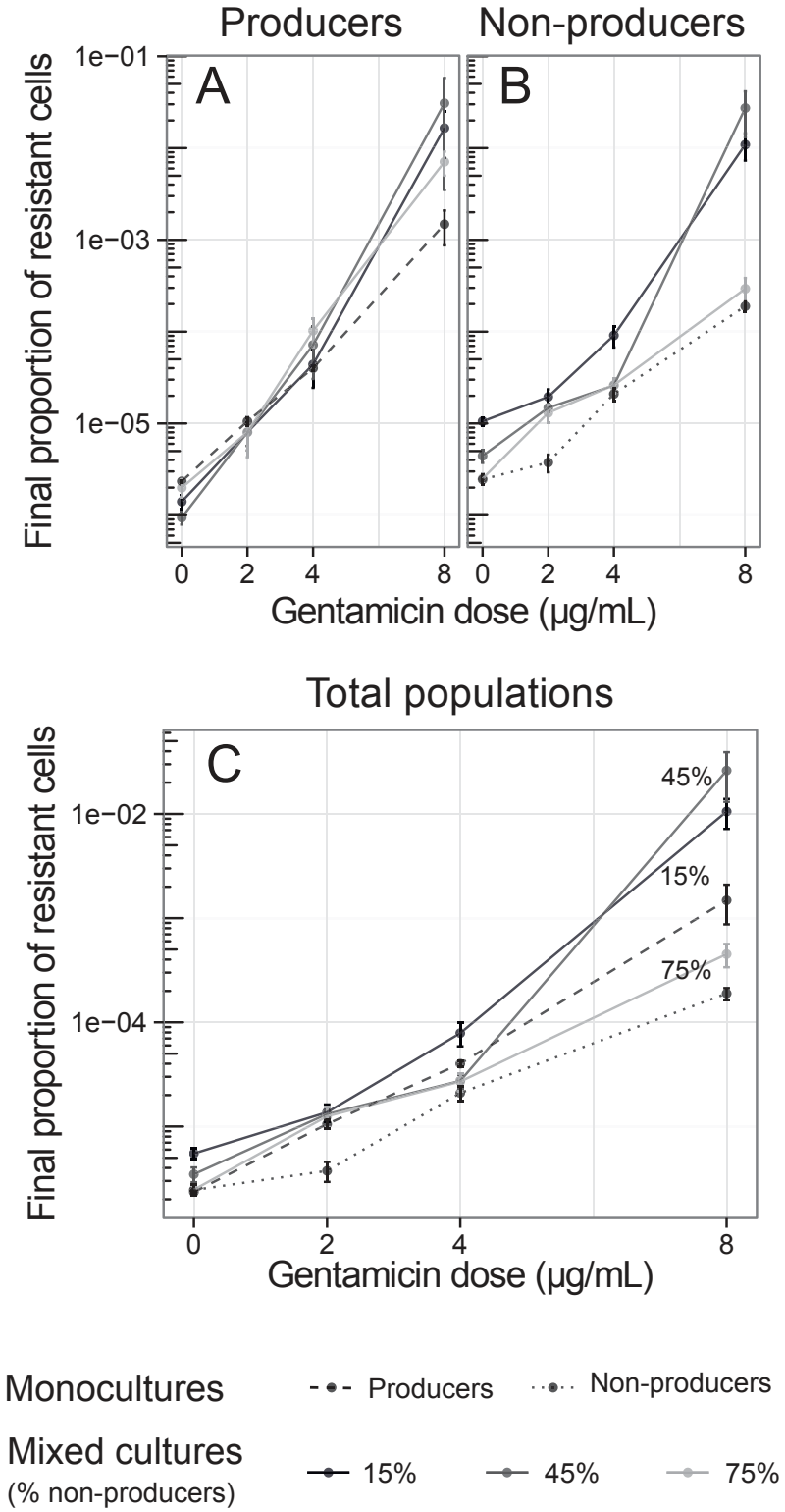


Figure 1.3: [Upper panel] Final proportion of resistant cells in producers (A) and in non-producers (B) in monocultures (dashed or dotted line, respectively) and in mixed cultures (solid line, grey scale corresponds to different initial frequencies of non-producers) depending on gentamicin dose. [Lower panel] Final proportion of resistant cells in total populations of monocultures and mixed cultures (C). Bars are standard errors of the mean.

Discussion

Previous work on cooperation under antibiotic pressure has essentially focused on cooperative or collective behaviours increasing antibiotic resistance or tolerance (Vega & Gore 2014, Meredith *et al.* 2015), such as enhanced biofilm formation (Kaplan 2011, Bleich *et al.* 2015) or secretion of antibiotic-degrading enzymes through quorum sensing regulation (Meredith *et al.* 2015) without addressing the potential conflicts emerging from these social behaviours. Our experiments in *P. aeruginosa* focused on the interplay between antibiotic selection and public goods cooperation. We found that the frequency of non-producers increased in mixed cultures under antibiotic pressure, especially at the highest dose. Moreover, the mixed cultures, mostly composed of non-producers after 48 hours, were better able to cope, both in terms of survival and resistance, with high antibiotic pressure than either monoculture. Our results emphasise the complex ecological and evolutionary dynamics of public goods behaviours and how these interact with biological stressors in the form of antibiotics.

In one of the few studies investigating interactions between antibiotics and social behaviours in bacteria, Diard and colleagues (2014) assessed the *in vivo* impact of ciprofloxacin on competition between virulent cooperative *Salmonella enterica* serovar Typhimurium and avirulent defectors. In the absence of the antibiotic, defectors outcompeted cooperators in the gut lumen, whereas the addition of the antibiotic reversed the outcome of the competition, leading to selection for the virulent cooperators. Indeed, only the virulent cells were able to invade host tissue and escaped the antibiotic killing all cells in the lumen. When antibiotic pressure decreased, the virulent strain reinvaded the lumen. Our results contrast with this conclusion as we observed that antibiotics led to the selection of non-producers relative to producers. We hypothesise that this is because of the difference in spatial structure: whereas the gut lumen is a highly structured environment, our *in vitro* studies were conducted under well-mixed conditions where the producers had no refuge from exploitation by non-producers.

In addition to antibiotics, several ecological antagonisms are likely to influence the interactions between public good producers and non-producers. In particular, the effect of competition has received much attention, but led to contrasting results. For instance, Celiker & Gore (2012) showed that interspecific competition may favour cooperation in the budding yeast *Saccharomyces cerevisiae*, whereas Harrison and colleagues (2008)

reported that the competitor *Staphyloccocus aureus* selected against siderophore cooperation in *P. aeruginosa*. These seemingly contradictory results likely arise from different ecologies in each system leading to differences in the nature and magnitude of costs and benefits of cooperation. For example, *S. cerevisiae* cooperators have preferential access to the public good, resulting in a competitive advantage when the public good is limiting. In contrast, Harrison and colleagues (2008) showed that competition for iron between *S. aureus* and *P. aeruginosa* leads to increased siderophore production in the latter, to the benefit of *P. aeruginosa* cheats in well-mixed environments. Employing computer simulations in a spatial setting, Mitri and colleagues (2011) further explored the effect of ecological competition on cooperative secretions and found that competition is more detrimental to secretors than to defectors when nutrients are limiting. They explained their results as follows: the investment in secretion limits growth and thereby competitive ability. This hypothesis likely applies to our experimental results: in the absence of antibiotics, producers grew slower than non-producers in mixed cultures because they invested in pyoverdin production to the benefit of every cell in the population. This may explain why producers were more affected than non-producers by antibiotic pressure. In other words, in the absence of a 'private benefit' to co-operators (expected to operate in spatially structured environments, for example), it is growth in the absence of antibiotics that, all else being equal, determines how well a strain can cope with an ecological antagonism. Moreover, antibiotics may affect producers more than cheats by directly incurring additional fitness costs to the former. This has been shown, for example, in *S. aureus*, where sublethal doses of ciprofloxacin, or mupirocin, or rifampicin induce the expression of the effector molecule regulating the quorum sensing system *agr* (Paulander *et al.* 2012).

Several studies have argued that ecological antagonisms should favour cooperation as a side effect of positive frequency dependence for resistance evolution: the most frequent type is more likely to acquire a resistance mutation and when the conferred benefit is higher than the cost of cooperation, the cooperative genes hitchhike with the resistance mutation leading to apparent selection for the more numerous cooperators (Morgan *et al.* 2012, Waite & Shou 2012, Quigley *et al.* 2012). This mechanism leads to the general expectation that the initially most frequent type in mixed cultures (i.e., having the most standing variation) should produce more resistant cells. In contrast, we observed that the resistant cells in mixed cultures were significantly more associated with non-producers, irrespective of their initial frequency. This indicates

that initial conditions are not sufficient to determine the evolution of resistance, nor the outcome of competition between producers and non-producers in the presence of antibiotics. Rather, our results suggest that initial standing variation may not have played an important role in adaptation to antibiotic stress. Under high antibiotic dose in mixed cultures, the non-producers benefitted from producer siderophores and grew better not only compared to their own monocultures (as expected), but grew even faster than monocultures of producers. We hypothesise therefore that even at low initial frequencies (15%), non-producer populations either already contained antibiotic resistant mutants or generated them more rapidly than did producers. This hypothesis is also supported by our finding of resistance being higher in producer monocultures compared to non-producer monocultures (where the latter grow at a lower rate), and an additional experiment (Appendix 3) in which we found that differences in growth and antibiotic resistance between producers and non-producers are mediated by siderophore availability and production: when populations grew under high iron availability conditions (where siderophores are not needed), the frequency of resistance was similar for both strains in mixed cultures and in both monocultures.

All else being equal, the population producing the most offspring (potential mutants) per unit time should reach higher resistance frequency. Hence, as non-producers exploited producers in mixed cultures, we expected the frequency of resistant cells in non-producers to be higher in mixed cultures compared to monocultures. Our results confirmed this prediction, in particular for the highest dose of antibiotics where the frequency of resistance increased by five orders of magnitude, except when the non-producers were initially very frequent in the mixed population (75%). In this latter case, the frequency of resistance was similar to the resistance in non-producer monocultures, arguably because the producers were not frequent enough to benefit the non-producers. Applying the same logic to producers, we should expect the frequency of resistance in producers to be higher in monocultures compared to mixed cultures. Surprisingly, while the frequency of resistance was lower or not significantly different from monocultures under the low antibiotic doses, the frequency of resistant cells in producers was higher in the presence of non-producers compared to monocultures under the highest dose. We hypothesised that these producers may show particular adaptations to both non-producers and antibiotic pressure. Although our results may reflect a transient state and these producers may ultimately disappear from the population; it is important to assess whether our selection regime has resulted in highly resistant

and fit cells. In a series of additional assays (Appendix 2), we further tested the level of resistance (minimum inhibitory concentration) and associated fitness costs of resistant producers from mixed cultures and from monocultures. Unlike non-producers, producers tended to be more resistant (higher level of resistance) in mixed cultures compared to monocultures ($p < 0.05$, Fig. 1.8). Moreover, this higher resistance did not come at an increased fitness cost (Fig. 1.9). Based on the growth assays, we did not detect significant differences in pyoverdin availability nor pyoverdin production per cell between resistant colonies from mixed cultures and from monocultures. This suggests that competition with non-producers did not select for decreased siderophore production in resistant producers.

Overall, our results indicate that ecological stressors and in particular antibiotics can play an important role in public goods social evolution by affecting the interactions between cooperative producers and cheating non-producers. In turn, these social interactions feed back to the population's ecological and evolutionary responses to the stressors, affecting survival and resistance. Our results have implications for treating bacterial infections with antibiotics, whereby, in selecting for non-producers, treatments with gentamicin are expected to lower bacterial virulence (Buckling *et al.* 2007, Kirienko *et al.* 2015). We observed nonetheless that highly resistant producers also arose in mixed cultures, which opens the question of how these may coexist with cheats (and lead to more virulent infections) as is observed *in situ* (Andersen *et al.* 2015). A leading hypothesis is spatial structure (Nadell *et al.* 2010, Allen *et al.* 2013a, Kümmerli *et al.* 2014). Future theory and experiments should test this prediction.

Appendices

Appendix 1

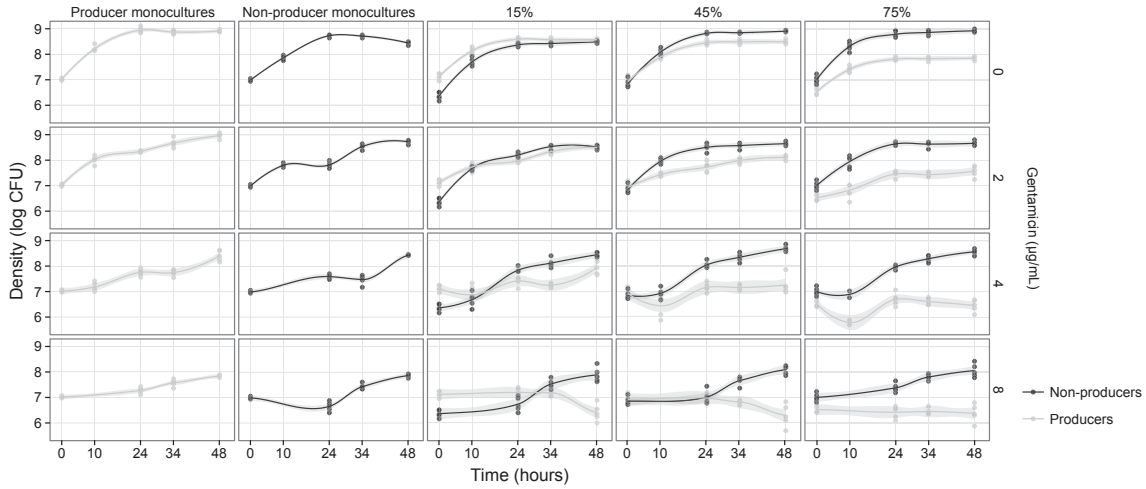


Figure 1.4: Densities of producers (grey) and non-producers (black) over the course of the experiment. Data are log-transformed. Lines are polynomial regression fitting and shaded regions are standard errors of the mean. Each row panel represents an antibiotic treatment: 0 μg/mL (first row), 2 μg/mL (second row), 4 μg/mL (third row) and 8 μg/mL of gentamicin (fourth row). The first two columns are monocultures and the last three columns are mixed cultures starting with 15%, 45% and 75% of non-producers, respectively.

Appendix 2

Repeated 48-hour experiment

We initiated 3 replicate populations of either monocultures of producers or monocultures of non-producers or mixed cultures with 15% non-producers. The experimental conditions were the same as in the main experiment. We estimated the densities (Fig. 1.6), frequencies of each bacterial type (Fig. 1.5) and proportion of resistant cells (Fig. 1.7) by plating at T_0 and T_{48}.

At the end of this 48-hour experiment, we arbitrarily chose 5 individual resistant colonies from each monoculture population, 5 resistant colonies of each bacterial type from each mixed culture, and 3 susceptible colonies of one replicate of each treatment. Additionally, 5 resistant and 5 susceptible colonies were isolated from each replicate ancestral population. The resistant colonies were isolated from KB agar plates with

10 μg/mL of gentamicin. The susceptible colonies were identified by introducing small samples of colonies onto KB agar and KB agar + 10 μg/mL gentamicin plates, simultaneously. Those that grew on the antibiotic-free plates but not on the antibiotic plates were scored as susceptible. Each isolated colony was inoculated in 200 μL of iron-limited CAA medium and incubated at 37 $^\circ$C overnight under constant orbital shaking (700 rpm) and then frozen in 20% glycerol at -80 $^\circ$C. These frozen stocks we then used in assays to evaluate resistance to gentamicin, population growth, and pyoverdin production.

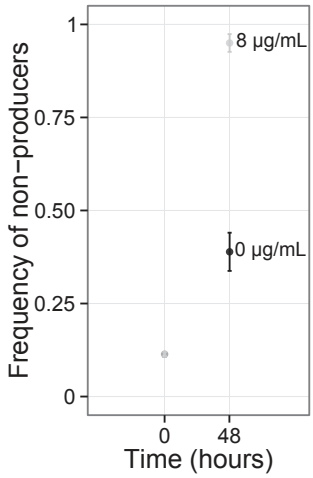


Figure 1.5: Change in non-producer frequencies between T_0 and T_{48} in antibiotic-free medium (black) and antibiotic-supplemented medium (8 μg/mL, grey). Bars are standard errors of the mean.

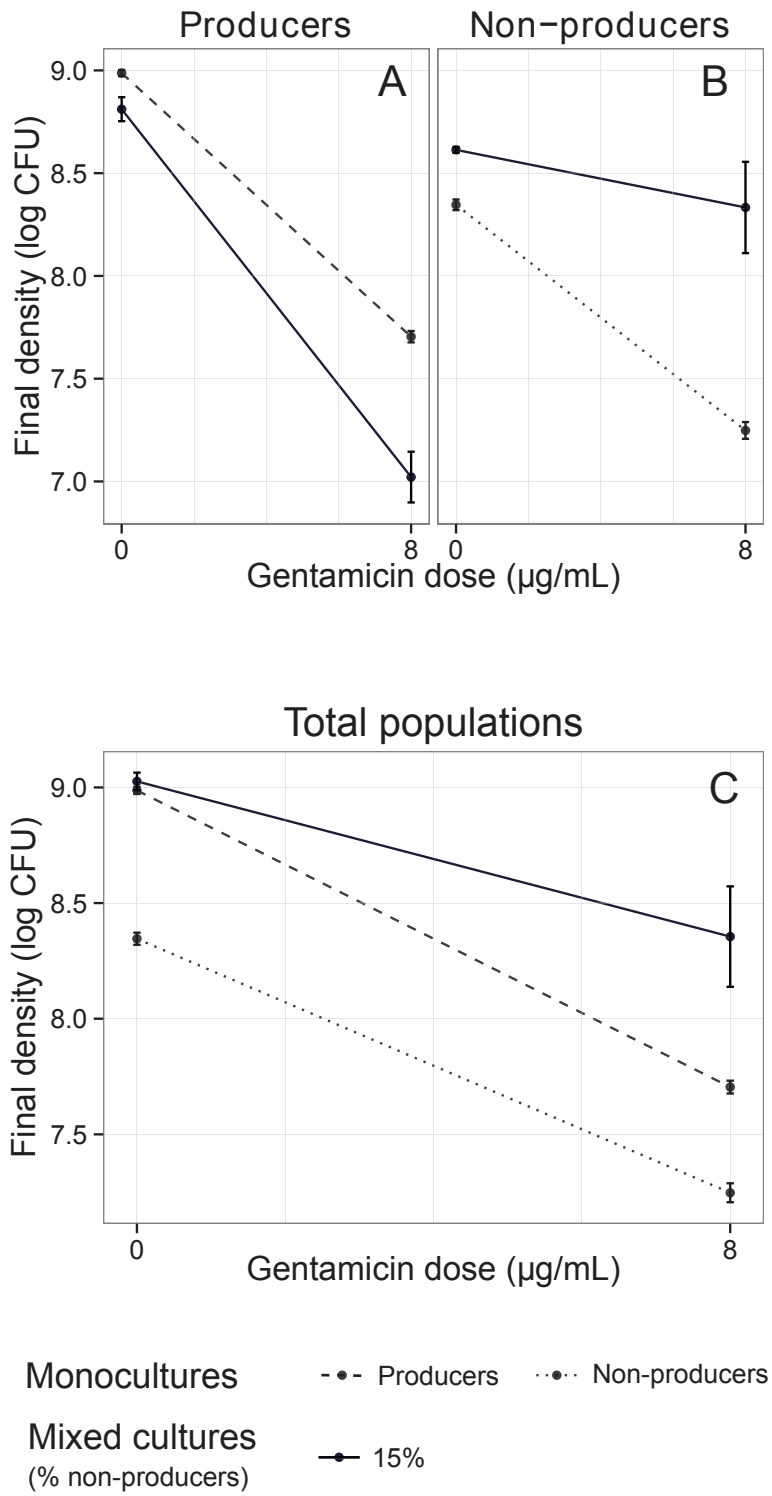


Figure 1.6: Final density of producers (A), non-producers (B), and total populations (C) depending on gentamicin dose and the initial non-producer frequency in the repeated competition experiment. The first two panels (A and B) present the final density of each clone in monocultures (dashed line=producers, dotted line=non-producers) and in mixed cultures (solid line). Panel C shows the final density of the total populations of monocultures and mixed cultures. Data are log-transformed CFUs and bars are standard errors of the mean.

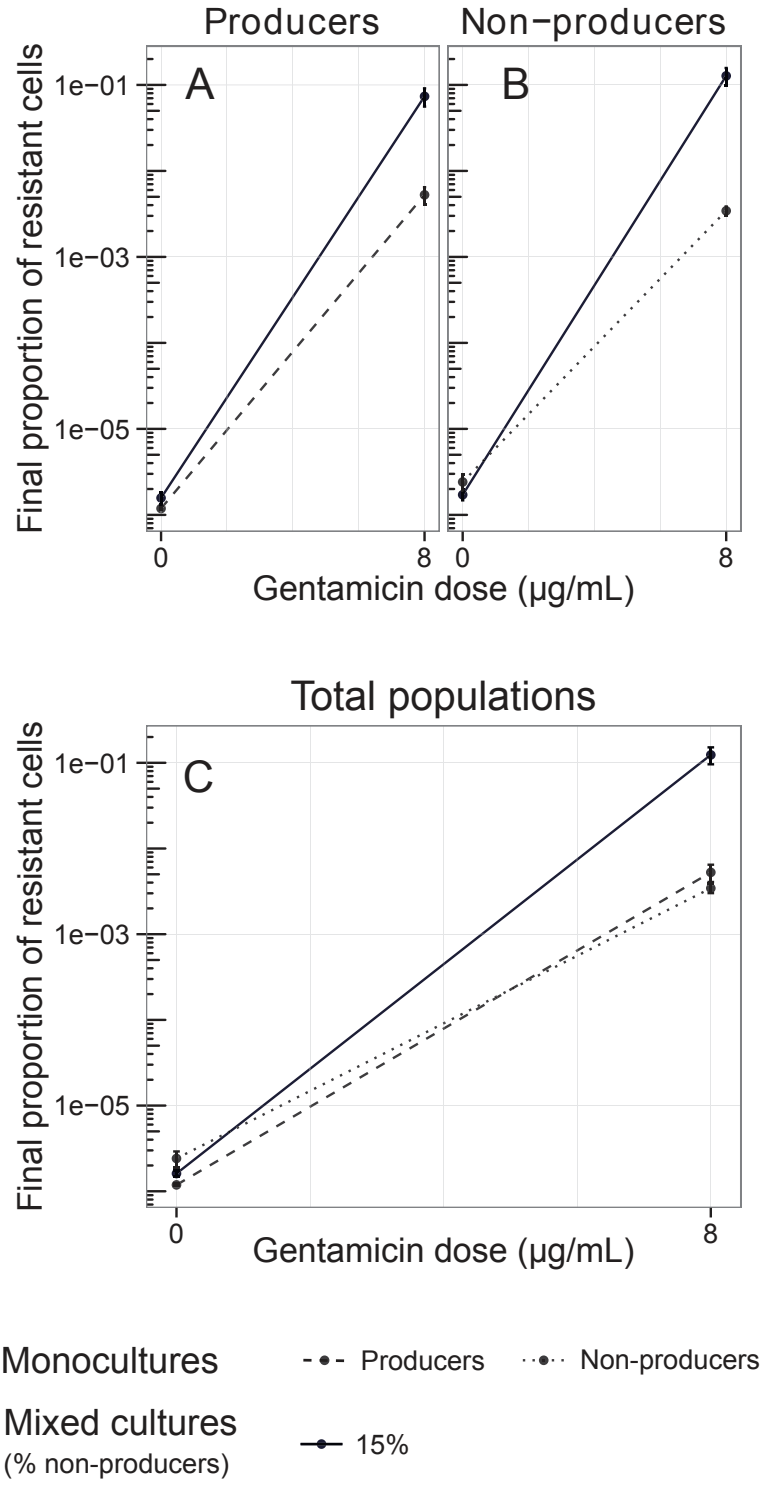


Figure 1.7: [Upper panel] Final proportion of resistant cells in producers (A) and in non-producers (B) in monocultures (dashed or dotted line, respectively) and in mixed cultures (solid line) depending on gentamicin dose, in the repeated competition experiment. [Lower panel] Final proportion of resistant cells in total populations of monocultures and mixed cultures (C). Bars are standard errors of the mean.

Level of resistance to gentamicin

The level of resistance to gentamicin was measured as the Minimum Inhibitory Concentration (MIC) at which no bacterial growth is detected. A sample of frozen stock from each previously isolated colony was inoculated with a sterile loop in 200 μL of iron-limited CAA medium and incubated at 37 $^\circ$C overnight under constant orbital shaking (700 rpm). All populations were then adjusted to an OD of 0.1 and 10 μL of each population was inoculated into 200 μL of iron-limited CAA medium with the antibiotic at two-fold increments (tested concentrations: 0, 2, 4, 8, 16 and 32 μg/mL gentamicin). After 24 hours of incubation at 37 $^\circ$C under constant shaking (700 rpm), we recorded the OD of each population using a spectrophotometer (ClarioSTAR microplate reader, BMG Lab Technologies) and scored growth inhibition as OD < 0.1. We assigned a rank value of MIC to each colony as follows: MIC of 2 μg/mL = rank 1, MIC of 4 μg/mL = rank 2, MIC of 8 μg/mL = rank 3, MIC of 16 μg/mL = rank 4, MIC of 32 μg/mL = rank 5 (Fig. 1.8).

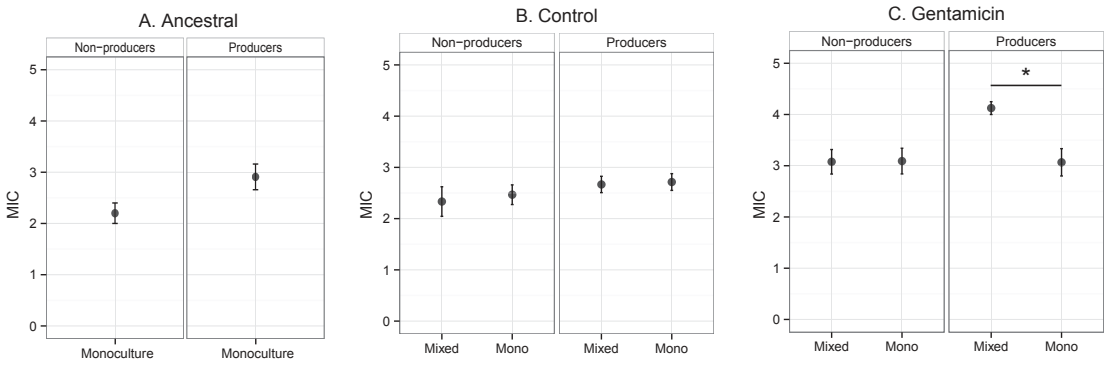


Figure 1.8: Minimum inhibitory concentration (MIC) of individual colonies of producers and non-producers, isolated from monocultures and mixed cultures. (A) Ancestral colonies, (B) colonies isolated from the evolved control populations, i.e. the populations that grew without gentamicin, (C) colonies isolated from the evolved treated populations, i.e. those growing with 8 μg/mL gentamicin. Data are ranked values of MIC and bars are standard errors of the mean. Stars indicate a significant difference between ranked values of MIC ($p < 0.05$).

Growth and pyoverdin production

We inoculated a loop of each frozen stock into 200 μL of iron-limited CAA medium and incubated the cultures at 37 $^\circ$C overnight under constant orbital shaking (700 rpm).

We adjusted all overnight populations to an OD of 0.1 with minimum salt solution (M9) and inoculated 10 μL of each population into an antibiotic-free iron-limited CAA medium and an iron-limited CAA supplemented with 8 μg/mL of gentamicin in clear-bottom black 96-well plates. Optical density (OD, emission: 600 nm) and fluorescence (relative fluorescence unit RFU, excitation: 400 nm, emission: 460 nm) were measured every 30 minutes using a spectrophotometer for 24 hours under constant shaking (700 rpm) at 37 $^\circ$ C.

Growth

We estimated the growth of each colony as the area under the curve (AUC) of OD over time in antibiotic-free and antibiotic-supplemented media (Fig. 1.9 and 1.10, respectively).

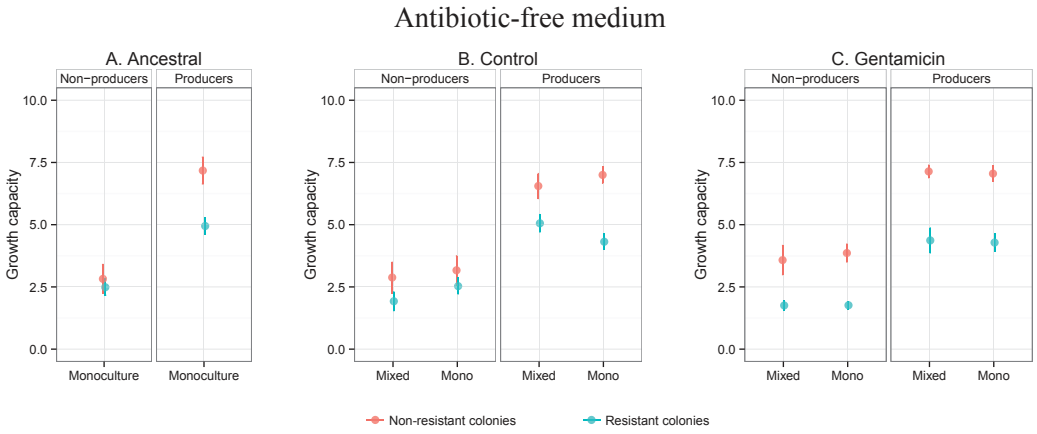


Figure 1.9: Growth in antibiotic-free medium of individual resistant (blue) and non-resistant (red) colonies of producers and non-producers, isolated from monocultures and mixed cultures. (A) Ancestral colonies, (B) colonies isolated from the evolved control populations, i.e. the populations that grew without gentamicin, (C) colonies isolated from the evolved treated populations, i.e. the populations that grew with 8 μg/mL gentamicin. Data are areas under the curves of OD_{600} over time and bars are standard errors of the mean.

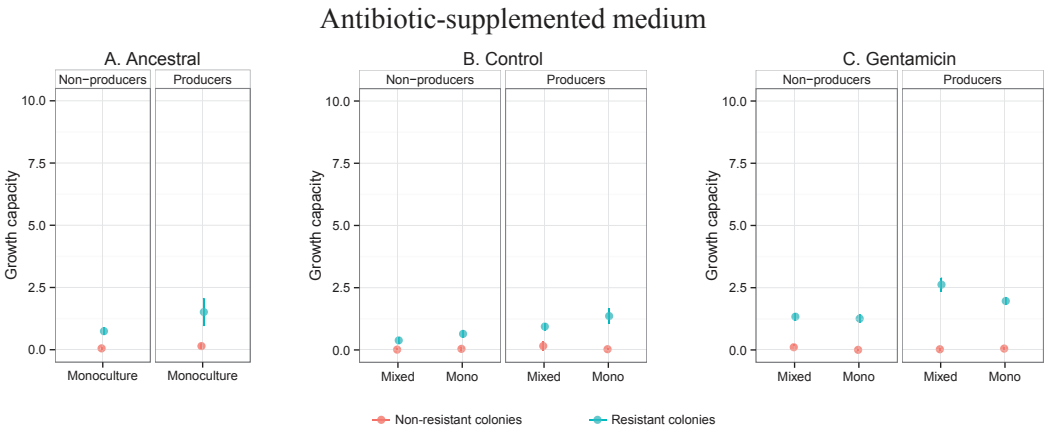


Figure 1.10: Growth in antibiotic-supplemented medium (8 μg/mL) of individual resistant (blue) and non-resistant (red) colonies of producers and non-producers, isolated from monocultures and mixed cultures. (A) Ancestral colonies, (B) colonies isolated from the evolved control populations, i.e. the populations that grew without gentamicin, (C) colonies isolated from the evolved treated populations, i.e. the populations that grew with 8 μg/mL gentamicin. Data are areas under the curves of OD_{600} over time and bars are standard errors of the mean.

Pyoverdin availability and production per producer cell

We calculated the pyoverdin availability per cell at each time point as the ratio of fluorescence to OD (RFU_t/OD_t, Fig. 1.11), and the rate of pyoverdin production as the ratio in the change in fluorescence to OD ($RFU_{t+1} - RFU_t)/OD_t$ (Fig. 1.12) (Ghoul 2014). We excluded the data for the first 5 hours, since the OD values were under the detection threshold of the spectrophotometer.

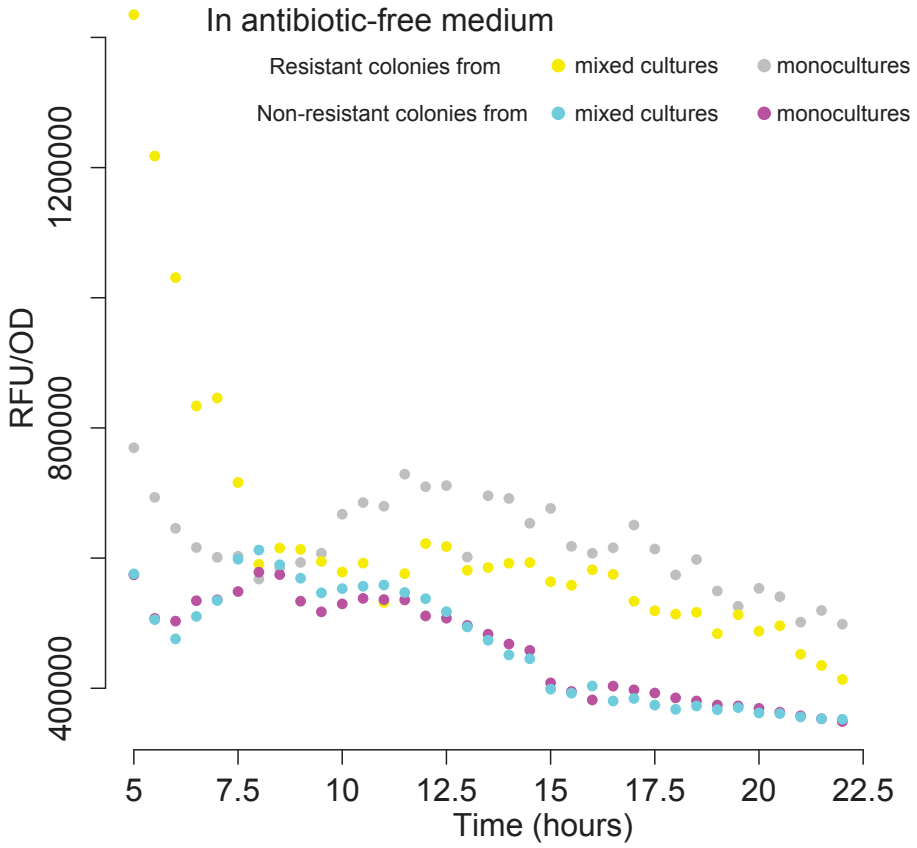


Figure 1.11: Siderophore availability per producer cell in antibiotic-free medium over a 23-hour growth assay. Data are the ratio of fluorescence (relative fluorescent unit RFU) to optical density (OD), averaged for 8 to 15 colonies isolated from monocultures (grey and pink) and mixed cultures (yellow and light blue), resistant (yellow and grey) and non-resistant (light blue and pink) to gentamicin.

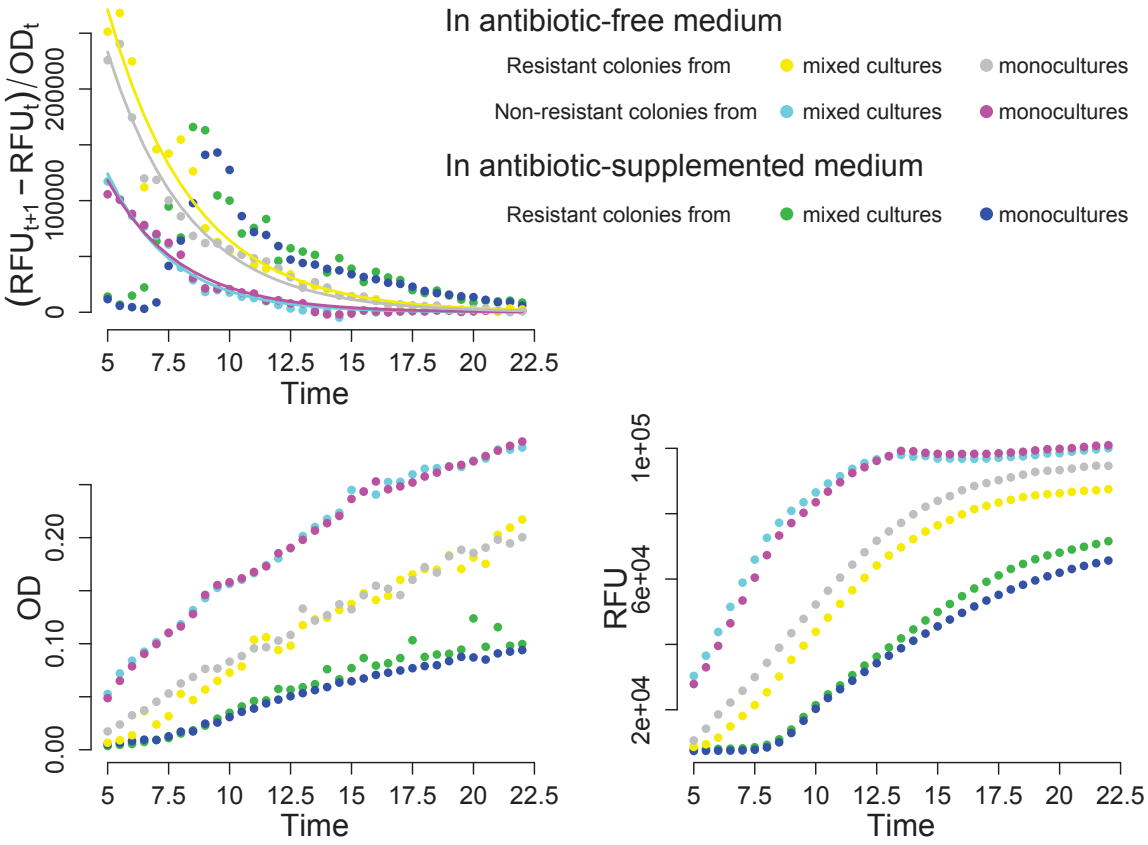


Figure 1.12: Siderophore (relative fluorescent unit, RFU) and growth (optical density, OD) data from a 23-hour growth assay in antibiotic-free (yellow, grey, light blue and pink) and antibiotic-supplemented (dark blue and green) media. Data are averaged for 8 to 15 colonies isolated from monocultures (grey and pink) and mixed cultures (yellow and light blue), resistant (yellow and grey, green and dark blue) and non-resistant (light blue and pink) to gentamicin. [Upper panel] Siderophore production per producer cell estimated as the ratio of the gradient of fluorescence (RFU_{t+1} - RFU_t) to optical density (OD). [Lower panel] Growth (OD, left panel) and fluorescence (RFU, right panel).

Appendix 3

48-hour experiment under iron-rich conditions

To assess the impact of the exploitation of the producers by the non-producers in mixed cultures on the change in the proportion of resistant cells, we repeated the 48-hour experiment under iron-rich conditions. Under these conditions bacteria do not rely on siderophore production as iron is directly available.

A pilot experiment showed that adding 8 μg/mL of gentamicin in the iron-rich medium led to the extinction of all non-producer populations and approximately one-third

of the producer populations. We determined that under these experimental conditions, any concentration above 6 μg/mL prevents sensitive *P. aeruginosa* growth. To limit the probability of population extinctions we therefore used gentamicin at a concentration of 5 μg/mL.

Three independent replicate colonies of both strains were inoculated in 6 mL of CAA in 30 mL Thermo-Fisher microcosms and incubated overnight under constant orbital shaking (200 rpm) at 37 $^\circ$C, before being used as inoculum for the 48-hour experiment. We initiated populations of either monocultures of producers or of non-producers, or mixed cultures (c 15% of non-producers initially) to a final density of c 10^7 bacteria per mL into 800 μL of CAA medium supplemented with 30 μM Fe(III)Cl_3 (ferric chloride, Sigma-Aldrich).

Each replicate population was then inoculated in an antibiotic-free control and in an antibiotic treatment containing 5 μg/mL of gentamicin. The 48-well plates were incubated at 37 $^\circ$C for 48 hours under constant shaking (350 rpm). We plated serial dilutions onto KB agar and KB agar + 6 μg/mL gentamicin plates at T_0 and T_{48} of each population to estimate the densities and the frequencies of each bacterial type, and proportion of resistant cells.

Appendix 4

Estimation of relative fitness

We assessed the effect of antibiotics on the relative fitness of non-producers as follows.

Classically, relative fitness is calculated as $w = 1 + s$ where s is the selection coefficient (Fisher 1930, Chevin 2011). When there is no change in frequency, the selection coefficient is null and the relative fitness is 1. The selection coefficient s describes the rate of change in the relative frequency of one strain compared to the other and is calculated as $s = d/dt(\ln(p/(1-p)))$, with p the proportion of the focal strain. This metric assumes that the frequencies change at a constant rate during the timeframe used to calculate s. In such a case, $\ln(p/(1-p))$ is linear over time (with slope s) for a given range of x values ($0.2 < x < 0.8$) (Fig. 1.13).

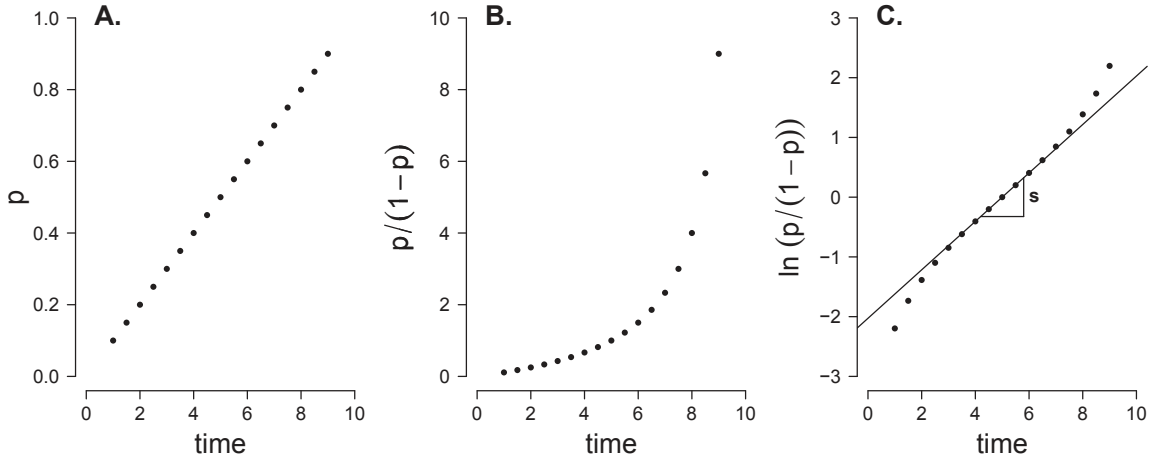


Figure 1.13: Hypothetical constant rate of changes in frequencies. (A) Change in the frequency (p) of the focal strain over time. (B) Relative change in the frequency of the focal strain compared to the competitive strain over time. (C) Linearization of the relative change in frequency over time and visualisation of the selection coefficient s (i.e., the slope).

However, if the frequencies do not change at a constant rate, then $\ln(p/(1-p))$ will be non-linear and the resulting calculation of s inaccurate (Fig. 1.14). This could occur when competition is frequency-dependent. For instance, in public goods scenarios such as that studied here, non-producer mutants have a greater fitness advantage when rare (Ross-Gillespie *et al.* 2009).

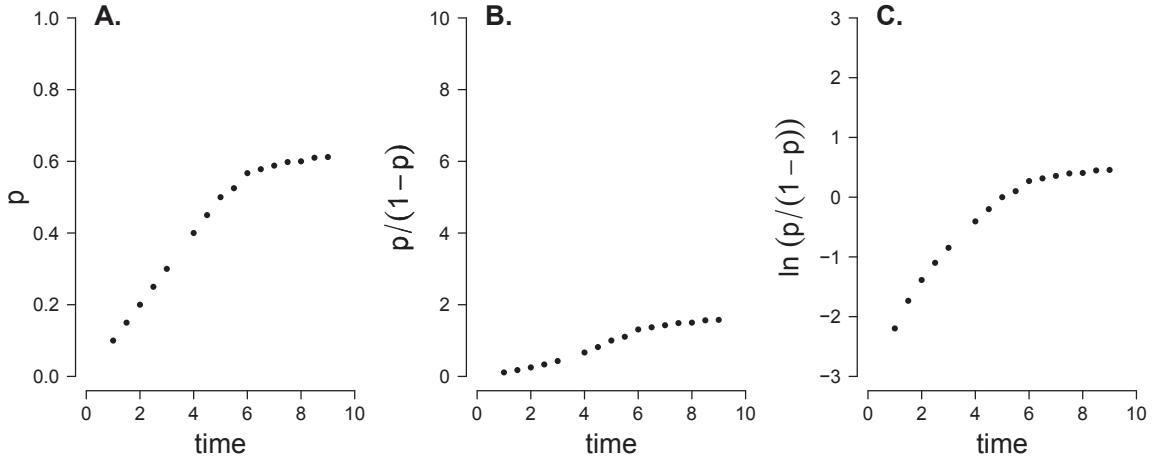
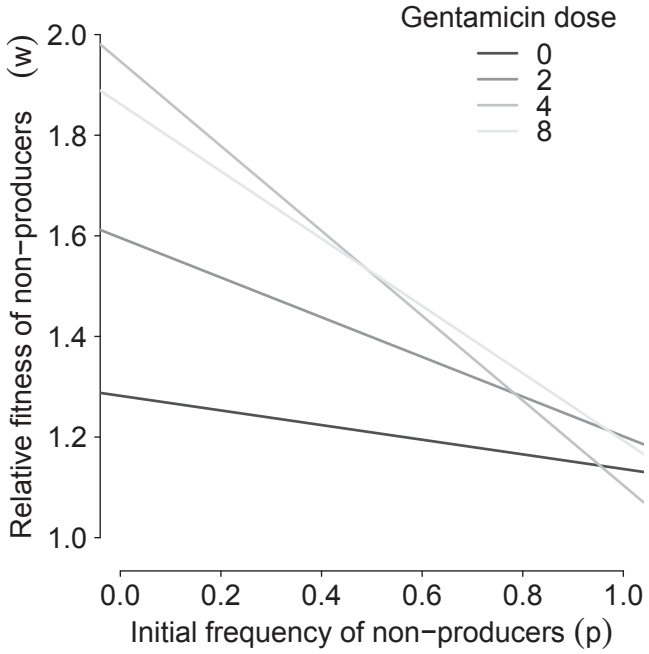


Figure 1.14: Hypothetical non-constant rate of changes in frequencies. (A) Change in the frequency (p) of the focal strain over time. (B) Relative change in the frequency of the focal strain compared to the competitive strain over time. (C) The logarithm of the relative change in frequency over time becomes non-linear.

Ribeck & Lenski (2015) recently addressed this issue and proposed an alternative method to calculate relative fitness, taking frequency dependence into account. They defined frequency-dependent relative fitness as $w(p) = 1 + s(1 - mp)$, where m is frequency-dependent selection term, and the slope ms describes the strength of frequency dependence. Expressing m and s according to initial and final relative frequencies, p_0 and p_f respectively, yields the following equation:

$$s = \frac{1}{(1-m)t}\left(\ln\left[\frac{p_f}{1-p_f}\frac{1-p_0}{p_0}\right] + m\ln\left[\frac{p_0}{1-mp_0}\frac{1-mp_f}{p_f}\right]\right)$$

where t is the number of generations. We used this equation to calculate the theoretical values of p_f for a range of p_0. Then, we employed Mathematica (Wolfram 10; code provided by N. Ribeck) to identify the values of m and s minimizing the mean square differences between the theoretical values and our empirical data (Fig. 1.15).



Gentamicin dose (μg/mL)	m	s
0	0.516	0.282
2	0.662	0.596
4	0.89	0.947
8	0.776	0.862

Figure 1.15: Frequency-dependent relative fitness of non-producers $w(p) = 1 + s(1 - mp)$ for different antibiotic doses and estimated values of m and s.

This method produces estimates of the selection coefficient and the strength and sign of frequency dependence (Fig. 1.15). However, in our experiment, antibiotic-induced mortality (Fig. 1.4) results in biases in the estimates of generation number (t), meaning that the method cannot accurately estimate relative fitness.

Given these difficulties, we took on an alternative approach to understand the fitness of a given strain compared to the fitness of the competing strain (i.e. its relative fitness) and represented (i) the dynamics of each strain when grown in mixed cultures (absolute fitness, Fig. 1.4) and (ii) how their absolute fitnesses translate into changes in population composition (changes in frequency, Fig. 1.1).

Moreover, in collaboration with Rob Noble, we are currently developing an alternative method to describe relative fitness with one metric, taking death into account. This is still ongoing work.

2

Environmental impacts on bacterial social dynamics: a modelling study of pyoverdin cooperation in antibiotic environments

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(*in preparation*) *authors contributed equally to this work

Introduction

In Chapter 1, we experimentally examined the interplay between antibiotic effects and siderophore production and cheating in *P. aeruginosa*. In particular, we investigated (i) the effects of different doses of the antibiotic gentamicin on the interactions between producers and non-producers and (ii) how siderophore cooperation affects antibiotic resistance. Briefly, we initiated monocultures and mixed cultures of producers and non-producers in well-mixed environments supplemented with 0, 2, 4 or 8 μg/mL gentamicin. We followed the densities and relative frequencies of producers and non-producers by plating samples of each population at the beginning of the experiment and after 10, 24, 34 and 48 hours. We estimated the frequency of resistant cells at the beginning and at the end of the experiment. Moreover, we repeated the experiment and isolated single colonies, resistant and susceptible to the antibiotic, from the evolved populations. These colonies were used in additional assays to estimate the cost of resistance, pyoverdin production and level of resistance.

We found two main results: (i) antibiotics increased the frequency of non-producers in mixed cultures in a dose-dependent manner and (ii) the frequency of resistant cells was higher in mixed cultures than in either monoculture. We hypothesised that the cost of pyoverdin production reduced the capacity of producers to cope with antibiotics, as they may have fewer metabolic resources available to invest in countering antibiotic stress.

Here, we examine this hypothesis and analyse the interactions between the effects of antibiotics and pyoverdin production and cheating using mathematical modelling. We show that a simple model assuming that producers are more affected by antibiotics than non-producers can explain the dynamics of relative frequencies in the different antibiotic environments. This model is then extended, by adding pyoverdin dynamics, to describe the growth of antibiotic resistance.

Effects of antibiotics on the interactions between producers and non-producers

We aim to understand how different doses of antibiotics affect non-producer frequencies over time. In Chapter 1, we found that the frequencies of non-producers increased more in the presence of antibiotics than in antibiotic-free controls (Fig. 2.1). Moreover, the dose of antibiotics affected the rates of change in frequencies: for the two lower doses (2 and 4 μg/mL), non-producer frequencies greatly increased during the first 24 hours before reaching a peak, whereas for the highest dose, this increase occurred during the second 24 hours. The changes in non-producer frequencies in the different antibiotic environments were qualitatively similar for the three initial frequencies of non-producers (0.15, 0.45 and 0.75).

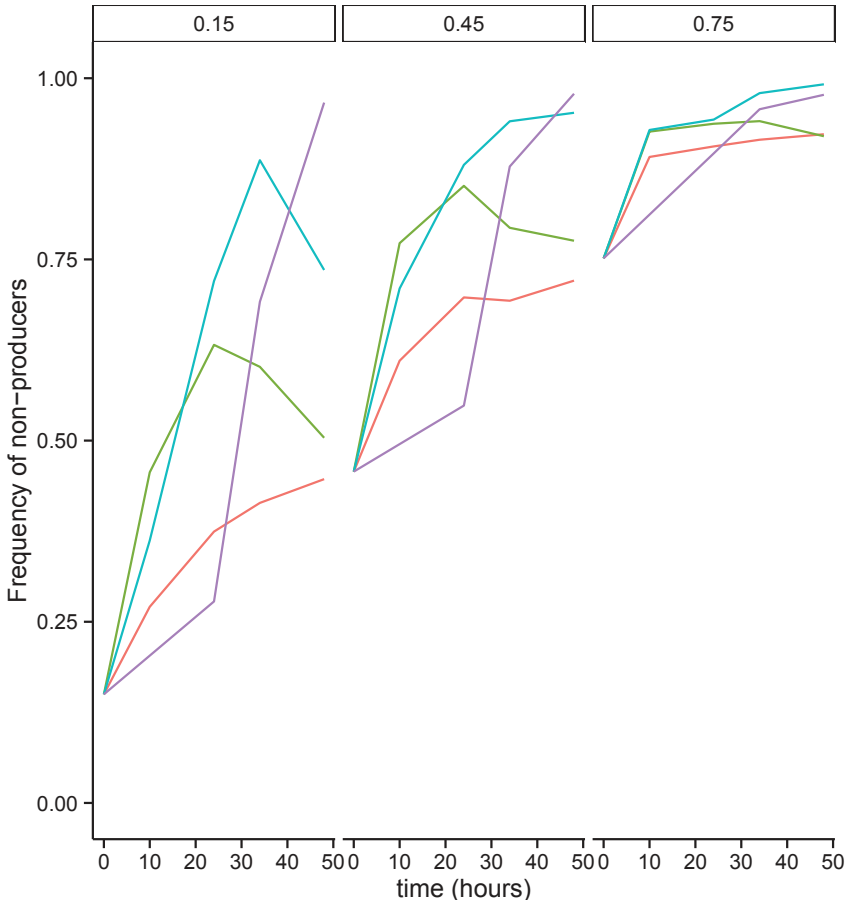

Figure 2.1: Experimental data from the competition experiments (Chapter 1). Change in non-producer frequencies between T_0 and T_{48}. The three panels correspond to the different initial frequencies of non-producers (15%, 45% and 75%). Colours are gentamicin doses (red = 0 μg/mL, green = 2 μg/mL, blue = 4 μg/mL, purple = 8 μg/mL).

Model 1

To investigate the effect of antibiotics on the dynamics of non-producer frequency, we consider a population containing four types of bacteria: producers (C = cooperators) and non-producers (D = defectors), each either completely resistant or susceptible to antibiotics.

We build the model based on five assumptions:

1. Pyoverdin dynamics can be neglected.

In a well-mixed environment, the absolute pyoverdin benefit for producers and non-producers should be equal. Moreover, the experimental results suggest that pyoverdin concentration does not substantially affect bacterial dynamics since the qualitative outcome of competition between producers and non-producers is not frequency dependent (Fig. 2.1).

2. Growth is density-dependent.

In our model, we assume logistic population growth.

3. Producers pay a fitness cost for producing pyoverdin, which affects the growth rate but not carrying capacity.

Our experimental data show that producers initially grew more slowly than non-producers in mixed cultures, but may reach the same carrying capacity (Fig. 1.4 in Chapter 1).

4. The main effect of the antibiotic is to slow reproduction.

Gentamicin is classified as a bacteriocide, but its killing effect is concentration-dependent and low doses may be bacteriostatic (Tam *et al.* 2006). As an approximation, we therefore consider that antibiotics modify the growth rate and the carrying capacity and assume no death.

5. The cost of antibiotic resistance affects the growth rate but not carrying capacity.

The experimental results (Fig. 2.12) suggest that carrying capacity should also be affected but the effect is negligible.

A model based on these five assumptions correctly predicts an increase in non-producer frequency over time for all antibiotic treatments. If we were to assume that

the only difference between producers and non-producers is the cost of pyoverdin production, the relative fitness of non-producers (estimated as the ratio of non-producer to producer growth rates) would be $1/(1-c)$, where c is the pyoverdin production cost. This relative fitness is greater than 1, meaning that the frequency of non-producers should increase. However, this model cannot explain why non-producer frequency actually increased more with low doses of antibiotics compared to the highest dose, nor the delay for the highest dose (Fig. 2.2). Indeed, because we assume that the antibiotic slows the reproduction rate (assumption 4) and thus lengthens the generation time, the non-producer frequency is predicted to increase more slowly in the presence of antibiotics compared to antibiotic-free controls (Fig. 2.2). In contrast, our experimental data showed that non-producer frequencies increased more rapidly under antibiotic treatments (Fig. 2.1).

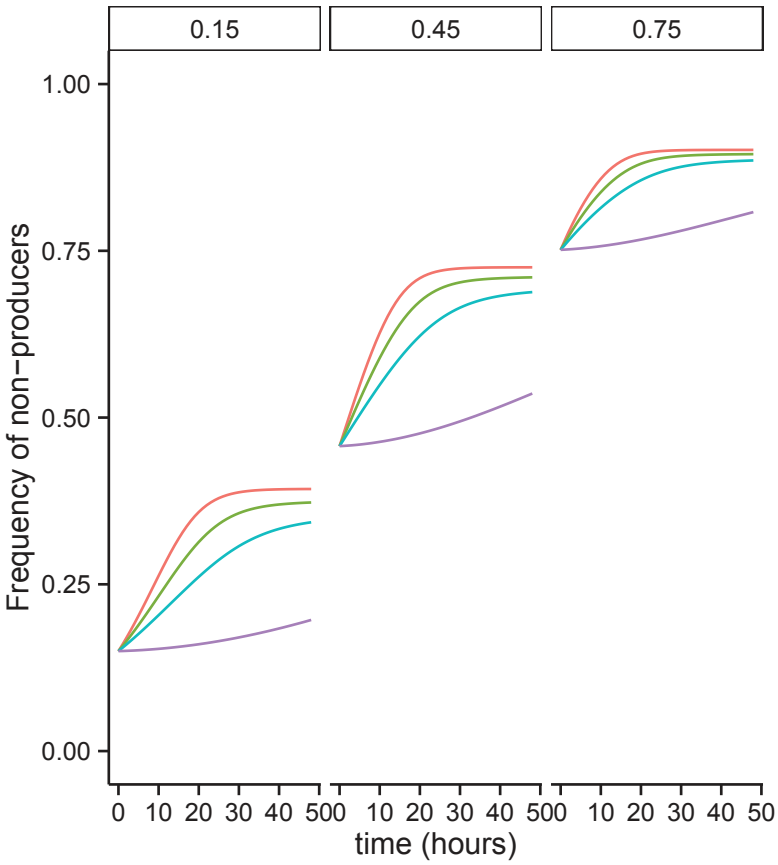

Figure 2.2: Change in non-producer frequencies for the model with equal antibiotic effects fitted to the experimental competition data. The three panels correspond to the different initial frequencies of non-producers (15%, 45% and 75%). Colours are gentamicin doses (red = 0 μg/mL, green = 2 μg/mL, blue = 4 μg/mL, purple = 8 μg/mL). Parameter values (rounded to two decimal places) are $b = 0.26$; $K = 4.7 \times 10^8$; $c = 0.3$; $c_R = 0.08$; $\alpha_1 = 0.12$; $\alpha_2 = 0.02$; $\mu = 0.004$; $\delta = 0$; $A = 11$.

Given these elements, we made two additional assumptions:

6. The antibiotic affects producers more than non-producers, and this effect is more pronounced at low antibiotic doses.

This relies on our hypothesis that the cost of pyoverdin production reduces the capacity of the producers to cope with antibiotics (Chapter 1). This assumption also accounts for how antibiotic dose affects changes in non-producer frequencies: at low doses, non-producer frequency increased more than in antibiotic-free controls, whereas this increase is initially lower than the control for the highest dose. The simplest way to implement this assumption is to make the difference in antibiotic effect between producers and non-producers decrease linearly with antibiotic dose (without changing

sign).

7. The effect of the antibiotic decreases over time.

This assumption can explain the observation that the changes in frequency under the highest dose of gentamicin, whereby the advantage to non-producers is initially reduced under the highest antibiotic dose compared to the control, but then increases after 24 hours. If the effect of the antibiotic decreases over time, after 24 hours the effect of the highest dose may be similar to lower doses and explain the rapid increase in non-producer frequency. A decrease in antibiotic effect may result from drug degradation, from bacterial adaptation, and/or from the accumulation of protective compounds (such as degrading enzymes). We formalised this assumption as an exponential decrease in the effective antibiotic concentration.

Model description

Model 1 is thus described by the following set of equations (Equations 2.1):

$$\begin{aligned}
\frac{\mathrm{d}p_C}{\mathrm{d}t} &= b\,(1-c)\left(1-\frac{n}{K}-\alpha_1 a\right)p_C,\\
\frac{\mathrm{d}q_C}{\mathrm{d}t} &= b\,(1-c-c_R)\left(1-\frac{n}{K}-\alpha_2 a\right)q_C,\\
\frac{\mathrm{d}p_D}{\mathrm{d}t} &= b\left(1-\frac{n}{K}-\alpha_1\phi_a a\right)p_D,\\
\frac{\mathrm{d}q_D}{\mathrm{d}t} &= b\,(1-c_R)\left(1-\frac{n}{K}-\alpha_2 a\right)q_D,\\
\frac{\mathrm{d}a}{\mathrm{d}t} &= -\mu a,
\end{aligned} \tag{2.1}$$

where p_C is the density of *susceptible* producers and q_C is the density of *resistant* producers. The subscript D indicates the same parameters, but for non-producers. The difference between the antibiotic effects on producers and non-producers is:

$$\phi_a = 1-\delta(1-a/A). \tag{2.2}$$

The variables and parameters are defined in Table 2.1.

Table 2.1: Variables and parameters of bacterial dynamics.

	Symbol	Definition
Variable	p_C	density of susceptible producers
	p_D	density of susceptible non-producers
	q_C	density of resistant producers
	q_D	density of resistant non-producers
	n	density of the total population
	a	effective antibiotic concentration
Parameter	b	baseline reproduction rate
	n_0	initial density of the total population
	K	baseline carrying capacity
	c	cost of pyoverdin production
	α_1	antibiotic effect on susceptible bacteria
	α_2	antibiotic effect on resistant bacteria
	δ	maximum relative reduction of antibiotic effect on non-producers
	c_R	cost of resistance to the antibiotic
	a_{max}	maximum antibiotic concentration used in the experiments (8 μg/mL)
	A	concentration at which the antibiotic affects producers and non-producers equally
	μ	rate of change in antibiotic effect

Model analysis

If the only effect of the antibiotic is to slow reproduction (assumption 5), then:

$$\alpha_i < \frac{1}{a_{max}} \left(1 - \frac{n_0}{K}\right).$$

If the antibiotic always has a lower effect on non-producers than on producers (assumption 6), then we must also assume $A > a_{max}$.

We calculate the relative fitness of non-producers by dividing their growth rate by the growth rate of producers:

$$w_D = \frac{\frac{dp_D}{dt}}{\frac{dp_C}{dt}} \frac{p_C}{p_D}. \tag{2.3}$$

Since the frequency of resistant cells is very low in all experimental populations (7% for the maximum resistance frequency, and << 1% for most populations), we only analyse the relative fitness of susceptible cells and ignore resistant ones.

If the population size is low relative to carrying capacity ($n << K$), then the relative fitness of non-producers is approximately:

$$\frac{1 - \alpha_1 \phi_a a}{(1 - c)(1 - \alpha_1 a)}. \tag{2.4}$$

To study how the relative fitness of non-producers varies with antibiotic concentration, we differentiate this expression with respect to a and find that the gradient of the relative fitness function changes sign when

$$a = \frac{1 \pm \sqrt{1 - \alpha_1 A}}{\alpha_1}.$$

It follows that if A is not much greater than a_{max} and α_1 not much smaller than $1/a_{max}$, then either the gradient is positive for all $a < a_{max}$ or the gradient becomes negative only when a is slightly less than a_{max}. Therefore, non-producer relative fitness is expected to increase with antibiotic concentration across all (or almost all) the range of concentrations used in the experiments (Fig. 2.3A).

To analyse the effect of antibiotics on the generation length, we use the ratio of the generation length in the presence of antibiotics to the generation length in their absence. When population size is low relative to carrying capacity ($n << K$), the relative generation length of non-producers is:

$$\frac{1}{1-\alpha_1\phi_a a}, \tag{2.5}$$

and relative generation length of producers is:

$$\frac{1}{1-\alpha_1 a}. \tag{2.6}$$

Therefore, antibiotics lengthen the time between divisions and, like the relative fitness of non-producers, the generation length increases superlinearly (i.e. faster than linearly) with antibiotic concentration (Fig. 2.3B).

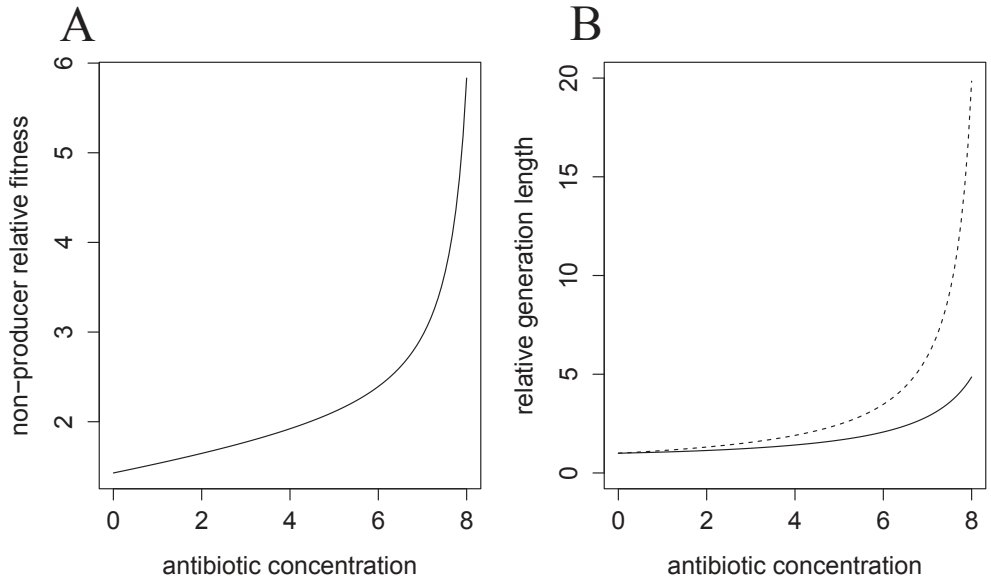

Figure 2.3: Antibiotic effects at the small-population limit fitted to experimental competition data in Model 1 (different antibiotic effects without siderophore dynamics). (A) Relative fitness of non-producers (Equation 2.4). (B) Generation length of producers (dashed line) and non-producers (solid line) relative to generation length in the absence of antibiotics (Equations 2.5 and 2.6).

Fitting method

The model was fitted using MCMC, implemented by the WBDiff package in WinBUGS (http://winbugs-development.mrc-bsu.cam.ac.uk/wbdiff.html).

Results

In this Model 1, when the population size is small compared to the carrying capacity, the relative fitness of non-producers generally increases with antibiotic concentration (Fig. 2.3A). The frequency dynamics, however, depend not only on the relative fitness but also on generation length, which also increases with antibiotic concentration (Fig. 2.3B). Therefore, the combination of these two opposing effects might be able to explain the observed dynamics.

When the model is fitted to the experimental population data (Fig. 2.4), the resulting frequency dynamics are indeed qualitatively consistent with observations (Fig. 2.5). Non-producer frequency is always higher at low or intermediate antibiotic concentrations than in the antibiotic-free controls. At higher antibiotic concentrations, non-producer frequency is initially lower than in the absence of antibiotic, but later increases. This pattern results from the antibiotic effects on both non-producer relative fitness and generation length (Fig. 2.3), such that the former effect is dominant at low antibiotic concentrations, and the latter dominates at higher concentrations. When the dose is low, the relative fitness of non-producers increases and the effect of antibiotics on the generation length is relatively small, such that non-producer frequency increases faster than in controls. However, at high doses the relative fitness of non-producers also increases, but the generation length is considerably longer so that the increase in non-producer frequency is slower than in controls. Because the antibiotic effect decreases over time (assumption 7), non-producer relative fitness under high doses eventually increases, becoming pronounced, after 24 hours.

According to the estimated parameter values, the antibiotic affects non-producers approximately 50% less at concentration 2 μg/mL, 40% less at 4 μg/mL, and 20% less at 8 μg/mL (Equation 2.2). In the model, the effective antibiotic concentration declines approximately 16% over the course of the experiment.

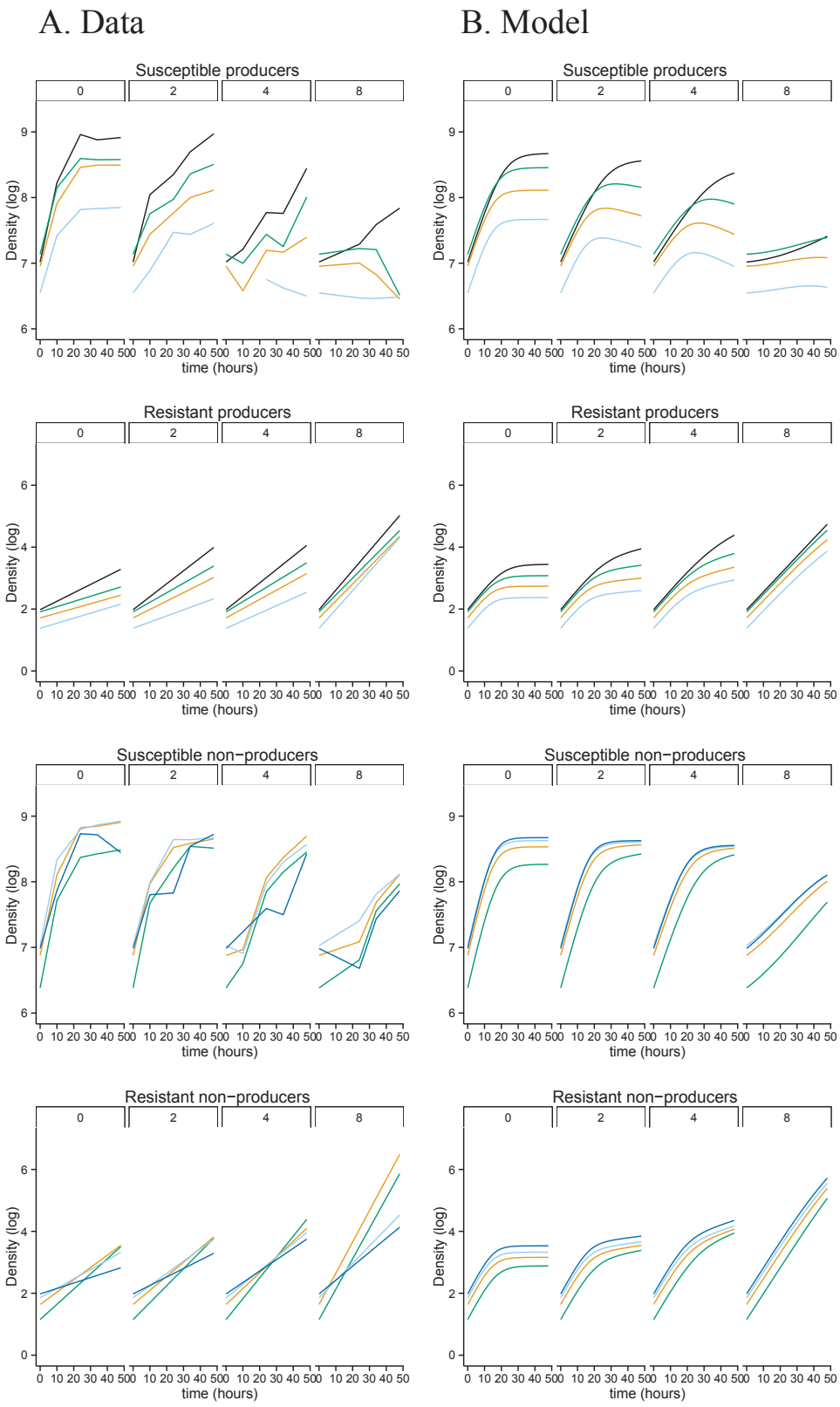

Figure 2.4: (A) Experimental data and (B) results fitted with Model 1 (different antibiotic effects without pyoverdin dynamics). Colours indicate initial non-producer frequencies (black = 0%, green = 15%, orange = 45%, light blue = 75%, dark blue = 100%). Parameter values (rounded to two decimal places) are $b = 0.26$; $K = 4.7 \times 10^8$; $c = 0.3$; $c_R = 0.08$; $\alpha_1 = 0.12$; $\alpha_2 = 0.02$; $\mu = 0.004$; $\delta = 0.6$; $A = 11$.

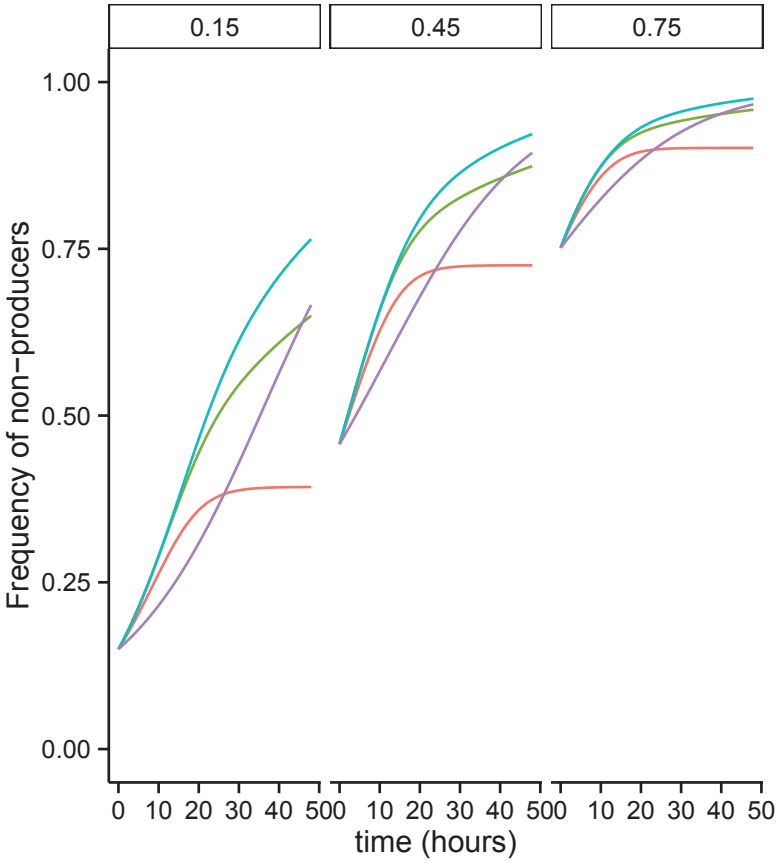

Figure 2.5: Change in non-producer frequencies for Model 1 (different antibiotic effects without pyoverdin dynamics) fitted to the experimental competition data. The three panels correspond to the different initial frequencies of non-producers (15%, 45% and 75%). Colours are gentamicin doses (red = 0 μg/mL, green = 2 μg/mL, blue = 4 μg/mL, purple = 8 μg/mL). Parameter values (rounded to two decimal places) are $b = 0.26$; $K = 4.7 \times 10^8$; $c = 0.3$; $c_R = 0.08$; $\alpha_1 = 0.12$; $\alpha_2 = 0.02$; $\mu = 0.004$; $\delta = 0.6$; $A = 11$.

Effects of initial non-producer frequency on antibiotic resistance

We then investigated the effects of the initial frequency of non-producers (and antibiotic dose) on the frequency of resistant cells in evolved populations. The experimental results show that, for the highest dose, the frequency of resistant cells is higher in mixed cultures compared to either monoculture, except when the non-producers initially represented 75% of the population (Fig. 2.6). Using the model of the previous section, however, we observe the opposite outcome (Fig. 2.7). We hypothesised that

non-producers reach higher frequencies of resistance in mixed cultures than in monocultures due to the presence of producers. In mixed cultures, non-producers benefit from pyoverdin availability without paying the cost of production so they grow faster and can better cope with the presence of antibiotics. When non-producers are initially very frequent, however, they reach resistance frequencies similar to when in monoculture and lower than in producer monocultures (Fig. 2.6). This suggests that pyoverdin may be limiting for the populations initiated with 75% non-producers and not for the populations started with 15% or 45% non-producers. Indeed, in these latter cases, the resistance frequency of non-producers is higher than in non-producer monocultures, as expected, but also higher than in producer monocultures (Fig. 2.6). These dynamics indicate that pyoverdin may play an important role in the emergence of resistance. We therefore extended the model to account for pyoverdin dynamics.

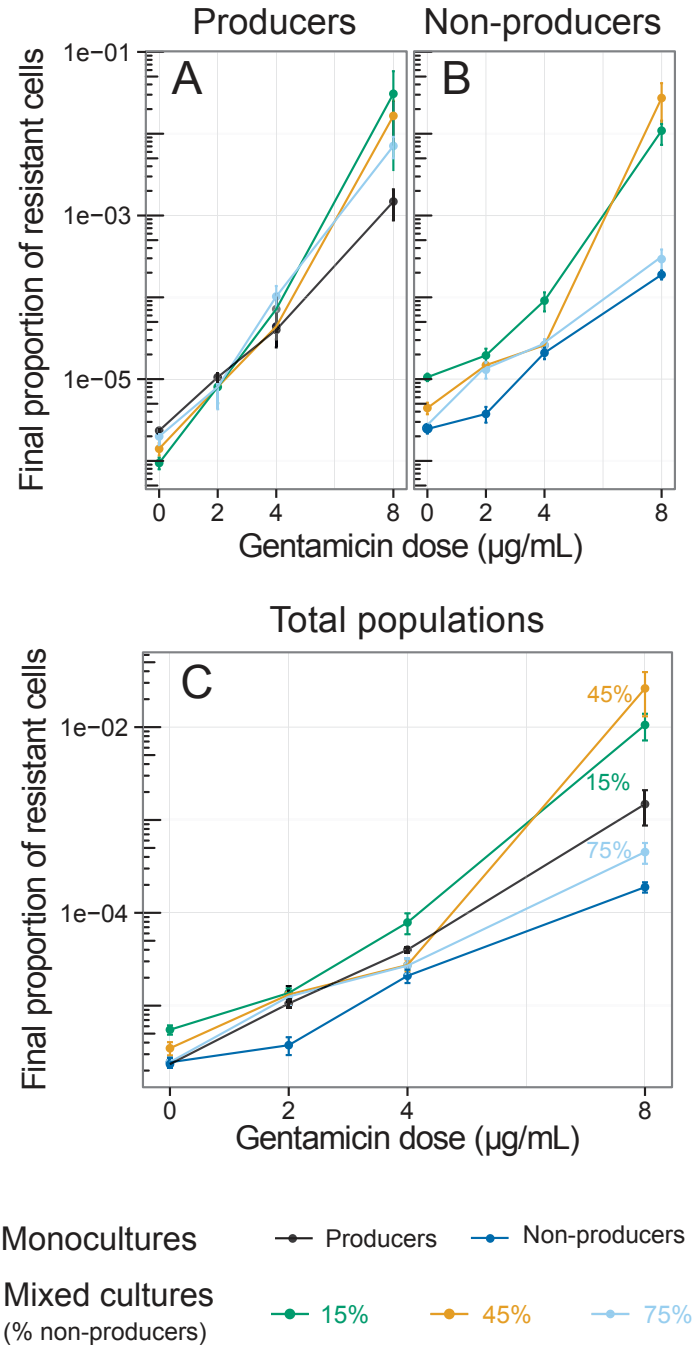

Figure 2.6: Experimental resistance data from the competition experiments (Chapter 1). [Upper panel] Final proportion of resistant cells in producers (A) and in non-producers (B) depending on gentamicin dose. [Lower panel] Final proportion of resistant cells in total populations depending on gentamicin dose (C). Colours indicate initial non-producer frequencies (black = 0%, green = 15%, orange = 45%, light blue = 75%, dark blue = 100%). Bars are standard errors of the mean.

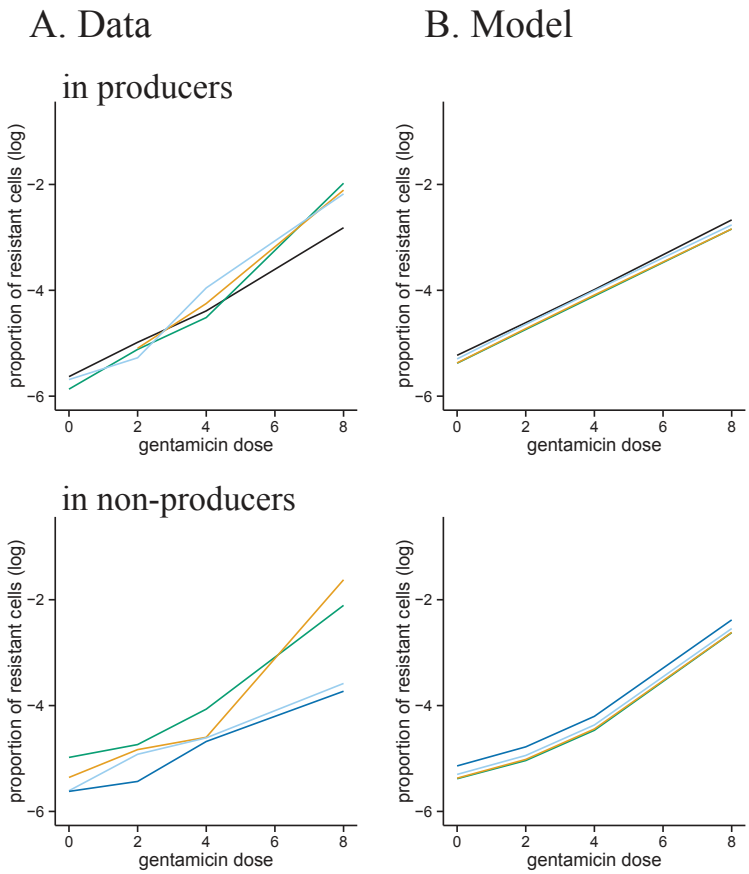

Figure 2.7: Final frequency of resistant cells in producers (upper panels) and non-producers (lower panels). (A) Experimental data and (B) results fitted with Model 1 (different antibiotic effects without pyoverdin dynamics). Colours indicate initial non-producer frequencies (black = 0%, green = 15%, orange = 45%, light blue = 75%, dark blue = 100%).

Model 2

We extend the Model 1 to include the following assumptions:

8. Pyoverdin concentration follows an S-shaped curve.

This may result either from pyoverdin consumption or degradation, or from a decrease of the production rate over time. Given that pyoverdin is reusable and durable over 48 hours (Kümmerli & Brown 2010), the latter hypothesis appears more plausible. Therefore, we assume that the production rate is inversely related to pyoverdin concentration.

9. The cost of pyoverdin production is proportional to the production rate.

10. The rates of bacterial reproduction depend on siderophore concentration.

The extended model describes bacterial dynamics as:

$$\begin{aligned}
\frac{\mathrm{d}p_C}{\mathrm{d}t} &= b\left(1 - c\left(1 - \frac{g}{G}\right) + \frac{rg}{g+H}\right)\left(1 - \frac{n}{K} - \alpha_1 a\right)p_C, \\
\frac{\mathrm{d}q_C}{\mathrm{d}t} &= b\left(1 - c\left(1 - \frac{g}{G}\right) - c_R + \frac{rg}{g+H}\right)\left(1 - \frac{n}{K} - \alpha_2 a\right)q_C, \\
\frac{\mathrm{d}p_D}{\mathrm{d}t} &= b\left(1 + \frac{rg}{g+H}\right)\left(1 - \frac{n}{K} - \alpha_1 \phi_a a\right)p_D, \\
\frac{\mathrm{d}q_D}{\mathrm{d}t} &= b\left(1 - c_R + \frac{rg}{g+H}\right)\left(1 - \frac{n}{K} - \alpha_2 a\right)q_D, \\
\frac{\mathrm{d}a}{\mathrm{d}t} &= -\mu a,
\end{aligned} \tag{2.7}$$

and the dynamics of pyoverdin concentration is:

$$\frac{\mathrm{d}g}{\mathrm{d}t} = v\left(1 - \frac{g}{G}\right)(p_C + q_C), \tag{2.8}$$

Table 2.2: Variable and parameters for pyoverdin dynamics.

	Symbol	Definition
Variable	g	pyoverdin concentration
Parameter	v	maximum siderophore production rate per cell
	r	maximum pyoverdin-dependent increase in growth rate
	H	half-velocity constant
	G	pyoverdin concentration at which production ceases

Model analysis

To analyse the effect of pyoverdin dynamics on the fitness of resistant cells relative to susceptible cells, we calculate the ratio of the relative fitness of resistant cells in the presence of pyoverdin to the relative fitness in the absence of pyoverdin.

The relative fitness of resistant producers, normalised by their relative fitness in the absence of pyoverdin is:

$$\frac{1-c}{1-c_R-c}\left(1 - \frac{c_R(g+H)}{\left(1 - c\left(1 - \frac{g}{G}\right)\right)(g+H) + rg}\right), \tag{2.9}$$

for non-producers, the equivalent expression is:

$$\frac{1}{1-c_R}\left(1-\frac{c_R(g+H)}{g+H+rg}\right). \tag{2.10}$$

Both expressions are independent of antibiotic concentration and population size.

To examine how this relative fitness of resistant cells varies with pyoverdin concentration, we differentiate these expressions with respect to g and obtain:

for producers

$$\frac{(1-c)(c(g+H)^2+GHr)}{G(1-c_R-c)\left(\left(1-c\left(1-\frac{g}{G}\right)\right)(g+H)+rg\right)^2}, \tag{2.11}$$

and

$$\frac{Hr}{(1-c_R)(g+H+rg)^2}, \tag{2.12}$$

for non-producers.

Since these derivatives are always non-negative, the relative fitnesses of resistant subpopulations of both producers and non-producers increase with pyoverdin concentration g.

Fitting method

The extended model was fitted using MCMC as described above. Although there were no experimental data on pyoverdin concentration for the competition experiments, we had pyoverdin accumulation data from the additional growth assays performed on the single antibiotic resistant and susceptible colonies isolated from evolved populations (for method details see Chapter 1, Appendix 2). The experimental conditions were different from the competition experiment with respect to the culture volume (200 μL for the assays and 800 μL for the competition experiment), the duration (24 hours for the assays and 48 hours for the competition experiment) and the type of populations (single colony for the assays and competition for the experiment). The growth rate was higher in the assays compared to the competition experiment and we do not know

whether the pyoverdin-related parameters also differed. Therefore, to avoid over-fitting, we maintained the parameter values previously estimated using the model without pyoverdin dynamics, except that we increased the cost of resistance to compensate for the additional siderophore effect. For the new pyoverdin-related parameters, we chose values that produce pyoverdin dynamics approximately consistent with what was observed in the additional growth assays. Based on measurements from these assays, we assume that initial pyoverdin concentration in producer monocultures is $G/10$ (that is, 10% of the maximum possible concentration). This set of parameters presents an example that is consistent with the values obtained by fitting the growth assay data. Moreover, the model analysis indicates that the dynamics remain qualitatively the same for different sets of pyoverdin-related parameters values.

Results

In the extended model, the dynamics of susceptible bacteria (Fig. 2.8) and of non-producer frequencies (Fig. 2.11) are not qualitatively different from these dynamics in the model without pyoverdin (Model 1). This is consistent with our assumption 1 that pyoverdin dynamics can be neglected as an explanatory variable in the dynamics of non-producer frequencies. Taking pyoverdin dynamics into account (Model 2), however, does change the dynamics of resistant bacteria (Fig. 2.8). Indeed, unlike in the model without pyoverdin dynamics, in the extended model the frequency of resistant cells in non-producer subpopulations is higher in mixed cultures than in monocultures (Fig. 2.9) and this effect is larger for the highest doses of antibiotics. In this model, the frequency of resistance increases with initial producer frequencies, because the relative fitness of the resistant subpopulation increases with pyoverdin concentration (Fig. 2.10; see model analysis for details).

Although the model correctly predicts that the effect of pyoverdin concentration is higher when the antibiotic dose is high, it cannot explain the difference in the experimental data between mixed cultures with 15% or 45% of non-producers initially on the one hand and monocultures or mixed cultures with 75% of non-producers initially on the other hand. Moreover, in the extended model, the frequency of resistant cells in producers is maximal in monocultures and decreases with increasing initial frequency of non-producers (Fig. 2.9).

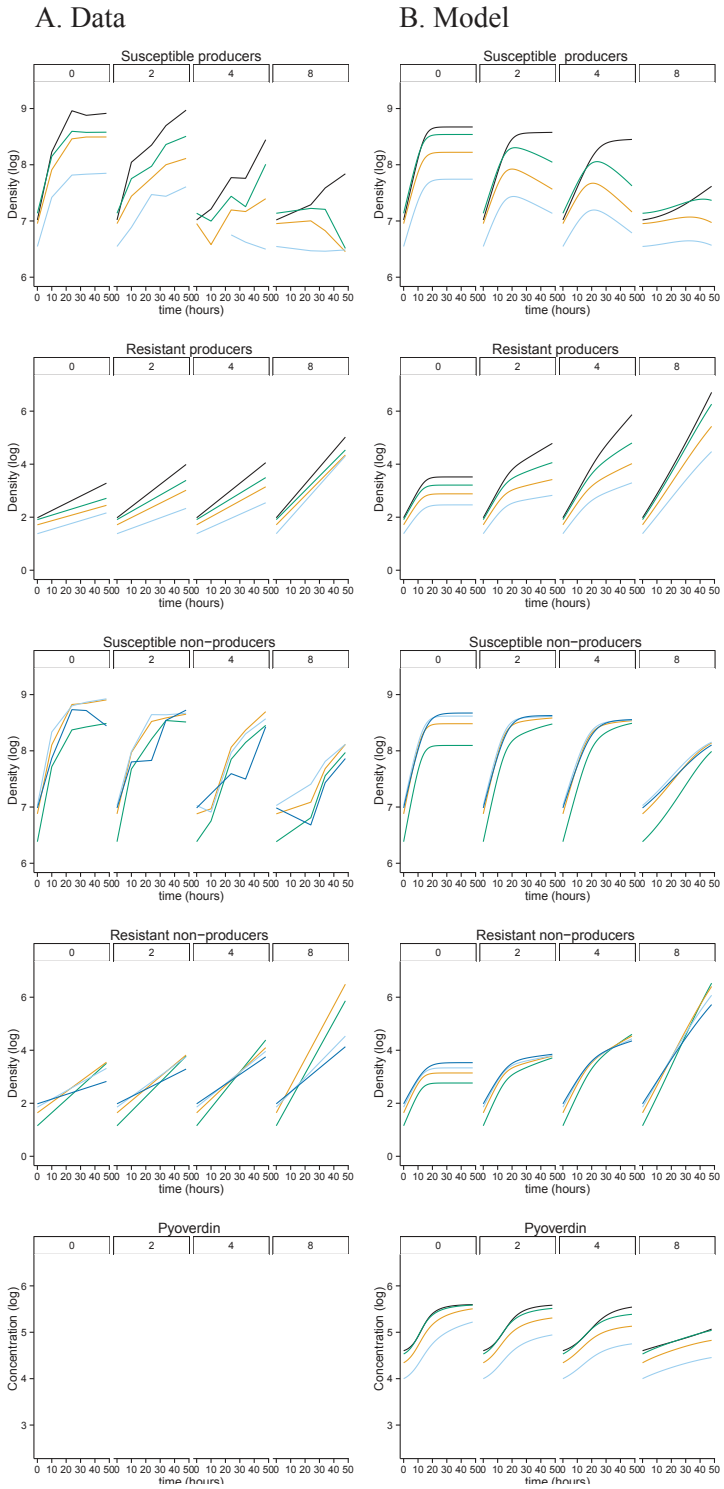

Figure 2.8: (A) Experimental data and (B) results fitted with Model 2 (different antibiotic effects with pyoverdin dynamics). Colours indicate initial non-producer frequencies (black = 0%, green = 15%, orange = 45%, light blue = 75%, dark blue = 100%). Parameter values (rounded to two decimal places) are the same as in Fig. 2.4 except that $c_R = 0.3$ and additional parameters are $r = 1$, $G = 4 \times 10^5$, $v = 1 \times 10^4$ and $H = 1 \times 10^5$. The initial siderophore concentration is 40000 times the initial producer frequency (the unit of pyoverdin concentration is arbitrary, but is chosen to correspond approximately to the unit of RFU measurements from the growth assays).

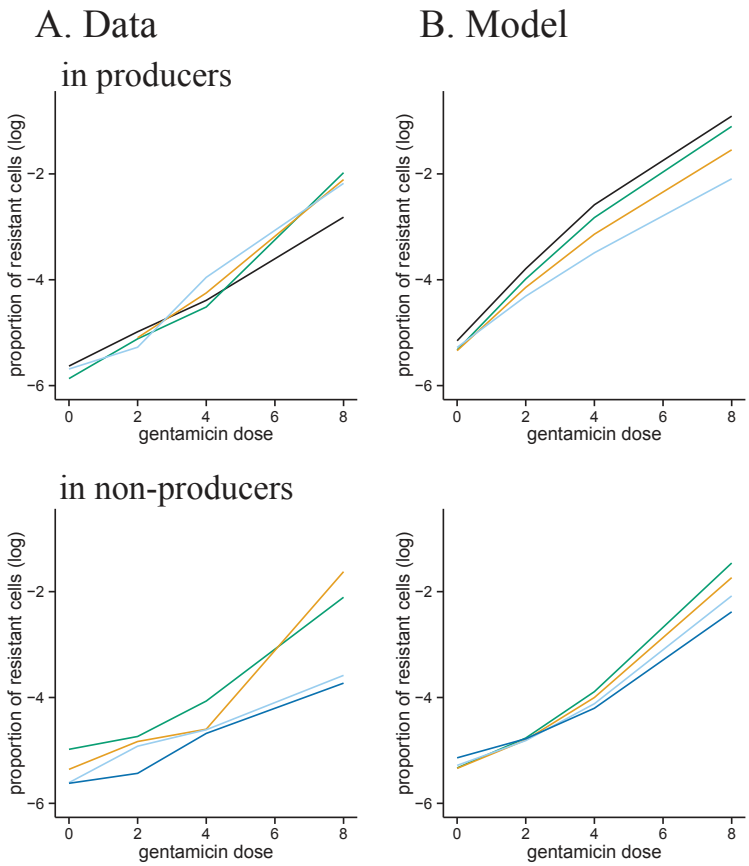

Figure 2.9: Final frequency of resistant cells in producers (upper panels) and non-producers (lower panels). (A) Experimental data and (B) results fitted with Model 2 (different antibiotic effects with pyoverdin dynamics). Colours indicate initial non-producer frequencies (black = 0%, green = 15%, orange = 45%, light blue = 75%, dark blue = 100%).

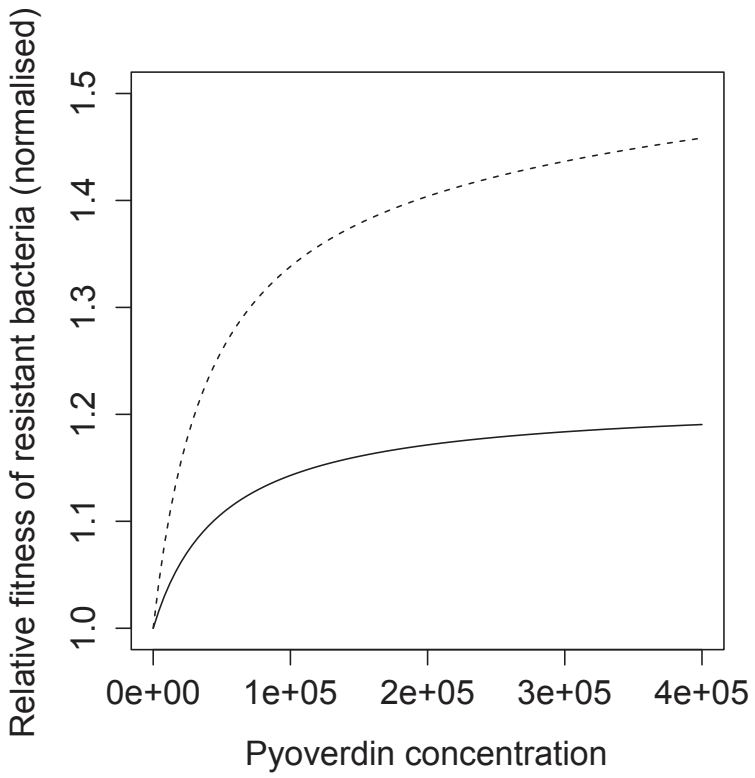

Figure 2.10: Effect of pyoverdin concentration on the relative fitness of resistant bacteria. Fitness is calculated according to Model 2 (different antibiotic effects with pyoverdin dynamics) for resistant producers (dashed line) and non-producers (solid line), relative to the corresponding susceptible subpopulations, and is normalised by the relative fitness in the absence of pyoverdin (Equations 2.9 and 2.10).

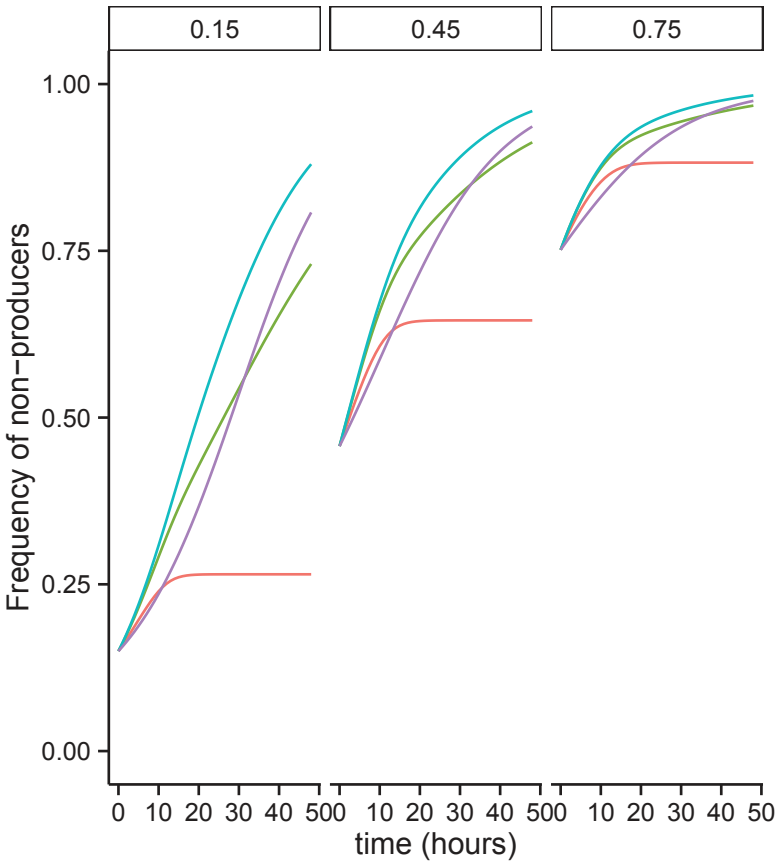

Figure 2.11: Change in non-producer frequencies for Model 2 (different antibiotic effects with pyoverdin dynamics) fitted to the experimental competition data. The three panels correspond to the different initial frequencies of non-producers (15%, 45% and 75%). Colours are gentamicin doses (red = 0 μg/mL, green = 2 μg/mL, blue = 4 μg/mL, purple = 8 μg/mL). Parameter values are the same as in Fig. 2.5.

Discussion

In this study, we used mathematical modelling to investigate the interactions between antibiotic pressure and siderophore cooperation in bacterial populations. In particular, we addressed (i) the impact of different antibiotic doses on competition between pyoverdin producers and non-producers and (ii) the consequences of pyoverdin interactions on the emergence of antibiotic resistance.

In competition experiments, the frequency of non-producers increased faster under antibiotic treatments than in controls. Mathematical modelling supports the hypothesis that this was due to non-producers being less affected by the antibiotic (espe-

cially at lower doses). As well as paying a cost of pyoverdin production in terms of reduced growth rate, it appears that producers might also pay an additional cost in harsh environments, under the assumption that they have fewer remaining metabolic resources to commit to counteracting antagonisms, such as antibiotics. This model outcome did not depend on public good dynamics (but it was robust to the inclusion of such dynamics, as shown in Fig. 2.11).

Siderophore dynamics appear to be more important in explaining the relative growth rates of resistant as opposed to antibiotic-sensitive bacteria. In our models, inclusion of an effect of pyoverdin on growth rates was sufficient to explain why the frequency of resistant non-producers becomes larger in mixed populations, compared to monocultures. The model, however, cannot explain the considerable increase in resistant non-producer frequency, under the highest antibiotic dose, in the mixed cultures initiated with 15% and 45% of non-producers compared to the mixed cultures with 75% of non-producers. In this latter case, the producers are initially rare in the population and pyoverdin production might not be sufficient to sustain the growth of the population. This is especially the case under high antibiotic pressure affecting producers more than non-producers. In contrast, for the mixed cultures initiated with a majority of producers, pyoverdin concentration appears sufficient to enable growth and as a consequence, the frequency of resistance increases compared to monocultures. Moreover, we may assume that the non-producers from the 15% or 45% mixed cultures and the non-producers from the 75% mixed cultures have different resistance mechanisms and/or different loci involved in resistance. As the 15% and 45% treatments grow faster, possibly due to a higher pyoverdin concentration (and in addition to the selective pressure exerted by the high antibiotic dose), they may have either evolved genetic resistance to the antibiotic, or expressed a relatively low cost, plastic response.

In the second model, the increase in growth rate is due not to mixed cultures *per se* but to pyoverdin concentration, which increases with initial producer frequency. Therefore, the model also predicts that resistant producers should attain higher frequencies in monoculture than in mixed cultures, whereas the opposite was in fact observed in experiments at the highest dose. This discrepancy between model and data requires further investigation. Indeed, we might expect that the presence of non-producers in mixed cultures would impede the spread of resistance in producers, consistent with the model predictions. This suggests that there may be some compensatory mechanisms in mixed cultures that enable the producers to reach higher resistant frequency than

in monocultures. The additional assays described in Chapter 1 (Appendix 2) indicate that producers from mixed cultures show higher levels of resistance than all other bacteria from the experiment, and apparently without an increased fitness cost. Alternatively, one possibility is that the observed trend results from the rapid decline of susceptible producers in mixed cultures at later time points under the highest antibiotic concentration. This could be due to bacterial death not accounted for in the model. According to this hypothesis, if resistant proportions had been measured earlier (e.g., at 24 hours), then they would have been more consistent with the model prediction.

We aimed to develop mathematical models that could recapitulate the observed ecological dynamics of siderophores and antibiotic resistance evolution, with the fewest possible assumptions and parameters. A better fit to the data might be obtainable by considering more details of the growth dynamics. For instance, some of the deviation between model predictions and data appear to be due to the logistic growth assumption. This can be seen when considering the growth of non-producer monocultures from the additional growth assays, in the absence of antibiotics. According to the model, because pyoverdin concentration is close to zero at all times, the non-producer densities in these experiments should follow simple logistic curves, approaching the constant carrying capacity K. In fact, the densities initially grow exponentially and then grow approximately linearly (Fig. 2.13). Producer densities follow similar curves, and continue to increase linearly even when the siderophore concentration is constant. We reduced the influence of this factor by fitting the model to log-transformed data. Future studies will include this modification to the logistic growth model.

Appendix

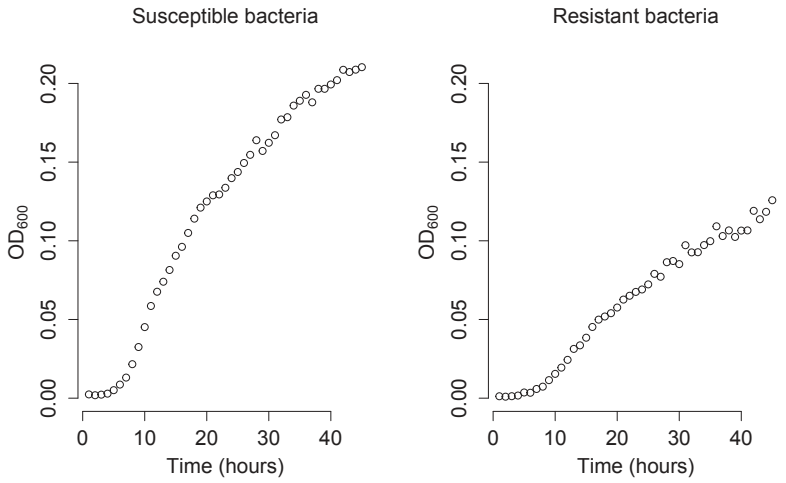

Figure 2.12: Growth in the antibiotic-free environment of susceptible (left) and resistant (right) bacteria isolated from the evolved populations of the competition experiment. Data are mean optical densities (OD_{600}) over time for producers and non-producers from both monocultures and mixed cultures.

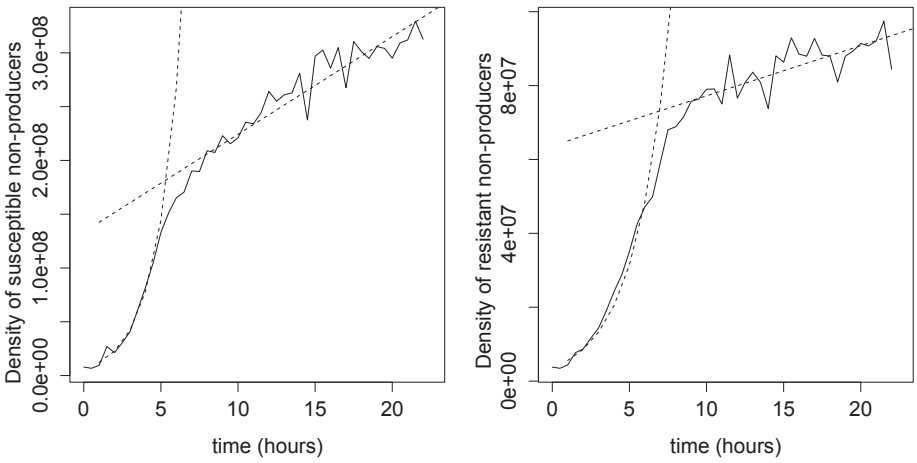

Figure 2.13: Experimental data from the growth assays (Chapter 1). Growth monocultures of susceptible (left panel) and resistant (right panel) non-producers in the absence of antibiotics. The growth curves (solid lines) are clearly not logistic, but instead are approximately exponential at earlier times and approximately linear at later times, as indicated by fitted regression curves (dashed lines). Data are mean optical densities (OD_{600}) converted into cell densities (with a standard curve).

3

Phage selection for bacterial cheats leads to population decline

Marie Vasse, Clara Torres-Barceló and Michael E. Hochberg. (2015) Proceedings of the Royal Society of London. Series B: Biological Sciences. 282(1818). pii: 20152207.

Abstract

While predators and parasites are known for their effects on bacterial population biology, their impact on the dynamics of bacterial social evolution remains largely unclear. Siderophores are iron-chelating molecules that are key to the survival of certain bacterial species in iron-limited environments, but their production can be subject to cheating by non-producing genotypes. In a selection experiment conducted over c 20 bacterial generations and involving 140 populations of the pathogenic bacterium *Pseudomonas aeruginosa* PAO1, we assessed the impact of a lytic phage on competition between siderophore producers and non-producers. We show that the presence of lytic phages favours the non-producing genotype in competition, regardless of whether iron use relies on siderophores. Interestingly, phage pressure resulted in higher siderophore production, which constitutes a cost to the producers and may explain why they were outcompeted by non-producers. By the end of the experiment, however, cheating load reduced the fitness of mixed populations relative to producer monocultures, and only monocultures of producers managed to grow in the presence of phage in situations where siderophores were necessary to access iron. These results suggest that public goods production may be modulated in the presence of natural enemies with consequences for the evolution of social strategies.

Introduction

Cooperation is a pervasive phenomenon in biological systems and despite considerable study, its establishment and maintenance are incompletely understood. Theoretical work starting with Hamilton's seminal papers (Hamilton 1964a;b) identified a number of key features that promote cooperation (Frank 1998, Nowak 2006, Gardner & Foster 2008). While many empirical studies have tested theory using social insects and cooperative birds and mammals (Rubenstein & Lovette 2007, Dobata & Tsuji 2013), an increasing number have employed microbes, given their rapid evolution and experimental control relative to metazoa (e.g., West *et al.* 2007a).

Numerous studies on both metazoan and microbial species show how ecological variables may influence social evolution, including resource supply (Baglione *et al.* 2006, Brockhurst *et al.* 2008), disturbance frequency (Rubenstein & Lovette 2007, Brockhurst *et al.* 2010), and spatial heterogeneity (Dumas & Kümmerli 2012), but also certain inter-individual effects stemming from social strategies (Kümmerli *et al.* 2009a), interspecific competition (Celiker & Gore 2012), and predation and parasitism (Mooring & Hart 1992, Garay 2009, Morgan *et al.* 2012). Yet, key evolutionary forces driving collective behaviour and group formation such as predation and parasitism (e.g., the selfish herd Hamilton 1971, Mooring & Hart 1992) have received limited attention as mediators of microbial social behaviours.

Predators and parasites may either be the basis of social behaviours, such as cooperative defence (e.g., Garay 2009, Jousset *et al.* 2009, Friman *et al.* 2013 but see Schädelin *et al.* 2012), or constitute a cost that potentially impacts other cooperative behaviours (e.g., resource access and sharing, quorum sensing Steiner & Pfeiffer 2007, Jousset *et al.* 2009, Jousset 2012). Such costs may differ between individuals adopting different social behaviours and include energy or time committed to defence or resistance (e.g., Van Buskirk 2000, Steiner & Pfeiffer 2007), or costs associated with trade-offs involved in evolved resistance to enemies (Jousset *et al.* 2009, Friman *et al.* 2013). Despite their ubiquitousness in nature and demonstrated importance in population ecology and evolutionary biology (e.g., Schmitz 2008, Sheriff & Thaler 2014), the impacts of natural enemies on the ecology and evolution of microbial cooperation remain largely unexplored.

Natural enemies could potentially impact cooperation through at least three non-

mutually exclusive processes: demography, plasticity and selection. First, predators and parasites may have a demographic effect (Gervasi *et al.* 2012), whereby reduced host or prey density either increases cooperation through lower local competition (e.g., West *et al.* 2002) and higher resource supply (Brockhurst *et al.* 2008), or decreases cooperation through less efficient signalling (Darch *et al.* 2012). Second, natural enemies may induce plastic changes in their host/prey behaviour either directly through, for example, physical contact or detection of stimuli (e.g., de Bono *et al.* 2002, Poisot *et al.* 2012), or indirectly (e.g., Sheriff & Thaler 2014) by affecting, for example, communication between neighbours (Zanette *et al.* 2011). Third, natural enemies may select for resistance that has pleiotropic and epistatic consequences on cooperation (Jousset 2012), or resistance that promotes the emergence of diversity generating mechanisms influencing cooperation. For example, viruses may select for higher mutation rates in certain bacterial populations (Pal *et al.* 2007, Jousset 2012 but see Gómez & Buckling 2013), which could decrease relatedness and favour the emergence of intermediate phenotypes with varying levels of investment in cooperation (Dumas & Kümmerli 2012).

What little is known about how natural enemies affect the social evolution of their prey or hosts in experimental settings comes from phage-bacteria systems. Morgan and colleagues (Morgan *et al.* 2012) investigated the impact of bacteriophage pressure on public goods production in the form of iron-chelating molecules (siderophores) in the bacterium *Pseudomonas fluorescens*. They found that phages prevent the emergence of initially rare non-producer mutants if the fitness gains of resistance mutations exceed those not producing siderophores. This is because numerically dominant producers are more likely to evolve resistance to phage by chance than less numerous non-producers. Their study also demonstrated positive frequency-dependence in the relative fitnesses of both producers and non-producers, but empirical investigation to a possible fitness advantage of the more frequent strategy is still lacking.

We investigated the experimental evolution of siderophore (pyoverdin) production in the pathogenic bacterium *Pseudomonas aeruginosa* PAO1 in the presence and absence of a bacteriophage. We compared the effect of phage under two experimental conditions: an 'iron-limited' environment in which the non-producers have access to iron through siderophore production by producers, and an 'iron-rich' environment in which iron availability is not limiting and non-producers do not gain from the presence of producers. To evaluate frequency-dependent interactions, we established mixed populations of *P. aeruginosa*, with different initial frequencies of the two strategy types.

We followed the relative fitness of each type and the production of public goods in the form of siderophores. We show that the fitness advantage to non-producers in competition is significantly increased in the presence of phage under both iron conditions. Moreover, we find evidence for negative frequency dependence of the non-producer strategy. Pyoverdin production by producers is significantly higher under phage pressure and provides a possible explanation for the dominance of non-producers in the presence of phage: augmented pyoverdin production comes at an added cost to producers and may constitute additional benefits to non-producers when iron availability is limited. However by the end of the experiment, whereas in iron-rich environments the advantage to non-producers led to overall growth in mixed populations, in the iron-limited environment the densities of mixed cultures actually decreased. We discuss these findings in the contexts of public goods dynamics and social evolution theory.

Materials and Methods

Strains and culturing conditions

We employed two isogenic strains of *Pseudomonas aeruginosa* (Ghysels *et al.* 2004) differing in their production of pyoverdin. The wild type PAO1 (ATCC 15692) is a pyoverdin producer whereas the mutant strain PAO1Δ*pvdD* is unable to produce this siderophore. The mutant strain was constructed by the knockout of the non-ribosomal peptide synthetase gene *pvdD* on the PAO1 wild type strain (Ghysels *et al.* 2004). Prior to our experiment, independent replicate colonies of both strains were inoculated in 30 mL microcosms containing 6 mL of King's B medium (KB, King *et al.* 1954) and incubated overnight under constant shaking (200 rpm). M9 minimal salt solution was used for all sample dilutions.

We used a stock of LKD16 phage (*Podoviridae* Ceyssens *et al.* 2006) amplified from a single plaque. Briefly, the phage plaque was introduced in an exponentially growing bacterial population of the ancestral *P. aeruginosa* PAO1 and incubated at 37 ˚C for 24 hours. Then, 10% chloroform was added to the culture to kill the bacteria. After vortexing and centrifugation (13000 rpm for 4 min), we recovered the phage-containing supernatant and stored it at 4 ˚C. This master stock was used for all experiments.

Evolution experiments were performed in the inner wells of 48-well plates to prevent

evaporation. A microcosm consisted of a well containing 1 mL of casamino acids medium (CAA; 5 g Casamino acids, 1.18 g $K_2HPO_4.3H_20$, 0.25 g $MgSO_4.7H_20$, per litre; BD Biosciences). Limited iron availability conditions were obtained by supplementing the CAA medium with sodium bicarbonate to a final concentration of 20 mM and 100 μgm/L of human apotransferrin (Sigma-Aldrich), a strong iron chelator, which binds free iron and prevents non-siderophore-mediated uptake of iron (Meyer *et al.* 1996). For high iron availability conditions, CAA medium was supplemented with 30 μM $Fe(III)Cl_3$ (ferric chloride, Sigma-Aldrich). To minimise the level of exogenous iron, all the solutions were prepared using millipore water. The 48-well plates were incubated at 37 $^\circ$C under static conditions.

Evolution experiment

We tested the impact of phage pressure on the siderophore production strategy by measuring the densities and relative frequencies of producers and non-producers under two conditions: a limited iron availability situation (hereafter called 'iron-limited' conditions) in which high siderophore production is required for iron acquisition, and a high iron availability situation (hereafter called 'iron-rich' conditions) in which a given amount of iron is directly available. Under the latter conditions, siderophore production is approximately 20 times lower than the former, and a previous study indicates that pyoverdin production ceases completely when iron supplementation is greater than 50 μM (Kümmerli *et al.* 2009b).

At the beginning of the experiment, iron-limited and iron-rich 1 mL microcosms were inoculated with c 5×10^5 cells from the overnight cultures. The treatments were each replicated 10 times and consisted of either monocultures (100% producers or 100% non-producers) or mixed cultures (11%, 28%, 62%, 76% and 91% non-producers), each either with or without phage. Half of the replicates were inoculated with c 5×10^3 particles of LKD16 phage and the other half were supplemented with the same volume of M9 minimum salt solution as a control (5 μL).

Every 24 hours, 10% of each population was transferred into fresh microcosms. The experiment was conducted for 5 transfers (approximately 15-20 bacterial generations), hereafter referred to as T_1-T_5 (T_0 corresponds to the ancestral populations, T_1 the first transfer -populations after 24 hours-, and T_6 the end of the experiment -24 hours after

transfer T_5).

At T_0, T_1 and T_6, populations were plated onto KB agar to estimate total densities and relative frequencies of producers and non-producers. Non-producers can be distinguished from the producers on KB agar, as the former produce white colonies, whereas the latter are yellow-green. To limit phage predation associated with phage treatments on KB agar, all populations were centrifuged once at 13000 rpm for 8 minutes. The supernatant containing phage was then discarded and the pellet resuspended in salt solution before being plated onto KB agar. This method does not remove all phages from the cultures, but decreases the phage to bacteria ratio sufficiently to permit accurate counts of colony forming units (CFUs) on Petri dishes.

Growth of each population was estimated by the Malthusian parameter $m = \ln(Nf/N_0)$ (Lenski *et al.* 1991) with N_0 and Nf being the initial and final densities, respectively. We calculated population growth at T_1 and T_6 to compare short and longer-term effects of phage and iron availability.

To express the relative performance of each strain in mixed populations, we calculated the change in relative frequency in non-producers over time as $v = [q_2(1 - q_1)]/[q_1(1 - q_2)]$ where q_1 and q_2 are, respectively, the initial and final proportions of non-producers (Ross-Gillespie *et al.* 2007). When $v > 1$ ($v < 1$), non-producers (producers) increase in frequency.

Phage-bacteria interactions

Bacterial resistance to phage

Resistance was measured as the proportion of bacterial colonies that grow in the presence of phage. 60 colonies of each strain (either producers or non-producers) were streaked against a line of phage (30 μL) on iron-rich CAA agar Petri dishes. After 24 hours of incubation, a colony sample was scored as sensitive to phage if there was clear inhibition of growth; otherwise it was scored as resistant. We used this method to test the resistance of ancestral bacteria to ancestral phage and the resistance of evolved T_6 bacteria to their sympatric T_6 phage (3 replicates).

Phage fitness in bacterial hosts

Following Wang (Wang 2006), we define phage fitness as $w = [\ln(P_t/P_0)]/t$, with P_0 and P_t being the densities of phage at times 0 and t after infection, respectively. We introduced phages (c 1000 particles/mL) and exponentially growing bacteria from each of six replicate populations of either producers or non-producers in 3 mL KB in 30 mL microcosms. After two hours of incubation, we vortexed a 1 mL sample of each population with 10% chloroform to kill bacteria, centrifuged at 13000 rpm for 4 minutes and recovered the supernatant to isolate the phage. The resulting phage solution was serially diluted and mixed with bacteria in soft KB agar (6 g/L agar in KB medium) and 1 mL was poured over a KB agar Petri dish (3 replicates per dilution). The Petri dishes were then incubated at room temperature overnight before being examined for plaque counting.

Pyoverdin production

We assayed pyoverdin concentration in each T_6 bacterial population by measuring fluorescence intensity (relative fluorescent units, RFU) in each well of the experimental plates at the end of the evolution experiment (i.e. 24 hours after the last transfer) with a spectrophotometer (excitation: 400 nm, emission: 460 nm, FluoSTAR Optima fluorescence microplate reader, BMG Lab Technologies).

As an additional test for the effect of phage on pyoverdin production, we followed bacterial growth and pyoverdin production of producer monocultures (using optical densities as a proxy for growth, and fluorescence as a proxy for pyoverdin quantity) in both iron-limited and iron-rich media for 12 hours. Single colonies were inoculated in 48-well plates, and after 6 hours of growth we introduced 10^7 phages into 5 of the 10 microcosms for each iron condition. The same volume (10 μL) of M9 minimum salt solution was introduced in the control microcosms.

Statistical analysis

All analyses were conducted with R software (R.3.1.1; http:// www.r-project.org/). Growth of producers and non-producers in monocultures (m) were compared across treatments with full factorial ANOVAs at T_1 and T_6 using iron availability, phage

(presence or absence) and strain as explanatory factors. Pairwise comparisons were made using the Student-Newman-Keuls (SNK) procedure (Steel *et al.* 1997) on iron-limited and iron-rich data, separately. Levels of resistance were compared between producers and non-producers using a mixed-effect model with replicate as a random factor. Relative fitness data (v) were first log-transformed to meet the assumptions of parametric analysis. We used a linear model to test whether the relative fitness of non-producers ($\log(v)$) changed significantly according to their initial frequency, iron availability and presence of phage. We estimated fluorescence and bacterial density using optical density readings over the duration of the 12 hours-growth experiment as area under the curve (MESS package Ekstrom 2011).

Results

Growth in monocultures in the absence and presence of phage

Consistent with previous studies (e.g., Griffin *et al.* 2004, Kümmerli *et al.* 2009b), monocultures of both producers and non-producers grew significantly less under iron-limited than under iron-rich conditions ($F_{1,24} = 77.91, p < 0.0001$ at T_1 and $F_{1,31} = 1590.87, p < 0.0001$ at T_6; Fig. 3.1). As expected, the presence of phage reduced the fitness of bacterial populations after 24 hours ($F_{1,24} = 76.67, p < 0.0001$), and this decrease was significantly different depending on iron availability and the bacterial strain (phage × iron × strain interaction $F_{1,24} = 24.08, p < 0.0001$). Specifically, under iron-limited conditions, the non-producers grew to significantly lower densities than the producers, both in the presence and absence of phage (Fig. 3.1A). In contrast, under iron-rich conditions, phage did not significantly decrease non-producer growth, but decreased producer growth such that the former grew to significantly higher densities than did the latter (Fig. 3.1B).

After five serial transfers in the iron-limited environment, producers reached significantly higher densities than non-producers, both in the presence and the absence of phage ($F_{1,15} = 158.95, p < 0.0001$, Fig. 3.1C). Moreover, phage significantly decreased densities of non-producers, whereas it did not affect densities of producers ($F_{1,15} = 83.14, p < 0.0001$, Fig. 3.1C). This finding is supported by a streaking assay that revealed a lower level of resistance in non-producers (17.8% ± 2.8%) than

in producers (97.8% ± 1.1%) ($F_{1,216} = 20.5, p < 0.0001$). By contrast, in iron-rich environments, there was no significant difference in growth between producers and non-producers ($F_{1,16} = 0.09, p = 0.77$; Fig. 3.1D) and both grew less in the presence than in the absence of phage ($F_{1,16} = 7.18, p < 0.05$). Yet, they both attained high densities, indicating that these bacteria may have evolved resistance to their sympatric phages. In the streaking assay, we detected high levels of resistance in both producers and non-producers (overall more than 97% of the tested colonies were resistant to sympatric phage), with resistance slightly higher in producers (99.4 ± 0.55 %) than in non-producers (95.5 ± 1.54%) ($F_{1,356} = 5.92, p < 0.05$).

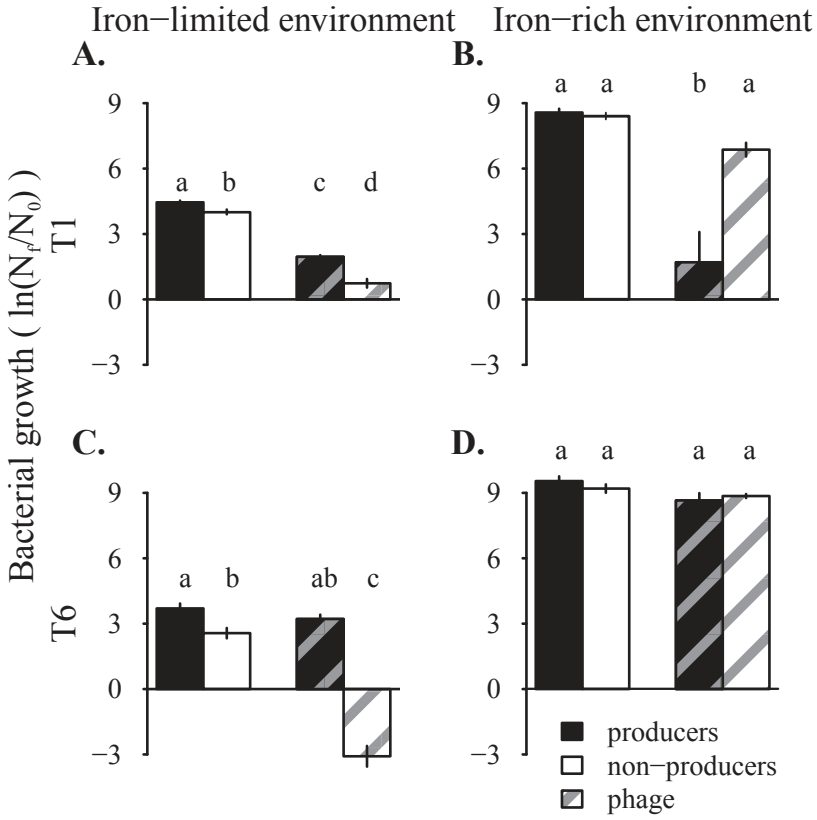

Figure 3.1: Growth of producers (black histobars) and non-producers (white histobars) in monocultures for the first 24 hours (upper panel; A, B) and over the 5 transfers (lower panel; C, D) in iron-limited (A, C) and iron-rich (B, D) environments. Data are logarithmically transformed. Grey hatched histobars represent populations with phage. Pairwise comparisons were made with Student-Newman-Keuls (SNK) tests. Means with the same letter do not significantly differ ($p > 0.05$). Bars are standard errors of the mean.

Relative fitness in the presence and absence of phage

As expected under iron-limited conditions and in the absence of phage, non-producers grew to higher densities when in competition with producers than they did in monocultures ($t_{14} = 3.95, p < 0.01$ at T_1 and $t_9 = 5.27, p < 0.001$ at T_6). In contrast, producers showed reduced growth in competition compared to in monocultures ($t_{27} = -7.23, p < 0.0001$ at T_1 and $t_{15} = -2.43, p < 0.05$ at T_6). These results indicate that non-producers benefitted from the presence of producers at a cost to the latter both at T_1 and T_6. Consistent with these findings, non-producers had a competitive advantage over producers when iron was limiting ($t_{24} = 10.22, p < 0.0001$ at T_1 and $t_{23} = 7.54, p < 0.0001$ at T_6; Figs. 3.4A and 3.2A). However, non-producers decreased in frequency under iron-rich conditions without phage, both at T_1 and T_6 ($t_{19} = -4.65, p < 0.001$ at T_1 and $t_{24} = -14.03, p < 0.0001$ at T_6; Figs. 3.4B and 3.2B). This result suggests that the pvdD gene may be involved in other fitness-related functions (Kümmerli *et al.* 2009b).

In the presence of phage in the iron-limited environment, the fitness of non-producers was higher in mixed cultures than in monocultures ($t_{14} = 5.84, p < 0.0001$ at T_1 and $t_{11} = 3.52, p < 0.01$ at T_6), whereas producers grew better in monocultures than in mixed cultures ($t_{27} = -4.64, p < 0.0001$ at T_1 and $t_{25} = -10.99, p < 0.0001$ at T_6). These results indicate that non-producers were cheating on producers under phage pressure, both at T_1 and T_6. Iron availability showed a significant interaction with the presence of phage both at T_1 and T_6, such that the impact of phage on the relative fitness of non-producers was different depending on iron conditions ($F_{1,84} = 50.51, p < 0.0001$ at T_1, $F_{1,88} = 69.15, p < 0.0001$ at T_6; Fig. 3.2). Under iron-rich conditions, phage presence reversed the outcome of competition between producers and non-producers, conferring an advantage to the latter, whereas they were outcompeted in the absence of phage ($t_{18} = 6.27, p < 0.0001$ at T_1 and $t_{23} = 7.41, p < 0.0001$ at T_6; Figs. 3.2B and 3.2D). Under iron-limited conditions, the presence of phage increased the advantage of non-producers at T_6 ($t_{31} = -2.94, p < 0.01$, Fig. 3.2C). At T_1, we found a significant interaction between the effect of phage and the initial frequency of non-producers ($F_{1,83} = 12.94, p < 0.001$, Fig. 3.2A). Under these conditions, the relative fitness of non-producers was negatively associated with their initial frequency under phage pressure ($F_{1,23} = 55.78, p < 0.0001$, $R^2 = 0.70$, Fig. 3.2A). The same trend indicative of negative frequency dependence was observed in the iron-rich environment

in the presence of phage, but was only statistically significant when a highly variable treatment (with initial frequency of 62% non-producers) was removed ($F_{1,13} = 12.8, p < 0.01$, $R^2 = 0.46$, Fig. 3.2B).

When iron availability was limited, mixed populations of producers and non-producers grew and reached high densities in the presence of phage at T_1 (Fig. 3.5A). After five transfers, however, these mixed populations were decreasing and only the producer monocultures actually grew under phage pressure ($t_{23} = 4.49, p < 0.0001$, Fig. 3.5C). Conversely, there was no significant difference in densities at T_6 between the mixed and the monocultures under iron-rich conditions (contrasts SNK, $p > 0.05$, Fig. 3.5D).

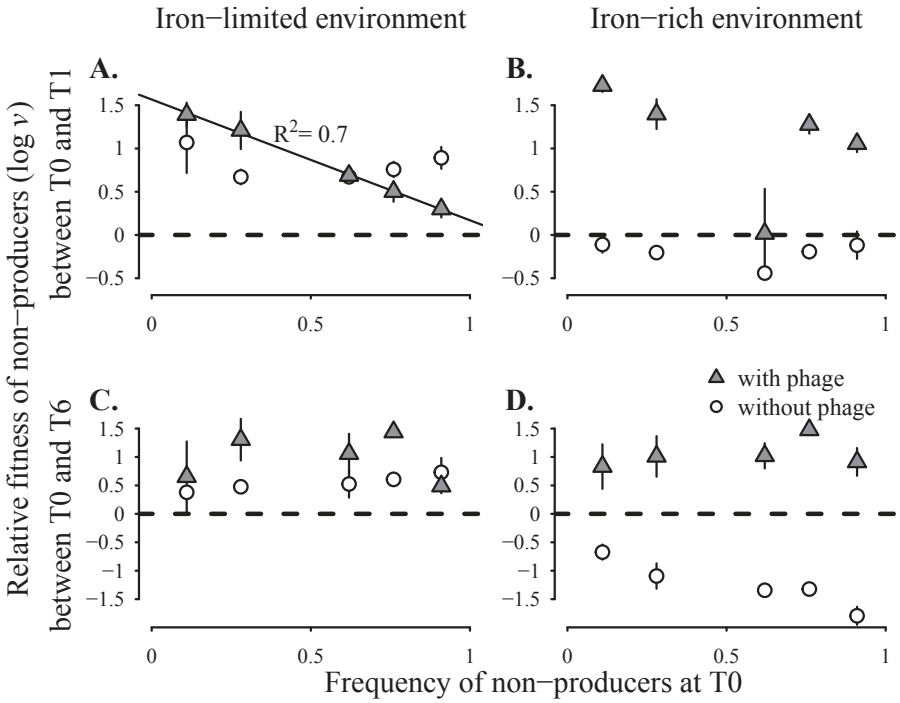

Figure 3.2: Relative fitness of non-producers between T_0 and T_1 (upper panel; A, B) and between T_0 and T_6 (lower panel; C, D) in iron-limited (A, C) and iron-rich (B, D) environments, in the absence (white circles) and presence (grey triangles) of phage. Data are logarithmically transformed ($\log v$). Regression is fitted according to the least squares method. When relative fitness ($\log v$) is positive, non-producers increased in frequency, whereas they decreased when negative. The dashed line represents the situation where the frequency of non-producers did not change between two time points. Bars are standard errors of the mean.

Testing hypotheses to explain the differential effect of phage on the producers

We tested two non-mutually exclusive hypotheses to explain our finding of phage having a larger impact on producer compared to non-producer populations. First we tested whether phages were initially either more infective and/or more productive on producers than on non-producers. In the streaking assay, no colony was scored as resistant for either ancestral bacterial strain against the ancestral phage. Furthermore, in the plaque assay to test phage production we found no evidence for higher phage fitness in the producers compared to the non-producers ($w_{producers} = 0.26 \pm 0.03$ sd and $w_{non-producers} = 0.29 \pm 0.02$ sd; $t_{10} = 1.9, p = 0.09$).

A second hypothesis to explain our finding is that phages imposed an additional cost on producers due to the over-production of pyoverdin. We measured pyoverdin in the T_6 microcosms, where the densities of producers in the presence and absence of phage in monocultures are similar ($t_{17} = 1.33, p = 0.2$), since a previous study showed that pyoverdin production is modified in response to cell densities (Kümmerli *et al.* 2009b). We found that in monocultures the amount of pyoverdin is higher in the presence than the absence of phage for both iron environments ($RFU_{\text{without phage}} = 10174 \pm 820$ sd, $RFU_{\text{with phage}} = 12099.4 \pm 298$ sd, $t_5 = -4.93, p < 0.01, n = 10$ in iron-limited environment and $RFU_{\text{without phage}} = 869 \pm 125$ sd, $RFU_{\text{with phage}} = 1210 \pm 91$ sd, $t_7 = -4.71, n = 10, p < 0.01$ in iron-rich environment).

We also tested the second hypothesis by following the density and pyoverdin production of ancestor producer monocultures. In support of this hypothesis, we observed higher fluorescence and therefore pyoverdin quantity in the populations with phage than without phage, under both iron conditions ($t_8 = -4.65, p < 0.005$ under iron-limited conditions and $t_8 = -7.42, p < 0.0001$ under iron-rich conditions; Fig. 3.3).

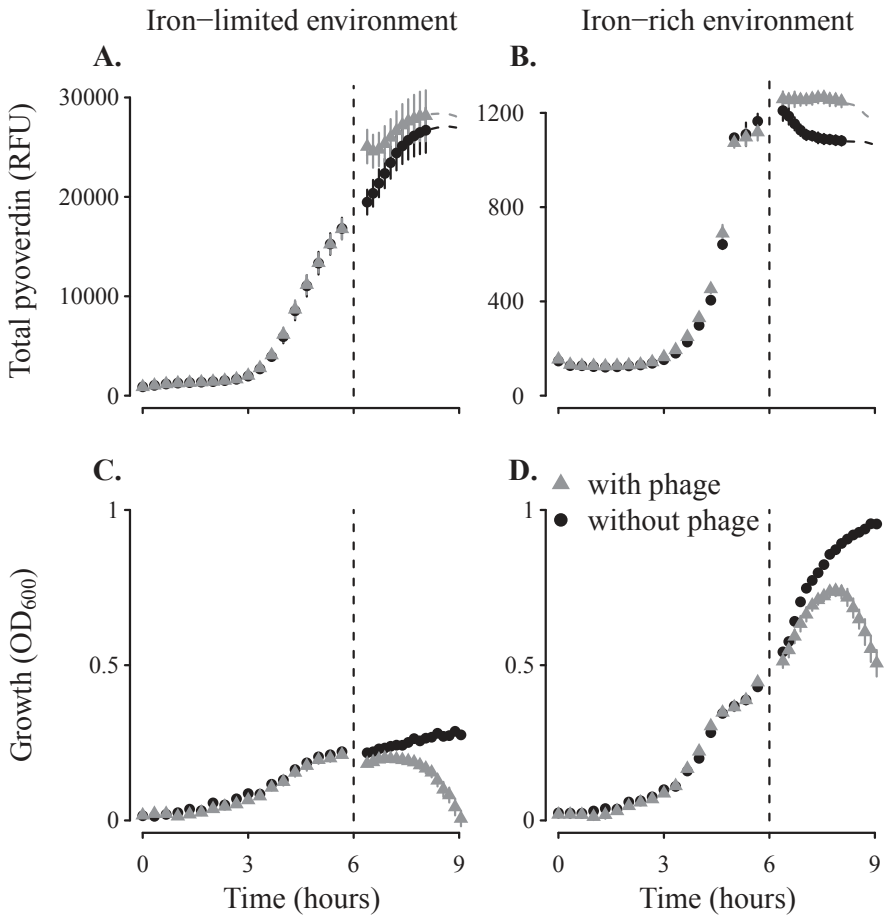

Figure 3.3: Pyoverdin accumulation (upper panel; A, B) and growth curves of producers (lower panel; C, D) in iron-limited (A, C) and iron-rich (B, D) environments in the absence (black circles) and presence (grey triangles) of phage. Phages were introduced after 6 hours (vertical dashed line). Bars are standard deviations of the mean.

Discussion

In this study, we investigated the impact of phage predation in both ecological and evolutionary time on bacterial public goods production strategies. We observed that the presence of phage increased the proportion of non-producing mutants, both when the production of siderophores is and is not beneficial for bacterial growth. Specifically, non-producers increased more in frequency in the presence than in the absence of phage in the iron-limited environment. Under iron-rich conditions, whereas the producers were at an advantage without phage, non-producers outcompeted them in the presence of phage. In the iron-limited environment with phage, the advantage to non-producers in mixed cultures ultimately led to decreased population densities, arguably due to

the lack of siderophores to sustain growth. These results highlight the importance of considering interspecific interactions under different environmental conditions when studying the evolution of public goods strategies and, more generally, social evolution and intraspecific cooperation and competition.

Previous studies have investigated how natural enemies may affect the outcome of public goods production in microbial systems. For example, predators have been shown to impact the interactions in *Pseudomonas* species in favour of producers due to the differential killing of non-producers (Jousset *et al.* 2009, Friman *et al.* 2013). In contrast, Morgan and colleagues (2012) reported that phage promote increases in the most frequent type regardless of its production strategy, because that type would be more likely to be associated with phage-resistant mutants. We found that phage always selected for an increase in non-producers, and found no evidence for a differential effect of phage on ancestral producers and non-producers in monocultures. Moreover, the relative fitness of non-producers in the presence of phage was significantly negatively frequency dependent during the first 24 hours, consistent with phage selecting against the more frequent siderophore production strategy. We therefore suggest that the overall impact of phage on temporal changes in the two bacterial types is influenced not only by initial conditions but also by subsequent, complex ecological and evolutionary dynamics. The seemingly contradictory outcomes of our work and the study by Morgan and colleagues (2012) may be explained by the use of a different phage–bacteria system, and/or the employment of different initial phage and bacteria densities, growth media and volume, and/or ranges of initial non-producer frequencies. In Morgan and colleagues (2012), non-producers decreased in frequency when initially at low frequencies ($\leq 1\%$), whereas the lowest initial frequency of non-producers in our protocol was 11%.

In our experiment, both the producers and non-producers have functional siderophore receptors. Therefore, even if phage were able to exploit those receptors as alternative binding sites, as observed in long-tailed phages of *Escherichia coli* (Letellier *et al.* 2004), this could not explain the higher effect of phage on producer densities. Moreover, we suggest that this phenomenon is unlikely in our system since the short-tailed phage LKD16 employs type IV pili as a receptor (Lammens *et al.* 2009) and the fluctuation selection dynamics it undergoes with bacteria is indicative of high specificity to this binding site (Betts *et al.* 2014).

Our results suggest that the dynamics of competition and producing strategies are mediated by siderophore availability. Higher overall production of pyoverdin leads to selection for non-producers by increasing the cost to benefit ratio for production. Harrison and co-workers (2008) reported that the presence of an interspecific competitor led to competition for iron. This in turn resulted in higher siderophore production, thus increasing the advantage to defecting mutants. Our results indicate that the presence of phage favoured non-producers in mixed cultures not only under iron-limited, but also under iron-rich conditions, due to an increase in pyoverdin production. This can be understood as follows. When iron availability is not limiting, there is little or no benefit to pyoverdin production for either producers or non-producers. However, because non-producers do not pay production costs, they would have a net advantage over producers, and this may explain why the former outcompeted the latter in mixed cultures and why producer recovery in monocultures is much slower than that of non-producers. As siderophores are not essential to import iron from the environment in our iron-rich experimental treatments, non-producers can grow in the absence of producers and all populations reached similarly high densities after five transfers. In contrast, when iron availability is limiting, pyoverdin production comes at a cost, but results in a net benefit to producers as it increases access to iron (e.g., Meyer *et al.* 1996, Griffin *et al.* 2004). Indeed, we found that producers evolved higher levels of resistance to phage in monocultures than did non-producers, suggesting that pyoverdin led to higher growth rates and increased the probability of resistance evolution to the phage. In mixed cultures, non-producers rely on siderophores from producers and the former are selected because they benefit without paying a cost. Their advantage, however, is negatively correlated with their initial frequency: when initially rare, the high pyoverdin to mutant ratio allowed mutants to increase rapidly to high frequencies as iron became more available, whereas when initially abundant, the amount of pyoverdin and thus of available iron per non-producer cell was lower and their fitness advantage was thereby decreased. Furthermore, while the advantage to non-producers in mixed cultures resulted in high population productivity in the presence of phage at T_1, the combined phage and cheating load was associated with decreases in density in these mixed populations by the end of the experiment. Indeed, the decrease in producer frequency as well as the dilution effect linked to serial transfers, both likely contributed to reduction in siderophore concentration resulting in insufficient iron availability to sustain population growth.

Moreover, previous studies together with our results suggest that the cost to producers in the presence of phage may be higher than that due to pyoverdin production alone, since phage may affect other traits such as motility, biofilm formation and pyocyanin production (Hosseinidoust *et al.* 2013c), and because pyoverdin is also a signalling molecule that may upregulate other costly behaviours (e.g., the production of exotoxins and endoproteases Lamont & Martin 2003, and biofilms Banin *et al.* 2005). Further research is needed to elucidate the complex interrelationships between putative social and other life-history traits.

Although we did not investigate the mechanism by which phage increased pyoverdin production, we propose three non-mutually exclusive hypotheses for upregulation. First, upregulation may be a form of 'terminal investment' (Poisot *et al.* 2012), whereby bacteria increase their survival chances in a stressful, uncertain environment. An increase in siderophore production may have both direct and indirect effects: it can favour growth through higher access to iron and/or regulate other potentially beneficial traits such as biofilm formation (e.g., Heilmann *et al.* 2012). Second, quorum sensing has been shown to influence phage-bacteria interactions (Høyland-Kroghsbo *et al.* 2013) and an increased activation of this system in the presence of phage may lead to the upregulation of public goods production and of pyoverdin in particular (e.g., Juhas *et al.* 2005 but see Dubern & Diggle 2008). Third, upregulation may be a consequence of phage manipulating bacteria to increase the former's own fitness, and there is some evidence that phage can impact host biology and behaviour (e.g., Wagner & Waldor 2002, Hargreaves *et al.* 2014a). Given that biological systems and natural environments of both bacteria and phage are generally poor in free ferric iron (Ratledge & Dover 2000), high local concentrations of siderophores would favour both bacterial growth and the fitness of phage progeny. Future study should test these and other hypotheses for siderophore upregulation (see also Poisot *et al.* 2012, Gómez & Buckling 2013 for associations between phage and increased bacterial growth).

Finally, although we focused on proximal bacterial siderophore production, our results have implications for the evolution of public goods cooperation. We found that in iron-limited monocultures, producer bacteria were able to recover from phage predation, suggestive of resistance evolution (Betts *et al.* 2014), whereas non-producers did not show this effect. In contrast, in iron-limited mixed cultures (which are the relevant situations for social evolution), phages initially selected for increasing frequencies of non-producers, apparently because producers were not able to evolve resistance. Inab-

ility to evolve resistance is likely to have been due to producers being rapidly reduced in numbers (and therefore evolutionary potential) by non-producers. These findings indicate that the phage selected against the producer strain and this, together with the relative advantage that non-producers had under iron-limiting conditions, resulted in higher non-producer frequencies in the 'social' environment. However, this advantage led to decreased population productivity arguably due to the combined phage and cheating load, consistent with our finding that only producer monocultures were able to cope with phage pressure under iron-limited conditions. Future studies should investigate the impact of phage predation on social evolution in spatially explicit systems, where our results would predict that the order of arrival of each population in a given patch will influence the social evolutionary dynamics in that patch, and at larger spatial scales, relative migration rates should have important effects on overall levels of cooperation and its spatial variation (Koella 2000).

Medical implications

Beyond the fundamental findings that phages influence public goods production, our work has implications for the possible medical use of phage. *P. aeruginosa* is a multidrug resistant bacterium responsible for many nosocomial infections and particularly, complications in cystic fibrosis patients. Combined therapies using both antibiotics and phage, although mostly tested *in vitro* (e.g., Torres-Barceló *et al.* 2014), have considerable potential in controlling certain human bacterial infections (Chhibber *et al.* 2013). Understanding the effect of phage on pyoverdin dynamics is of particular interest given the role of siderophores in bacterial virulence (Meyer *et al.* 1996). Our results show that phage can select for non-siderophore producing bacterial variants, suggesting that colony virulence should be lessened compared to colonies dominated by producers (Meyer *et al.* 1996). This advantage to non-producers was observed under two medically relevant iron conditions: an iron-limited environment wherein hosts scavenge and retain iron using iron-binding proteins as an innate immune response to bacterial infections (Skaar 2010), and an iron-rich environment, as observed in chronically infected tissues (Reid *et al.* 2002). However, we also showed that phage upregulate siderophore production, and therefore suggest that in viscous medium where siderophores do not readily diffuse (as would be expected in many *in vivo* situations), this would select for producers and lead to higher virulence (Meyer *et al.* 1996). These points highlight the

importance of understanding the complex interplay between public goods dynamics and phage predation in the design of phage therapies.

Appendix

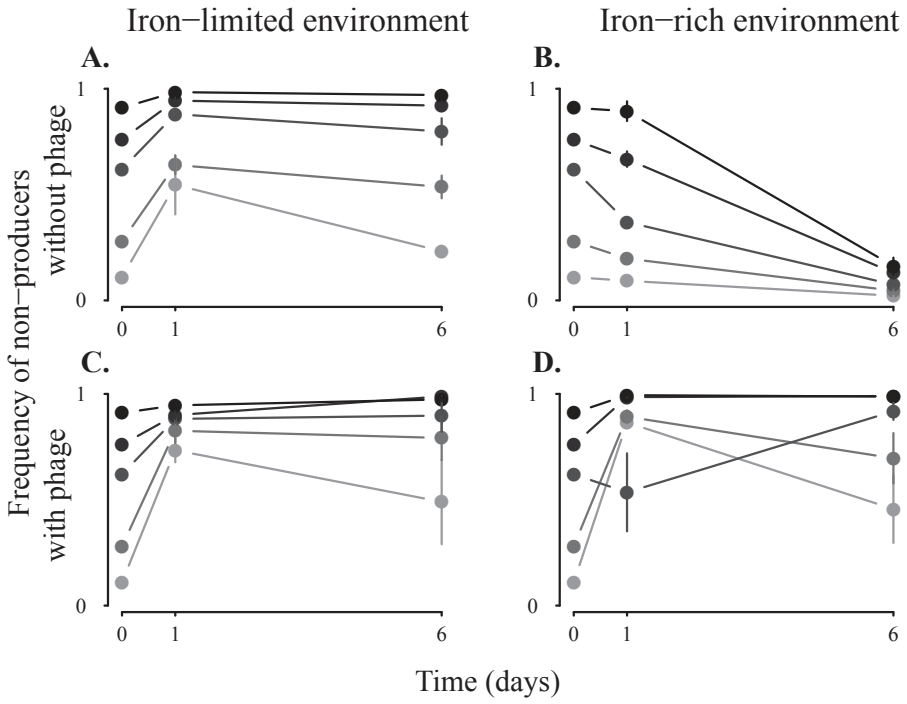

Figure 3.4: Changes in the frequency of non-producers in mixed populations in the absence (upper panel; A, B) and presence (lower panel; C, D) of phage in iron-limited (A, C) and iron-rich (B, D) environments. Different colours represent different initial frequencies of non-producers. Bars are standard errors of the mean.

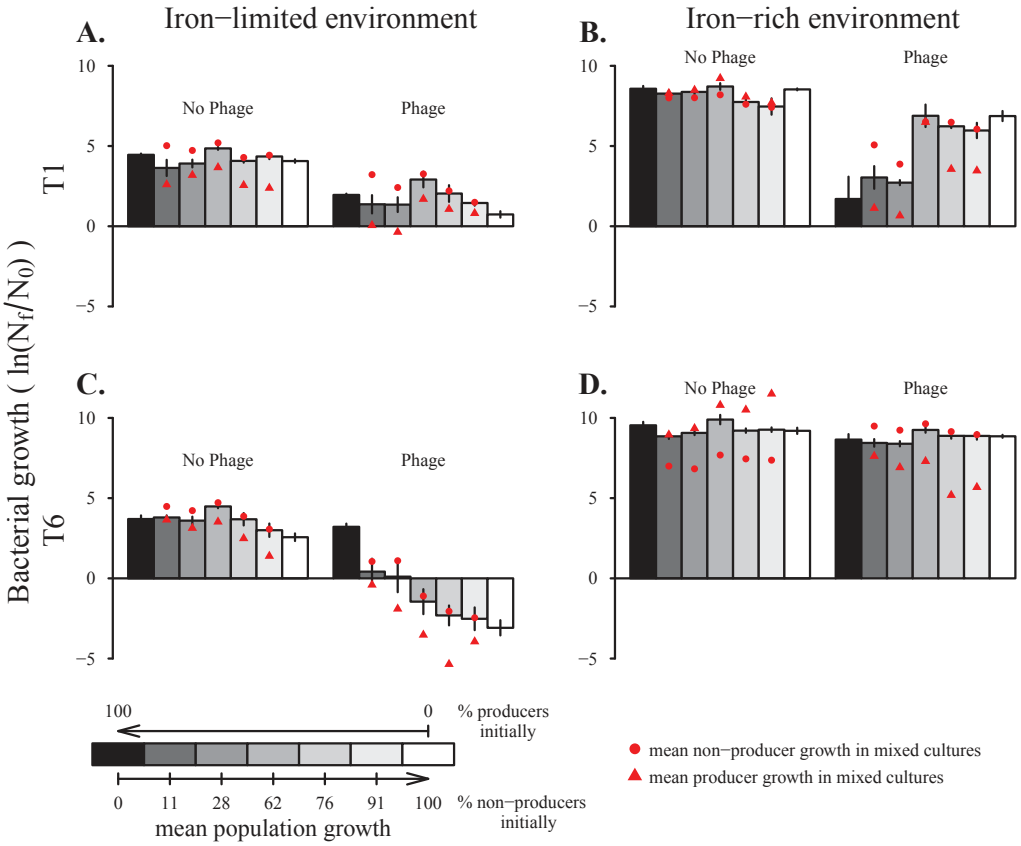

Figure 3.5: Total population growth (producer monocultures: black histobars; non-producer monocultures: white histobars; mixed cultures: grey scaled histobars corresponding to different initial frequencies of non-producers) for the first 24 hours (upper panel; A, B) and over the full 5 transfers (lower panel; C, D) in iron-limited (A, C) and iron-rich (B, D) environments. Red symbols are mean growth of producers (triangles) and non-producers (circles) in mixed cultures, respectively. Data are logarithmically transformed. Bars are standard errors of the mean.

Part II

Effects of phages and antibiotics as therapeutic agents on bacterial pathogens

4

A window of opportunity to control the bacterial pathogen *Pseudomonas aeruginosa* combining antibiotics and phages

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Clara Torres-Barceló, Flor I. Arias-Sánchez, Marie Vasse, Johan Ramsayer, Oliver Kaltz and Michael E. Hochberg. (2014) PLoS ONE 9 (9): e106628

Abstract

The evolution of antibiotic resistance in bacteria is a global concern and the use of bacteriophages alone or in combined therapies is attracting increasing attention as an alternative. Evolutionary theory predicts that the probability of bacterial resistance to both phages and antibiotics will be lower than to either separately, due for example to fitness costs or to trade-offs between phage resistance mechanisms and bacterial growth. In this study, we assess the population impacts of either individual or combined treatments of a bacteriophage and streptomycin on the nosocomial pathogen *Pseudomonas aeruginosa*. We show that combining phage and antibiotics substantially increases bacterial control compared to either separately, and that there is a specific time delay in antibiotic introduction independent of antibiotic dose, that minimises both bacterial density and resistance to either antibiotics or phage. These results have implications for optimal combined therapeutic approaches.

Introduction

Antibiotic resistant bacteria are a widespread problem that threatens human health. Due to the rapid adaptation of bacteria to old and new antibiotics there is an urgent need to develop alternative treatments (Rodriguez-Rojas *et al.* 2013, Garcia-Quintanilla *et al.* 2013, Allen *et al.* 2013b). Phage therapy, the use of parasitic viruses as antibacterial agents is attracting renewed attention due to their host specificity, innocuity for treated patients, and potential for evolution to outpace bacterial resistance (Pirnay *et al.* 2012). Despite considerable research on single or combined therapies involving phage (Wright *et al.* 2009, Chan *et al.* 2013), the underlying evolutionary processes remain poorly understood.

Evolutionary theory predicts that combined therapies can be more effective than a single component agent for preventing or limiting the evolution of antibiotic resistance (Bourguet *et al.* 2013), and this approach has gained attention in the control of pathogenic microbes (Lu & Collins 2009, Escobar-Páramo *et al.* 2012). Specifically, adaptive trade-offs can emerge due to fitness costs associated with resistance to more than one antimicrobial agent, as shown in the evolution of resistance to multiple antibiotics (Ward *et al.* 2009). Despite their potential, combined antimicrobial therapies are subject to the evolution of resistance due to convergent mechanisms of resistance if they target similar pathways, and the specific combination will determine the speed of resistance evolution (Yeh *et al.* 2009). Synergistic drug combinations, where joint antimicrobial effectiveness is greater than the individual effects, are more efficient and can be employed at lower doses, although selection for resistance can be substantial (Michel *et al.* 2008, Yeh *et al.* 2009). Antagonistic drugs have a combined effect that is lower than predicted, and although they generally slow the evolution of resistance are rarely used in a clinical context (Michel *et al.* 2008, Yeh *et al.* 2009).

The actual implementation of antibiotic therapies also has important implications for the development of resistance (Gumbo *et al.* 2007, Zinner *et al.* 2013). For instance, antibiotic dose can have an important effect on the evolution of resistance, but the mechanisms involved differ between low and high doses. In general, lower doses select for low cost resistance mutations that can be crucial to the stepwise acquisition of higher dose resistance, and higher doses impose stronger selection for resistant alleles (Canton & Morosini 2011, Read *et al.* 2011). Another factor influencing the short and

long-term efficiency of combined therapies is the timing of application, especially for antibiotics and phage, where phage replication and antibiotic effect are both density-dependent (Ryan *et al.* 2011). Phage population dynamics will be determined by the number of hosts in which they can replicate, with consequences for the amplification of phage densities and the therapeutic effectiveness (Levin & Bull 1996). If phages are administered at low bacterial densities or bacteria non-amenable physiologically, then the increase in phage densities will be lower and recurrent application of phages may be necessary (Levin & Bull 1996).

We challenged the opportunistic pathogenic bacterium *Pseudomonas aeruginosa* PAO1 with a lytic bacteriophage and the antibiotic streptomycin, with the aim of uncovering the effects of independent and combined treatments. This nosocomial pathogen species represents a particular danger to cystic fibrosis patients, and is known to readily evolve antibiotic resistance (Breidenstein *et al.* 2011). The antibiotic streptomycin has been shown to act synergistically when used with other chemical antimicrobials and is commonly used to treat *P. aeruginosa* infections (Zembower *et al.* 1998, Poole 2005). By studying *in vitro* bacterial density dynamics, we show that phages and streptomycin have a synergistic negative effect against bacteria. We also find a specific window of opportunity in the addition time of the antibiotic, enhancing the suppression of populations already treated with phage. Antibiotic dose did not significantly affect bacterial density, contrary to conventional clinical practice of using high antibiotic doses (Read *et al.* 2011). Finally, we find no evidence that the synergistic effect of the combined treatments is driven by genetic trade-offs between resistances to the phage and to the antibiotic. A more likely explanation is a demographic feedback produced by phage addition, limiting the capacity of the bacteria to resist antibiotic exposure. Our study provides an evolutionary basis for the optimization of combined treatments.

Materials and methods

Bacterium, phage and media

We used the bacterium *Pseudomonas aeruginosa* PAO1 and the phage LUZ7, from the *Podoviridae* family (Ceyssens *et al.* 2010). The experiment was carried out in 24-well

plates, with bacteria growing in King's B (KB) medium at 37 $^\circ$C without agitation. M9 medium was used for dilutions. The antibiotic streptomycin (Sigma-Aldrich) was added to liquid medium at either 100 or 240 μgm/L, known to represent sub-lethal and MIC concentrations for PAO1, respectively (Ward *et al.* 2009). The phage stock was prepared as described in Betts and colleagues (2013). Briefly, 10% vol/vol chloroform was added to phage-containing bacterial cultures, vortexed and centrifuged. Phage-containing supernatants were carefully recovered and stored at 4 $^\circ$C. This LUZ7 stock (10^7 PFU/mL) was used as the ancestral phages for all the experiments.

Experimental design

Six hours prior to the start of treatments, the 120 bacterial replicate populations were initiated from a *P. aeruginosa* PAO1 overnight culture, by adding 15 μL of culture to 1.5 mL of KB in 24-well plates. Phages were added (10^5 LUZ7 phages/mL) after 6 hours (T_0), when bacterial populations were growing exponentially and therefore vulnerable to phage attack. We used a concentration of phages high enough to affect the bacterial population dramatically (decreasing density by 6 orders of magnitude), but without producing complete extinction. We established single treatments, with only phage or only antibiotic added, as well as combined phage-antibiotic treatments (Fig. 4.1), named single-phage, single-strep and phage-strep, respectively. The antibiotic was added at one of three time points: simultaneously with the phage (+0 hours), with a delay of +12 hours, or with a delay of +24 hours. Two antibiotic doses were tested: 100 or 240 μg/mL. For each treatment we established 9 replicate populations, 108 total: 2 phage treatments (yes/no) $\times$ 2 antibiotic doses $\times$ 3 addition times$\times$ 9 replicates. Six control replicate lines were established for the single-phage treatment and for untreated control lines.

Density measurements and resistance assays

Bacterial density was measured at different time points (T_0, T_{14}, T_{45}, T_{70} respectively 0, 14, 45, and 70 hours post phage inoculation), by counting the number of growing colonies (colony-forming units, CFU) from samples plated on KB agar at appropriate dilutions.

At the end of the experiment (T_{70}), we assessed the surviving populations' resistance to streptomycin. 1 μL of the final populations were inoculated on to 250 μL of fresh KB containing streptomycin at different concentrations (12, 25, 50, 100, 200, 400 or 800 μg/mL). After 24 hours, bacterial density was measured by means of optical density (OD) at 600 nm (FluoSTAR, Optima fluorescence microplate reader, BMG Lab Technologies). Resistance was taken as the Minimum Inhibitory Concentration (MIC), defined as the streptomycin concentration at which no bacterial growth was detected. For populations even resisting the highest concentration (800 μg/mL) the MIC was arbitrarily set to 1600 μg/mL.

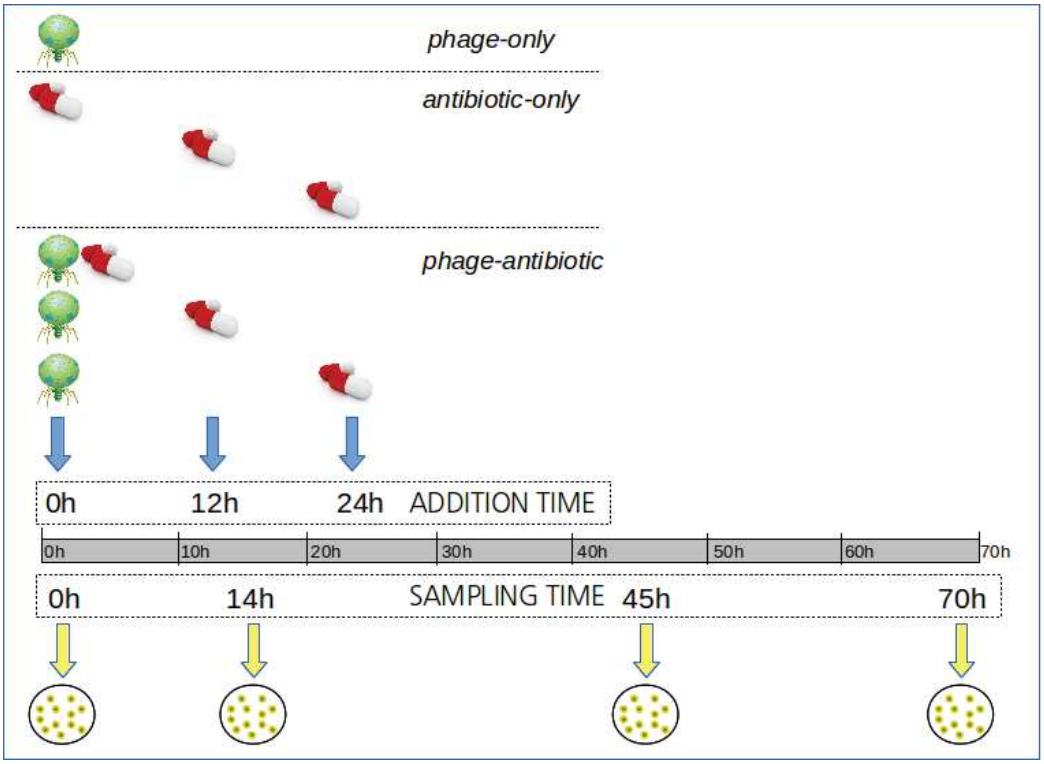

Figure 4.1: Overview of the experimental design. Exponentially growing bacteria were treated with (i) only phage (at 0 hours), (ii) only antibiotic (1 dose at 0 hour, 12 hours, or 24 hours) or (iii) first phage and then antibiotic (1 dose at 0 hours, 12 hours, or 24 hours). Replicate populations of the bacteria were sampled at 0 hours, 14 hours, 45 hours and 70 hours, for density and resistance measurements. All antibiotic treatments were repeated for two streptomycin doses (100 and 240 μg/mL).

To measure phage resistance, 1 μL of final bacteria was added to 250 μL of media containing ancestral phage (c 10^5 phages) and OD recorded after 24 hours. Phage resistance was taken as a quantitative trait, calculated as the difference in OD obtained with and without phage added. The same assay was performed with evolved phage. To this end, the 9 replicates from the last time point of a given treatment were pooled

and evolved phages extracted as described above. Thus, bacteria were confronted with a mix of phages from their own treatment. Bacteria from treatments without phage (single-strep, control, ancestral bacteria) were confronted with evolved phage from the +0 hours phage addition time treatment with 100 μg/mL of streptomycin. All OD values were corrected for absorbance of blank wells; replicates for which positive control wells without phage showed zero growth were not used for analysis.

Statistical analysis

Using the JMP statistical package (SAS 2012), we employed General Linear Model (GLM) techniques to analyze variation in bacterial density (CFU/mL, log10-transformed), antibiotic resistance (MIC, square-root-transformed) and phage resistance (OD difference between bacteria challenged with phage and not). In the main analyses, we tested fully factorial models, containing phage treatment (yes/no), antibiotic dose as explanatory factors and antibiotic addition time as a covariate. To test for non-linear effects of addition time, we also fitted its second-order polynomial term (addition time^2). Minimal adequate models were established through backward elimination of non-significant terms in the model. Where appropriate, analyses were carried out separately for single and combined treatments; additional tests compared evolved and ancestral bacteria.

To calculate expected final densities (70 hours) in combined phage-antibiotic treatments, we paired single-phage with single-strep replicates. For both replicates in a pair, we calculated the reduction in bacterial density relative to the untreated controls (difference in CFU/mL). We then added together the two single density reductions to obtain the expected density in a hypothetical combined phage-antibiotic treatment. Specifically, for each combination of antibiotic dose and addition time, 36 of the possible 81 (9×9 replicates from single treatments) pairs were arbitrarily chosen and the density difference calculated relative to each of the two untreated control lines. This gave a total of 72 expected values that were to be compared with the corresponding observed values in the true combined phage-antibiotic treatment.

Results

Bacterial density

We challenged *P. aeruginosa* with either single or combined treatments of the phage LUZ7 and two doses of the antibiotic streptomycin ('strep'), administered at different time points. Bacterial population density was tracked over 70 hours (Fig. 4.1) to test the hypothesis that the use of phages can contribute to reduce antibiotic doses below the MIC, and that simultaneous or sequential administration of the two antimicrobials have different consequences on bacterial densities. Both single-phage and single-strep treatments strongly reduced bacterial density over the first 24 hours, by up to 6 orders of magnitude (Fig. 4.2). However, densities rebounded and nearly reached the levels of untreated controls by the end of the experiment (70 hours) in all populations (Fig. 4.2, 4.3A).

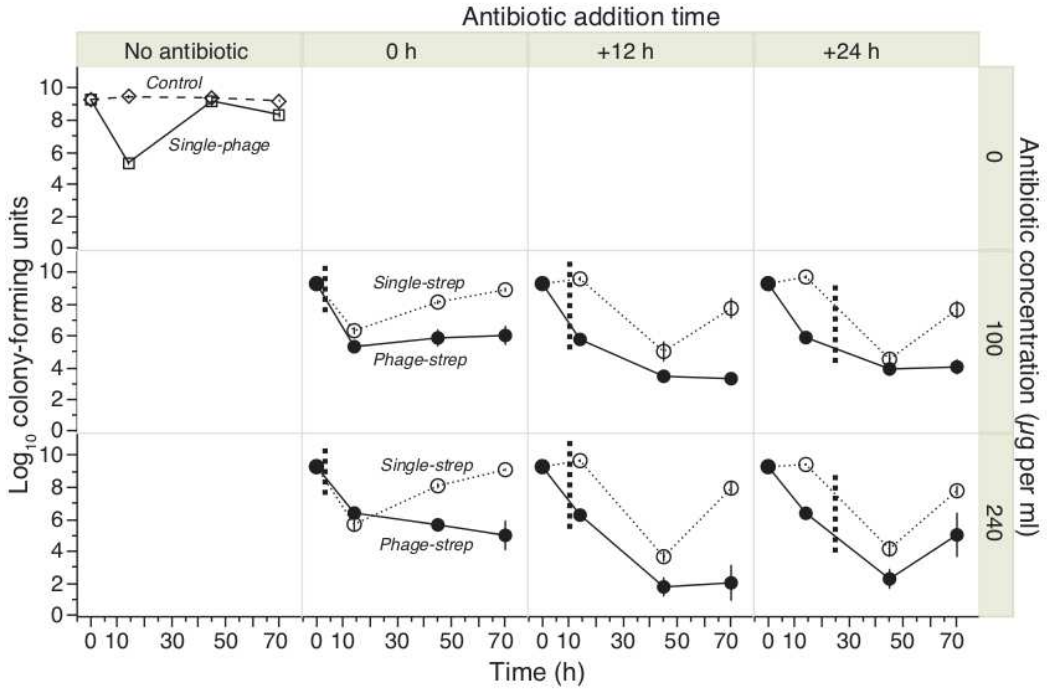

Figure 4.2: Changes in bacterial density over the course of the experiment. The six lower panels show the single antibiotic treatment ('single-strep') and the combined phage-strep treatment, for different addition times (dotted lines) of the antibiotic streptomycin and for two antibiotic doses. The top-left panel shows the single-phage treatment and the unexposed control lines.

The combined phage-strep treatment caused a significantly stronger reduction in

density compared to either single treatment (vs. single-phage: $t_{58} = 3.60$, $p = 0.001$; vs. single-strep: $t_{105} = 9.43$, $p < 0.0001$; Fig. 4.3A). Unlike in the single treatments, almost 60 % (30/54) of the populations did not recover from the combined treatment and showed strongly suppressed final densities ($< 10^5$ CFU/mL). We then evaluated the relative effects of simultaneous (0 hours) or delayed (+12 hours, +24 hours) addition of streptomycin to populations containing phage (Fig. 4.1). We found that bacterial density reduction at the end of the experiment was maximal when the antibiotic was added with a +12 hours delay (phage × strep addition time^2 interaction: $F_{1,95} = 5.03$, $p < 0.05$). Streptomycin dose (100 vs. 240 μg/mL) had no significant effect on final density, nor were there significant interactions with other treatments (all $p > 0.25$; Fig. 4.2).

We further assessed whether the combined action of phage and antibiotic was additive or synergistic. To this end, we extrapolated outcomes in combined treatments from added effects on final bacterial density in the single treatments. Final densities were significantly lower than expected ($F_{1,477} = 278.0$, $p < 0.0001$; Fig. 4.3B), indicating a positive synergistic action of phage and antibiotic. This positive synergy was most pronounced for the +12 hours antibiotic addition time (expected/observed×strep addition time interaction: $F_{2,477} = 14.06$, $p < 0.0001$; Fig. 4.3B). Thus, an intermediate time delay in antibiotic addition in the combined treatment resulted in the strongest negative impact on bacterial population density.

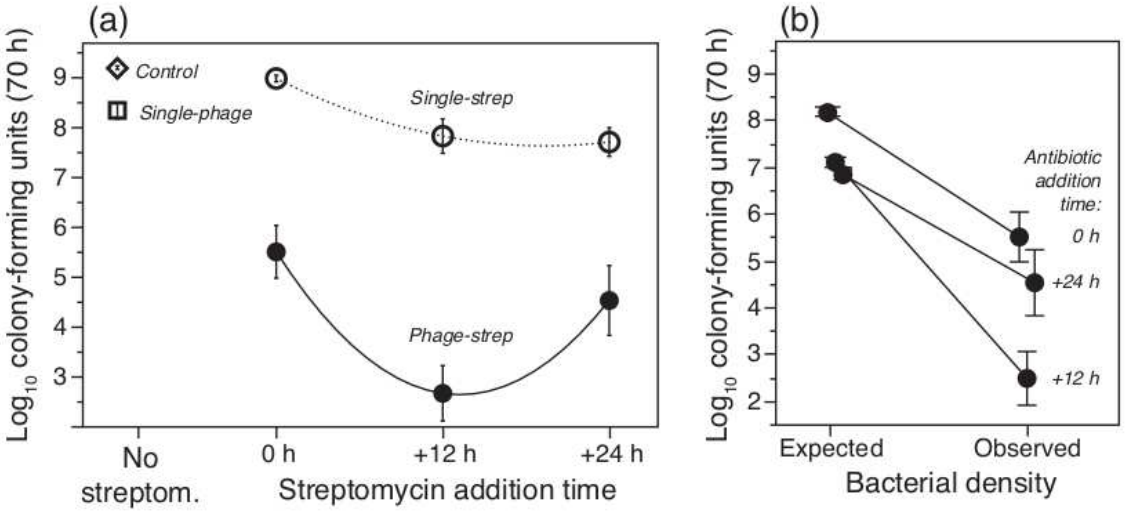

Figure 4.3: Final bacterial densities. (a) Effects of single treatments (single-strep, single-phage) and combined treatments (phage-strep), for different addition times of the antibiotic streptomycin in mean (± SE) final bacterial densities (70 hours). Control lines were untreated, and ancestral bacteria regrown from frozen stocks for the assay. Note that the lines connect final densities for independently tested addition times, and do not represent time series of bacterial density. (b) Expected and observed density in the combined treatments, for the different antibiotic addition times. Expected density extrapolated from single treatments, assuming additive action of antibiotic and phage.

Antibiotic and phage resistance

For the final bacterial populations (70 hours) we analyzed variation in resistance to (ancestral) phage and to the antibiotic, the latter measured as the Minimum Inhibitory Concentration (MIC) of streptomycin. Bacteria from streptomycin treatments generally evolved very high levels of resistance (MIC $\geq$ 800 μg/mL, Fig. 4.4A). In the single-strep treatment, resistance reached maximum levels, whereas antibiotic resistance was lower in the combined phage-strep treatment ($F_{1,79} = 27.6$, $p < 0.0001$), but nonetheless higher than in the single-phage treatment ($F_{1,40} = 47.41$, $p < 0.0001$, Fig. 4.4A). Resistance values were lower for populations where streptomycin was added to the phage with a +12 hours delay (treatment $\times$ strep addition time interaction: $F_{1,79} = 4.48$, $p < 0.05$) compared to the other treatments. Note that while ancestral bacteria were fully susceptible to streptomycin, moderate increases in resistance were detected for bacteria from the single-phage treatment and for totally unexposed controls (Fig. 4.4A).

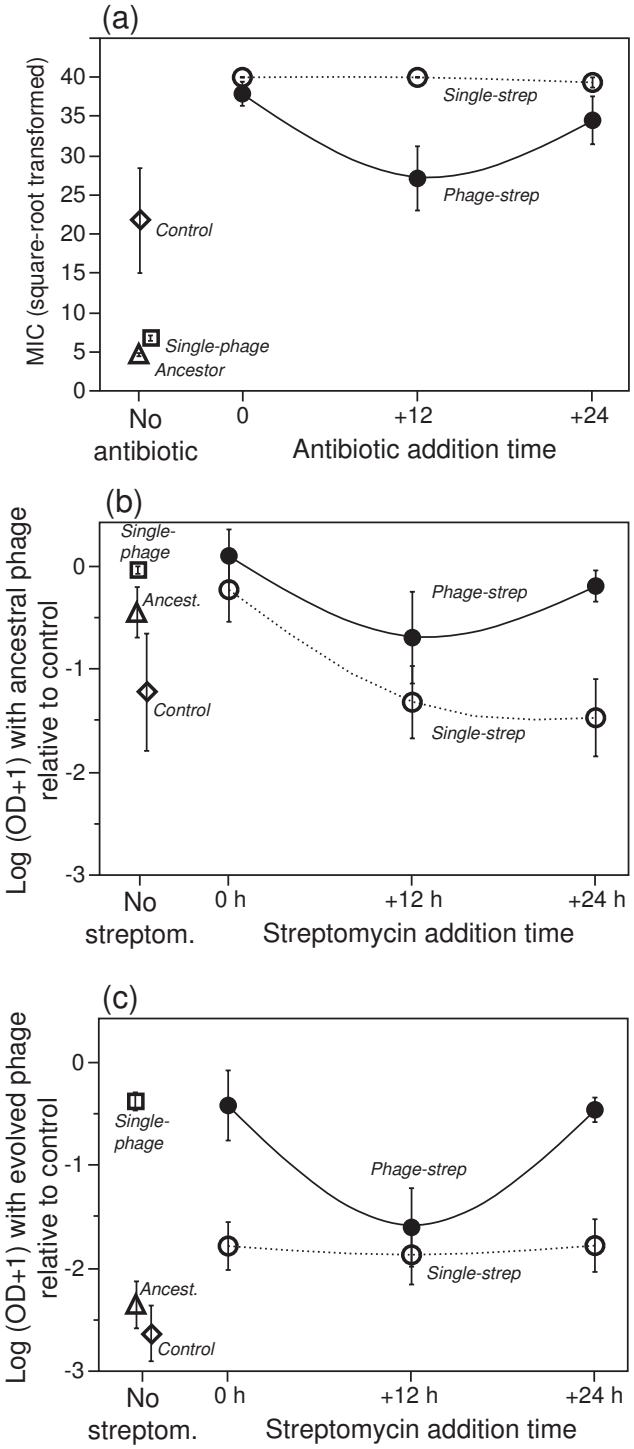

Figure 4.4: Mean (± SE) resistance of final bacterial populations (70 hours) from single (strep or phage) and combined (phage-strep) treatments. Control lines were untreated, and ancestral bacteria regrown from frozen stocks for the assay (a) Streptomycin resistance, assessed as the Minimal Inhibitory Concentration (MIC, in μg/mL). (b) Resistance to ancestral phage, calculated as the difference in optical density (OD, log-transformed) (+1) of bacteria in the presence and absence of phage, measured in a 24 hours growth assay. (c) Resistance to evolved phage, as measured in (b). Note that the lines connect final densities for independently tested addition times, and do not represent time series of bacterial density.

As expected, bacteria from phage treatments were more resistant to phage than bacteria from no-phage treatments ($F_{2,110} = 5.90$, $p < 0.01$; Fig. 4.4B). Specifically, the absence of phage tended to produce a decrease in resistance, suggesting a possible cost of resistance. Similar to the finding for antibiotic resistance, increases in phage resistance in the combined treatment tended to be minimal when strep was added with a +12 hours delay (strep addition time: $F_{1,32} = 2.97$, $p = 0.0947$). In the single-strep treatments, we observed a loss of phage resistance significantly affected by the antibiotic addition time ($F_{1,45} = 7.22$, $p = 0.01$). Very similar results were obtained when resistance was measured against evolved (from 70 hours) rather than ancestral phage, with a clear minimum when adding the antibiotic at +12 hours ($F_{1,32}$= 5.20, $p <$ 0.05, Fig. 4.4C). Resistance against ancestral phage was generally higher than against evolved phage (across all replicates: $t_{33} = 3.62$, $p = 0.001$), suggesting adaptation of phage to contemporary bacteria.

Finally, we found no evidence for a trade-off between antibiotic resistance and phage resistance in the combined treatment. In fact, the across-population correlation between the two traits was positive rather than negative ($r = 0.41$, $n = 34$, p < 0.05), indicating that higher levels of phage resistance were associated with higher antibiotic resistance.

Discussion

There is increasing attention on alternative treatments against bacterial pathogens, due to the inevitability of antibiotic resistance and difficulties in developing new antibiotics (Arias & Murray 2009). The combination of antibiotics and phages for clinical or environmental applications is a tantalizing possibility, but it is not known whether phage therapy alone or in combination with antibiotics will improve on antibiotics alone in the short term, and reduce or prevent resistance in the long term. In this work, we study the combined effect of an antibiotic and phages on *P. aeruginosa* bacterial population density and levels of evolved resistance, testing different application sequences and antibiotic doses. We show that combined treatments result in synergistic suppression of bacterial density and less resistance than either treatment alone, but also that the application sequence of both antimicrobials, and not antibiotic dose, is key to minimise the levels of resistance.

We found that combining phage and antibiotic results in lower bacterial density than expected from the addition of the respective single treatment effects (Fig. 4.3). This positive synergism is consistent with previous observations in *P. fluorescens* (Escobar-Páramo *et al.* 2012, Zhang & Buckling 2012), and probably due to resistance mutation limitation as a result of lowered bacterial population size (Martin *et al.* 2013). A possible explanation for this synergistic effect is a demographic feedback produced by the addition of phage (Levin & Bull 1996), limiting the capacity of the bacteria to resist antibiotic exposure, as suggested by the detailed bacterial density dynamics (Fig. 4.2). Bacteria were most affected when the antibiotic was applied when the phages themselves had their strongest impact on bacterial population density (+12 hours addition time), suggesting an optimal window of opportunity in the implementation of combined therapies to restrain pathogens. For the other two application times, the synergistic effect was reduced. When the two agents are applied simultaneously, streptomycin is likely to constrain the efficacy of phages due to intensive host damage by means of protein synthesis inhibition (Kohanski *et al.* 2010). If inhibition reduces the per-host cell output of phage, then overall phage titer may not be sufficient to cause massive reductions in bacterial cell density. Conversely, when the antibiotic is applied 24 hours after the phage, we argue that bacterial populations recover before being submitted to the antibiotic. This demographic feedback mechanism is consistent with 'evolutionary rescue', which links the demographic dynamics of population decline with the genetic dynamics of adaptation under rapid environmental deterioration (Ramsayer *et al.* 2013). Our results suggest that understanding the population dynamics and evolutionary biology of multiple interactive agents is important for the success of new therapies (Levin & Bull 1996).

A possible evolutionary risk of antimicrobial compounds producing a synergistic effect in a combined treatment is that resistance mutations have a larger selective advantage compared to the single treatments, and as such the rate of adaptation will be higher (Chait *et al.* 2007, Hegreness *et al.* 2008). In our combined treatments, resistance to both phage and antibiotic increased relative to the ancestral bacteria, indicating positive responses to selection by both agents (as already shown separately for phages and antibiotics in *P. aeruginosa*; Brockhurst *et al.* 2005, Hall *et al.* 2011b, respectively). However, resistance levels did not exceed those of single treatments: they were equal or even lower (Fig. 4.4). This suggests that our combined treatments did not lead to faster adaptation of the bacteria. Consistent with previous work (Drenkard

& Ausubel 2002), we observed an increase in antibiotic resistance in non-treated control populations relative to ancestral bacteria, possibly associated with biofilm formation, adaptation to the nutrient media or the emergence of low frequency antibiotic resistant mutants in the large populations occurring in our microcosms ($> 10^9$ cells/mL).

Interestingly, we show that an intermediate time delay between application of phage followed by an antibiotic leads to lower levels of bacterial resistance to either agent, as compared to shorter or longer delays, or to the application of either agent separately. Indeed, the sequential application of combined therapies has been suggested to generate lower levels of resistance compared to simultaneous addition, especially when the antimicrobial agents have different bacterial targets, as in a recent study employing phages that use different host receptors (Hall *et al.* 2012). We also provide experimental support that in synergistic combinations it is possible to reduce antibiotic doses and still reduce bacterial populations significantly whilst limiting resistance (Yeh *et al.* 2009). Given the prediction that demographic and genetic changes interact, it is not unexpected that the effects of addition time on resistance mirrored those on bacterial density. The capacity of treated bacterial populations to recover and attain high densities is directly related to the increasing frequency of resistant mutants, and thus the higher mean population resistance levels observed at the end of our experiment. Nevertheless, in another study using distinct combinations of phages against bacteria, Hall and colleagues (2012) argue that the effectiveness of multiphage therapy depends on the order and type of phages combined, indicating a more mechanistic constraint rather than the demographic one suggested here.

More generally, combined phage-antibiotic therapy may be expected to have an advantage over antibiotic cocktails of less cross-resistance because phage and antibiotics are fundamentally different regarding cellular mechanisms affected and the genetic changes resulting in resistance (Levin & Bull 1996). In particular, streptomycin resistance mutations typically involve the ribosomal protein S12 and 16S rRNA (Springer *et al.* 2001), whereas resistance mutations to a *Podovirus* such as LUZ7 usually require the alteration of lipopolysaccharide (LPS) components, the phage receptor on the bacterial outer membrane (Springer *et al.* 2001). Here, we observed a general positive association between mean levels of phage and antibiotic resistance for the combined treatments, suggesting potentially unconstrained multiple resistance evolution, or in other words, that trade-offs between phage and antibiotic resistance do not appear to play a role (De Paepe & Taddei 2006). It should be noted, however, that we measured

resistance at the population level; a more precise analysis of the relationship would require measurements of individual bacterial genotypes to establish genetic correlations.

As an antimicrobial agent, bacteriophages are different from antibiotics in that the former can evolve or even coevolve with the bacteria, and therefore potentially limit resistance evolution during treatment (Levin & Bull 1996, Escobar-Páramo *et al.* 2012, Zhang & Buckling 2012). How this (co)evolutionary component influences the efficiency and predictability of treatment outcomes is still largely unclear (Cairns *et al.* 2009, Hall *et al.* 2012, Betts *et al.* 2013). Here, we find evidence for the evolution of bacterial resistance to phage, but also evolutionary change in phage infectivity, in agreement with recent study (Betts *et al.* 2013). Overall, bacterial resistance to phage from the end of the experiment was lower than that to the ancestral phage, clearly suggesting evolution of the phage towards increased infectivity. Levels of resistance to ancestral and evolved phage were highly correlated, indicating considerable coherence in treatment effects regarding resistance evolution. Future study needs to evaluate to what extent these patterns involve coevolutionary specificity, and what the implications are for longer-term pathogen control.

We acknowledge that *in vitro* studies such as ours will be limited in predicting outcomes in a clinical context, in which other important drivers of selection for pathogenic microbes include the host immune system and its spatial structure (Brown *et al.* 2013), and bacterial densities might be significantly lower (Leggett *et al.* 2012). In addition, we should expect that resistance to either phages and/or antibiotics will entail fitness costs for bacteria that could be accentuated *in vivo* (Zhang & Buckling 2012). Interestingly, phage resistance may lead to selection for less virulent bacterial variants, for example, through the loss of surface phage receptors that are also virulence determinants, as in the case of *Yersinia pestis* (Filippov *et al.* 2011). This possibility should be explored both *in vitro* and in hospital settings to evaluate if combined approaches at disinfection are also able to reduce the pathogenicity of bacteria surviving such treatments.

The use of combined antimicrobial therapies for the treatment of highly resistant pathogens has been applied in the clinic, for example, to combat *Mycobacterium tuberculosis*, HIV, and the malaria pathogen *Plasmodium falciparium* (Fischbach 2011). Even though it has achieved considerable success, the potential to reduce the rate of evolution of resistance to combination therapies need to reach more problematically

resistant infectious diseases in the future (Cottarel & Wierzbowski 2007). A better understanding of the pharmacodynamics of combining phages and antibiotics will be vital to the eventual implementation of new therapeutic strategies targeting multiresistant nosocomial infections (Breidenstein *et al.* 2011). Our work shows that at an intermediate application time there is a window of opportunity, where mortality due to the antibiotic results in lower absolute populations and reduced resistance levels because bacterial populations are at low density, and both antibiotic and phage resistance mutations are less likely to be present (Ramsayer *et al.* 2013).

5

Long-term effects of single and combined introductions of antibiotics and bacteriophages on populations of *Pseudomonas aeruginosa*

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Evolutionary Applications (*in revision*) *authors contributed equally to this work

Abstract

With escalating resistance to antibiotics there is an urgent need to develop alternative therapies against bacterial pathogens and pests. One of the most promising is the employment of bacteriophages (phages), which may be highly specific and evolve to counter anti-phage resistance. Despite an increased understanding of how phages interact with bacteria, we know very little about how their interactions may be modified in antibiotic environments and, reciprocally, how phage may affect the evolution of antibiotic resistance. We experimentally evaluated the impacts of single and combined applications of antibiotics (different doses and different types) and phages on *in vitro* evolving populations of the opportunistic pathogen *Pseudomonas aeruginosa* PAO1. We also assessed the effects of past treatments on bacterial virulence *in vivo*, employing larvae of *Galleria mellonella*. We find a strong synergistic effect of combining antibiotics and phages on bacterial population density and in limiting their recovery rate. Our long-term study establishes that antibiotic dose is important but that effects are relatively indifferent to antibiotic type. From an applied perspective, our results indicate that phages can contribute to managing antibiotic resistance levels, with limited consequences for the evolution of bacterial virulence.

Introduction

Despite widespread bacterial resistance and dwindling discovery of new molecules, antibiotics are still overwhelmingly the principal agent used against bacterial infections (Laxminarayan *et al.* 2013). Alternatives are needed and it has been argued that antimicrobial approaches and more generally chemotherapies often ignore insights coming from evolutionary biology (Read *et al.* 2011, Pena-Miller *et al.* 2013). Specifically, study has shown that the goal of minimizing or eliminating pathogen populations through high-dose therapies can be counterproductive, since it will select for resistant or refractory phenotypes (Hughes & Andersson 2012, Ramsayer *et al.* 2013), which will repopulate the infection, and potentially spread into the environment.

A promising alternative to antibiotics is the use of phages. Phage therapy employs highly specific isolates as one or more applications of potentially perpetuating, coevolving anti-bacterial agents (Viertel *et al.* 2014). The majority of assessments of phage therapy comes from observational study (Fruciano & Bourne 2007), and with a few notable exceptions (Bruttin & Brüssow 2005, Wright *et al.* 2009, Sarker *et al.* 2012), most controlled experimental tests have been conducted *in vitro* or in animal models (Fu *et al.* 2010, Lood *et al.* 2015, Scanlan *et al.* 2015). Although some of these studies show support for the efficacy of phage therapy, one pervasive shortcoming is existing or evolved bacterial resistance (Bikard & Marraffini 2012, Seed *et al.* 2014).

Given the large population sizes attainable within a single bacterial infection (Leggett *et al.* 2012), it is not surprising that rare benefitial mutations have a higher probability of fixing, meaning that resistance evolution to single agents is a pervasive issue in control (Read *et al.* 2011, Gonzalez *et al.* 2013, Orr & Unckless 2014). Ecological and evolutionary theories provide testable hypotheses for when multiple control agents should be more effective at control than any subset (Hendry *et al.* 2011). Amongst alternatives to single agent, high dose therapies, increasing attention is being focused on combinations between two or more antibiotics (Hagihara *et al.* 2012), or antibiotics and phage (Lu & Collins 2009) as strategies for reducing or eliminating bacterial pathogen resistance. Given the potential diversity of bacteriophages predating a given bacterial strain (Kwan *et al.* 2005, Weitz *et al.* 2013), combinations of phages and antibiotics can be identified which will attack different bacterial targets and, should resistance mutations be present, these would be different for each agent thereby redu-

cing the probability of the emergence of resistance mutations to either or both agents (Escobar-Páramo *et al.* 2012, Hall *et al.* 2012). Studies have demonstrated improved efficacy of associating phages and antibiotics to treat methicillin resistant *Staphylococcus aureus* (MRSA) (Kirby 2012, Chhibber *et al.* 2013), *Pseudomonas aeruginosa* (Hagens *et al.* 2006, Knezevic *et al.* 2013, Torres-Barceló *et al.* 2014), and *Escherichia coli* strains (Ryan *et al.* 2012, Coulter *et al.* 2014). For example, Torres-barcelo and colleagues (2014) recently showed that in sequential therapies against *P. aeruginosa*, the specific timing between phage and antibiotic introductions provides a window of opportunity for control. Some of these studies indicate a synergistic effect between both antimicrobial agents in preventing bacterial growth (Hagens *et al.* 2006, Kirby 2012, Knezevic *et al.* 2013, Torres-Barceló *et al.* 2014), that is, that the effect of the combination is greater than the sum of effects produced by each agent separately (Loewe 1953). Whereas recent work suggests that double-resistant bacteria would be strongly selected if using antibiotic cocktails (Pena-Miller *et al.* 2013), antibiotic and phage combinations indicate the opposite effect (Verma *et al.* 2009, Kirby 2012, Zhang & Buckling 2012), although the underlying mechanisms remained unexplored. Moreover, little is known about the effects of antibiotic doses on the evolutionary process in combination therapies (e.g., for antibiotics and phages see Hagens *et al.* 2006, Torres-Barceló *et al.* 2014). Also, with few exceptions (Kirby 2012), long-term therapeutic effects, which may be more representative of *in vivo* situations, have not been explored. These two issues, dose and duration, are central in understanding resistance evolution (Read *et al.* 2011), and constitute a major challenge in employing combined therapies.

The continuous emergence of antibiotic resistance is especially important in Gram-negative bacteria, which cause approximately 70% of the infections in intensive care units (Hernandez *et al.* 2013). The bacterium used in the present study, *P. aeruginosa*, is a leading cause of nosocomial infections and chronic lung infections in cystic fibrosis patients (Mesaros *et al.* 2007). *P. aeruginosa* is intrinsically resistant to many antibiotics because of the limited permeability of its outer membrane and efflux pump systems (Breidenstein *et al.* 2011). It also has a high potential for resistance adaptation through mutational mechanisms, including increased efflux pump activity and enzymatic antibiotic modifications (Breidenstein *et al.* 2011). To date, antibiotic therapy is the principal means for controlling *P. aeruginosa* infections, and although combination therapies have been investigated involving multiple antibiotics (Traugott *et al.* 2011, Paul & Leibovici 2013), antibiotic-phage associations have not been extensively invest-

igated beyond the relative order of introduction of the antimicrobials (Holt & Hochberg 1997, Escobar-Páramo *et al.* 2012, Torres-Barceló *et al.* 2014), and limited work has considered the effect of combining different concentrations of antibiotics or phages on bacterial virulence (Hosseinidoust *et al.* 2013c). These are important questions, since it is not clear to what extent initially intense ecological interactions, longer-term evolutionary effects, and/or their interactions influence outcomes.

In this study we compare the effects of a range of doses of three antibiotics representing different modes of action on *P. aeruginosa* populations in the presence or absence of phages. With the aim of assessing long-term efficacy, we exposed bacteria to the different treatments for 7 days. We evaluated important aspects of bacterial evolutionary potential such as adaptation rate, final density, antibiotic resistance, resistance to phage, and virulence. We find that for all antibiotics tested, phages and antibiotics have negative synergistic effects on bacterial populations, and this is positively correlated with antibiotic dose. Even when single and combined treatments had similar effects on bacterial densities, recovery rates were slower for all the combined antibiotic-phage conditions. Interestingly, combination treatments limited antibiotic resistance levels compared to antibiotic treatments alone, whereas antibiotics did not have this reverse effect on phage resistance. Finally, both single and combined treatments reduced bacterial virulence in wax moth larval hosts compared to untreated ones, but phage treated bacteria attenuated the magnitude of the reduction in virulence. We discuss the relevance of our findings for future research aimed at treating bacterial infections in human health care.

Materials and Methods

Bacteria and phages

We used the bacterium *P. aeruginosa* PAO1 (F.E. Romesberg's strain Cirz *et al.* 2006) and the lytic phage LKD16, from the *Podoviridae* family (Ceyssens *et al.* 2006). All experiments were carried out in 96-well plates, with bacteria growing in 200 μL of King's B (KB) medium at 37 $^\circ$ C without agitation. M9 medium was used for dilutions. The phage stock was prepared as described elsewhere (Betts *et al.* 2013). Briefly, 10% vol/vol chloroform was added to phage-containing bacterial cultures, vortexed and

centrifuged. Phage-containing supernatants were carefully recovered and stored at 4 °C. This LKD16 stock was titered (1.12×10^7 PFU/μL) and used as the ancestral phage for all the experiments.

Treatments and experimental evolution

Individual clones of *P. aeruginosa* PAO1 were isolated in KB solid agar plates from the clonal stock stored at -80 °C. Three microcosms with 6 mL of KB were used to each amplify a different, arbitrarily selected clone under continuous shaking (200 rpm) for 6 hours to obtain an exponential phase culture of c 10^6 cells/mL. These microcosms were then mixed and used as the inoculum for the evolution experiment.

To study the long-term effects of different phage-antibiotic treatments, c 2×10^3 cells of *P. aeruginosa* PAO1 (from a mix of the three microcosms) were inoculated into 200 μL of fresh media containing phage-only, antibiotic-only or phage-antibiotic conditions. We used the antibiotics carbenicillin, gentamicin and trimethoprim (Sigma-Aldrich), belonging to the following families (bacterial pathways targeted): β-lactam (inhibits cell wall synthesis); aminoglycoside (blocks protein synthesis); sulfamide (interferes with nucleic acid synthesis). These antibiotics were added to liquid medium at the required concentrations to inhibit 5%, 50% and 95% of bacterial growth, hereafter referred to as IC5, IC50 and IC95, respectively (see Table 5.1 for the specific concentrations). Phage dose was determined in pilot studies using the same bacterial starting density (c 10^4 cells/mL) as that achieving 90% bacterial growth inhibition relative to a control after 24 hours (1 PFU/ 100 bacteria). This concentration of a moderately virulent phage allowed us to follow bacterial population dynamics under conditions where both bacterial extinction and complete resistance were unlikely.

Table 5.1: Concentrations of the different antibiotics for ID5, ID50, ID95 and MIC, in mg/L.

Antibiotic concentrations (mg/L)				
	IC5	IC50	IC95	MIC
Carbenicilin	9	42	67	102
Gentamicin	27	42	61	70
Trimethoprim	64	162	444	512

Six replicates of each treatment were distributed arbitrarily in three 96-well plates, for a total of 108 populations (6 replicates × 3 antibiotics × 3 doses × 2 phage (presence or absence)). An equivalent number of replicates per plate with phage-only treatments

and non-treated bacteria (positive controls) were also established (6 replicates × 2 controls × 3 plates: 36 populations). The total number of populations was 144. Negative controls with media only were distributed in all plates to monitor for the occurrence of possible contamination.

Bacterial density was measured every 24 hours by means of optical density (OD) at 600nm (FluoSTAR, Optima fluorescence microplate reader, BMG Lab Technologies). So as to homogenise cultures, thorough pipette mixing of each well was performed before transferring each population into a new well containing either fresh media under the same treatment conditions (antibiotics and/or ancestral phages) or under control conditions. A volume corresponding to 10% of each population was calculated and transfered, to minimise possible bottleneck processes. This same protocol was carried out for seven consecutive days. After being transferred, the remainder of the microcosm was stored in 20% glycerol at -80 ° C for further analysis.

Phage effect on bacterial growth

To measure bacterial resistance to phage, we compared the growth of either evolved or ancestral bacteria, with or without ancestral phage. Final bacterial populations and the ancestral stock, all stored at -80 ° C, were streaked on solid agar KB plates to isolate colonies. Four replicate populations of each treatment were used and one colony of each was amplified in 1 mL of KB in 48-well plates at 37 ° C. After overnight incubation, all cultures were centrifuged at 13 000 rpm, the supernatant discarded and the pellet resuspended in M9. This procedure eliminated most previous phage present in the evolved bacteria. OD was then standardised to the same level for all tested populations, c 10^6 cells added to 200 μL of KB with and without the ancestral LKD16 phage (1 PFU/ 100 bacteria), and OD recorded every 20 minutes for 24 hours at 37 ° C. To minimise the effects of condensation and biofilm interference on OD readings, 10 seconds of agitation at 200 rpm was programmed to occur before each measurement.

Determination of antibiotic resistance levels

Antibiotic resistance level was measured as the Minimum Inhibitory Concentration (MIC) at which no bacterial growth was detected. Approximately c 2×10^3 cells of each final treatment population were inoculated into media containing concentrations

of each respective antibiotic at two-fold increments. After 24 hours, we scored growth inhibition as OD < 0.1. Antibiotic resistance was scored for the evolved populations relative to that of the ancestor (normalised to MIC=1). Thus, a value of 2 reflects a doubling in the MIC relative to the ancestral clone, 3 is twice the MIC of 2, and 4 twice the MIC of 3.

Virulence assays

The virulence of evolved bacteria and ancestral clones was measured by inoculating a sample from each of the frozen final populations and the frozen ancestral stock, respectively, into fresh media and incubating at 37 ° C until obtaining exponential phase cultures (OD between 0.4 and 0.7). Bacterial replicates from the same treatment were then pooled, centrifuged and resuspended in M9 buffer and the density adjusted through dilution to *c* 5×10^3 cells/mL. 20 μL of each bacterial treatment was injected into each of 12 wax moth (*Galleria mellonella*) larvae of homogeneous size (*c* 2-3 cm) with a micro-injector. Negative control treatments injecting only M9 were also performed. Larvae were kept at 37 ° C. The virulence of evolved and ancestral bacteria was assessed as mortality rate per treatment, established by observing larval mortality every hour for 48 hours.

Statistical analysis

All statistical analyses were conducted using R statistical software (R 3.1.1; http://www.r-project.org/). For the main analysis, we applied fully factorial models by including phage treatment (yes/no), antibiotic type and antibiotic dose as explanatory factors. Repeated measures through time were included as random factors when present. Where appropriate, analyses were carried out separately for single and combined treatments.

Specifically, we performed Linear Mixed Effects models for the analysis of bacterial density through time and t-tests to compare densities between treatments for final (day 7) densities. To calculate the rate of adaptation over the seven days of the experiment, a non-parametric and non-linear approach was carried out as follows. First, the deviation from the mean OD of the untreated control of each plate at each time (which varied significantly between plates and in time $F_{4,158} = 1181.731, p < 0.001$) was subtracted

from the observed OD values to correct for "plate" and "time" effects. In a second step, a model was fitted for each replicate via a logistic-type nonlinear least squares function (Kahm *et al.* 2010):

$$y(t) = \frac{A}{1 + e^{\left(\frac{4\mu}{A}(\lambda - t) + 2\right)}}.$$

A smoothed cubic spline was used to estimate the lag-phase λ, the maximum slope μ, and maximum growth A ('grofit' package, Bates & Chambers 1992). Once statistically fit, we calculated the rate of adaptation (r) from the following equation:

$$r = \partial_t \log[y(t)] = \frac{4\left(1 - \frac{1}{1+e^{\left(2+\frac{4\lambda\mu}{A}\right)}}\right)\mu}{A},$$

and used ANOVAs to analyze the effect of the different treatments on r.

We investigated whether combined treatments were subadditive, additive or superadditive (i.e., synergistic) by pairing random couples of single-phage with single-antibiotic replicates at each time-point of the evolution experiment for each dose and antibiotic type. For both replicates in a pair, we calculated the reduction in bacterial density relative to the untreated controls. We then added the two single treatment reductions to obtain the expected density in a hypothetical combined treatment. We performed this operation for every replicate in the experiment. The effect was scored as synergistic if $\Delta\text{OD}_{\text{phage}} + \Delta\text{OD}_{\text{ab}} < \Delta\text{OD}_{\text{phage+ab}}$, where Δ is the absolute decrease in optical density (OD), and phage+ab is the combined treatment. The maximum additive effects were limited to the untreated control OD values, the biological meaningful threshold (i.e. maximum reduction is the density of the untreated control). We then compared densities between observed and expected replicates in a factorial model. A non-parametric Spearman correlation was used to test the linear dependence between antibiotic dose and synergy (calculated as observed values subtracted from expected values) variables.

We employed General Linear Model techniques to analyze variation in antibiotic resistance levels (MIC). To study the possibly inhibitory effect of ancestral phages in the final populations, we considered growth over 24 hours and the final OD after 24 hours. The area under the curve (AUC) was estimated for each OD dynamic with

the R package 'MESS' (Ekstrom 2011). The estimated AUCs were compared between treatments using ANOVAs. Multinomial analyses were used to analyze the effect of treatments on the final OD.

Time-dependent larval survival was estimated using the Kaplan-Meier technique (Bland & Altman 1998). A multivariate Cox model was then applied to compare the survival curves due to bacteria isolated from different treatments.

Results

Analysis of bacterial population dynamics over 7 days

As expected, the addition of phage or antibiotic had a strong negative effect on bacterial densities during the experiment (Linear Mixed-Effect Model, phage: $F_{1,42} = 340.19, p < 0.0001$; antibiotic: $F_{1,42} = 143.85, p < 0.0001$; Fig. 5.1). Across time, there were significant antibiotic type and dose effects and interactions between these and the addition of phage (LME, antibiotic type: $F_{2,709} = 19.90, p < 0.0001$; antibiotic dose: $F_{1,32} = 184.68, p < 0.0001$; phage × antibiotic type: $F_{2,709} = 2.29, p < 0.05$; phage × antibiotic dose: $F_{1,32} = 1.91, p < 0.01$; Fig. 5.1), meaning that the specific combinations of different antibiotics with phage had an impact on bacteria density dynamics. At final bacterial densities (day 7) however, the effect of antibiotic type disappears and only the addition of phage and antibiotic dose are significant (LME, antibiotic type: $F_{2,66} = 28.93, p = 0.2530$; phage: $F_{1,32} = 60.97, p < 0.05$; antibiotic dose: $F_{1,32} = 55.23, p < 0.0001$; Fig. 5.1). Specifically, bacteria treated with phage and antibiotics reached an average final optical density (OD) of 0.92 ± 0.07, whereas antibiotic-only treated bacteria had an OD of 1.32 ± 0.05. Final density values showed a significant negative correlation with antibiotic dose (Pearson's test: t = -6.92, df = 142, $p < 0.0001$). These results indicate that the addition of phage combined with high doses of any of the antibiotics reduces the long-term growth of *P. aeruginosa* PAO1.

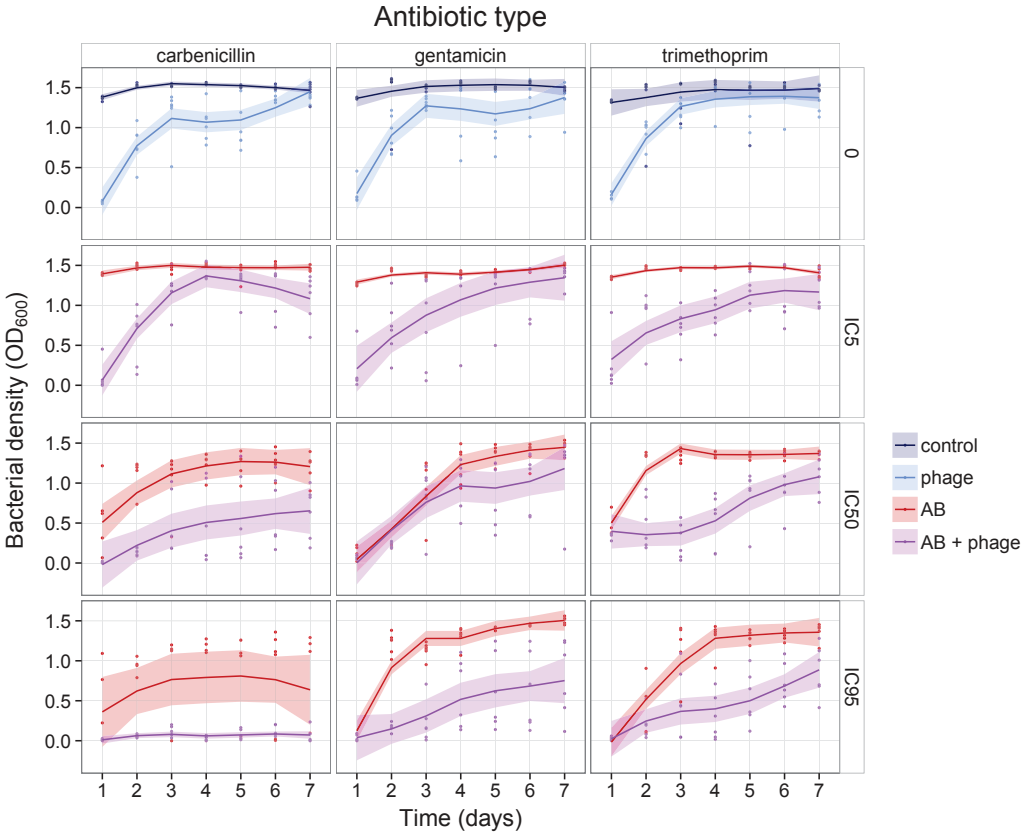

Figure 5.1: Bacterial density dynamics measured every 24 hours for 7 days. The horizontal panels represent the different concentrations of antibiotics relative to the resulting % inhibition of growth (IC: inhibitory concentration), and the vertical panels represent the different antibiotic types used. 'Control' corresponds to untreated bacteria, 'phage' to phage only, 'AB' to antibiotic only and 'AB + phage' to combined phage and antibiotic treatments. Solid lines represent the means of 8 populations. Shaded regions are 95% confidence intervals.

We find that bacterial density recovery rates (slopes of bacterial density over 7 days of treatment) are marginally affected by the addition of phages and significantly by antibiotics (ANOVA: $F_{1,134} = 3.27, p = 0.0660$; $F_{2,134} = 13.80, p < 0.01$, respectively). On average, slopes calculated with a non-parametric and non-linear approach (see Material and Methods) increase with antibiotic dose ($F_{1,48} = 29.45, p < 0.0001$) under antibiotic-only treatments (2.08 ± 0.36) (Left caption, Fig. 5.7). In contrast, in the double treatments we observe smaller recovery rates (1.85 ± 0.22), higher at the lowest antibiotic doses, and decreasing as the concentration of antibiotic increases, indicating a significant interaction between the addition of phages and antibiotic dose (ANOVA antibiotic dose: $F_{1,48} = 11.73, p < 0.01$; antibiotic dose × phage: $F_{1,98} = 39.13, p < 0.0001$; Right caption, Fig. 5.2). These results thus show a pronounced effect of high antibiotic dose when combined with phages in limiting bacterial population recovery,

whereas bacteria recover faster with high dose of antibiotics alone. Furthermore, we detect significant effects of using different antibiotic types in single treatments, but not in the presence of phages (ANOVA antibiotic type: $F_{2,48} = 6.40, p < 0.01$; $F_{2,48} = 1.87, p = 0.1659$, respectively).

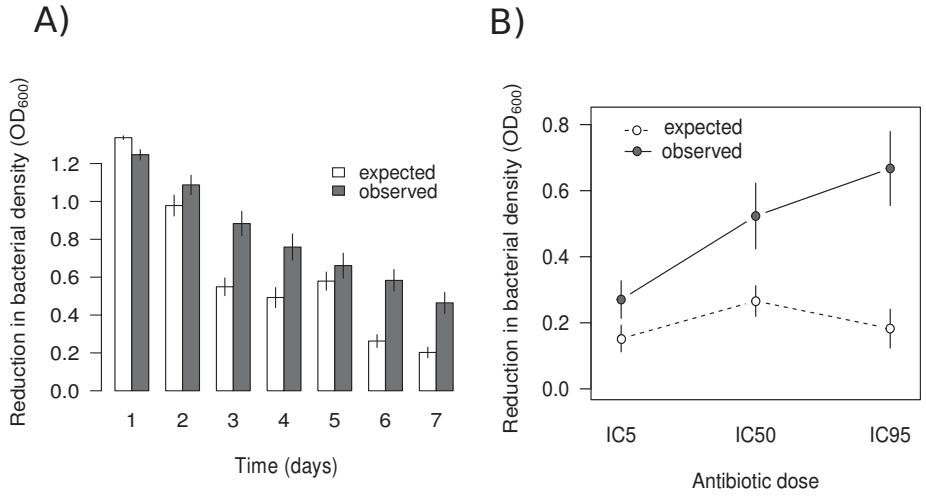

Figure 5.2: Synergistic effects of treatments: expected additive versus observed effects of combined phage-antibiotic treatments in preventing growth in bacterial populations. Effects on growth are measured as decreases in density relative to the control. The expected additive effect was calculated by summing the individual effects of phage and antibiotic treatments. A synergistic effect was identified when the observed OD difference was significantly higher than the expected additive effect. Panel A: Synergistic effects through time. Antibiotic types and doses were combined. Panel B: Synergistic effects in final bacterial populations (day 7) as a function of antibiotic dose (% inhibition of growth IC). Antibiotic types were grouped. For both panels bars represent standard errors.

A synergistic effect was identified when the recorded combination OD difference (observed) was significantly higher than the expected additive effect (see Material and Methods for more details). Overall, the combination of phages and antibiotics had a synergistic effect on reductions in bacterial density (LME: $F_{1,34} = 58.71, p < 0.0001$; Fig. 5.2). However, we see no such effect over the first 2 days of the experiment (LME: $F_{1,34} = 0.08, p = 0.7839$; Fig. 5.2A), presumably because of the initial strong ecological effect of each antimicrobial separately. Beyond two days, the combination of phages and antibiotics resulted in a considerable extra (i.e., synergistic) decrease in the populations compared to a simple additive effect (LME: $F_{1,34} = 74.18, p < 0.0001$; Fig. 5.2A). There were significant differences between antibiotic types in the synergistic effect through time (LME: $F_{2,34} = 19.69, p < 0.0001$; Fig. 5.4). Nonetheless, analogous to the density results, the antibiotic type effect vanishes by the end of the experiment

(ANOVA: $F_{2,34} = 3.09, p = 0.0507$), whereas the antibiotic dose component plays an important role for the duration of the experiment and at the final time-point (LME: $F_{1,34} = 144.74, p < 0.0001$; day 7 ANOVA: $F_{1,34} = 16.11, p = 0.0001$; Fig. 5.2B). Indeed, there is a strong positive correlation between antibiotic dose and the synergistic effect (Pearson's correlation: t= -5.24, d.f. = 334, $p < 0.0001$; Fig. 5.2B). Comparing the final observed and expected effects, carbenicillin produced the maximum synergistic effect, with a 3-fold difference between expected and observed values in the reduction of bacterial density, compared to the 2-fold difference observed for gentamicin and trimethoprim (Fig. 5.4).

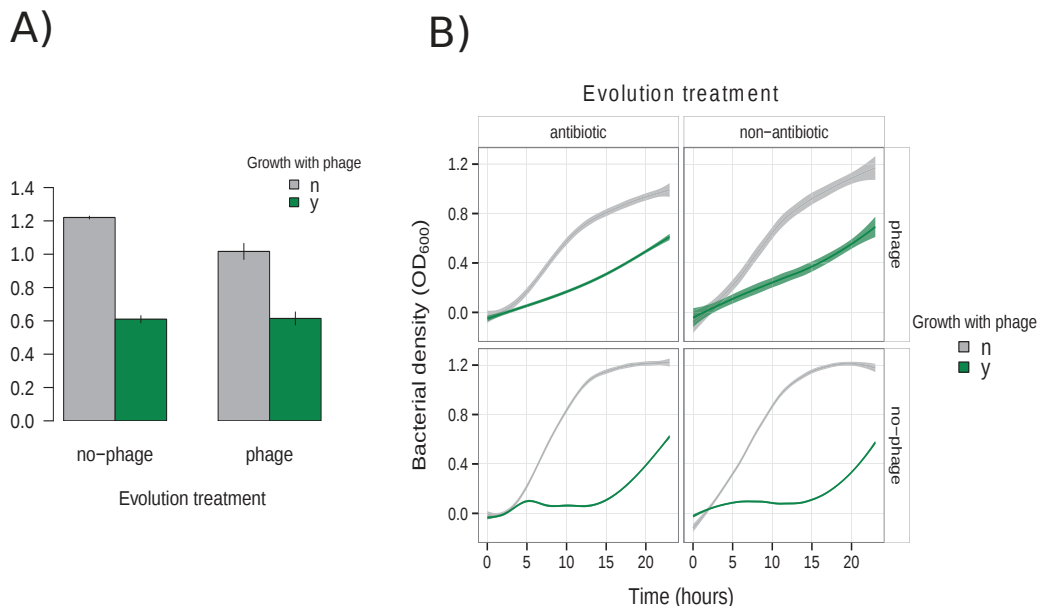

Figure 5.3: Effects of reintroducing ancestral phage into final bacterial populations. Panel A represents the final OD attained by bacteria in 24 hours depending on if they were evolved with or without phages (x-axis). The legend indicates re-exposure or not to the ancestral phage (green and grey, respectively). Bars are standard errors. Panel B shows the OD values over 24 hours used to determine the AUC (area under the curve) in the presence or absence of ancestral phage (green and grey, respectively). The different vertical panels represent treatments of bacteria evolved with antibiotics or not. Horizontal panels divide treatments where phage was or was not introduced as a treatment. Antibiotic types and doses were grouped.

In summary, bacterial densities are significantly affected by the combination of phages and antibiotics compared to either separately. The higher the dose of antibiotic combined with phage, the more populations decreased and the slower they recovered. In contrast, with antibiotics only, the higher the dose, the faster the rate at which bacteria recovered. This is also reflected in the combined effects of antibiotics and phages

being synergistic and dependent on the antibiotic dose used.

The synergistic effect observed between antibiotic and phages suggests that either double-treated bacteria were not capable of achieving complete resistance to phages or antibiotics, or that the surviving (resistant) populations carried costs that impaired their normal growth. To investigate these possibilities, we measured: i) ancestral phage effects on bacterial growth capacity, ii) antibiotic resistance levels and (iii) growth capacity of the final bacterial populations.

Analysis of final treated bacteria

Resistance to phages

To estimate the effect of previous treatment on bacterial growth in the presence of ancestral phages, we isolated colonies from the end of the experiment (day 7) and inoculated them with the ancestral phage. We then measured final densities after 24 hours to assess the inhibitory effect of phages and also the Area Under the Curve (AUC) as an estimate of the overall bacterial growth capacity (Fig. 5.3).

We observed a strong effect of ancestral phage in decreasing bacterial densities after 24 hours (1.12 $\pm$ 0.03 reduced to 0.62 $\pm$ 0.02; ANOVA: $F_{1,70} = 52.13, p < 0.0001$). Surprisingly, this reduction was not significantly different between bacterial populations previously treated with or without phage (ANOVA: $F_{1,75} = 0.019, p = 0.8920$; Fig. 5.3A). The final bacterial density in the presence of ancestral phage did not differ depending on previous treatment with different antibiotic types or doses (ANOVA, antibiotic type effect: $F_{2,75} = 1.10, p = 0.364$; antibiotic dose: $F_{1,75} = 0.02, p = 0.881$). These results suggest that, independent of treatment, evolved bacteria were still highly susceptible to the ancestral phage.

A deeper analysis of the effect of the ancestral phage on overall 24 hours bacterial growth measured as the AUC (Area Under the Curve) revealed slightly different effects. When confronted with ancestral phage, bacteria that had been previously treated with phage showed a linear increase and higher AUC values than non-phage treated bacteria, the latter exhibiting an initial lag phase with a small peak, followed by exponential recovery (ANOVA: $F_{1,60} = 9.32, p < 0.01$; Fig. 5.3B). Nonetheless, single antibiotic treatments or combined treatments with phage did not differ in overall growth with

ancestral phages ($F_{1,80} = 1.08, p = 0.302$). In single treatments, neither antibiotic type nor dose significantly affected the AUC in the presence of ancestral phage ($F_{2,60} = 1.55, p = 0.2200$; $F_{1,60} = 0.09, p = 0.7720$; respectively). In contrast, antibiotic type was found to be significant in combined treatments, whereas there was no effect of antibiotic dose ($F_{2,20} = 5.06, p < 0.01$; $F_{1,20} = 0.04, p = 0.8524$; respectively). However, when combined and only-phage treatments were analyzed separately, none of the three antibiotics had a significant effect on phage resistance (carbenicillin, $F_{1,20} = 0.48, p = 0.4907$; gentamicin, $F_{1,20} = 0.18, p = 0.6757$; trimethoprim, $F_{1,20} = 3.49, p = 0.0618$). These results show that treatments with and without phages have different growth dynamics in the presence of the ancestral phage, even though the final outcome (final OD) was apparently unchanged, indicating the expression of partial resistance. Finally, as expected in the absence of ancestral phage, the growth capacity was significantly limited for bacteria resulting from combined phage-antibiotic treatments compared to either single treatment, both for final OD and the AUC ($F_{1,75} = 21.47, p < 0.0001$; $F_{1,60} = 23.60, p < 0.0001$; respectively; Fig. 5.3). This difference suggests a cost of resistance in double treatments, constraining them to sub-optimal growth even in the absence of antimicrobials.

Resistance to antibiotics

We then tested whether the combined treatments limited the final level of antibiotic resistance (MIC) acquired by the surviving evolved bacteria compared to ancestral levels. We also determined the magnitude of the change in antibiotic resistance generated by the different antibiotic doses and types. Treatments with antibiotics resulted in different levels of increase in the MIC compared to controls, but the addition of phages attenuated this effect (Multinomial analysis, antibiotic addition; $\chi^2 = 13.01$, d.f. $= 5$, $p < 0.05$; phage addition: $\chi^2 = 22.482$, d.f. $= 5$, $p < 0.001$). We found evidence for an antibiotic type effect, but not an antibiotic dose effect on the MIC increase relative to the ancestral bacteria (MNA, antibiotic type: $\chi^2 = 192.56$, d.f. $= 10$, $p < 0.0001$; Fig. 5.4; antibiotic dose: $\chi^2 = 7.01$, d.f. $= 5$, $p = 0.2201$). These results suggest that phages constrain the evolution of antibiotic resistance in combined treatments.

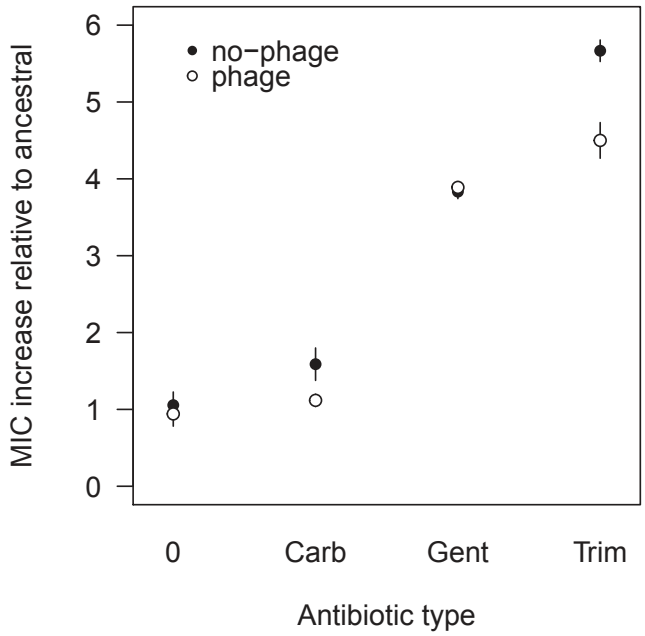

Figure 5.4: Antibiotic resistance increases in final bacterial populations relative to the ancestor (assigned as MIC = 1) regarding the presence and type of antibiotic. 'No-phage' and 'phage' indicate whether bacteria evolved with phages. Antibiotic types are assigned as '0' for non-antibiotic treatments, 'Carb' for carbenicillin, 'Gent' for gentamicin and 'Trim' for trimethoprim. Different antibiotic doses were grouped. Bars are standard errors.

Virulence

We then analysed the consequences of the 7-day treatments on bacterial virulence *in vivo*, inoculating the evolved bacteria into wax moth larvae (*Galleria mellonella*) and measuring their life-span. Supplementing phages in addition to antibiotics daily *in vitro* reduced *P. aeruginosa* final virulence in wax moth larvae compared to non-treated controls, but the reduction was smaller than that of bacteria treated with antibiotic alone (combined treatments vs controls: Z = -15.7, d.f. = 3, $p < 0.0001$; combined treatments vs antibiotic-only: Z = 4.89, d.f. = 5, $p < 0.0001$; Fig. 5.5). Phage only treatments produced bacteria that were on average 17 % more virulent than the non-treated controls (Z = 2.76, d.f. = 1, $p < 0.01$), but still significantly less virulent than ancestral bacteria (Z = -5.46, d.f. = 1, $p < 0.0001$). In general, all evolved bacteria (controls and treatments) were less virulent relative to ancestral bacteria (non-treated vs ancestral: Z = -4.90, d.f. = 1, $p < 0.0001$), and we found statistically significant

differences depending on the antibiotic type and dose used (Z for type = 10.31, d.f. = 2, $p < 0.0001$; for dose: Z = 12.86, d.f. = 2, $p < 0.0001$, Fig. 5.4), with low doses reducing virulence compared to higher doses.

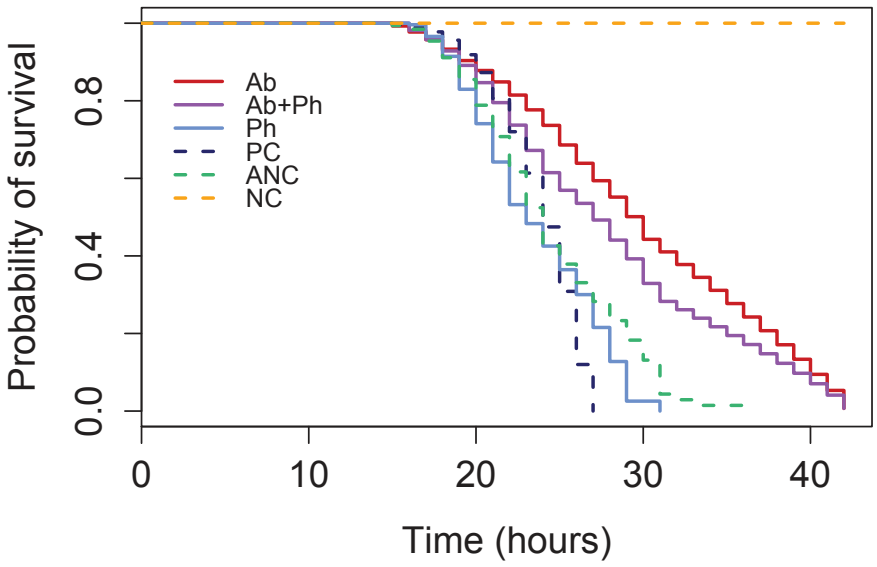

Figure 5.5: Probability of survival of *Galleria mellonella* larvae inoculated with ancestral or final bacterial populations from the different treatments. Antibiotic doses and types are grouped. Ab (only antibiotic), Ab+Ph (antibiotic and phages), Ph (only phages), PC (evolved without any treatment), ANC (ancestral bacteria), NC (larvae inoculated with buffer only).

Discussion

Previous study has considered the separate effects of phages and antibiotics on bacterial population sizes and resistance (Hall *et al.* 2012, Pena-Miller *et al.* 2013), and several recent investigations have assessed simultaneous and/or sequential applications of these antimicrobials (Hagihara *et al.* 2012, Escobar-Páramo *et al.* 2012, Zhang & Buckling 2012, Knezevic *et al.* 2013, Torres-Barceló *et al.* 2014). Whereas theory indicates that other mitigating factors may be important in the evolutionary process, specifically the force of selection (Holt & Hochberg 1997) and interactions between control agents (Kim *et al.* 2014), these basic theoretical predictions have not been empirically tested. In this work, we determined the short and long-term impacts of either

phages and/or antibiotics (three different types at three different doses) on populations of the pathogenic bacterium *P. aeruginosa*.

Our findings confirm previous study showing that phage-antibiotic combinations typically result in larger reductions of bacterial populations and the same or less resistance than either antimicrobial introduced in isolation (Escobar-Páramo *et al.* 2012, Zhang & Buckling 2012, Torres-Barceló *et al.* 2014). Nevertheless, we found that certain factors may mitigate the selective impact of antibiotics on bacterial populations. Our results can be summarised as follows. First, increasing antibiotic dose resulted in greater reductions in bacterial population densities and adaptive potential. Second, at least for the antibiotic types we tested that are associated with different bacterial targets, significant synergistic effects with phage were observed. Third, we show that *in vitro* antibiotic resistance levels are significantly reduced by the presence of phages, generalizing previous results (Verma *et al.* 2009, Kirby 2012, Zhang & Buckling 2012) to other antibiotic classes. Finally, we found that bacterial virulence is generally reduced by the treatments, but the effects of antibiotics are significantly greater than phage. We discuss these and other findings in more detail below.

Evolutionary theory suggests that, all else being equal, higher doses and longer exposure times to chemotherapeutic agents should increase selection pressure for resistance (Read *et al.* 2011), but empirical study shows that many mitigating factors may reduce or reverse this prediction (Kouyos *et al.* 2014). One important factor is the probability of obtaining resistance mutations associated with different costs and study has shown, for example, how low dose strategies may increase the fixation of low cost resistance mutations, partially compromising therapeutic outcome (Kouyos *et al.* 2014). In the present work, higher doses of antibiotic-only treatments for three different antibiotics led to more rapid bacterial recovery (Fig. 5.2). A recent study found a similar effect (i.e., 'evolutionary rescue') when testing different doses of streptomycin with *P. fluorescens*, possibly due to different emergence times or different relative benefits of favorable mutations (Ramsayer *et al.* 2013). Conversely, when combined with a lytic phage, we find that the higher the antibiotic dose, the slower the bacteria recover in terms of population density (Fig. 5.2). Since the initial effect of reducing population densities did not differ significantly for the highest doses of single and combined treatments, it would appear that the initial probability that a population generates beneficial mutations cannot explain this result. More likely explanations include mechanistic trade-offs between double resistance to antibiotics and phages, and

costly compensatory mutations that would be less readily fixed under these conditions compared to the single antimicrobial treatments. This last hypothesis is supported by the lower growing capacity recorded in the absence of the phage and antibiotic for the double treatments compared to single antimicrobial conditions (Fig. 5.3).

A second conclusion from our study is that synergistic effects with phage are observed for all three antibiotics (Fig. 5.3), indicating some level of generality across their different targeted mechanisms of action. It has been suggested (Zak & Kradolfer 1979) that the different cellular morphologies induced by these antibiotics play a role in interactions with phages leading to synergy. Gentamicin induces cell enlargement and carbenicillin and thimetroprim cause elongation and filamentation (Zak & Kradolfer 1979). These phenotypes have been associated with increased rate of phage production, probably due to the altered physiological state and sensitivity to lysis (Comeau *et al.* 2007). However, we found that carbenicillin was particularly synergistic in combination with phages, suggesting that cell wall disruption is somehow involved in the enhancement of phage predation. Perturbations in the peptidoglycan layer produced by low doses of β-lactam antibiotics have been suggested to not only alter cell morphology, but also to accelerate cell lysis produced by different phages in *E. coli* (Comeau *et al.* 2007). We do not know to what extent, however, such a mechanism may be acting in our system, involving a different microbial host and higher antibiotic concentrations. Moreover, our results indicate that for all three antibiotics, synergy was not observed during the initial phases of the interactions, but rather only at the end of the experiment (Fig. 5.2A), suggesting that it was associated with bacterial evolution. One possible explanation is that during the first hours of contact, both single and double treatments considerably decrease bacterial density, whereas after 6 serial transfers (c 50 bacterial generations), simultaneous resistance to antibiotics and phages entails larger costs than to either mortality agent separately. The net result is lower than expected populations in the combined treatments, but only once costly resistance emerges as a result of selection and evolution. Consistent with this explanation, several studies have shown that phages limit the emergence of antibiotic resistant variants in combined treatments (Verma *et al.* 2009, Escobar-Páramo *et al.* 2012, Kirby 2012, Zhang & Buckling 2012, Torres-Barceló *et al.* 2014). Additional research is necessary however to understand how and why relative times of introduction affect this phenomenon.

Third, whereas *in vitro* antibiotic resistance levels are significantly reduced by the presence of phages, the corresponding resistance to phages did not differ between treat-

ments in the presence or absence of antibiotics (Fig. 5.3). Betts and colleagues (2013) previously showed that when bacteriophage LKD16 was passaged on *P. aeruginosa*, phage resistance levels attained *c* 80% after 6 transfers, and subsequent study indicated that resistance to this phage is costly, since it is partially lost when bacteria and phages coevolved (Betts *et al.* 2014). Different constraints on the independent evolution of antibiotic and phage resistances could explain why phages affected antibiotic resistance levels but not vice versa, suggestive of different pleiotropic effects. Further research is needed to investigate the mechanisms behind this asymmetric effect.

Finally, past studies have found different relationships between elevated antibiotic resistance and virulence expression, the most frequent being negative (Beceiro *et al.* 2013). For example, the loss of porins increases antibiotic resistance but decreases virulence in many bacterial species, whereas effects in bacterial strains showing up-regulation of efflux pumps and virulence are less clear (Beceiro *et al.* 2013). On the other hand, while lysogenic phages can transfer virulence factors (Fortier & Sekulovic 2013), the majority of previous studies show that bacteria resistant to lytic phages have attenuated virulence on the host, whether arthropods, fish, plants, mice or humans (Evans *et al.* 2010, Filippov *et al.* 2011, Hall *et al.* 2012, Laanto *et al.* 2012, Seed *et al.* 2014). Costly resistance to phages and/or antibiotics can impact relevant pathogenicity factors and/or lessen bacterial growth rates, resulting in less harm to the host (Beceiro *et al.* 2013, Seed *et al.* 2014). Our results showing that antibiotics alone reduce virulence are in line with most previous experimental studies, but we find that phages lower virulence less than antibiotics (Evans *et al.* 2010, Laanto *et al.* 2012). Specifically we found that *in vivo* inoculation of wax moth larvae with carbenicillin-treated bacteria resulted in the least virulent bacteria (Fig. 5.2), possibly due to resistance mutations in the peptidoglycan synthesis pathway, this being implicated in bacterial pathogenesis (Godlewska *et al.* 2009). Comparing the doses applied, the weakest dose of antibiotics (IC5) of single antibiotic treatments decreased the time to death of the larvae significantly more than higher dose treatments. Because this effect was not significant for all antibiotic types, this suggests specificity in either the action or resistance mechanisms associated with gentamicin and trimethoprim. For example, Haddadin and colleagues (2010) showed that some antibiotics at sub-MIC levels interfere with bacterial biofilm virulence expression (Haddadin *et al.* 2010). Nevertheless, our results suggest that combined therapies do not result in the largest reduction in virulence, and that antibiotic dose produces a counterintuitive effect, with

low doses reducing virulence more than higher ones. Consistent with this effect, it has been demonstrated that sub-MIC levels of antibiotics interfere with the expression of bacterial virulence-associated genes depending on the type and concentration of the antibiotic used (Haddadin *et al.* 2010, Andersson & Hughes 2014). Therefore, we conclude that parameters that tend to reduce both bacterial populations and minimise resistance appear not to be those that result in lower virulence. Our results provide important insights for the potential use of phage-antibiotic combinations in controlling bacterial pathogens and pests. The strong bacterial density reduction caused by the combined action of phages and high doses of antibiotics, together with the reduced probability of bacterial resistance evolution under these conditions, support the choice of high doses of antibiotics combined with lytic phages as an evolution-minimizing approach against bacterial pathogens and pests. Regulatory requirements for the employment of phages are challenging (Verbeken *et al.* 2014), and their use will not extend to all kinds of infection or all scenarios for a given infection type, but results such as those reported here suggest promising future research avenues for understanding and applying combined phage-antibiotics therapies.

Appendix

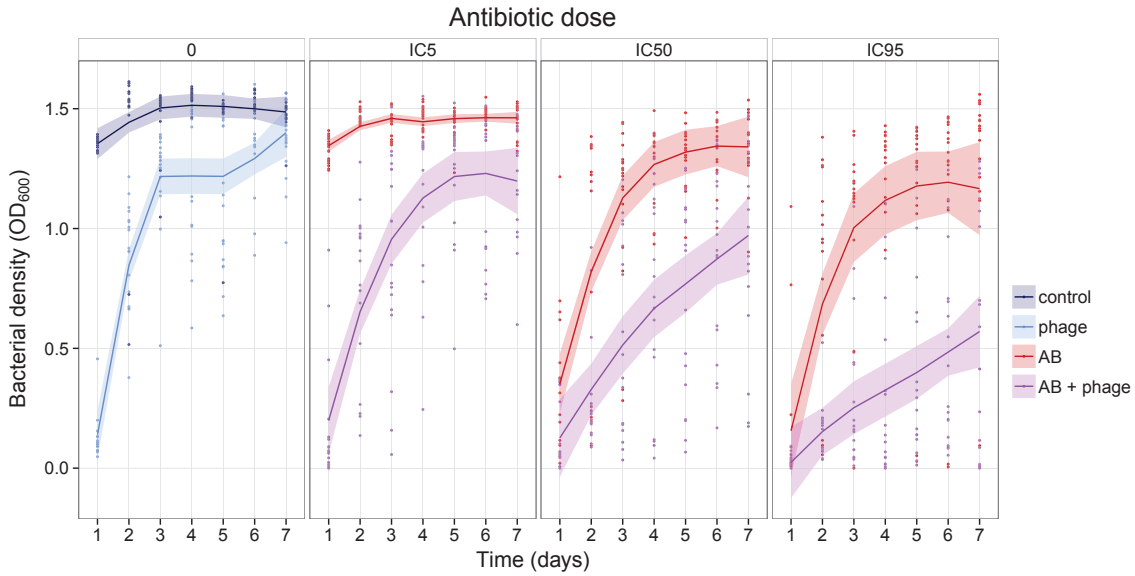

Figure 5.6: Bacterial density dynamics measured every 24 hours for 7 days. Different panels represent the different concentrations of antibiotics relative to the resulting % inhibition of growth (IC: inhibitory concentration). 'Control' corresponds to untreated bacteria, 'phage' to phage only, 'AB' to antibiotic only and 'AB + phage' to combined phage and antibiotic treatments. Antibiotic types were grouped. Shaded regions are 95% confidence intervals.

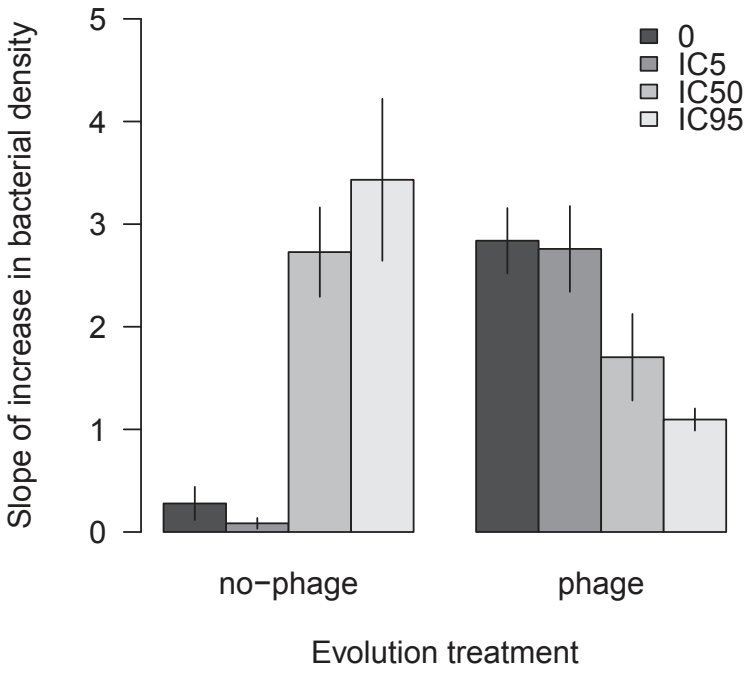

Figure 5.7: Slopes of bacterial density with and without phages, for each antibiotic dose. Slope estimates the rate of bacterial population density recovery over the 7 days of the experiment. Numbers indicate antibiotic doses employed as % growth inhibition (IC). The different antibiotic types were grouped. Bars represent standard errors.

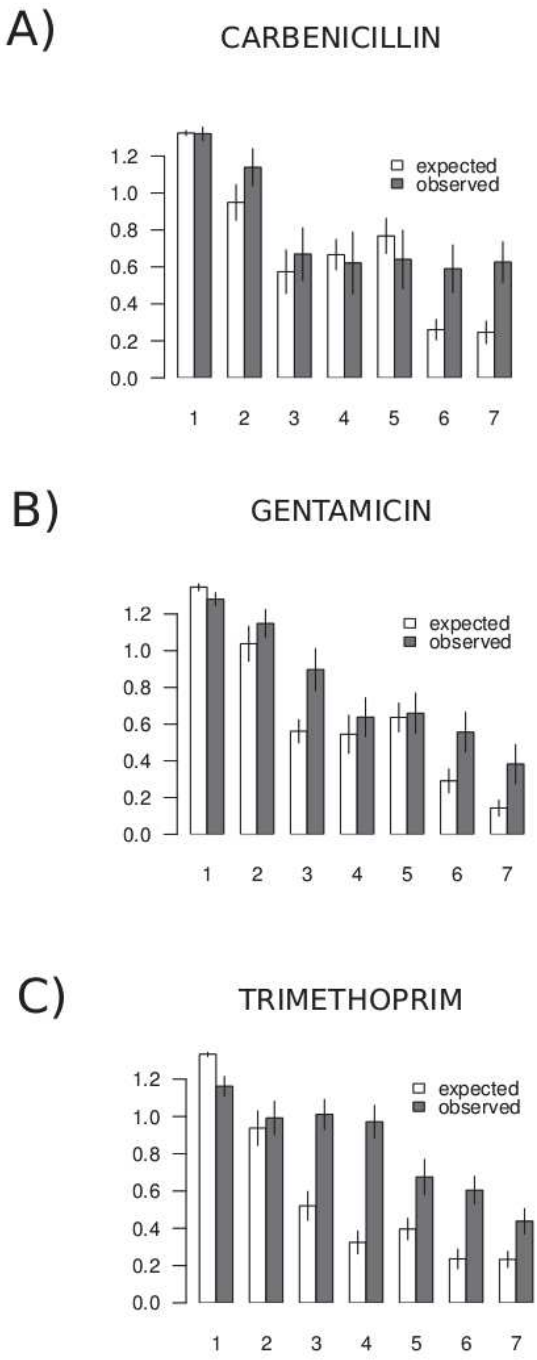

Figure 5.8: Synergistic effects of treatments through time depending on antibiotic type (A, carbenicillin; B, gentamicin; C, trimethoprim). Expected additive versus observed effects of combined phage-antibiotic treatments in preventing growth in bacterial populations. Effects on growth are measured as decreases in density relative to the control. The expected additive effect was calculated by summing the individual effects of phage and antibiotic treatments. Different antibiotic doses were grouped. Bars are standard errors.

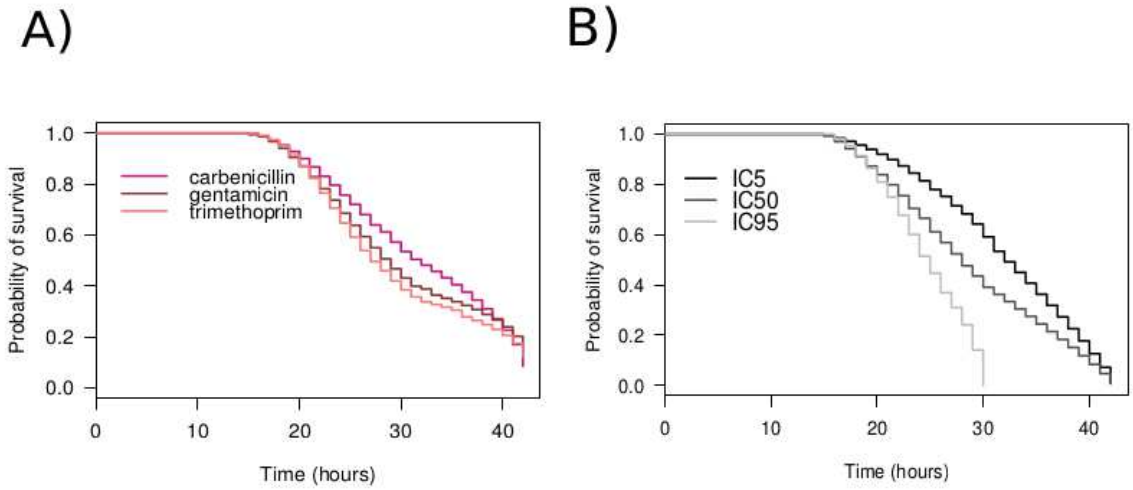

Figure 5.9: Probability of survival of *Galleria mellonella* larvae inoculated with final bacterial populations. Panel A compares the different antibiotic types. Antibiotic doses and treatments with and without phage were grouped. Panel B compares the different antibiotic doses employed. Antibiotic types and treatments with and without phage were grouped. In both panels virulence produced by untreated and ancestral strains of bacteria, or negative controls are not shown.

General conclusion

Overview of the main results

Public goods production appears to be a widespread solution for bacteria to exploit, modify and cope with their environment. For instance, the focal public goods of this thesis, siderophores, enable bacteria to use otherwise unavailable environmental iron. By modifying their environment, bacteria may favour other group-level traits, such as dispersal by secreting biosurfactants (Kearns 2010), or increase local carrying capacity and/or growth rate when the public goods give access to more resources (Platt & Bever 2009). Understanding how social behaviours originate and translate into changes in absolute and relative fitnesses is particularly important when studying bacterial responses to ecological antagonisms. As argued in this thesis, in addition to avoidance behaviours such as certain forms of swarming or biofilm formation, public goods production likely shapes the impact of antagonisms through its effects on population growth. All else being equal, we expect large, growing populations to be more likely to find resistance mutations and/or plastic responses to counteract antagonisms.

In Chapters 1 and 3, we submitted experimental populations of *P. aeruginosa*, either in monocultures or in mixed cultures of siderophore producers and non-producers, to different antibiotic doses (Chapter 1) and to phages (Chapter 3). In the presence of antagonisms, we showed that (i) cooperation is associated with higher resistance frequency in producer monocultures compared non-producer monocultures, and (ii) cooperation in mixed cultures is exploited by non-producing cheats, which generally attain higher resistance frequencies than in monocultures, leading to higher overall resistance in mixed populations compared to either monoculture. We cannot exclude the possibility that standing variation and mutation rate affect these resistance dynam-

ics, but our results suggest that they play a lesser role compared to increased growth rate due to enhanced iron availability. Indeed, we found no evidence for higher resistance frequency in the initially larger populations. In contrast, we did observe that the faster-growing strain generally reached higher resistance frequencies, and hypothesise that this reflects evolutionary potential associated with more replication events (Orr & Unckless 2014). The importance of siderophore dynamics in the emergence and spread of resistance is further illustrated by the mathematical model in Chapter 2. By assuming that bacterial growth rate depends on siderophore concentration, the model correctly predicts that resistance frequency in non-producers is higher in mixed cultures than in monocultures. Our longer-term experiment with phages showed, however, that the advantage to cheats may be short-lived if public goods are lacking in the non-producer-dominated mixed cultures. Indeed, as the producer frequency decreases, public goods availability is reduced and the population may eventually decline. This indicates that, while public goods cooperation may enable pure producer populations to survive and resist antagonisms, the social conflict within mixed populations may accelerate their demise.

The impact of ecological antagonisms on public goods cooperation

We have shown how both the social environment (availability of free iron, initial frequencies of producers and non-producers) and environmental harshness (presence or absence and dose of antibiotics; presence or absence of phages) interact to influence the ecology and evolution of a bacterial population. Our central result is harsh environments increase the fitness advantage of non-producers in mixed cultures, our hypothesis for this being that cooperators need to cope with both the antagonism and the effects of a superior competitor (i.e., the cheat), and may have less metabolic resources to persist. Our mathematical model (Chapter 2) provides further support for this hypothesis. Moreover, we found that, as expected, the non-producer's competitive advantage depends on the availability of the public good. When producers are very rare (or initially common but subsequently outcompeted by non-producers) the concentration of public goods may not (or no longer) sustain population growth. The population is then more vulnerable to antagonisms and may eventually decline, as illustrated by our

experiments under phage pressure in Chapter 3.

Taken together, we suggest that antagonisms favour public goods cheats when the good is a resource, in the short-term. In the longer term, mixed populations as a whole may decrease in density, and neither cheats nor cooperators manage to persist. How can we explain the persistence of public goods producers, given the ubiquity of antagonisms in bacterial systems?

Below, I propose that cooperation can persist in the presence of ecological antagonisms by positive frequency dependent selection, assortment and spatial structure, and/or compensatory mechanisms.

Positive frequency dependent selection

Morgan and colleagues (2012) proposed that producers should be favoured in mixed cultures by positive frequency dependence. The argument is that larger population size is associated with a higher probability to obtain a resistance mutation through higher standing variation and/or more division events. It follows that when producers are more numerous than non-producers, they are also more likely, all else being equal, to evolve resistance. If the resistance mutation confers a greater fitness benefit than the social mutation, then the resistance mutation hitchhikes with cooperative gene(s) and cooperation spreads in the population. Morgan and colleagues' (2012) experimental test of this hypothesis shows that under phage pressure, non-producers need to be sufficiently rare in the initial population (<1%) for cooperators to be favoured. Under these circumstances, while non-producers can still benefit from public goods, their numbers are likely to be insufficient to obtain a resistant mutation. Consistent with our results, beyond an initial frequency threshold (10% in Morgan *et al.* 2012), non-producers invade the population in the presence of phages. Subsequent theoretical study showed that this threshold criterion depends on the cost of cooperation (Quigley *et al.* 2012).

Assortment and spatial structure

We found that under iron limiting conditions, while the non-producing populations declined in density in the presence of phages, cooperator populations grew, having at-

tained almost complete resistance (Chapter 3). Moreover, our results indicate that, in the presence of antagonisms, mixed patches of producers and non-producers should be rapidly dominated by the latter and eventually decline. This suggests that, in the long-term, patches dominated by obligate cheats may go locally extinct. If cooperators are able to disperse at sufficient rates compared to cheats and vacant patches emerge sufficiently frequently (e.g., due to local extinctions), then cooperation can persist globally in harsh environments (see also Koella 2000). These basic verbal predictions although compelling, are overly simple, in part because they do not take into consideration the spatial dynamics of the antagonism. Future models and analysis will be necessary to uncover the conditions promoting cooperator persistence, and associated temporal and spatial dynamics.

Compensatory mechanisms

Cooperators may also increase their relative fitness by reducing their costs with compensatory mechanisms including pleiotropy, non-social mutations and specific plastic behaviours. Pleiotropy in particular has been shown to favour the maintenance of cooperation in the presence of antagonisms. For instance, quorum-sensing producers form denser biofilms than non-producers under predation pressure in *P. fluorescens* (Friman *et al.* 2013). Biofilms not only provide shelter to avoid predators, but also create a structured environment favouring resource exploitation and limiting the spread of cheats (but see Rainey & Rainey 2003, Brockhurst *et al.* 2006). Another example comes from public good-producing *S. enterica* serovar Typhimurium that are outcompeted by cheats in the absence of antibiotics, but are maintained under antibiotic pressure since they can invade host tissue, thereby escaping both the stressor and the cheats (Diard *et al.* 2014). Non-social mutations can also contribute to the maintenance of cooperation by increasing the relative fitness of cooperators. In *P. aeruginosa* for example, a mutation on a transcriptional repressor gene *psdR* enhances intracellular metabolism and increases the fitness of the *psdR*-mutants without affecting public goods production (Asfahl *et al.* 2015). The *psdR*-mutant cooperators, however, remain vulnerable to *psdR*-mutant cheats but show a higher tolerance to partial cheats (that participate less in public goods production). Finally, antagonisms may induce plastic behaviours including swarming motility, which enables dispersal to new habitats (e.g., Shen *et al.* 2008, Butler *et al.* 2010), and may lead to the colonisation of free patches

by cooperators.

These mechanisms are not mutually exclusive and may be mitigated in more complex social systems where cooperation involves a continuum of producing strategies and cheating may be facultative. Future study should evaluate the relative importance of frequency dependent selection, spatial structure and compensatory mechanisms in natural environments and medical settings, where they most likely occur. Moreover, ecological pressure in natural environments may vary both in space and time depending on the presence and intensity of isolated or multiple antagonisms, and result in increased local heterogeneity contributing to the persistence of cooperation. Relative migration rates of cooperators and cheats and variability in local densities would then be crucial for social dynamics (Wakano *et al.* 2009), as well as 'higher-level' cooperation such as physical protection against antagonisms (e.g., biofilms Hosseinidoust *et al.* 2013a, Kaplan 2011 and mucoids Scanlan & Buckling 2012) and complex signalling (Diggle *et al.* 2007a;b). Such a research programme will open exciting avenues of thinking about cooperation in different taxa and for different fitness-determining behaviours (e.g., escaping antagonisms, cooperative breeding, exploiting resources) when confronted with environmental challenges.

Towards medical applications: the use of ecological antagonisms as control agents

The observation of the detrimental effects of phages and antibiotics as ecological antagonisms on bacterial populations has led to their therapeutic use for the control of bacterial infections both in medicine and agriculture. While antibiotics and phages have long been applied in single treatments, recent studies highlight the great potential of phage-antibiotic combined therapies, in particular in the context of minimising antibiotic resistance (Hagihara *et al.* 2012, Escobar-Páramo *et al.* 2012, Zhang & Buckling 2012, Knezevic *et al.* 2013, Torres-Barceló *et al.* 2014). The design of combined therapies requires understanding the biology and evolutionary ecology of bacterial populations under phage and antibiotic pressure as well as the potential interactions (and their effects) between the two agents. In Chapters 4 and 5, we studied the impacts of a panel of phage-antibiotic combinations on bacterial survival, evolution of resistance and virulence. Specifically, we compared single and combined treatments seeking to

unravel the conditions for synergistic effects by testing the impact of antibiotic dose, inoculation sequence, and phage and antibiotic modes of action.

Recent work has examined how our understanding of social traits can be applied to the control of bacterial pathogens (e.g., Brown *et al.* 2009). Our results that harsh environments select for non-producing cheats, taken together with studies showing that siderophores and other public goods may constitute virulence factors (e.g., adhesive polymers, quorum sensing molecules, toxins; Rumbaugh *et al.* 2009, Leggett *et al.* 2014, Pollitt *et al.* 2014), indicate that antagonisms should reduce bacterial virulence. We predict, and empirical studies show (e.g., Diard *et al.* 2014), that spatial structure promotes public good producers, which in turn should lead to more acute infections. We suggest that future study explore how multiple antagonisms should improve bacterial control, reduce virulence, and minimise resistance.

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Résumé

Introduction

Les bactéries comptent parmi les formes de vie les plus anciennes sur Terre et occupent la quasi-totalité des écosystèmes dont elles constituent le socle fonctionnel, jouant un rôle primordial dans divers processus tels que la décomposition de la matière organique ou le cycle de l'azote (DeLeon-Rodriguez *et al.* 2013). Elles participent également à une multitude d'interactions avec d'autres organismes allant d'associations symbiotiques au parasitisme. Chez les humains, et les autres mammifères, les bactéries colonisent par exemple la peau, les voies nasales, les poumons et le tube digestif. Environ 10^{14} cellules microbiennes, au moins deux fois le nombre de cellules humaines, vivent sur nous et en nous (Whitman *et al.* 1998, Turnbaugh *et al.* 2007). Ces microbes, principalement des bactéries, constituent notre microbiote et peuvent avoir des conséquences positives ou négatives considérables sur la santé humaine et la physiologie. Parmi les effets positifs, on rapporte le rôle du microbiote intestinal dans le développement et le fonctionnement du système immunitaire (Chow *et al.* 2010) et la digestion (Bäckhed *et al.* 2005). En revanche, au niveau de la population humaine, les bactéries peuvent être la cause d'épidémies telles que la tuberculose, le cholera et la pneumonie (Brachman & Abrutyn 2009) et la source de certaines infections nosocomiales (Peleg & Hooper 2010). La conception de traitements antibactériens efficaces nécessite l'identification des facteurs écologiques et évolutifs à l'origine de la pathogénèse ainsi que la compréhension des mécanismes impliqués dans les réponses des populations bactériennes et l'évolution de la résistance.

Malgré leur incontestable succès au sein de nombreux écosystèmes, les bactéries sont confrontées à une grande diversité d'antagonismes écologiques. Ces antagonismes

sont des contraintes externes qui réduiraient la valeur sélective des bactéries par des effets létaux ou sous-létaux définis selon un continuum de réponses à court et long termes. En effet, un antagonisme peut entrainer un stress à court terme mais être létal à plus long terme si l'organisme touché ne parvient à répondre à la pression de sélection. Historiquement, les hommes ont tiré parti de certains antagonismes dans le but de contrôler les bactéries pathogènes. Si les réponses des cellules individuelles ont fait l'objet d'études approfondies, les conséquences écologiques et évolutives des antagonismes au niveau populationnel restent encore à explorer.

Pourtant, de récentes études rapportent que les populations bactériennes sont capables de répondre à des pressions environnementales grâce à des comportements collectifs (Lee *et al.* 2010, Meredith *et al.* 2015). Ces comportements collectifs sont largement répandus chez les bactéries et pourraient jouer un rôle fondamental dans leurs interactions avec les antagonismes. La vie en groupe dans les populations bactériennes peut résulter d'une reproduction essentiellement locale et d'une migration réduite mais également de pressions exercées par des antagonismes tels que des prédateurs (Hosseinidoust *et al.* 2013a, Claessen *et al.* 2014). Ce mode de vie implique des interactions compétitives mais aussi des comportements sociaux modulés par les contacts et une communication de cellule à cellule (e.g., Crespi 2001, West *et al.* 2007a). La dynamique et l'évolution des populations bactériennes peuvent être modifiées par les interactions sociales notamment si la croissance requiert la recherche collective de ressources ou le partage de composés produits individuellement mais accessibles à tous («biens publics»). Les comportements sociaux peuvent aussi affecter la répartition des populations en augmentant la capacité de dispersion au niveau populationnel (par essaimage par exemple) et permettre la colonisation de nouveaux habitats (Velicer & Yu 2003). Ils sont également impliqués dans les interactions interspécifiques, notamment par le biais de composés antimicrobiens ciblant des espèces compétitrices (Gardner & West 2006, West & Gardner 2010, Hawlena *et al.* 2010).

Ce travail de thèse est centré sur l'effet de deux types d'antagonismes en particulier : les bactériophages (ou phages) qui sont des virus spécifiques de bactéries et agissent en prédateurs pouvant (co)évoluer avec les bactéries; et les antibiotiques en tant qu'agents stressants dont l'action peut éventuellement mener à la mort des bactéries. Les phages et les antibiotiques peuvent induire des réponses chez les bactéries ou causer leur mort. Ainsi, des concentrations suffisamment élevées d'antibiotiques et de phages peuvent être létales alors que des doses sous-létales ou des phages incapables d'achever leur cycle

lytique peuvent constituer des stresseurs et déclencher des réponses bactériennes telles que l'évitement ou la tolérance phénotypique (e.g., Poisot *et al.* 2012, Bernier & Surette 2013, Andersson & Hughes 2014). Cette thèse comporte deux parties, adressant chacune un problème scientifique concernant les interactions entre antagonismes environnementaux et écologie évolutive des populations bactériennes. La première partie présente mon principal travail de recherche sur les interactions entre les pressions de sélection exercées par les phages ou les antibiotiques et les comportements sociaux dans les populations de la bactérie *Pseudomonas aeruginosa*. La seconde partie est un travail collaboratif, visant à une meilleur compréhension des effets des antibiotiques et des phages sur les populations de *P. aeruginosa* dans le contexte des thérapies simples (un seul agent de contrôle) et combinées (phages et antibiotiques combinés).

Problème 1 : Etant donnée l'ubiquité des comportements sociaux au sein des populations bactériennes et leur importance pour la dynamique de ces populations, il est très probable qu'ils soient affectés par des antagonismes écologiques tels que des prédateurs (phages) ou des composés d'origine microbienne ou humaine (antibiotiques). Comprendre les conséquences de ces interactions sur la biologie des populations requiert à la fois l'analyse de l'impact des antagonismes sur les comportements de coopération et l'étude des effets de ces comportements sur la réponse de la population aux antagonismes.

Problème 2 : Les réponses bactériennes face aux antagonismes sont également une question centrale pour la santé humaine. L'évolution de la résistance aux antibiotiques est progressivement devenue un problème crucial et il est aujourd'hui essentiel d'étudier les conséquences de thérapies alternatives. Ceci exige l'identification des conditions selon lesquelles des mesures thérapeutiques simples ou combinées réduisent la valeur sélective, la taille des populations bactériennes et leur potentiel évolutif.

Modèle biologique

Toutes les expériences présentées dans ce manuscrit ont été conduites sur des populations de la bactérie *P. aeruginosa*. Cette bactérie est très versatile et vit dans des niches écologiques extrêmement variées telles que le sol, l'eau, les plantes, les animaux et la plupart des habitats créés par les hommes (Lister *et al.* 2009). De nombreux comportements sociaux ont été observés et décris au sein de populations de *P. aeruginosa*

et notamment des comportements dépendants de la sécrétion collective de molécules (biens publics) dans un environnement partagé. Cette bactérie est devenue un exemple classique pour l'étude de l'évolution de la socialité bactérienne et un grand nombre de travaux empiriques rapportent la production de biofilms, de molécules de signal, de bactériocines et de composés permettant l'accès à des nutriments (Rainey & Rainey 2003, Griffin *et al.* 2004, Diggle *et al.* 2007b, Xavier *et al.* 2011, Ghoul *et al.* 2015).

Dans la première partie de ma thèse, j'étudie les interactions entre des conditions environnementales difficiles dues à la présence de phages ou d'antibiotiques et la coopération fondée sur les biens publics en utilisant l'exemple de la production de sidérophores par *P. aeruginosa*. Les sidérophores sont des molécules extracellulaires qui chélatent le fer environnemental non soluble et sont produites de manière facultative par certaines bactéries lorsque la disponibilité en fer soluble est limitante (Guerinot & Yi 1994). Ces molécules sont sécrétées dans l'environnement et, si les conditions permettent leur partage avec les cellules du voisinage, elles fonctionnent comme des biens publics (West & Buckling 2003). Ce modèle biologique présente différents avantages pour l'étude des impacts des antagonismes sur les comportements de coopération. Premièrement, la production de sidérophores est facultative et est modifiée par les caractéristiques environnementales. L'impact de facteurs écologiques sur cette production a fait l'objet de nombreuses études centrées sur la théorie de la coopération. Deuxièmement, la production de sidérophores est coûteuse; il y a donc un avantage à tricher, c'est- à-dire à profiter des biens publics sans participer à leur production (Griffin *et al.* 2004). L'apparition spontanée d'individus non-producteurs («tricheurs») a été décrite dans des populations de producteurs de sidérophores à la fois *in vitro* (Jiricny *et al.* 2010) et *in vivo* (Vos *et al.* 2009, Andersen *et al.* 2015). D'un point de vue pratique, les bases génétiques et les voies métaboliques impliquées dans la production de sidérophores sont bien étudiées et comprises (e.g. Crosa 1989, Meyer *et al.* 1996, Visca *et al.* 2007), et des collections de mutants sont disponibles. Enfin, la production de sidérophores a des conséquences sur la virulence de *P. aeruginosa*. En effet, les sidérophores peuvent être considérés comme des facteurs de virulence non-spécifiques car ils favorisent l'acquisition de nutriments et promeuvent ainsi la croissance bactérienne (Buckling *et al.* 2007, Cornelis & Dingemans 2013). Le sidérophore principal de *P. aeruginosa*, la pyoverdine, est également une molécule signal liée à la régulation d'autres facteurs de virulence tels que les exotoxines et les protéases (Lamont *et al.* 2002). En outre, il a été montré récemment que la pyoverdine peut elle-même agir comme une toxine en

créant des dommages sur les mitochondries et en modifiant l'homéostasie du fer chez *Caenorhabditis elegans* et les cellules de mammifères (Kirienko *et al.* 2015).

De plus, *P. aeruginosa* est un pathogène humain opportuniste et représente une des causes majeures de maladies nosocomiales telles que la pneumonie, les infections du système urinaire et du système sanguin (Aloush *et al.* 2006, Lister *et al.* 2009). Cette bactérie induit des complications médicales chez les patients immunodéprimés et les brûlés, et se révèle particulièrement dangereuse pour les malades de la mucoviscidose (Schurek *et al.* 2012). Les hauts niveaux de résistance à de nombreux antibiotiques tels que certaines pénicillines, fluoroquinolones et glucopeptides ainsi que l'impressionnante capacité d'évolution de résistance au cours des traitements rendent difficile la conception de thérapies appropriées et efficaces (Lister *et al.* 2009). L'utilisation des phages présente une alternative prometteuse pour le contrôle des pathogènes bactériens, en particulier en combinaison avec des antibiotiques (Verma *et al.* 2009, Torres-Barceló *et al.* 2014). La théorie de l'évolution prédit une efficacité supérieure à deux pressions de sélection différentes qu' à chacune séparément et soutient ainsi les thérapies combinées. Néanmoins, alors que de nombreuses études ont testé l'effet des phages et des antibiotiques *in vitro* et *in vivo* avec des résultats encourageants, les mécanismes sous-jacents demeurent majoritairement inconnus. Des travaux supplémentaires sont nécessaires afin d'explorer des caractéristiques cruciales des traitements telles que les doses et le moment d'inoculation ainsi que les effets à long terme des combinaisons sur l'évolution de la résistance et la virulence. Ces questions font l'objet de la seconde partie de ma thèse.

Partie 1
Impact des antagonismes écologiques sur la coopération et les biens publics

La production de biens publics apparait comme une solution répandue chez les bactéries pour exploiter, modifier et faire face à leur environnement. Les sidérophores, qui constituent l'objet d'étude de cette thèse, permettent par exemple aux bactéries d'utiliser le fer environnemental qui leur est autrement inaccessible. Ces modifications de l'environnement peuvent favoriser l'expression de différents traits de groupe tels que la dispersion collect-

ive par la sécrétion de biosurfactants (Kearns 2010), mais aussi augmenter la capacité du milieu ou le taux de croissance des bactéries si les biens publics permettent l'accès à davantage de ressources (Platt & Bever 2009). Par conséquent, la production de biens publics peut affecter l'impact des antagonismes en facilitant les comportements collectifs d'évitement (biofilms et dispersion par exemple) et par ses effets sur la croissance bactérienne. Toute chose étant égale par ailleurs, on s'attend à ce que de denses populations en croissance aient davantage de chances de trouver une mutation conférant une résistance ou des réponses plastiques pour contrer les antagonismes. Il est alors particulièrement important pour l'étude des réponses bactériennes aux antagonismes écologiques de comprendre l'origine des comportements sociaux et la manière dont ils se traduisent en changement de fitness absolues et relatives.

Au cours des Chapitres 1 et 3, nous avons soumis des populations expérimentales de *P. aeruginosa*, en monocultures ou en cultures mixtes de producteurs et de non-producteurs de sidérophores, à différentes doses d'antibiotiques (Chapitre 1) et à des phages (Chapitre 3). Nous avons montré qu'en présence de ces antagonismes, la coopération est associée à une fréquence supérieure de résistance dans les monocultures de producteurs comparée aux monocultures de non-producteurs. De plus, cette coopération est exploitée par les non-producteurs dans les cultures mixtes et ceux-ci atteignent de plus fortes fréquences de résistance qu'en monocultures. Il en résulte une fréquence globale de résistance dans les populations plus élevée en cultures mixtes que dans chacune des monocultures. Si nous ne pouvons exclure la possibilité que la variabilité génétique initiale et le taux de mutation affectent ces dynamiques de résistance, nos résultats suggèrent qu'ils jouent un rôle mineur comparé à l'augmentation du taux de croissance grâce à une disponibilité supérieure en fer. Nous avons ainsi observé que la souche ayant la croissance la plus rapide atteignait généralement des fréquences de résistance supérieures et nous pouvons émettre l'hypothèse que ceci reflète le potentiel évolutif associé à davantage d'évènements de réplication (Orr & Unckless 2014). L'importance de la dynamique des sidérophores dans l'émergence et l'expansion de la résistance est également illustrée par le modèle mathématique du Chapitre 2. En faisant l'hypothèse que la croissance bactérienne est modulée par la concentration de sidérophores, le modèle prédit que la fréquence de résistance parmi les non-producteurs est plus élevée en cultures mixtes qu'en monocultures. Notre expérimentation sur plus long terme avec des phages montre, cependant, que l'avantage des tricheurs ne perdure pas si les biens publics viennent à manquer. En effet, si la fréquence de producteurs

diminue, la disponibilité des biens publics diminue également et aboutit au déclin de la population. Ceci suggère que, si la coopération peut favoriser la survie des populations pures de producteurs, les conflits sociaux au sein des populations mixtes peuvent accélérer leur disparition.

Nous avons montré que l'environnement social (disponibilité en fer, fréquence initiale de producteurs et non-producteurs) et les antagonismes écologiques (présence or absence d'antibiotiques et de phages ainsi que leurs concentrations) interagissent pour influencer l'écologie et l'évolution des populations bactériennes. Notre résultat central est que les environnements difficiles augmentent l'avantage sélectif des non-producteurs en cultures mixtes, notre hypothèse explicative étant que les coopérateurs doivent faire face à la fois aux antagonismes et à la pression de compétiteurs supérieurs (les tricheurs) and disposer de moins de ressources métaboliques pour se maintenir. Notre modèle mathématique (Chapitre 2) soutient cette hypothèse. De plus, conformément à nos attentes, l'avantage sélectif des non-producteurs dépend de la disponibilité des biens publics. Lorsque les producteurs sont très rares (ou initialement fréquents mais par la suite éliminés au profit les non-producteurs) la concentration de biens publics peut ne pas (ou ne plus) permettre le maintien de la croissance de la population. La population est alors davantage vulnérable envers les antagonismes et peut décliner, ainsi que le montrent nos expériences présentées dans le Chapitre 3. Suite à nos observations, nous suggérons que les antagonismes favorisent les tricheurs à court terme, lorsque le bien public est une ressource ou permet l'accès à une ressource. A plus long terme, les densités globales des populations mixtes peuvent décroitre et ni les tricheurs ni les coopérateurs ne persistent. Comment alors expliquer la persistance des coopérateurs étant donnée l'ubiquité des antagonismes dans les systèmes bactériens ?

Nous proposons que la sélection fréquence-dépendante positive, la structuration spatiale et les mécanismes compensatoires puissent participer au maintien de la coopération en présence d'antagonismes écologiques.

Premièrement, il a été proposé par Morgan et collaborateurs (2012) que la sélection fréquence-dépendante positive exercée par un antagonisme peut favoriser les producteurs en cultures mixtes. L'argument est le suivant : les densités bactériennes élevées sont associées à une plus forte probabilité de mutation conférant une résistance grâce à la fois à une variabilité initiale supérieure et à davantage d'évènements de réplication. Il s'ensuit que lorsque les producteurs sont plus nombreux que les non-producteurs,

ils ont également davantage de chances, toute chose étant égale par ailleurs, d'évoluer une résistance. Si cette mutation de résistance confère un avantage sélectif supérieur comparé à la mutation du comportement social, alors l' (les) allèle(s) de coopération augmentent en fréquence par autostop génétique avec la mutation de résistance. Le test expérimental de cette hypothèse présenté dans l'étude de Morgan et collaborateurs (2012) montre qu'en présence de phages, les non-producteurs doivent être suffisamment rares dans la population initiale (moins de 1%) pour que les coopérateurs soient favorisés. Dans ces conditions, alors que les non-producteurs peuvent toujours bénéficier des biens publics, leur nombre semble insuffisant pour obtenir une mutation de résistance. En accord avec nos résultats, au-del à d'un certain seuil de fréquence initiale (10% dans l'étude de Morgan *et al.* 2012), les non-producteurs envahissent la population en présence de phages. Une étude théorique ultérieure a montré que ce seuil dépend du coût de la coopération (Quigley *et al.* 2012). Deuxièmement, nous avons observé que lorsque la disponibilité en fer était limitée, alors que les populations de tricheurs décroissent en présence de phages, les populations de coopérateurs croissent et atteignent une résistance quasi-complète (Chapitre 3). De plus, nos résultats indiquent qu'en présence d'antagonismes, les patchs mixtes de producteurs et de non-producteurs sont rapidement dominés par ces derniers et finissent par décliner. Ceci suggère qu' à long terme les patchs dominés par des tricheurs obligatoires pourraient disparaitre localement. Si la dispersion des coopérateurs atteint des taux suffisamment hauts comparés aux tricheurs et que des patchs vides émergent assez fréquemment (suite à des extinctions locales par exemples), alors la coopération peut se maintenir à l'échelle globale dans des environnements difficiles (voir aussi Koella 2000). Ces arguments verbaux, quoiqu'importants sont excessivement simplifiés, notamment car ils ne tiennent pas compte de la dynamique spatiale des antagonismes. De futurs modèles et analyses sont nécessaires afin de comprendre les conditions favorisant le maintien des coopérateurs ainsi que leurs dynamiques temporelles et spatiales associées.

Troisièmement, la valeur sélective relative des coopérateurs peut également augmenter en réduisant leurs coûts grâce à des mécanismes compensatoires tels que la pléiotropie, les mutations non-sociales et certains comportements plastiques. La pléiotropie, en particulier, peut favoriser le maintien des coopérateurs en présence d'antagonismes. Ainsi, chez *P. fluorescens*, les producteurs de molécules de quorum sensing forment des biofilms plus denses que les non-producteurs sous pression de prédation (Friman *et al.* 2013). Les biofilms constituent non seulement une protection contre les prédateurs mais

participent aussi à la structuration de l'environnement et favorisent ainsi l'exploitation des ressources tout en limitant la propagation des tricheurs (voir cependant Rainey & Rainey 2003, Brockhurst *et al.* 2006). Les mutations non liées aux comportements sociaux peuvent également contribuer à la persistance de la coopération en augmentant l'avantage sélectif relatif des coopérateurs. Ceci a, par exemple, été montré chez *P. aeruginosa* : la mutation d'un gène régulant le métabolisme intracellulaire augmentent la valeur sélective des mutants sans affecter la production de biens publics et confère un avantage aux producteurs mutants face aux non-producteurs (Asfahl *et al.* 2015). Enfin, les antagonismes peuvent induire des comportements plastiques tels que la dispersion par essaimage, qui permet la migration vers de nouveaux habitats (Shen *et al.* 2008, Butler *et al.* 2010), et peut conduire à la colonisation de patchs vides par les coopérateurs.

Ces mécanismes ne sont pas mutuellement exclusifs et peuvent être modulés dans les systèmes sociaux plus complexes dans lesquels la coopération implique un continuum de stratégies de production de biens publics et dans lesquels tricher peut être facultatif. De futures études pourraient évaluer l'importance relative de la sélection fréquence-dépendante positive, de la structuration spatiale et des mécanismes compensatoires dans des environnements naturels et dans un cadre médical, o ù ils sont davantage susceptibles de s'exprimer. De plus, dans les environnements naturels, la pression écologique peut varier dans le temps et l'espace selon la présence d'antagonismes multiples ou isolés et l'intensité de la sélection qui en résulte, menant ainsi à un accroissement de l'hétérogénéité locale qui contribue au maintien de la coopération. Les taux relatifs de migration des coopérateurs et des tricheurs et les variations locales de densités seraient alors des déterminants cruciaux de la dynamique sociale (Wakano *et al.* 2009), ainsi que des comportements «supérieurs» de coopération tels que la protection physique contre les antagonismes (les biofilms par exemple) ou les signaux complexes (Diggle *et al.* 2007a;b). Une telle recherche ouvrirait de passionnantes pistes de réflexion sur la coopération chez différents taxons dans des contextes écologiques difficiles.

Partie 2

Vers des applications médicales : l'utilisation des antagonismes écologiques comme agents de contrôle

L'observation des effets néfastes des phages et des antibiotiques sur les populations bactériennes a mené à leur usage thérapeutique pour contrôler les infections bactériennes en médecine et en agriculture. Alors qu'ils ont longtemps été utilisés séparément dans des traitements simples, de récentes études soulignent le remarquable potentiel des thérapies combinant les phages et les antibiotiques, en particulier en vue de minimiser la résistance aux antibiotiques (Hagihara *et al.* 2012, Escobar-Páramo *et al.* 2012, Zhang & Buckling 2012, Knezevic *et al.* 2013, Torres-Barceló *et al.* 2014). La conception des thérapies combinées requiert la compréhension préalable de la biologie et de l'écologie évolutive des populations bactériennes en présence de phages et d'antibiotiques ainsi que des interactions potentielles (et leurs effets) entre les deux agents. Dans les Chapitres 4 et 5, nous avons étudié les impacts d'un panel de combinaisons phages-antibiotiques sur la survie des bactéries, l'évolution de la résistance et la virulence. Plus particulièrement, nous avons comparé les traitements simples (impliquant un seul agent) aux traitements combinés afin de mieux appréhender les conditions qui favorisent des effets synergiques en testant l'impact de la dose d'antibiotiques, la séquence d'inoculation des deux agents et leurs modes d'action. Nos travaux confirment les avantages des thérapies combinées en termes d'évolution de la résistance et de la virulence et s'inscrivent dans un ensemble d'études théoriques et empiriques sur les potentialités de ces thérapies comme traitements des infections microbiennes.

Conclusion

De récents travaux ont proposé d'utiliser notre compréhension des traits sociaux des bactéries pour améliorer le contrôle des bactéries pathogènes (Brown *et al.* 2009 par exemple). Nos résultats montrent que phages et les antibiotiques peuvent sélectionner favorablement les tricheurs. Ceci, ainsi que des études montrant que les sidérophores et d'autres biens publics peuvent constituer des facteurs de virulence (Rumbaugh *et al.* 2009, Leggett *et al.* 2014, Pollitt *et al.* 2014), indiquent que les antag-

onismes devraient réduire la virulence bactérienne. Nous proposons, et des études expérimentales le montrent (par exemple Diard *et al.* 2014), que la structuration spatiale de l'environnement favorise les producteurs de biens publics, et pourrait alors mener à des infections plus sévères. De futures études pourraient explorer comment de multiples antagonismes peuvent améliorer le contrôle des bactéries, réduire la virulence et minimiser la résistance.

Evolutionary ecology of social bacterial populations under antibiotic and bacteriophage pressure

Bacteria are the basis of virtually all ecosystems and examining their dynamics in the face of biotic and abiotic perturbations is essential to understanding their persistence, evolution and diversification. This thesis is directed towards a better understanding of the impact of phage and antibiotic pressure on the evolutionary ecology of bacterial populations and, in particular, on the evolution of bacterial social behaviours. First, using a combination of mathematical modelling and experimental evolution, we studied how antagonisms in the form of antibiotics (Chapters 1 and 2) and phages (Chapter 3) affect the dynamics of public goods production and strategies, and the evolution of resistance in populations of the bacterium *Pseudomonas aeruginosa*. We found that both phages and antibiotics favour cheats over cooperators in well-mixed environments. While the advantage to cheats led to population growth and even increased resistance frequency in the short-term (Chapter 1), the cheat-dominated populations eventually declined in the presence of phage predators, arguably due to the combination of antagonist pressure and cheating load (Chapter 3). Second, based on the evolutionary prediction that multiple control agents will be more efficient at controlling bacterial populations and reducing the evolution of resistance, we investigated *in vitro* the complex interactions between phages and antibiotics in the context of combined therapies. We showed that the combination of phages and antibiotics decreased population survival and resistance evolution significantly more than either alone. While this main result may be mitigated by several factors such as antibiotic dose (Chapters 4 and 5), the timing of inoculation (Chapter 4), and antibiotic mode of action (Chapter 5), it is also obtained in longer-term assays (Chapter 5). Our results highlight the complexity of the interplay between the negative effects exerted by antibiotics and phages and the evolutionary ecology of bacterial populations, and bring new insights both to the understanding of social evolution and to the potential therapeutic use of phages and antibiotics.

Keywords: social evolution, *Pseudomonas aeruginosa*, experimental evolution, antibiotics, bacteriophages, resistance

Ecologie évolutive des populations bactériennes sociales sous la pression de bactériophages et d'antibiotiques

Les bactéries constituent le socle de presque tous les écosystèmes et l'étude de leurs dynamiques face aux perturbations biotiques et abiotiques est essentielle à la compréhension de leur maintien, de leur évolution et de leur diversification. Cette thèse vise à une meilleure appréhension de l'impact des bactériophages et des antibiotiques sur l'écologie évolutive des populations bactériennes et, plus particulièrement, sur l'évolution de leurs comportements sociaux. Dans une première partie, nous avons étudié comment les antibiotiques (Chapitres 1 et 2) et les phages (Chapitre 3) affectent les interactions fondées sur la production de biens publics ainsi que l'évolution de la résistance dans les populations de *Pseudomonas aeruginosa*, en combinant modélisation mathématique et évolution expérimentale. Nous avons montré que les phages et les antibiotiques favorisent les tricheurs face aux coopérateurs dans les environnements homogènes. Alors que l'avantage des tricheurs permet la croissance de la population et augmente la fréquence de résistance à court terme (Chapitre 1), les populations dominées par les tricheurs finissent par décliner en présence de phages, vraisemblablement suite aux pressions combinées des phages et des tricheurs (Chapitre 3). Dans une seconde partie, nous avons exploré *in vitro* les interactions complexes entre les phages et les antibiotiques dans le contexte des thérapies combinées. Conformément à la prédiction de la théorie de l'évolution selon laquelle plusieurs moyens de contrôle combinés sont plus efficaces que chacun séparément, nous avons montré que l'usage simultané de phages et d'antibiotiques réduit davantage la survie et la résistance des populations. Si ce résultat principal peut être modulé par différents facteurs tels que la dose d'antibiotiques (Chapitres 4 et 5), le moment d'inoculation (Chapitre 4), et le mode d'action des antibiotiques (Chapitre 5), il persiste sur le long terme (Chapitre 5). Nos résultats soulignent la complexité des interactions entre les effets négatifs des phages et des antibiotiques et l'écologie évolutive des populations bactériennes et apportent de nouveaux éléments à la fois à la compréhension de l'évolution de la socialité et à l'usage thérapeutique potentiel des phages et des antibiotiques.

Mots clés : évolution sociale, *Pseudomonas aeruginosa*, évolution expérimentale, antibiotiques, bactériophages, résistance