THE EVOLUTIONARY ECOLOGY OF SPITEFUL BACTERIOCIN PRODUCTION

R. Fredrik Inglis

Hertford College

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ABSTRACT

Understanding the conditions that favour the evolution and maintenance of spiteful bacteriocin production combines two important questions from the fields of social evolution and microbiology. Spiteful behaviours, though, initially thought to be rare represent an important class of interactions between bacteria through the production of bacteriocins. Bacteriocins can be considered spiteful as they are costly to produce (in many cases requiring lysis) and are costly to sensitive bacteria (i.e. they are lethal). However, much about the ecology of spiteful behaviours and bacteriocin production remains unclear. Mathematical models have given us important insights into some conditions that should favour bacteriocin production, but few empirical studies exist supporting these results. In this thesis I use the bacterium *Pseudomonas aeruginosa* (a prolific producer of bacteriocins), to examine conditions that favour bacteriocin production. I also investigate more specific elements about this system and toxin production in general. I find that bacteriocin production in *P. aeruginosa* closely follows predictions made from mathematical models under a range of different conditions (e.g. frequency, scale of competition, multiple social traits). I also find that resistance can evolve to bacteriocins and biological mechanisms such as the neutralisation of one's own toxin can have important consequences. Finally, I consider bacteriocin as a policing trait testing predictions about the role that linkage plays in policing. This work represents a comprehensive study into the importance of bacteriocin production in bacteria.

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Finally the most important person I have to thank is my wife Jen. This thesis is dedicated to you.

STATEMENT OF AUTHENTICATION

The work presented in this thesis is, to the best of my knowledge, original as acknowledged in the text. I hereby declare that I have not submitted any part of this material for a degree at this or any other institution

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R. F. Inglis

STATEMENT OF AUTHORSHIP

Chapter 2 is a modified version of Inglis *et. al.* (2009). I designed and carried out all the experimental work and statistical analyses. Dr. Andy Gardner developed and analysed the mathematical models used in this chapter, as well as providing comments on the manuscript. Dr. Pierre Cornelis provided the strains used in this experiment and gave comments on a draft of the manuscript. Dr. Angus Buckling provided assistance in writing the manuscript and guidance in experimental design. I believe that I performed the greater part of the work in the creation of this chapter, in accordance with the Exam Regulations 2009-2010, as stipulated on page 872, Chapter 17, Section B, Heading 4, Sub-heading 4.

In Chapter 3, I designed and carried out all the experimental work and statistical analyses. Dr. Andy Gardner developed and analysed the mathematical models used in this chapter, as well as providing comments on the manuscript. Dr. Angus Buckling provided assistance in writing the manuscript and guidance in experimental design. I believe that I performed the greater part of the work in the development of this chapter, in accordance with the Exam Regulations 2009-2010, as stipulated on page 872, Chapter 17, Section B, Heading 4, Sub-heading 4.

In Chapter 4, I designed and carried out all the experimental work and statistical analyses. Dr. Sam Brown developed and analysed the mathematical models as well as providing comments on the manuscript. Dr. Angus Buckling provided assistance in writing the manuscript and guidance in experimental design. I believe that I performed the greater part

of the work in the development of this chapter, in accordance with the Exam Regulations 2009-2010, as stipulated on page 872, Chapter 17, Section B, Heading 4, Sub-heading 4.

The remaining five chapters constitute wholly my own work.

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INTRODUCTION

1.1 SOCIAL EVOLUTION

1.1.1 Background

What is social evolution? Simply put, it is the study of how intraspecific social interactions evolve and their importance in shaping the biology of living organisms. An interaction can be considered 'social' if it has fitness consequences for both the actor and the recipient (Hamilton 1970), and this provides a basic framework with which to classify different types of behaviours. There are four main classes of behaviour, based on how they affect the fitness of the individuals involved (mutual benefit, selfishness, altruism, and cooperation) and can be illustrated in a simple pay-off matrix (Figure 1.1). In the first row the actor benefits from its own action and either helps the recipient (mutual benefit) or harms him (selfishness).

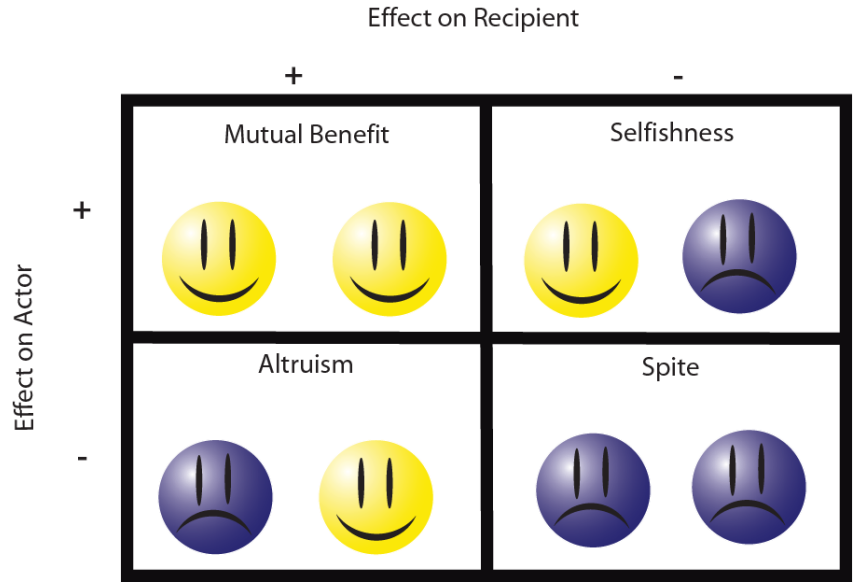

Figure 1.1: The four basic social behaviours. Mutual benefit: both actor and recipient benefit; Altruism: recipient benefits at the expense of the actor; Selfishness: actor benefits at the expense of the recipient; Spite: both actor and recipient suffer a loss.

Understanding why behaviours such as these (mutual benefit and selfishness) evolve is conceptually easy, as they involve direct fitness benefits to the actor. Individuals

that perform these types of behaviour will, therefore, be fitter than those that do not and should spread in the population. However, interactions such as altruism, an action that harms the actor but benefits the recipient, and spite, an action that harms both the actor and recipient (seen in second row interactions in Figure 1.1) are more puzzling, as in both these cases the actor is suffering a loss in fitness (Hamilton 1964; Hamilton 1972; Gardner and West 2006). This raises the question of how these types of behaviours can evolve if individuals that perform them are less fit than their competitors?

One possible solution utilises the social partner's genetic similarity to explain these interactions and is termed kin selection. This is famously captured in Hamilton's rule: $rb>c$, where an altruistic behaviour can be favoured when the cost to the actor ($c>0$) is smaller than the benefit (b) to the recipient multiplied by the relatedness of the recipient to the actor (r) (Hamilton 1963), where relatedness measures genetic similarity of social partners relative to the average member of the competing population, relative to a reference population. In this sense relatedness refers to the extent to which an actor's genotype predicts that of a recipient and can be measured as a regression coefficient (Queller 1994; Frank 1998). Now the action is viewed in the broader context of the interacting population, and the determining factor for the evolution of the behaviour is the effect on the inclusive fitness of the actor (fitness of both the individual and that of other highly related members of the population). Behaviours which are harmful to the actor (such as altruism) can, therefore, evolve if they provide a large enough benefit (b) to closely related individuals (high r).

Kin selection provides a simple yet powerful theoretical framework for understanding altruistic cooperation within species. There have been numerous theoretical

and empirical studies conducted, examining different aspects of ecology and evolution in relation to the role of kin selection in altruistic cooperation, across a range of different organisms(Hamilton 1972; Taylor 1992a; Van Baalen and Rand 1998; Mitteldorf and Wilson 2000; West *et al.* 2001; West *et al.* 2002; Queller *et al.* 2003; Velicer 2003; West and Buckling 2003; Griffin *et al.* 2004; Wenseleers and Ratnieks 2006; Buckling *et al.* 2007; Diggle *et al.* 2007; West *et al.* 2007; Buckling and Brockhurst 2008; Kümmerli *et al.* 2009; Gardner 2010). These have been reviewed extensively in a variety of different contexts (West *et al.* 2002; Lehmann and Keller 2006; West *et al.* 2007; Buckling and Brockhurst 2008; Velicer and Vos 2009). Hamilton's rule does, however, make several assumptions which must be fulfilled before it is applied. These include 1) Weak selection (mutants behave only slightly different from the wild type) 2) additivity of costs and benefits and 3) transmission of traits unaltered to offspring (Frank 1998).

1.1.2 Spite

Using Hamilton's rule, one can also show that spiteful behaviours (interactions where both the actor and recipient suffer harm from the action) should be favoured when the cost to the actor ($c>0$) is smaller than the product of the negative benefit ($b<0$) to the recipient and negative relatedness ($r<0$) of the recipient to the actor *(Gardner et al. 2007)*. In this type of interaction the actor is able to increase its inclusive fitness by actively harming competitors, thereby reducing the intensity of competition and increasing resource availability. This can also be thought of as indirect altruism, where an actor harms a recipient to help a relative, and now involves three parties instead of two (West and Gardner 2010). Defining spite in this way requires the relatedness (r) to be negative when

describing the relationship between the actor and recipient. Positive relatedness describes a scenario where two individuals share more genes than average, and so negative relatedness describes a relationship where fewer genes are shared than on average.

One of the major problems that negative relatedness presents is how to discriminate between negative and positive related individuals, and then direct spiteful behaviours towards the negatively related ones. One mechanism proposed to overcome this is kin discrimination, where the actor is able to recognise its own kin who are statistically more related than average, and those that are not kin who are less related than average (Hamilton 1970; Gardner *et al.* 2004). If relatedness to the victims of spite is sufficiently negative, then spite can be favoured by kin selection. This negative relatedness is expected to be very small in large populations, unless the actor can identify a huge number of kin. However, if the level of competition is local then relatedness measured relative to the average competitor can be strongly negative even in very large populations (Gardner and West 2006).

An alternative mechanism, similar to kin discrimination, is when individuals can estimate genetic relatedness directly, in the absence of kinship cues. If a gene coding for a distinctive trait (i.e. a green beard) is genetically associated with the spite gene, then the actor can pick out its positive and negative relations using this distinctive marker as a guide (Dawkins 1976). By limiting spiteful behaviours to individuals that do not share the same marker, the actor ensures that its victims are sufficiently negatively related. Green beards, however, are predicted to be rare because selection will favour other genes in the genome to disrupt this mechanism (Hamilton 1964). Positive-frequency dependent selection acts on spiteful green beards either driving them to extinction or fixation, and if everyone in the

population has a green beard then there is no one to be spiteful to. This leads to a situation where spite is only a transient strategy over evolutionary time (Hamilton 1970).

However, this assumption, that greenbeards cannot persist in nature, seems incorrect as a number of both altruistic and spiteful greenbeards have been identified. These include *Dictyostelium discoideum*, where aggregating cells form cooperative fruiting bodies depending on the presence of a specific gene (*csa*) (Queller *et al.* 2003) and the *Gp-9* gene, in *Solenopsis invicta* fire ants, which allows workers to identify related queens based on their odour (Keller and Ross 1998). Furthermore, it has recently been shown that greenbeards can also be divided based on whether they are obligate (always expressed) or facultative (only expressed in the presence of reciprocal greenbeards), which can affect their dynamics (Gardner and West 2010).

1.1.3 Examples of Spite in Nature

Compared to altruistic cooperation, there is a relative paucity of empirical and theoretical studies examining the evolution and ecology of spiteful interactions. This may in part be due to spite's chequered history since its inception by Hamilton in 1963 (Hamilton 1963). There have been a few documented examples of spiteful behaviours in a range of different organisms, but often these behaviours can be shown to be exhibiting merely selfish behaviours (Foster *et al.* 2001). One of the major problems when interpreting spite in large, charismatic organisms, such as birds and mammals, is disentangling the myriad other benefits the action could have. This led Keller *et al.* to conclude that real examples of spiteful behaviour were still lacking (Keller *et al.* 1994).

Another problem that has perhaps hindered the study of spite, stems from Wilson's definition of spite which includes a third party term to describe the benefit of the behaviour (Wilson 1975). In Wilson's explanation a non-interacting third party that is closely related to the spiteful actor receives the benefit. Classically this has led to a distinction between Hamiltonian and Wilsonian spite. This, however, seems to be a mere semantic difference. As previously mentioned, using a third party is, perhaps, conceptually easier, but the outcome is still the same and a third party remains implicit in the two party definition (Gardner *et al.* 2007).

These problems aside, there have been some well documented examples of spiteful behaviour occurring in nature. Social insects provide many cases of spite, for example worker policing in hymenopterans (bees, ants, and wasps) (Foster *et al.* 2001). Workers in these species have functioning ovaries which enables them to produce males, since unfertilised eggs are male and fertilised eggs are female (haplodiploid sex determination). However, in most species the queen dominates male production (Bourke 1988). One reason for this is the tendency of non-reproductive workers preventing others from reproducing by aggression or egg eating. This can be considered spiteful as workers are investing time and energy to kill other workers' sons at no direct benefit to themselves (Foster *et al.* 2000). Workers are more related to the sons the queen produces than those of other workers, thereby providing the direct benefit to more related individuals. This type of behaviour has been observed in honeybees (Ratnieks and Visscher 1989) and Vespinae wasps(Foster and Ratnieks 2000).

Another similar example is sex allocation biasing by hymenopteran workers. Workers are more related to their sisters than their brothers because of haplodiploid sex

determination. Therefore, workers are expected to try to increase female production, whereas the queen's optimum would an equal sex ratio (Trivers and Hare 1976). Male killing by social insect workers to increase resources for females has been observed in several species (Sundström 1994; Evans 1995). This can also be viewed as spite as there is little or no direct benefit to the workers harming their brothers, but their more related sisters benefit from increased resource availability.

One of the most well publicized cases of spiteful behaviour is arguably the queen killing in the fire ant *Solenopsis invicta* (Keller and Ross 1998). Heterozygous workers (*Bb*) that carry a (*b*) allele at the *Gp-9* (general protein-9) locus kill queens (*BB*) in the colony that lack it. This is an example of the aforementioned green beard, as workers are able to directly identify queens that do or do not possess the *b* gene. In this manner workers are able to directly measure their relatedness with the queen. The actual mechanism of this mutation, is to change the odour of the queen, which the workers can detect (Keller and Ross 1998).

The larval forms of polyembryonic parasitoid wasps are also thought to display spiteful behaviours (Gardner *et al.* 2007). In this hymenopteran family, Encrytidae, a fraction of the larvae develop precociously as soldiers, giving up their own future reproduction in order to kill competitors, including the opposite sex siblings, developing in the same host (Giron *et al.* 2004). The male soldiers preferentially direct their spite towards less related individuals, feeding on the extraembryonic membrane surrounding each larva during its development in their host (Giron and Strand 2004; Giron *et al.* 2004). They are also under strong local selection for host resources, another requirement for spiteful behaviours to evolve. Modeling by Gardner *et al.* has shown that the male soldiers main

role is to defend against other parasitoid species and to provide other benefits for the brood at a cost to themselves (Gardner *et al.* 2007).

Cytoplasmic incompatibility caused by the bacterium *Wolbachia* can be considered a spiteful trait (Hurst 1991). In this system when uninfected *Culex pipiens* females mate with infected males, their eggs are rendered sterile. *Wolbachia* is transmitted vertically through the egg cytoplasm, so infected males provide no route for transmission. By sterilising the progeny of non-infected females, the bacteria are increasing the frequency of infected females in the population. This comes at a cost to bacteria in the males as they have no opportunity to transmit but benefits their clone mates in the female bacteria (Hurst *et al.* 1996). The exact molecular mechanism by which this sterlisation works has yet to be elucidated, but is thought to work via some lock-and-key interaction where *Wolbachia* produces a "lock" that binds to the paternal nucleus and can only be rescued by a "key" component produced by bacteria present in the egg (Poinsot *et al.* 2003).

Finally, bacteriocin production in bacteria can be regarded as spiteful. It is costly to the individual cell that produces it and also costly to the recipient, which is killed. The individual cost of bacteriocin production may be the diversion of resources from other cellular functions, however in many gram negative bacteria such as *Escherichia coli*, cell death is required for the release of bacteriocins (Riley and Wertz 2002). Individuals from the same lineage are protected from the toxic effects of bacteriocins as a result of genetic linkage between the bacteriocin gene and an immunity gene that encodes a factor that deactivates the bacteriocin (Riley and Wertz 2002). Bacteriocin production can provide an indirect fitness benefit by reducing the level of competition experienced by relatives.

1.2 THE EXPERIMENTAL SYSTEM

1.2.1 Experimental Evolution

Studying the social lives of bacteria, as opposed to mammals or birds, provides many experimental advantages (West *et al.* 2007; Buckling *et al.* 2009). Individual bacteria display highly sophisticated behaviours and are able to communicate and to cooperate to enable activities such as dispersal, niche construction, reproduction, warfare, and signalling (Griffin *et al.* 2004; Harrison and Buckling 2005; Brockhurst *et al.* 2006; Diggle *et al.* 2007; Xavier and Foster 2007). Their small size, large populations, and short generation times allow one to observe evolution in real time. Mutations, for example, arise quickly, which can drastically affect social behaviours, such as the evolution of social "cheats" who no longer participate in the behaviour but reap all the benefits.

Populations can be studied for hundreds or even thousands of generations, and these populations can be stored in suspended animation (Lenski *et al.* 1991). This provides a living fossil record, allowing direct comparisons to be made between ancestors and descendants. Conditions in the lab can be specifically and accurately manipulated to test defined ecological conditions. However, studies are not limited to the laboratory and naturally occurring bacteria can be studied in the soil (Vos and Velicer 2009) or in infections (Köhler *et al.* 2009). Finally, as full genome sequencing and mutant libraries are available, this holds the potential for directly comparing phenotypes and genotypes in social evolution (Foster *et al.* 2007).

In vitro evolution studies have already been successfully used to augment our understanding of many areas of evolution and ecology, including adaptation (Lenski and Travisano 1994; Burch and Chao 1999; Burch and Chao 2000; Rozen *et al.* 2002), adaptive

radiation (Rainey and Travisano 1998; MacLean 2005; MacLean *et al.* 2005), co-evolution (Chao *et al.* 1977; Buckling and Rainey 2002a; Buckling and Rainey 2002b; Vos *et al.* 2009), and altruistic cooperation (Griffin *et al.* 2004; Harrison and Buckling 2005; Buckling *et al.* 2007; Diggle *et al.* 2007; Kümmerli *et al.* 2009), where bacteria, phage, yeast, and amoebae have all been used as experimental systems.

Microbial systems provide a powerful tool to test specific hypotheses, using several replicate populations, growing in defined conditions, over many generations. However, there are still limitations to these types of studies. Microbes are often grown under unnatural conditions in the laboratory (high densities in well mixed, nutrient rich media), and so may behave differently, thereby, limiting the scope of the results. There is also a potential for experimental conditions to be over simplified and contrived, but while this may sometimes be the case, this type of system does allow one to investigate interactions which would be intractable in complex systems and can act as an important test of existing theory. Another often proffered criticism is that results derived from microorganisms cannot be applied to larger organisms. Although, it is true that not all results can be applied to macrobiota, microbes are interesting and important organisms in their own right. By looking, however, for broad ranging principles such as social behaviours, one may be able to form general principles about bacterial interactions that are able to hold true across a range of species and habitats, and also lend a starting point to tease apart system specific interactions.

1.2.2 Bacteriocins in an Evolutionary/Ecological Context

To date little experimental evidence exists confirming that bacteriocin production is a truly spiteful interaction. This is not to say that bacteriocins have not been studied in an ecological or evolutionary context. On the contrary, much is known both experimentally and theoretically about bacteriocin production. One of the earliest studies looking at the ecological and evolutionary role of bacteriocin and spiteful behaviours is that of Chao and Levin (Chao and Levin 1981). In their study, they examined the effect of bacteriocin production in structured versus non-structured habitats. They found that bacteriocin producers were only at an advantage when fairly common in non-structured habitats, whereas in the structured habitats they have an advantage even when initially rare. When a bacterium produces bacteriocin in a structured environment it is more likely that related individuals will benefit from the resulting zone of clearance. This is in contrast to a non-structured environment where the bacteriocin producing bacteria would be randomly distributed with respect to their genotypes, so conspecifics would be unlikely to benefit from the killing of competitors when rare. They conclude, therefore, that for the evolution of bacteriocin a structured habitat is necessary.

Understanding the conditions that favour bacteriocin production and spiteful behaviours has been further analysed theoretically. Gardner *et al.* have modelled the evolution of bacteriocin production, showing that it should be favoured when producers are at intermediate relatedness in the population (Gardner *et al.* 2004). When the producer strain is at low frequency, the benefits of reducing competition and freeing up resources will be shared by the sensitive strain as much as producing strains, hence there is less net benefit to producing (relatedness is only weakly negative). Similarly at high frequency

producing has less benefit because there are few competitors to kill, and hence more available resources to exploit. Only at intermediate frequencies will bacteriocin production confer the greatest fitness advantage by killing competitors and thereby "freeing-up" resources, both by preventing competitors from growing into unexploited patches and by removing them from their current ones.

Furthermore this spiteful behaviour can be seen to affect parasite virulence. Many models predict a tragedy of the commons to occur when two different strains are in direct competition leading to greater virulence (Van Baalen and Sabelis 1995; Frank 1996a; Gandon *et al.* 2001). However, spiteful interactions are predicted to reduce overall virulence when the producing strain is at intermediate frequencies in the population (Gardner *et al.* 2004). This can be best understood if overall bacterial load contributes to virulence as spiteful interactions will reduce the numbers of sensitive bacteria. Both these predictions are purely theoretical and have only been partially tested using *Photorhabdus spp.* infections in caterpillars, finding that single strain infections were more virulent than mixes of bacteriocin producers and sensitive species (Massey *et al.* 2004). Further work looking at the effect of migration on virulence in a bacteria/nematode system found that virulence is reduced at high migration rates, leading to more local diversity and, therefore, more bacterial interference competition (Vigneux *et al.* 2008).

Another important ecological and evolutionary aspect of bacteriocin production is the opportunity for rock-paper-scissors dynamics to occur in spatially structured environments (Kerr *et al.* 2002; Czárán and Hoekstra 2003). Given the strong selective pressure imposed by bacteriocins, it is unsurprising that bacteriocin resistance readily evolves. This can lead to scenarios where bacteriocin-producing, sensitive, and resistant

strains are in competition, where the producers are able to out compete sensitives, resistants out compete producers, and sensitives out compete resistants (rock-paper-scissors). Costly resistance is also likely to explain why there is considerable diversity in bacteriocin types maintained within microbial species. It is probably too costly to be resistant to all bacteriocins at once and the probability of resistance will be lowest with respect to rare bacteriocins. As such, rare bacteriocin alleles will increase in frequency, allowing a diversity to be maintained.

More recently work on yeasts infected with double stranded RNA viruses has helped to further elucidate the ecological conditions that favour the production of anti-competitor toxins. These viruses can be considered similar to bacteriocins as there is no known horizontal transmission of the virus between yeast cells: daughter cells are instead infected via the cytoplasm of their parents (Magliani *et al.* 1997). In *Saccharomyces cerevisiae*, the K1 toxin system requires two viruses, one that encodes the toxin and immunity proteins and a second that is involved in encapsulating the virus. Unlike most bacteriocins, lysis is not required to release the toxin, but there is a pronounced fitness cost when carrying the virus (Wloch-Salamon *et al.* 2008).

Using this system, Grieg and Travisano (2008) were able to show the importance of density in determining the success of toxin-producing strains. Density dependence has previously been shown to be an important ecological determinant in allelopathic interactions (Brown *et al.* 2006), but experimental work has focused on the frequency of the producing and sensitive strains. Grieg and Travisano (2008) demonstrated that toxin producers, even when at intermediate frequencies, were unable to invade if the overall microbial density was low. This effect was also observed in structured environments, which

tend to favour toxin production. When both producers and sensitive strains are rare there will only be limited interaction between them and there will be little benefit in producing toxins. Instead toxin producers will be at a disadvantage due to the metabolic and pleiotropic costs of producing toxins.

Nutrient availability, dispersal, and their effect on interference competition in yeast have also been considered experimentally. It has been suggested that toxin production might act as strategy of last resort among starving cells by killing competitors for nutrients(Ivanovska and Hardwick 2005). Wloch-Salamon *et al.* (2008) grew *S. cerevisiae* toxin-producing strains in competition with sensitives under low and high nutrient conditions. Nutrient availability was manipulated by varying the rate of nutrient replacement, following initial growth to carrying capacity (therefore controlling initial densities). Wloch-Salamon *et al.* (2008) found that toxin producers were able to out-compete sensitive cells only in high nutrient environments and were out-competed when grown under low nutrient conditions. They argued that the lack of toxin production seen in the low nutrient environment is unlikely to be solely due to a nutritional constraint, but rather due to a physiological change in toxin production or resistance developing after the nutrients have been depleted (Wloch-Salamon *et al.* 2008).

They conclude that this acts as support for Frank's (1994) theoretical prediction that toxin production has evolved to occur as a competitive strategy under conditions where resources are abundant and growth is allowed (Frank 1994). This makes it unlikely that toxin production is used to acquire resources from sensitive cells, but is more important in the invasion of high nutrient patches. They also looked at the effect on dispersal, finding that limited dispersal favoured toxin producers. A benefit was still observed in treatments

with high dispersal, but it was insufficient to compensate for the resource cost of carrying the toxin producing virus. These results are consistent with Chao and Levin's (1981) results in spatially structured environments as dispersal will reduce the chances that related cells will profit from toxin production.

1.2.3 Introduction to Pyocins

By considering bacteriocin as a spiteful behaviour this opens an intriguing avenue of research by allowing the combination of social evolutionary theory with microbial systems. All bacteria produce a wide arsenal of killing agents, with 99% being able to make at least one bacteriocin (Klaenhammer 1988). There is really no universally accepted definition for what a bacteriocin is, but it generally contains various protein based molecules that inhibit bacterial growth (Jack *et al.* 1995). The most commonly accepted definition is based on colicin work in the gram positive *E. coli* (Riley and Wertz 2002). In this model colicins have a narrow killing spectrum and are only toxic to closely related strains. Colicins are encoded on plasmids composed of a colicin gene, which encodes the toxin, an immunity gene (that provides immunity by binding to and inactivating the toxin proteins), and a lysis gene which codes for the protein involved in colicin release through the lysis of the producer cell (Cascales *et al.* 2007). Colicin expression is SOS regulated and toxin production is lethal for the producing cell and any neighbouring sensitive cells. Killing functions range from pore formation in the cell membrane to destruction of genetic material.

The bacterium *Pseudomonas aeruginosa*, is a gram negative bacillus, that inhabits soil, fresh water, marine habitats, and is able to infect humans. Most strains that have been

isolated both from clinical and environmental sources produce bacteriocin (Michel-Briand and Baysse 2002). Bacteriocins were first discovered in *P. aeruginosa* by François Jacob in 1954, and he named them pyocins as they were similar to the already discovered colicins. During the next 50 years three different classes of pyocin were discovered and defined (Kageyama and Egami 1962; Takeya *et al.* 1967; Ito *et al.* 1970; Kuroda and Kageyama 1979; Sano and Kageyama 1993; Parret and De Mot 2002). Most strains produce pyocins but the spontaneous production level of pyocins is quite low unless mutated (Michel-Briand and Baysse 2002). Pyocins act via a single hit mechanism, whereby only one particle is required to kill a cell. However not all pyocins are active, so in some cases up to 300 adsorbed pyocins are required to kill a sensitive cell (Michel-Briand and Baysse 2002). Pyocinogenic strains are insensitive to their own pyocins, although one exception has been published (Goodwin *et al.* 1972).

P. aeruginosa produces 3 basic types of pyocin: R, F, and S. They are divided by their structure and mode of function. Strains often produce more than one pyocin type with R- and F-type pyocins being produced in more than 90% and S-type in 70% of characterised isolates (Smith *et al.* 1992). PAO1, for example, produces R2, F2, and S2 pyocins (Parret and De Mot 2002). Unlike colicins, pyocins are located on the bacterial chromosome. R- and F-type pyocins require cell lysis for their release, whereas S-type has no associated lysis protein gene, however the lytic systems described for R- and F-type pyocins may be shared by S-type pyocins (Parret and De Mot 2002).

R-type pyocins were the first class of pyocins to be described and are particulate, resembling a bacteriophage (Kageyama and Egami 1962; Nakayama *et al.* 2000). To date there have been 8 R-type pyocins described: R-1, C9, R2, R3, R4, R5, 21, and 430c

(Kageyama 1964; Higerd *et al.* 1967; Ito *et al.* 1970; Govan 1974; Takeda and Kageyama 1975). All R-type pyocins are nuclease and protease resistant. R1 pyocin is morphologically similar to a T-even phage tail. It has a rod like structure of a double hollow cylinder which consists of a sheath and core (120nm long, 15 nm wide) with one bacterium being able to produce 200 R pyocin particles (Ishii *et al.* 1965; Shinomiya 1972). R-pyocins bind to bacteria using lipopolysaccharide fibres (Ikeda and Egami 1973) and are able to bind to a range of different bacteria such as *Neisseria gonorrhoeae*, *Neisseria meningitidis*, and *Haemophilus influnzae (Michel-Briand and Baysse 2002)*. Once bound to a receptor, its sheath contracts, and the core penetrates the outer membrane. After adsorption R-type pyocins arrest synthesis of macromolecules in sensitive bacteria, resulting in death, due to pore formation and subsequent depolarisation in circa 20 minutes (Ohsumi *et al.* 1980; Uratani and Hoshino 1984).

F-type pyocins were the second class of pyocin discovered (Takeya *et al.* 1967). The "F" prefix derives from its flexuous rod appearance when viewed using an electron microscope. In total there are three F-type pyocins, 1-3 (Kuroda and Kageyama 1979). The structure of F1 pyocin is a flexuous, non-contractile rod of 106nm in length with a width of 10nm which resembles a flexible phage tail (Kuroda and Kageyama 1981). The specific activity of F-type pyocins is different according to their class. Both F- and R-types are structurally similar to viruses but do not replicate within host cells. The infectivity of some phages can be neutralised by adding sera of pyocins (Michel-Briand and Baysse 2002). An interesting side note is that the G+C% is similar in R-and F-type pyocins to the whole chromosome, indicating that their phage origin is probably not a recent event (Michel-Briand and Baysse 2002).

The final class of pyocins are S-type, or soluble pyocins. Four types of S-type pyocins have been purified (S1, S2, S3, and AP41 (Ito *et al.* 1970; Holloway *et al.* 1973; Duport *et al.* 1995)), and a further two more have been predicted from gene sequences studies (Parret and De Mot 2000). S-type pyocins are organised into four domains arranged linearly. The N-terminal domain is involved in the recognition of the cell surface receptor, domain II has an unknown function and is dispensable for killing activity, the third domain is responsible for pyocin translocation and penetration, and the C-terminal domain carries the lethal activity (Sano *et al.* 1993; Michel-Briand and Baysse 2002). S-type pyocins are protease sensitive, unlike R- and F-type, and are composed of two proteins. The larger protein is involved in killing whereas the smaller protein, which shares sequence homology with the colicin E2, is thought to be a type of immunity protein which acts to protect the host (Sano and Kageyama 1993). The cause of cell death is DNA breakdown due to the endonuclease activity of the pyocin (Sano *et al.* 1993; Duport *et al.* 1995). S1 and S2 pyocins inhibit phospholipid synthesis within 5 minutes under iron limited conditions, and is probably the primary mode of the killing action (Ohkawa *et al.* 1975; Sano *et al.* 1993). The smaller protein provides immunity by binding to the C-terminal end of the large protein which is involved in DNase activity (Sano and Kageyama 1993).

Under iron-limited conditions S-type pyocins adsorption and killing are increased (Ohkawa *et al.* 1980). Iron limitation causes the iron-regulated outer membrane proteins to appear on the outer membrane, which are known to be the binding sites of S2 and S3 as well as the receptors for ferripyoverdine (Ohkawa *et al.* 1980; Meyer 2000). Translocation into the cell is thought to occur via the tonB system like that of siderophores (Schalk *et al.* 2001). S2 is known to bind to type 1 ferripyoverdine receptor FpvA (Denayer *et al.* 2007),

whereas S3 binds to type 2 ferripyoverdine receptor FpvA (2a or 2b) (Tümmler and Cornelis 2005). Bacteria cannot have S3 and S2 sensitivity at the same time. S1 kills independently of what type of ferripyoverdine receptor is present. All S2 producers have the type 1 *FpvA* gene. Some strains with type 1 receptors have shown resistance to S2 pyocins, but they either have S1 or S2 immunity genes (both confer immunity) or are not producing pyoverdine (and so no receptors are expressed) (Denayer *et al.* 2007). PA14 has a type 1 receptor but remains immune, similarly to the PAO29P strain, but both have a mutation in residue 46 of the FpvA receptor (valine to isoluecine). This mutation is, however, not sufficient to provide immunity in engineered strains and it is thought that another mutation must be present in the translocation system (Denayer *et al.* 2007). A brief pictorial summary illustrating the interactions between pyocins and pyoverdines can be seen in Figure 1.2.

The exact physiological role for pyocins remains unclear, and this may differ depending on when they are expressed and their mode of action. Production is inducible through DNA damage, but this seems an unlikely source of regulation (Michel-Briand and Baysse 2002). Pyocinogenity appears more often in clinical situations than in the environment (Govan 1986), and under iron limited conditions, such as the cystic fibrosis lung, S-type pyocins might be extremely important in mediating bacterial interactions (Brown *et al.* 1984). Pyocins have also been considered as a means of therapy against infectious *P. aeruginosa*, with fusion constructs of multiple pyocins being tested (Michel-Briand and Baysse 2002; Gillor *et al.* 2005; Kirkup Jr. 2006).

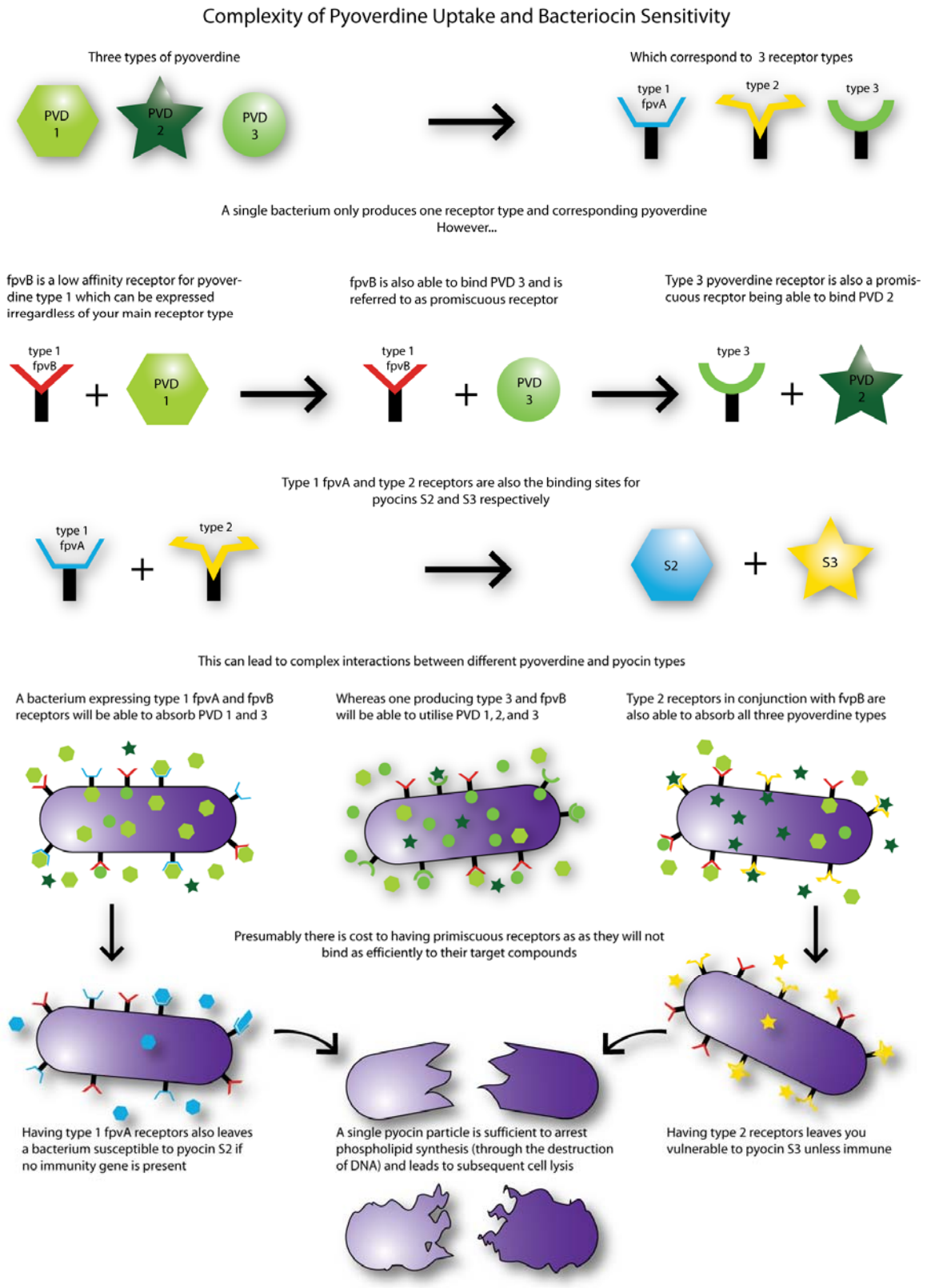

Figure 1.2: A brief pictorial summary of pyocin and pyoverdine interaction in *P. aeruginosa* as described in (Ghysels *et al.* 2004; Denayer *et al.* 2007)

1.3 AIMS

Pyocins in *P. aeruginosa* provide a promising model system to study social interactions. Pyocins and siderophores (e.g. pyoverdine) have already been shown to form an important antagonism from a molecular perspective. This system also provides large scope for studying social behaviour, not only spiteful ones, but also how spite and cooperation (in the form of siderophore production) interact.

In this thesis, I plan to:

1. First consider what basic conditions favour spiteful behaviours:
    a. Such as frequency of the spiteful individuals in the population and how this relates to virulence (Chapter 2)
    b. And how the scale of competition can affect this relationship (Chapter 3)
2. Consider how spite can be altered by other social traits such as cooperation (Chapter 4)
3. Investigate more specifics about bacteriocin production and the ecological role that neutralising one's own toxin can play (Chapter 5)
4. Explore how resistance to bacteriocins can evolve (Chapter 6)
5. Examine further the functional linkage between absorbing pyocin and pyoverdine, in what can be considered a policing type behaviour. (Chapter 7)

Although the implications of my results will be specifically associated to the ecology and evolution of pyocins and bacteriocins, they will hopefully be extended to spiteful behaviours in general and the role of sociality in disease virulence.

CHAPTER 2

SPITE AND VIRULENCE IN THE BACTERIUM *PSEUDOMONAS AERGUGINOSA*

SUMMARY

Social interactions within populations of pathogenic microbes may play an important role in determining disease virulence. One such ubiquitous interaction is the production of anticompetitor toxins: an example of a spiteful behaviour, because it results in direct fitness costs to both the actor and recipient. Following from predictions made by mathematical models, we carried out experiments using the bacterium *Pseudomonas aeruginosa*, to test under what social conditions toxin (bacteriocin) production is favoured and how this in turn affects virulence in the larvae of the wax moth, *Galleria mellonella*. Consistent with theory, we found that the growth of bacteriocin producers relative to sensitive, non-producers is maximised when toxin producers are at intermediate frequencies in the population. Furthermore, growth rate and virulence in caterpillars was minimised when bacteriocin producers have the greatest relative growth advantage. These results suggest that spiteful interactions may play an important role in the population dynamics and virulence of natural bacterial infections.

2.1 INRODUCTION

In recent years there has been a growing interest in understanding the evolution of social behaviours in microbes (West *et al.* 2007). While the evolution of cooperation (behaviours which benefit the recipient) has received considerable theoretical and empirical attention, the evolution of spite (behaviours that harm both the actor and recipient) has been relatively neglected. Conditions that favour the evolution of spite can be understood in terms of selection maximising an individual's inclusive fitness (transmission of one's own genes, and one's own genes in other individuals). Spiteful behaviours can, therefore, theoretically evolve when they target individuals that are less likely to share the same genes as the actor than an average member of the competing population. That is, the relatedness between the recipient and the actor is negative (Hamilton 1970; Hamilton 1972; Hamilton 1996; Foster *et al.* 2001; Gardner and West 2004b; Brown *et al.* 2006).

Spiteful behaviours found in nature are surprisingly common and one well documented example is the production of bacteriocins. Bacteriocins are extracellular antimicrobial compounds produced by almost all bacteria (Riley and Wertz 2002). They can be considered spiteful as they are costly to produce and kill susceptible cells via a range of mechanisms, including enzyme inhibition and the breakdown of DNA and cell membranes. The costs of production can be suicide (in *Escherichia coli*, for example, cell lysis is required to release the bacteriocins), but even where cell death is not required there will be an inevitable metabolic cost which is likely to be greater than the direct fitness benefits: bacteriocins are highly diffusible, hence the producing cell is unlikely to experience the benefit of killing a competitor (Michel-Briand and Baysse 2002; Riley and Wertz 2002). Crucial for the evolutionary maintenance of bacteriocin production is that

bacteriocins specifically target non-related individuals while doing no harm to the bacteriocinogenic cells, usually due to immunity factors that are genetically linked to the toxin (Riley and Wertz 2002). Note that relatedness in this context specifically refers to similarity at the bacteriocin loci between interacting individuals, rather than average similarity across the whole genome. In this sense, bacteriocins can be viewed as spiteful green beards, whereby the same gene complex is capable of directing spite towards individuals which do not have the gene complex for the spiteful behaviour (Brown and Buckling 2008).

A number of theoretical and empirical studies identify ecological conditions that favour the maintenance of spite (12-18) (Chao and Levin 1981; Frank 1994; Kerr *et al.* 2002; Czárán and Hoekstra 2003; Gardner *et al.* 2004; Greig and Travisano 2008; Wloch-Salamon *et al.* 2008). Assuming individuals possess mechanisms to distinguish between related and unrelated individuals (Gardner and West 2004b), spiteful behaviours are predicted to evolve to maximal levels when the frequency of individuals with the same spiteful trait makes up some intermediate frequency of the population (Gardner *et al.* 2004). If the spiteful group is at a high frequency in the interacting population, spite will be less favoured because the reduction in the competition resulting from the spiteful action will be small compared to the costs of being spiteful. Conversely, if the spiteful group is at a low frequency in the interacting population, the few individuals that are targeted will be on average no less related than the individuals that are targeted. Hence relatedness will be zero or weakly negative. This leads to the prediction that spite will be most favoured when the spiteful genotype is at an intermediate frequency in the interacting population. Note that in a previous paper we refer to frequency of a particular genotype within the interacting

population as "kinship" (Gardner *et al.* 2004). This has a different meaning to relatedness, which refers to similarity between actor and recipient relative to the competing population as a whole.

An explicit test of the predicted unimodal relationship between spite and the frequency of spiteful genotypes has yet to be carried out. Existing empirical studies are, however, consistent with this prediction. Specifically, it has been shown *in vitro* that toxin producers can invade sensitive populations only when they are above a threshold starting frequency in both *Escherichia coli* and the yeast *Saccharomyces cerevisiae* (Chao and Levin 1981; Greig and Travisano 2008).

Understanding how the genetic population structure of microbial pathogens affects production of bacteriocins has important applied implications, most notably in terms of the amount of harm infection causes their host (virulence). Attenuated virulence is predicted to coincide with maximal levels of spite (Gardner *et al.* 2004), because under these conditions the growth rate of the infecting population will be lowest, as a result of increased killing and investment into the spiteful behaviours.

Here we use the opportunistic human pathogen, *Pseudomonas aeruginosa*, and a caterpillar model to explicitly test the predictions that 1) bacteriocin production is most favoured when the spiteful genotype is at intermediate frequencies in the interacting population, and 2) this results in minimal *in vivo* population growth rate and virulence. We also extend our previous evolutionary mathematical models to confirm that the qualitative predictions still hold in the ecological context of this experimental system.

2.2 MATERIALS AND METHODS

2.2.1 Model

2.2.1.1 *Bacteriocins*

We consider two strains of bacteria growing under resource competition, with the focal strain making a relative investment *c* into bacteriocin production and the competitor strain making no such investment. We assume that the focal strain is immune to its bacteriocin, but a proportion *pk* of cells of the competitor strain is killed, where *p* is the proportion of the focal strain in the local medium.

The 'per-capita' growth of the focal (producing) strain (the growth scaled to that of a non-producer strain in pure culture) is given by

$$G_P = \frac{1-c}{1-a\left(pc+(1-p)pk\right)},$$

where *a* is the extent of local competition for resources (e.g. the degree of soft selection), and the growth of the competitor (non-producing) strain is

$$G_N = \frac{1-pk}{1-a\left(pc+(1-p)pk\right)}.$$

The total growth is given by

$$G_T = \frac{1-\left(pc+(1-p)pk\right)}{1-a\left(pc+(1-p)pk\right)}.$$

Thus, in the extreme of complete local competition ($a = 1$), the total growth is fixed at $G_T = 1$.

The growth of the focal (producing) strain is independent of its local frequency p in the absence of resource competition ($a = 0$), and is given by $G_P = 1\text{-}c$. Here, the bacteriocin producer always exhibits lower growth than a pure culture of the non-producing strain (i.e. $1\text{-}c < 1$). In the presence of local competition for resources ($a > 0$), the growth of the producing strain is dependent on its local frequency; the derivative

$$\frac{\mathrm{d}G_P}{\mathrm{d}p} = \frac{a(1-c)(c+k-2pk)}{\left(1-a\left(pc+(1-p)pk\right)\right)^2}$$

takes the same sign as $c+k-2pk$, i.e. $\mathrm{d}G_P/\mathrm{d}p > 0$ when $p < (c+k)/2k$ and $\mathrm{d}G_P/\mathrm{d}p < 0$ when $p > (c+k)/2k$. Thus, the growth of the producing strain is a monotonically-increasing function of its frequency if $c > k$, and a unimodal-shaped function of its frequency if $c < k$. In particular, the growth of the producing strain is $G_P \to 1\text{-}c$ as $p \to 0$, and $G_P \to (1\text{-}c)/(1\text{-}ac)$ as $p \to 1$. Note that $(1\text{-}c)/(1\text{-}ac) < 1$ so, if $c > k$, the growth of the producing strain is always less than that achieved by a pure culture of the non-producing strain. If $c < k$ then growth of the producing strain is maximised at the $p^* = (c+k)/2k$, and here it is given by $G_P = 4(1\text{-}c)k/(4k\text{-}a(c+k)^2)$, which exceeds the growth of the non-producing strain in pure culture if a

$> 4ck/(c+k)^2$. Note that c (the cost to the producer) must less than k (the maximum cost experienced by the recipient) for pyocin production to be maintained by natural selection.

Assume that the above growth is occurring in a single subpopulation of a much larger structured population where the producing strain is vanishingly rare, and that the focal subpopulation is representative of all the subpopulations in which the producing strain is located. Then the local frequency (p) of the producing strain is equivalent to the kin selection coefficient of relatedness (r) describing the genetic similarity of cells of the producing strain to the other cells growing in its locality. The producing strain is expected to invade from rarity if its growth is greater than the average in the whole metapopulation (non-producing strain in pure culture), i.e. when $G_P > 1$. This yields the condition

$$\frac{1-c}{1-a\left(pc+(1-p)pk\right)}>1,$$

which may be re-expressed as

$$\left(-\frac{ap}{1-ap}\right)\left(-(1-p)k\right)>c$$

which is of the form $RB>C$ where $R = -ap/(1-ap)$ is Queller's (1994) (Queller 1994) form of relatedness (genetic similarity of social partners relative to competitors), and is equivalent to equation (A2) in Gardner, West & Buckling (2004).

2.2.1.2 *Virulence*

Now consider that each subpopulation represents a single host individual carrying a bacterial infection. Assume that the virulence of the bacterial infection is proportional to its growth, i.e.

$$V = bG_T .$$

Under the extreme of complete resource competition ($a = 1$), bacterial growth is $G_T = 1$ and virulence is fixed at $V = b$. With less intense resource competition ($a < 1$), virulence is dependent on the frequency of the producing strain within the infection; the derivative

$$\frac{\mathrm{d}V}{\mathrm{d}p} = -b\frac{(1-a)(c+k-2pk)}{\left(1-a\left(pc+(1-p)pk\right)\right)^2}$$

has the opposite sign of $c+k-2pk$, i.e. $\mathrm{d}V/\mathrm{d}p < 0$ when $p < (c+k)/2k$ and $\mathrm{d}V/\mathrm{d}p > 0$ when $p > (c+k)/2k$. The sign of $\mathrm{d}V/\mathrm{d}p$ is always opposite of that of $\mathrm{d}G_P/\mathrm{d}p$, and so virulence is monotonically decreasing with the frequency of the producing strain when $c > k$ and is a U-shaped function of the frequency of the producing strain when $c < k$. In particular, virulence is maximised in the absence of bacteriocin production ($p = 0$), and is minimised when the producing strain is at the intermediate frequency $p^* = (c+k)/2k$.

2.2.2 Bacterial Strains

Pseudomonas aeruginosa strain PAO1, was employed as the bacteriocin producer and serotype O:9 as the bacteriocin sensitive competitor. PAO1 is a known producer of pyocin S2, whereas serotype O:9 is sensitive to S2 pyocins (Smith *et al.* 1992; Denayer *et al.* 2007). PAO1150-2, a transposon, bacteriocin-knockout mutant of *psy2*, acted as a non-producing, isogenic control strain (Jacobs *et al.* 2003). Bacteriocin production in *P. aeruginosa* can involve lysis, but it is not clear if it is essential for the release of the soluble pyocins that are the focus of this study (Michel-Briand and Baysse 2002). Bacteriocin production, sensitivity, and insensitivity were confirmed using a simple plate assay where the production of relevant bacteriocin is determined by overlaying bacteria mixed in semi-solid agar on plates that have been spotted with bacteria of another strain, as described by Fyfe *et al.* (Fyfe *et al.* 1984). If the strain inoculated on the plate produces bacteriocin that kills the strain mixed with semi-solid agar, a halo shaped zone of clearing can be observed in the bacterial lawn after incubating at 37°C for 18 hours. The absence of a clear halo indicates that either the overlaid strain is insensitive to the bacteriocin producer or the inoculated strain does not produce any bacteriocin.

2.2.3 Competition Assays

Overnight cultures of each strain were grown shaking at 0.65g and 37°C for 18 hours and then diluted to an OD_{600nm} of 1.8 to ensure similar numbers of bacteria per millilitre. These cultures were subsequently grown on agar plates to determine the number of bacteria present using colony forming units (CFUs) as an approximate measure. 30ml glass universals containing 6ml of Kings Media B broth were inoculated with a total of 10^4

cells with different starting frequencies of the individual strains. PAO1 and O:9 were competed against each other at starting frequencies of, 99%, 90%, 50%, 10%, 1%, and 0.1%. This exact design was replicated in the PAO 1150-2 and O:9 competition. Cultures were propagated in a shaking incubator at 0.65g and 37°C and sampled at 48 and 96 hours, allowing time for the effect of the bacteriocin to be observed.

At each time point (48h and 96h) we calculated the relative growth of the producer to sensitive and non-producer to sensitive at the different starting frequencies. This was done by plating the various treatments on KB agar plates and counting the number of CFUs for each strain. All strains were easily distinguishable from one another because of unique colony morphology and size. At the more extreme frequencies antibiotic plates were required to give better resolution of colony counts, and this was possible due to the different antibiotic resistance profiles of the assorted strains (PAO1 resistant to streptomycin 1250 µg/ml, O:9 resistant to rifampicin 312.5 µg/ml, and PAO 1150-2 resistant to tetracycline 312.5 µg/ml). Selection coefficients (*S*) were used to estimate at what frequency bacteriocin production is favoured in PAO1 relative to 1150-2 using the common competitor O:9, where $s = (m_{PAO1/1150\text{-}2} - m_{O:9})/ m_{O:9}$, and (*m*) refers to ln(final density/starting density) (Lenski *et al.* 1991). All frequencies were replicated 6 times, and statistical analyses performed in Minitab 15. Selection coefficients were preferable to simply using growth rates (*m*), to control for between tube variation.

2.2.4 In Vivo Virulence Bioassay

Virulence assays were performed as described by Harrison *et al.* (Harrison *et al.* 2006). Briefly, overnight cultures of PAO1, O:9, and PAO1150-2 were diluted in minimal

salt solution. Fifth instar waxmoth (*Galleria mellonella*) larvae (Livefood UK; http://www.livefood.co.uk) were randomly allocated to be inoculated with 10^4 CFUs of PAO1/O:9 and PAO 1150-2/O:9 mixtures. The starting frequencies of the bacterial combinations consisted of 99%, 50%, and 1% PAO1 and PAO1150-2 to O:9. Larvae were swabbed with 70% ethanol to prevent contamination of the injection site and injected into the abdomen using Terumo 1ml disposal syringes and BD Microlance 30G ½" needles. The injection volume was 50µl in all cases. Twenty larvae were assigned to each treatment, and a further 20 larvae were injected with 50µl of minimal salt solution as negative controls. Larvae were then incubated at 37°C and monitored for death at 30 minute intervals between 10 and 14 hours and again at 24 hours post-inoculation. Larvae were scored as dead if they failed to respond to mechanical stimulation of the head.

Overall density of the different bacterial strains within the caterpillar hosts was also measured. Caterpillars were inoculated as previously described and incubated for 8 hours at 37°C. Larvae were then weighed, dipped in 70% ethanol to kill surface contaminants, and homogenised in 500µl minimal salt solution using a plastic pestle. Homogenates were centrifuged at 3000 rpm for 3 minutes to pellet the solid, and aliquots of diluted homogenate plated onto KB agar. Agar plates were supplemented with 15µg/ml ampicillin to select against growth of native larval-gut bacteria (this concentration of ampicillin does not affect the growth of *P. aeruginosa*). Plates were incubated overnight at 37°C and subsequently scored for CFUs.

2.3 RESULTS

A simple mathematical model was developed to describe the ecological conditions that favour bacteriocin production. In this model a focal lineage of bacteriocin producing bacteria compete with sensitive bacteria, and remain insensitive to the bacteriocins of its sensitive competitors. Under these conditions (which are described in more detail in the methods section) bacteriocin production provides the greatest advantage when producers are at intermediate frequency in the population and virulence is reduced at these frequencies. Examples of the growth rate of the producing strain at different frequencies and extent of local competition are shown in Figure 2.1. Note that that same qualitative relationships hold if the y-axis displays the selection coefficient of the producing strain (the difference between the growth of the producing strain and sensitive strain).

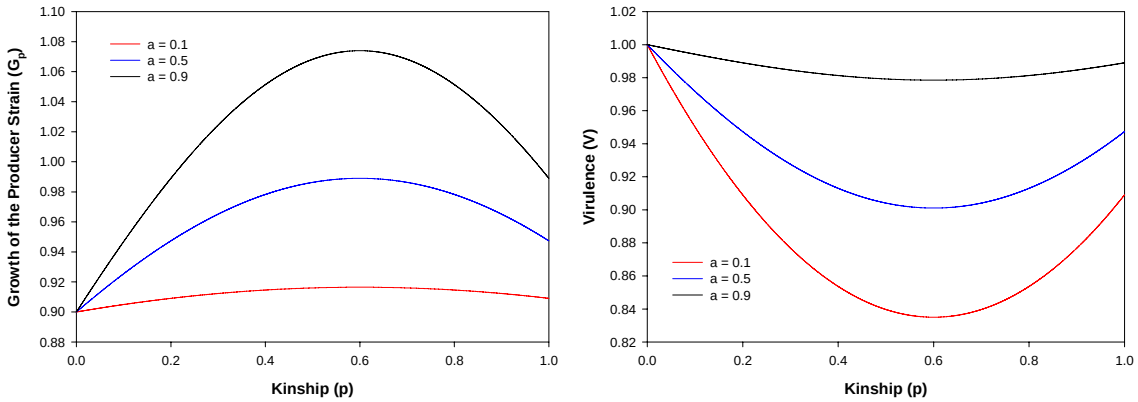

Figure 2.1: Modelling the Relative Growth and Virulence of a Producer. Output from our mathematical models across a range of resource competition (a), when $c = 0.1$ and $k = 0.5$, showing that producers growth is maximised at intermediate frequencies and at these frequencies virulence is attenuated.

In our *in vitro* experiments we manipulated densities of both a bacteriocin producing strain (PAO1, producer of pyocin S2) and a sensitive competitor (O:9) to create a range of different starting frequencies with respect to the producer (between 0 and 1)

(Denayer *et al.* 2007). As a control, we established the same range of starting frequencies for an isogenic mutant (PAO1150-2) of PAO1 that did not produce a bacteriocin that could affect the sensitive strain. Selection coefficients were used to estimate the fitness of the producing and isogenic non-producing strain relative to the sensitive strain. As predicted by the mathematical model the fitness of the producing strains showed a unimodal relationship with starting frequency (Figure 2.2), peaking at intermediate values (linear term $F_{1, 32} = 20.76$, $p<0.001$; quadratic term $F_{1, 31} = 29.64$, $p<0.001$). By contrast, the isogenic non-producing strain showed a weakly negative relationship with starting frequency (Figure 2.2), with a slope of -0.0975 (linear term $F_{1,34} = 11.34$, $p<0.002$; quadratic term $F_{1,33} = 0.13$, $p>0.721$). The ratio of the selection coefficients of the producing and isogenic non-producing strain also displayed a significant unimodal relationship (linear term $F_{1,32} = 23.44$, $p<0.001$; quadratic term $F_{1,31} = 25.85$, $p<0.001$).

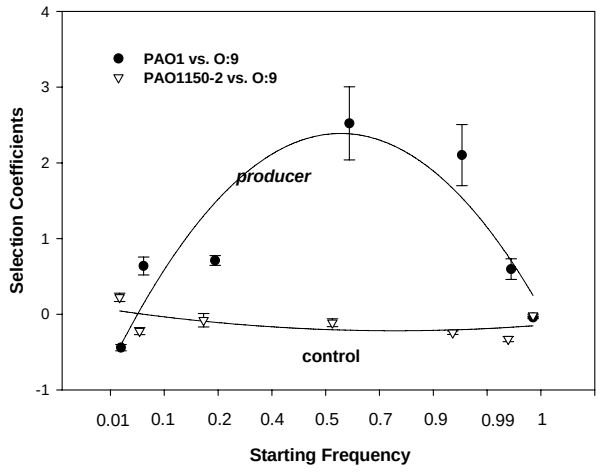

Figure 2.2: Relative Growth of Bacteriocin Production. Relative growth rate of PAO1 vs. O:9. (producer, black circle) compared to relative growth rate of PAO1150-2 vs. O:9 (control, white triangle) along a range of different starting frequencies used to manipulate relatedness. PAO1 vs. O:9 shows a distinct peak in relative growth at intermediate frequencies (linear term $F_{1, 32} = 20.76$, $p<0.001$; quadratic term $F_{1, 31} = 29.64$, $p<0.001$).

The effect of bacteriocin production on growth of the producer strain is dependent on the amount of time the bacteria spend competing. Specifically, only a weak relationship is observed between relative growth and starting frequencies before 96 hours of growth, probably owing to the time it takes for PAO1 to reach sufficient densities for pyocin to have an effect on O:9. Allowing bacteria to grow for longer periods of time does not qualitatively change the results but only makes detection of both PAO1 and O:9 harder at treatments with vey high and low producer frequencies, as the overall numbers of bacteria are decreasing though increasing build up of waste metabolites and depletion of resources. This dynamic is consistent with our model and is captured by the intensity of local competition ('*a*' parameter).

We next determined the relationship between virulence (as measured by time to death of infected caterpillars) and frequency of the producing strain. We manipulated the infecting bacterial populations to give high (99%), intermediate (50%), and low (1%) frequencies of the producing strain relative to the susceptible strain. Consistent with the above (and other (16)) theory, intermediate frequencies resulted in much longer time to death than the high and low frequencies (linear term $F_{1,58} = 52.47$, $p<0.001$; quadratic term $F_{1,57} = 55.85$, $p<0.001$; Figure 2.3a). The proposed mechanism behind this reduction in virulence at intermediate frequencies is reduced growth rate of the population as a whole, resulting from the high mortality rates of the susceptible strain. Consistent with this view, we found that bacterial density prior to the death of the insects showed an inverse unimodal relationship with frequency of the bacteriocin producer, such that density was lower for the 50% treatment (Figure 2.3b; linear term $F_{1,59}= 4.5$, $p<0.038$; quadratic term $F_{1,58}= 7.99$, $p<0.007$). Note that when the non-producing strain was competed with the susceptible

strain there was no significant difference in virulence between the high, intermediate, and low starting frequency treatments ($P > 0.25$ for both linear and quadratic terms; Figure 2.4a), and only a linear relationship exists between density and starting frequency of the non-producer (linear term $F_{1,58} = 16.24$, $p<0.001$; quadratic term $F_{1,57} = 2.66$, $p>0.108$; Figure 2.4b).

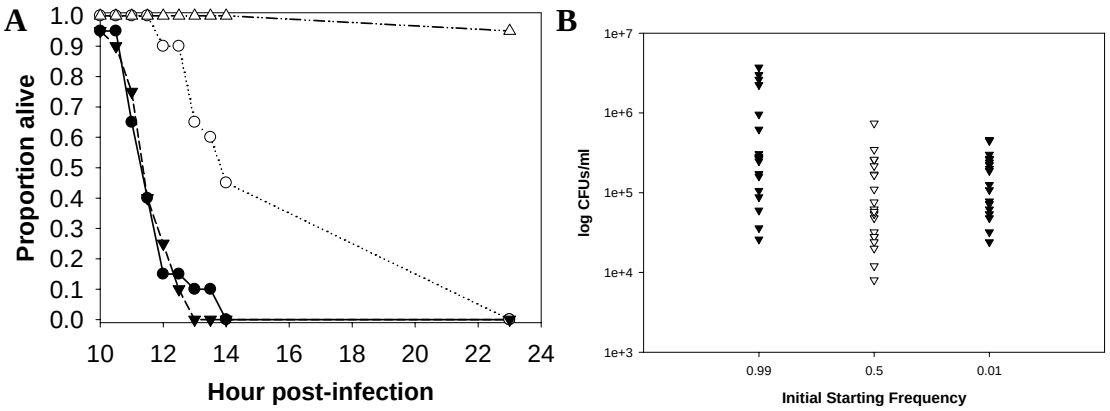

Figure 2.3: Virulence and Density Affected by Frequency of Bacteriocin Producer. A. Time to death of caterpillars inoculated with PAO1 / O:9 mixtures. Initial starting frequencies of PAO1 are indicated on the graph and correspond to the adjacent line. At the intermediate starting frequency death is significantly delayed (linear term $F_{1,58} = 52.47$, $p<0.001$; quadratic term $F_{1,57} = 55.85$, $p<0.001$). B. The average total bacterial density of PAO1 and O:9 is indicated for the three different starting frequencies of the bacteriocin producer. A significant reduction in overall density occurs after 8 hours of growth in the intermediate frequency treatment of PAO1 vs. O:9 where bacteriocin producer and sensitive are inoculated at initially near equal densities.

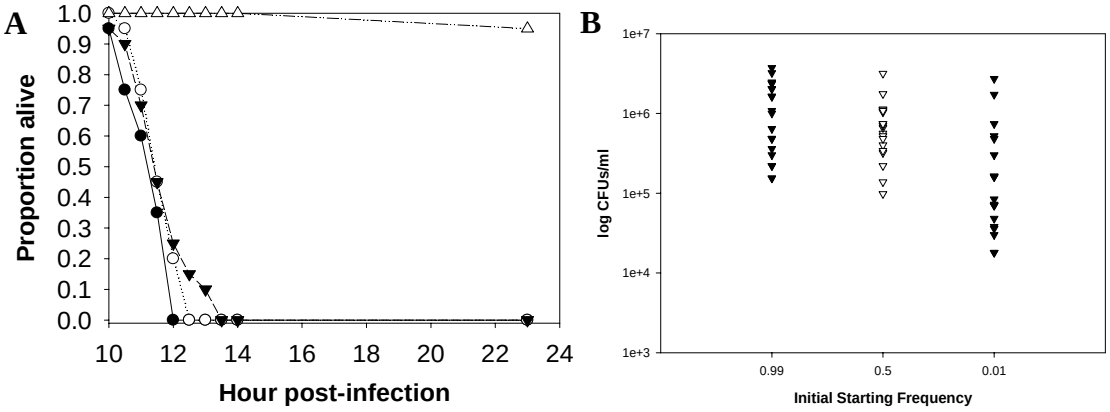

Figure 2.4: Virulence and Density Unaffected by Frequency When Bacteriocins are not Produced. A. Time to death of caterpillars inoculated with PAO1150-2 / O:9 mixtures. Initial starting frequencies of PAO1150-2 are again indicated on the graph and correspond to the adjacent line, but in this case there is no significant difference delay in time to death (linear term $F_{1,58} = 1.28$, $p>0.263$; quadratic term $F_{1,57} = 0.65$, $p<0.422$) B. The average total bacterial density PAO1150-2 and O:9 is indicated for the three different starting frequencies of the bacteriocin-negative mutant. There is no significant difference in overall density after 8 hours of growth in the caterpillars between the different starting frequencies.

2.4 DISCUSSION

In this study, we show a unimodal relationship exists between the growth of spiteful, toxin-producing bacteria when competing with susceptible strains, and their starting frequency. Furthermore, we demonstrate that conditions that favour spiteful behaviours result in minimal virulence in caterpillar hosts, as a result of reduced population growth rate. Finally, we show theoretically that this unimodal relationship between the fitness of bacteriocin producers holds in both ecological and evolutionary contexts.

It is necessary to emphasise that in our experiments the producer differs from the sensitive strain in other ways than pyocin production and susceptibility, as the strains are not isogenic. This, however, does not alter our interpretation of the data, as the non-producer, which is isogenic to the producer, shows only a weak negative density dependent relationship between its growth and frequency, probably due to slightly different resource use of the different strains (Brockhurst *et al.* 2006). Furthermore virulence at intermediate frequencies is attenuated in our producer strain whereas there is no difference in any treatment with the non-producer.

Our model suggests some very simple mechanisms to explain our results. When the producer is at low frequency, the benefits of reducing competition and freeing up resources will be shared by the sensitive strain as much as producing strains, hence there is little net benefit to the producing strain (relatedness is only weakly negative). Similarly, bacteriocin production has less benefit at high frequencies, because there are few competitors to kill, and hence there are fewer resources to be gained from costly bacteriocin production. Only at intermediate frequencies will bacteriocin production confer the greatest fitness advantage

by killing competitors and thereby "freeing-up" resources, both by preventing competitors from growing into unexploited patches and by removing them from their current ones.

The impact of spatial structure on the fitness of bacteriocin producers can also be understood in terms of the frequency of the producer in a competitive arena. In Chao and Levin's (Chao and Levin 1981) experiments using *E. coli*, spatial structure was manipulated to give two scenarios: mass habitat and structured habitats. In mass habitats there was a frequency dependent relationship to the success of bacteriocin production, where bacteriocin producers were only able to invade if relatively common. In structured habitats, however, bacteriocin producers are able to invade even when at low starting frequencies. Spatial structure makes individuals interact locally, and through stochastic processes can result in higher local frequencies of the producer. This creates conditions where the producer is at a high enough frequency and relatedness is sufficiently negative to allow bacteriocin producers to dominate. In our experiment we used homogeneously mixed environments, similar to "mass habitats" of Chao and Levin (Chao and Levin 1981), but by considering a range of different relatedness structures we are able to show that frequency, facilitated by habitat structure, is driving this dynamic.

Consistent with theoretical results, virulence was greatly attenuated when mixing bacteriocin producers with sensitive bacteria. This result is consistent with recent studies showing that: 1) a mixture of one bacteriocin producing strain and sensitive strains of *Photorhabdus* and *Xenorhabdus* spp. resulted in lower virulence in caterpillars than the respective single strain infections (Massey *et al.* 2004); and 2) mixing of *Xenorrhabdus nematophila* and its symbiotically associated nematode reduce virulence and increased susceptibility to bacteriocins (Vigneux *et al.* 2008).Here, we clarify these results by

demonstrating that mixed infections show reduced virulence, but only when the bacteriocin producers are at an intermediate frequency in the infecting population. Furthermore, we determine that attenuation of virulence at intermediate frequencies is almost certainly explained (as predicted) by a reduced growth rate of the infecting population as a whole, resulting from the high mortality rate of the susceptible strain. Density may not be the only important factor in determining virulence as intrinsic genetic differences between the various strains may also have a notable effect.

The specific shape (inverse unimodal) of the relationship between virulence and strain frequency of the infecting population is likely to be entirely dependent on the spiteful interactions (Buckling and Brockhurst 2008). When other types of social interactions are more important than spite in determining the outcome of competition, different relationships are predicted. First, a monotonic negative relationship is predicted when bacteria are simply competing for resources, because high diversity results in greater resource competition leading to rapid host-exploitation and increased virulence (Frank 1996a). Second, a positive relationship between virulence and diversity is predicted when bacteria need to cooperate to grow, because cooperation is most likely to be favoured when diversity is low (Brown *et al.* 2002; West and Buckling 2003). We are currently investigating, both theoretically and empirically, how the relationship between virulence and strain frequency is affected when multiple social interactions are important to the outcome of competition.

This study has provided novel experimental evidence of how strain frequency, and in turn relatedness, affects bacteriocin production. Note however that we have specifically measured local fitness and not global fitness under conditions that favour bacteriocins. We

have also ignored any evolution of non-producing resistance types (that would lead to a 'rock-paper-scissors' interaction (Kerr *et al.* 2002)), as we are only competing producing versus sensitive or non-producing versus sensitive strains. However, non-producing resistance in competition with producing resistance represents a form of social cheating, and hence we can apply this kin selection framework to understand this problem in the future.

Here we have shown that spiteful behaviours, or more specifically, bacteriocin (pyocin) production is crucially affected by the frequency in the population of a given strain. We have also shown that pyocin production can have a major impact on the virulence of *P. aeruginosa* infections. The study may ultimately have practical applications in terms of manipulating the competitive arena such that toxin producers are favoured and hence reduce virulence. Pyocin production in *P. aeruginosa* is also likely to be important in a clinical setting, especially in diseases such as cystic fibrosis where pyocin producing strains are commonly found (Govan 1986) and different strains are often out competed as the disease progresses.

CHAPTER 3

SPITE AND THE SCALE OF COMPETITION IN *PSEUDOMONAS AERUGINOSA*

SUMMARY

Scale of competition has been shown to be an important factor in shaping the evolution of the social interactions. Although many theoretical and experimental studies have examined its impact upon altruistic cooperation, relatively little research effort has been focused upon spiteful behaviours: actions that harm both the actor and recipient. In this study, we expand upon the existing theory, and we investigate experimentally how scale of competition mediates selection for spite in the bacterial pathogen *Pseudomonas aeruginosa*, under different genetic relatedness structures. Consistent with our theoretical results, we find in our experiments that spiteful greenbeards are favoured under conditions of relatively low genetic relatedness and under both local and global competition.

3.1 INTRODUCTION

Spiteful behaviours are those that reduce the fitness of both the actor and recipients (Hamilton 1970; West *et al.* 2007), and have been identified in a range of organisms from bacteria to wasps (Hurst 1991; Foster *et al.* 2000; Gardner *et al.* 2004; Gardner *et al.* 2007; Inglis *et al.* 2009). Understanding the selective forces that drive the evolution of spiteful behaviours is likely to be of particular relevance to disease-causing organisms, where spiteful interactions can have a profound effect on disease severity (Buckling and Brockhurst 2008). Genes for spite can potentially be favoured by natural selection when actor and recipients are statistically less genetically similar than are random individuals in the same population (Hamilton 1970; Hamilton 1972; Grafen 1985; Hamilton 1996; Foster *et al.* 2001; Gardner and West 2004b; Gardner *et al.* 2004; Brown *et al.* 2006; Brown *et al.* 2009). This is formalized in Hamilton's (1963, 1964, 1970) rule of kin selection $rb\text{-}c>0$, where: r is the coefficient of relatedness between actor and recipient, with respect to the genes responsible for the trait; c is the fitness cost to the actor; and b is the benefit to the recipient. In the context of spite, actor and recipient both suffer a cost ($c > 0$, $b < 0$), and hence Hamilton's rule can be satisfied only when relatedness is sufficiently negative ($r < 0$, and $rb\text{-}c > 0$). Negative relatedness can only emerge if individuals can be discriminated on the basis of sharing the appropriate genes in common, either indirectly (e.g., through kin recognition) or directly, via the 'greenbeard' mechanism, whereby a gene (or a number of tightly-linked genes) encodes both the spiteful behaviour and also a phenotypic 'marker' that allows carriers and non-carriers of the spite gene to be discriminated (Hamilton 1970; Gardner and West 2004b; Gardner and West 2010; West and Gardner 2010). Note that spite can also be viewed as indirect altruism (Gardner and West 2006; Lehmann *et al.* 2006;

Gardner *et al.* 2007), where the benefit of the spiteful action is shared among individuals which also possess the spiteful allele. For both spite and altruism, the behaviour is favoured because it shifts reproductive value away from individuals who share fewer genes in common with the actor, and towards individuals who share more genes in common with the actor, increasing the actor's inclusive fitness.

Selection acting upon social traits may crucially depend upon population structure. This issue has received considerable theoretical and (more recently) empirical attention in the context of the evolution of indiscriminate altruism (Taylor 1992a; Taylor 1992b; Wilson *et al.* 1992; Frank 1998; Van Baalen and Rand 1998; Mitteldorf and Wilson 2000; West *et al.* 2001; West and Buckling 2003; Gardner and West 2004b; Griffin *et al.* 2004; Rousset 2004; Lehmann and Keller 2006; El Mouden and Gardner 2008; Johnstone 2008; Kümmerli *et al.* 2009; Wild and Fernandes 2009; Gardner 2010). Population viscosity (i.e. where dispersal is very local), can increase the genetic structure of populations (F_{ST}) which acts to favour cooperation among individuals within patches, but at the same time increases local competition between individuals sharing the same alleles, which acts to disfavour cooperation. By contrast, high rates of dispersal decrease the genetic similarity of neighbours, but allow the growth-rate benefits of being in a cooperative group to be exported throughout the population. Note that the genetic structure of the population is distinct from the relatedness coefficient entering into Hamilton's rule: the former describes the tendency for the same genes to occur in close proximity, rather than spread evenly over the whole of the population (Rousset 2004); whereas the latter describes the genetic similarity of two individuals engaging in a particular type of social interaction (Grafen 2006). This distinction is important when social interactions are not simply mediated by

spatial proximity, as might be the case with kin recognition or greenbeards (Gardner and West 2010).

These opposing effects of population viscosity can theoretically also influence the evolution of spiteful behaviour. First, genetic structuring, which is often determined by dispersal patterns, can dramatically alter selection for spite. Both theory and experiments have shown that spiteful behaviour is maximally favoured when the local frequency of genes for spite is intermediate (Gardner and West 2004; Gardner *et al.* 2004; Inglis *et al.* 2009; Gardner and West 2010) (Gardner and West 2004b; Gardner *et al.* 2004; Inglis *et al.* 2009; Gardner and West 2010). Here, there is access to potential victims who do not carry the spite gene and, to the extent that the harming of victims frees up resources for individuals in their local neighbourhood, carriers of the spite gene will be among the latter. However, if the local frequency of the spite gene is low, any locals who do benefit from the freeing up of resources are unlikely to carry the gene, whereas if the local frequency of the gene is high, spiteful individuals have little access to potential victims who do not carry the gene. Second, theoretical work (Gardner and West 2004; Gardner *et al.* 2004; Gardner and West 2010) also suggests that spite genes are most likely to be favoured when competition is local (e.g. owing to low dispersal). This is because the third-party beneficiaries of spite are locals, who may carry the spite gene more often than individuals from the wider population. As competition becomes increasingly global, it is individuals from the wider population who stand to benefit most from resources freed up by spite, and they only infrequently carry the spite gene. However, this result may be largely driven by the assumption in these evolutionary models that the spiteful greenbeard is vanishingly rare, and, therefore, is by definition at low frequencies in the global (but not necessarily the

local) population. No theoretical work, to date, has addressed how the scale of competition affects selection for spite under a range of possible frequencies of the spiteful allele and, moreover, if and how these variables interact with genetic structuring. We address this in the current work. Consideration of a range of starting frequencies is crucial if spiteful alleles do not necessarily reach equilibrium frequencies, which would result when, for example, population viscosity changes through time.

No empirical studies have determined the effect of scale of competition on the evolution of spite, although there is a growing body of empirical work investigating the importance of genetic structuring (Chao and Levin 1981; Massey *et al.* 2004; Inglis *et al.* 2009). A recent study reported that global versus local dispersal (low and high population viscosity) favoured spiteful behaviours in bacteria associated with symbiotic nematodes, but these manipulations altered kinship and the scale of competition in a correlated manner: low dispersal both increased kinship and resulted in more local competition (Vigneux *et al.* 2008). Here we use the opportunistically pathogenic bacterium, *Pseudomonas aeruginosa*, to investigate how scale of competition affects the success of spiteful versus non-spiteful strategies, under conditions of both high and low genetic structure.

P. aeruginosa is an excellent study system for spiteful behaviours, as it produces bacteriocins (specifically pyocins), proteinaceous, anti-competitor toxins which kill susceptible bacteria (reduce fitness of recipient) and are costly to produce as cell lysis is required for their release (reduce fitness of actor) (Nakayama *et al.* 2000; Michel-Briand and Baysse 2002; Cascales *et al.* 2007; Denayer *et al.* 2007). In this context bacteriocin production can be considered to be a spiteful greenbeard, as bacteriocin producing bacteria are able to specifically harm social partners that do not share the same bacteriocin gene

complex (allowing negative relatedness to occur) (Gardner *et al.* 2004; Gardner and West 2010). Previous studies using this bacterium have confirmed that the fitness of spiteful genotypes is maximized at intermediate frequencies (Inglis *et al.* 2009). Moreover, the same bacterium has been used in an analogous way to the current study in testing the importance of scale of competition and genetic structure on the evolution of public goods production (Griffin *et al.* 2004).

3.2 MATERIALS AND METHODS

3.2.1 Bacterial Strains

Pseudomonas aeruginosa strain PAO1, was used as the bacteriocin producer and serotype O:9 as the bacteriocin sensitive competitor (Inglis *et al.* 2009). PAO1 is a known producer of pyocin S2, whereas serotype O:9 is sensitive to S2 pyocins (Smith *et al.* 1992; Denayer *et al.* 2007). This sensitivity can be readily confirmed using simple plate assays (Fyfe *et al.* 1984; Inglis *et al.* 2009). Bacteriocin production in *P. aeruginosa* involves only a few cells in the population which actively lyse to release the toxin. Although it is not clear whether lysis is required for the release of soluble pyocins, which are the focus of this study (it has been suggested that it is (Nakayama *et al.* 2000)), there is a metabolic cost to production (Michel-Briand and Baysse 2002). PAO1 and O:9 have different genetic backgrounds, so the isogenic, S2 pyocin knockout mutant, PAO1150-2, was also used to determine that the difference in fitness between PAO1 and O:9 is due solely to pyocin production/sensitivity (Jacobs *et al.* 2003; Inglis *et al.* 2009)(Figure 3.2a and b).

3.2.2 Competition Experiments

Short term competition experiments were carried out to confirm both the generality of the results and that the observed differences in fitness are in fact due to pyocin production and sensitivity (Figure 3.2a and b). Overnight cultures of each strain were grown with shaking at 0.65g at 37 °C for 18 h and then diluted to an OD600 of 1.8 to ensure similar numbers of bacteria per milliliter. These cultures were subsequently grown on agar plates to determine the number of bacteria present, with colony forming units (CFUs) as an approximate measure. 30ml glass universals containing 6ml of Kings Media

B broth were inoculated with a total of 10^4 cells with different starting frequencies of the individual strains. PAO1and O:9 were competed against each other at starting frequencies of 99%,90%, 50%, 10%,1%, and 0.1%. The isogenic, knockout mutant, PAO1150-2, was competed against O:9 at a frequency of 50% and in isolation. Cultures were propagated in a shaking incubator at 0.65g at 37 °C and sampled after 96 h, allowing time for the effect of the bacteriocin to be observed. We calculated the relative growth of the producer to sensitive and non-producer to sensitive at the different starting frequencies. This was done by plating the various treatments on KB agar plates and counting the number of CFUs for each strain. All strains were easily distinguishable from one another because of unique colony morphology and size. At the more extreme frequencies, antibiotic plates were required to give better resolution of colony counts, and this was possible due to the different antibiotic resistance profiles of the assorted strains (PAO1 resistant to 1,250 g/ml streptomycin; O:9 resistant to 312.5 g/ml rifampicin; and PAO 1150-2 resistant to 312.5 g/ml tetracycline). Relative fitness (w) was used to estimate at what frequency bacteriocin production is favored in PAO1 relative to 1150-2 using the common competitor O:9, where $w = (m_j/m_i)$, and m refers to ln(final density/starting density) of strain j (in this case either PAO1 or 1150-2) and strain i (O:9) (Lenski *et al.* 1991). All frequencies were replicated 6 times.

3.2.3 Experimental Design

Overnight cultures of both strains (PAO1 and O:9) were grown in 30 ml glass universals containing 6ml of King's medium B (KB), shaking at 0.65g and 37°C for 18 hours and were subsequently diluted and then plated onto KB agar petri dishes. These

plates where then incubated for a further 18 hours at 37°C, and the bacterial colonies from the agar plates were used to start the experiment. We independently manipulated genetic structure and the scale of competition using a two-way full-factorial ANOVA design. Replicates contained one population divided into six subpopulations. Each subpopulation was grown in a tube of KB broth. All treatments were inoculated with bacterial cores, containing 10^6 cells of bacteria for both strains, obtained by stabbing colonies from the agar plates with a 1 ml sterile pipette. Bacterial cores were also serially diluted and plated to confirm that the densities were in fact the same (results not shown). In the high genetic structure treatments, three tubes were inoculated by colony cores from either PAO1 or O:9 (1:1 overall ratio of spiteful to sensitive). Low genetic structure treatments initially comprised six tubes inoculated with colony cores of each strain (1:1 mix). Cultures were grown for 10 hours in a 37°C orbital incubator, shaking at 0.65g. Local competition cultures were then individually plated onto KB agar, whereas equal volumes from each culture, within a global competition treatment, were mixed together before plating (Griffin *et al.* 2004). Plates were incubated for 18 hours at 37°C, and after determining the relative frequencies of the two strains, random colony cores were inoculated into fresh KB tubes: low genetic structure tubes were inoculated with two clones and high genetic structure tubes with one. This selection procedure was repeated for five transfers. Every round of selection we scored the frequencies (based on their distinct colony morphologies) of the spiteful and sensitive bacteria growing on the agar plates, and the relative proportions inoculated into the next set of tubes. We use the proportion of spiteful bacteria inoculated into the next generation as the response variable in our analyses and in our figure. The whole experiment was carried out six times.

3.2.4 Analyses

We analysed the data using a linear mixed effects model in R (version 2.9.2). For all the analyses on the proportion of bacteriocin producers, the proportion was arcsine square root transformed before the analysis, and the normal distribution subsequently confirmed by graphical means and the Shapiro-Wilkinson test. Our minimal adequate model was as follows: (transformed) proportion of bacteriocin producers~genetic structure + scale of competition + time + genetic structure:time + scale of competition:time, random effect = strain replicate, accounting for temporal autocorrelation.

3.2.5 Model

We consider an infinite population made up of patches, within which all social interactions, and a proportion a of competitive interactions, occur. We consider two strains of bacteria are present in the population: a producer (P) strain which suffers a relative growth cost c in order to synthesize a toxin, to which it is immune, and a nonproducer (N) strain which suffers a growth cost k in the presence of the toxin. The population frequency of producer cells is denoted p.

In the first variant of the model, we assume that all patches are founded by a single bacterial cell ($r = 1$). Hence, there are two types of patch: P patches occur with frequency p, and N patches occur with frequency $1-p$. The growth of producers is given by $g_P = 1-c$, and the growth of nonproducers is $g_N = 1$, owing to the latter never coming into contact with the toxin. The population average growth is $g = pg_P + (1-p)g_N$, and the fitness of the producer strain is given by its growth relative to that of its competitors:

$$w_P = \frac{g_P}{a g_P + (1-a) g}. \quad (1)$$

Since $g_P < g$, we have $w_P \leq 1$ for all $a \leq 1$. Hence, the producer strain is never selectively favoured when patches are clonal ($r = 1$). Producer fitness w_P is a monotonically increasing function of scale of competition a: it increases from a minimum $w_P = g_P/g$ at $a = 0$ to a maximum $w_P = 1$ at $a = 1$.

In the second variant of the model, we assume that each patch is founded by two independently-chosen bacterial cells ($r = 1/2$). Hence, there are three types of patch to consider: PP patches occur with frequency p^2, PN patches occur with frequency $2p(1\text{-}p)$, and NN patches occur with frequency $(1\text{-}p)^2$. Growth of producers is $g_{P|PP} = g_{P|PN} = 1\text{-}c$ in both PP and PN patches, whereas growth of nonproducers is $g_{N|PN} = 1\text{-}k$ in PN patches and $g_{N|NN} = 1$ in NN patches. The population average growth is $g = p^2 g_{P|PP} + p(1\text{-}p)(g_{P|PN}+g_{N|PN}) + (1\text{-}p)^2 g_{N|NN}$, and the fitness of the producer strain is:

$$w_P = p\frac{g_{P|PP}}{a g_{P|PP} + (1-a) g} + (1-p)\frac{g_{P|PN}}{a(g_{P|PP} + g_{P|PN})/2 + (1-a) g}. \quad (2)$$

Depending upon parameter values, the fitness of the producer strain may be less than or greater than 1, and so can be favoured or disfavoured by selection. For ease of analysis, we will use a linear approximation for fitness (neglecting higher order terms of c and k):

$$w_P \approx 1 + (1-p)\left(a\frac{c+k}{2} + (1-a)pk - c\right), \quad (3)$$

which is monotonically decreasing with c and monotonically increasing with k for all $0 < a$, $p < 1$, i.e. a smaller growth cost of toxin production and a greater toxicity against sensitives increases the likelihood of the producer strain being favoured by selection.

Fitness is a linear function of a, monotonically increasing if $p < (c+k)/2k$ and monotonically decreasing if $p > (c+k)/2k$. Hence, so long as $k > c$ (a necessary condition for the producer strain to be favoured), then producer fitness increases with scale of competition at $p = ½$, i.e. the condition imposed in the main experiment. Note that $w_P \approx 1+(1-p)(pk-c)$ in the absence of local competition ($a = 0$), and the condition for the producer strain to be favoured here is $p > c/k$ (this is equivalent to the condition given in row 4 of Table 2 in Gardner & West 2010). Hence, spiteful toxin production can be favoured in the absence of local competition ($a = 0$). Also note that there is no impact of scale of competition at the point $p = (c+k)/2k$. Assuming that bacteriocin-mediated killing is efficient ($k >> c$), then this occurs at $p \approx ½$, i.e. here we expect scale of competition to have minimal impact on fitness of producers when producers and non-producers are approximately equally frequent in the population.

Finally, fitness is a quadratic function of p, and inspection of the second derivative (which is never positive) shows that this is a dome-shaped function; the maximum is always less than 1, but may be greater than or less than zero. Hence, over biologically feasible parameter space, fitness is either dome shaped or monotonically decreasing with producer frequency p.

3.3 RESULTS

In this study, we investigate how scale of competition and genetic structure affects selection for spiteful toxin production in pathogenic bacteria. We first expand previous theoretical work in this area, to investigate how selection for spite is affected by the starting frequency of the spiteful lineage. Our theoretical results are consistent with previous work investigating genetic structure and the scale of competition on spite. First, spite is favoured at intermediate genetic structure (50%) relative to high genetic structure (100%), with spite never favoured in the latter case. Second, increasingly global competition selects against spite when the starting frequency of the spiteful allele in the metapopulation is rare (Figure 3.1). However, the effect of increasing scale of competition on the fitness of the spiteful allele is dependent on both its starting frequency and the ratio of the fitness cost to the actor (c) and recipient (k). Specifically, increasing scale of competition will decrease the fitness of the spiteful allele when the frequency, $p < (c+k)/2k$. In our model, with patches founded by two independent bacterial strains, increasing scale of competition will therefore always decrease the fitness of the bacteriocin allele when its frequency is less than 50% (and greater than 0%). However, the effect of scale of competition (i.e the difference in fitness of the spiteful allele) will typically be less (or no different) at 50% starting frequencies than at lower frequencies. Finally, under high frequencies of the spiteful lineage the genetic similarity to the third-party beneficiaries of spite are not necessarily dependent upon the scale of competition, and spite can, therefore, be favoured under entirely global competition in some circumstances.

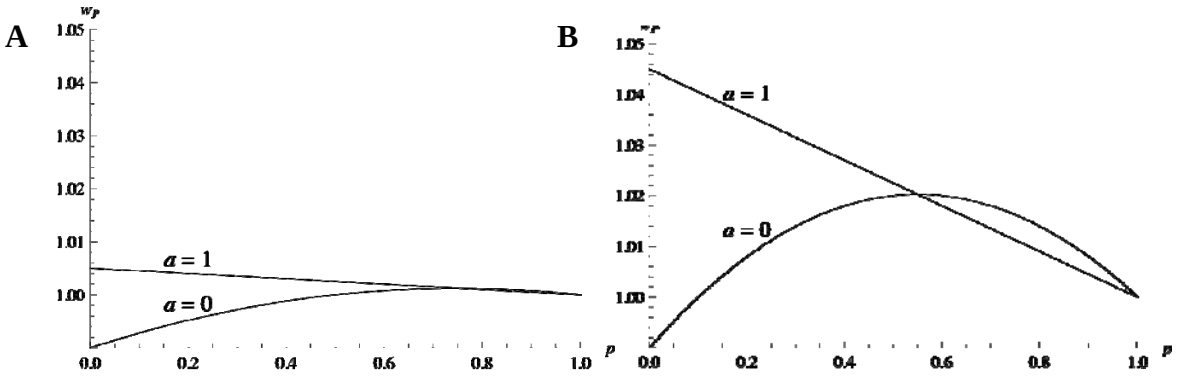

Figure 3.1: Model results. The fitness of a bacteriocin producer as a function of starting frequency in the metapopulation, under conditions of entirely local (a = 1) or global (a = 0) competition. Two individuals are present in each patch. (A) c (cost to actor) = 0.01, k (cost to recipient) = 0.02 (and hence k/c = 2). (B) c = 0.01, k = 0.10 (and hence k/c = 10). Note that there is no effect of scale of competition (a) on bacteriocin producer fitness (wP) at the point p = (c+k)/2k. Assuming efficient bacteriocin action k >> c, then this occurs at p ≈ 0.5.

We carried out short term experiments to establish qualitative consistency between our experimental and theoretical work, as well as to investigate the importance of starting frequency for the fitness of the spiteful lineage. We first identified a cost to bacteriocin production by comparing the growth rates of our producing strain with that of an isogenic mutant (PAO1150-2 which does not produce pyocin S2) when grown in isolation (Figure 3.2a and b). We also show that the relative growth rate in isolation of the susceptible strain (which is not the same genetic background as the producing strain) is greater than that of the producing strain, but does not differ from the pyocin-negative mutant (Figure 3.2a and b). This suggests that the different growth rates of the producing and susceptible strains can be explained to some extent by pyocin production. When we competed the pyocin-negative and susceptible strain in the same tube at equal starting densities, we found the susceptible strain had no significant growth rate advantage (Figure 3.2b). By contrast, the pyocin producer showed a massive fitness advantage against the susceptible strain under these conditions (Figure 3.2a). These results confirm that pyocin production confers an absolute

growth rate cost, but can confer a large fitness advantage when in direct competition with susceptible strains, as assumed in our theoretical work.

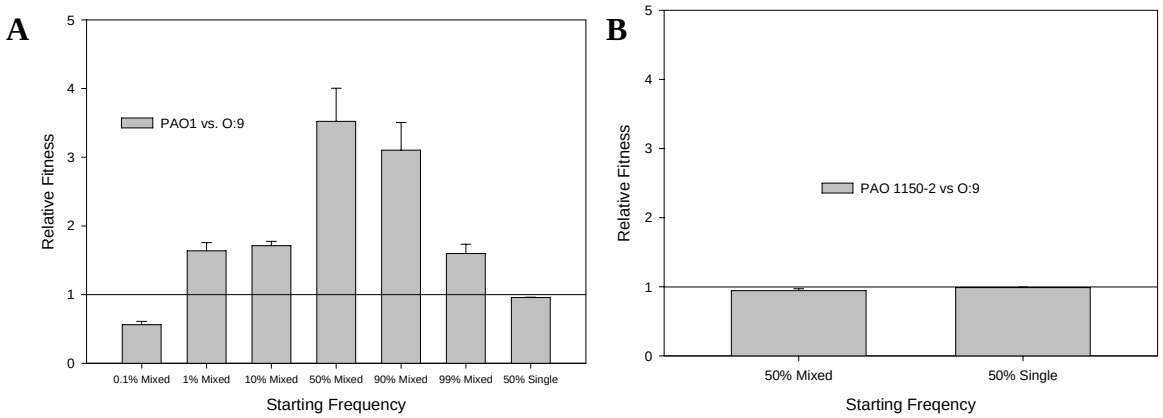

Figure 3.2: A) Invasion of spiteful greenbeards (PAO1) under a range of different starting frequencies when in competition with the sensitive strain (O:9). The spiteful greenbeards are able to invade when starting at 1% of the population but show the greatest relative fitness at 50:50 frequencies. The figure also illustrates that when spiteful individuals are disadvantaged under conditions where they are not in direct competition with the sensitive individuals (50% single). **B) Invasion of an isogenic, spiteful knock-out mutant (PAO1150-2 that does not produce pyocin S2) in mixed and single competitions**. This illustrates that the ability of the spiteful greenbeard to invade is due to pyocin production (spite) as fitness in both mixed and single competitions are statistically the same ($t = -1.99$, $p>0.05$ and $t = -2.1$, $p>0.05$ respectively). Error bars are standard errors of the means for both graphs. Stars indicate p values for two tailed t-tests looking at the difference between the mean of each treatment from 1, after a sequential Bonferroni procedure has been applied (Holm 1979) (** $p<0.01$, * $p<0.05$, no star $p>0.05$)

We next investigated how the relative fitness of the spiteful lineage when in direct contact with the susceptible lineage varied with starting frequency (Figure 3.2a). Consistent with previous work, we found a large fitness advantage at all but very low within-population starting frequencies; in this latter case, the relatively small reduction in competitor frequency is outweighed by the cost of pyocin production. Given the qualitative consistency of results at all but very low frequencies, we chose a single starting frequency of the spiteful lineage (50%) for the larger metapopulation. This frequency was chosen both for experimental ease (similar starting frequencies allow more accurate estimates of changes in frequency), as well as for the sake of parsimony: our theoretical results

demonstrate that 50% frequency should minimise effects of scale of competition compared to lower frequencies

In our main experiment we independently manipulated the scale of competition and genetic structure in experimental metapopulations. To achieve relatively high genetic similarity between social partners, we initiated each subpopulation with a single bacterial clone: in the first generation half the subpopulations in a treatment were initiated with the spiteful strain (PAO1) that produces bacteriocins, and the other half with the sensitive strain (O:9) (Figure 3.3). To contrast this, we imposed relatively low genetic similarity by initiating each subpopulation, in the other half, with two genetically different bacterial clones: in the first generation all of the subpopulations were initiated with a 1:1 mix of spiteful and sensitive individuals and that genetic similarity is measured with respect to the spiteful trait (bacteriocin production). We imposed relatively global competition by mixing the cultures from all of the subpopulations in a treatment before plating, and then transferring random colonies from this single plate to initiate new subpopulations (Figure 3.3). This procedure allows productivity in a tube to determine the genetic contributions to subsequent generations, increasing the relative importance of global competition (competition between patches). In contrast, we imposed relatively local competition by allowing every subpopulation in a treatment to provide equal numbers of colonies in the next generation. This removes the advantage of being in a more productive tube, and hence increases the importance of local competition (competition within a patch).

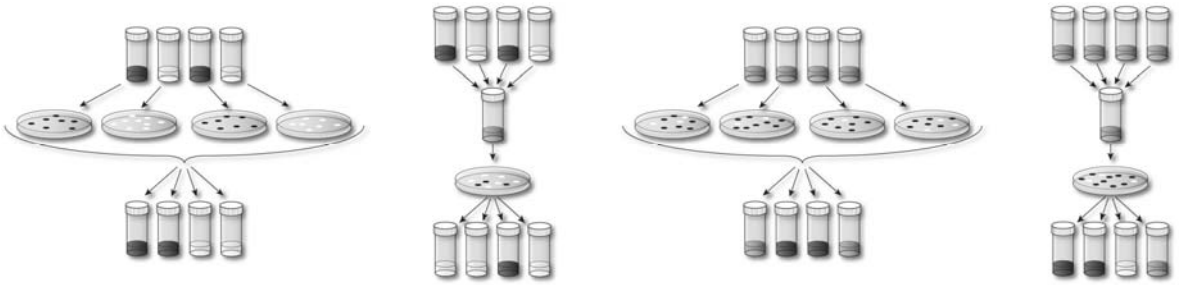

Figure 3.3: Experimental design. Genetic relatedness was varied among interacting individuals by inoculating each subpopulation with either a single bacterial clone (relatively high genetic relatedness) or two bacterial clones (relatively low genetic relatedness). Scale of competition was manipulated by either mixing all the subpopulations from a treatment before plating (relatively global competition) or by allowing each subpopulation, in a treatment, to provide equal numbers of colonies to the next generation (relatively local competition). Here, black represents the spiteful strain (PAO1), white represents the sensitive strain (O:9), and grey represents a mix of the two.

There was no significant interaction between the scale of competition and genetic structure ($F_{(1,117)} = 2.36$, $P>0.14$, this interaction term was subsequently discarded from the model) (Figure 3.4), although there is a trend that the scale of competition has a more pronounced effect when sub-populations are initiated with single clones (high genetic structure). However, the mean success of the spiteful genotype was greater under local and global competition ($F_{(1,117)} = 6.08$, $P<0.015$) and under low versus high genetic structure ($F_{(1,117)} = 43.16$, $P < 0.0001$) (Figure 3.4). Indeed, under conditions of high genetic structure and global competition the sensitive strain went to fixation within all the experimental populations. This is because the sensitive strain does not pay the cost of producing the bacteriocin, hence its intrinsic growth rate is higher, allowing it to outcompete the bacteriocin producer when it is not directly interacting with the spiteful individuals. By contrast, the spiteful genotype had the greatest advantage under local competition and low genetic structure, because under these conditions it will frequently encounter sensitive competitors, and reductions in intrinsic growth rates do not have fitness

consequences. Note that there was not a significant change from the 50% starting frequency of the non-spiteful genotype under high genetic structure and local competition, because there was no opportunity for fitness differences. In this case, the treatment acts as a control to illustrate there are no underlying biases in this type of selection regime. These findings are entirely consistent with our theoretical predictions.

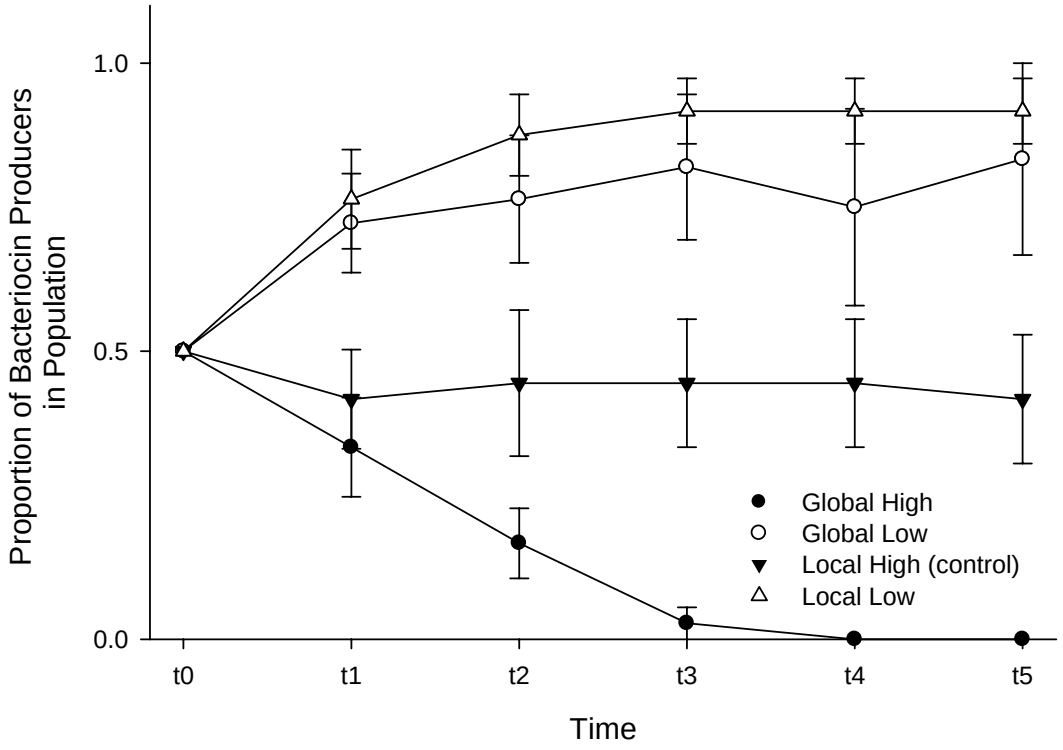

Figure 3.4: The evolution of spiteful greenbreads in response to the scale of competition and genetic relatedness. Error bars show standard errors of the means. Spite is favoured under conditions of low genetic relatedness under both global and local scales of competition.

3.4 DISCUSSION

Our results provide a clear experimental demonstration of how population viscosity influences the evolution of spiteful behaviours, both in the form of scale of competition and genetic structure. As previously suggested (Griffin *et al.* 2004), the scale of competition is likely to be of particular importance in microbes, as their life cycle involves stages with limited movement and, hence, relatively local growth and competition, but also relatively long distance dispersal stages (Velicer 2003; West *et al.* 2007). Overall, scale of competition is likely to vary continuously across microbial species depending on dispersal rates, and in the context of bacterial pathogens, this variation will occur as a result of within-host growth (local) and transmission to new hosts (relatively global).

Both the scale of competition and genetic structure can be affected by dispersal, with often opposing effects on selection for social behaviours (Hamiltion and May 1977; Crespi and Taylor 1990; Koenig *et al.* 1992; Perrin and Lehmann 2001). Specifically, dispersal decreases genetic structure (favouring spite), but makes competition more global (disfavouring spite). However, unlike social acts which do not involve greenbeard recognition, bacteriocin production can theoretically be favoured under a range of genetic structures (> 0% and < 100%) and under all scales of competition, except global competition in infinite populations (Lehmann *et al.* 2009). As populations are of course never infinite, this suggests that only conditions of zero dispersal will inevitably select against spiteful acts. This view is consistent with a previous study using a bacteria/nematode system that reported greater interference competition (bacteriocin production) under conditions of high compared with low dispersal (Vigneux *et al.* 2008).

In summary, our study provides the first empirical support that the scale of competition can predictably affect the evolution of spiteful behaviours. Moreover, we show both theoretically and empirically that global competition can select against spite when spiteful genotypes are at high starting frequencies in the global populations; in addition to when the spiteful genotypes are vanishingly rare, as in previous theoretical work (Gardner and West 2004; Gardner *et al.* 2004; Gardner and West 2010). Spite is likely to be a generally important social interaction in bacteria, as nearly all bacterial isolates have been found to produce bacteriocins (Riley and Wertz 2002), and also in a clinical setting, as many *P. aeruginosa* clinical isolates have been found to produce pyocins (Govan 1986). Understanding complex social behaviours and how they interact is an important area of scientific discovery which has the potential to benefit our understanding of how infections change in their virulence and may lead to innovative therapies.

CHAPTER 4

SPITE AND COOPERATION IN *PSEUDOMONAS AERUGINOSA*

SUMMARY

Social interactions have been shown to play an important role in bacterial evolution and virulence. However, the majority of studies conducted have only considered social traits in isolation. This simplistic approach, although extremely useful in indentifying conditions where individual traits can be favoured, may miss potentially important synergies or antagonisms between social traits that are expressed simultaneously. In this study we develop new theory and conduct experiments to elucidate how two social traits (spite and public goods cooperation) can interact in the bacterium *Pseudomonas aeruginosa*, and how this in turn can affect virulence. Consistent with our theoretical predictions, we find that spite and public goods cooperation work together in synergistic manner, fundamentally changing the ecological conditions where these two traits can be favoured. Furthermore, this interaction between traits greatly impacts bacterial virulence in our caterpillar model system. These results confirm the importance of considering multiple social traits coincidentally, especially under natural conditions where traits can contribute equally to bacterial fitness.

4.1 INTRODUCTION

Social behaviours in pathogenic bacteria are ubiquitous, and include interactions such as communication, altruistic cooperation and spiteful toxin production (West *et al.* 2007; Brown and Buckling 2008). Recent years have seen a proliferation of empirical and theoretical studies investigating the selective forces acting on social traits in pathogenic bacteria, and the resulting impact on virulence (Bremermann and Pickering 1983; Frank 1992; Frank 1996a; West and Buckling 2003; Gardner *et al.* 2004; Massey *et al.* 2004; Harrison *et al.* 2006; Brown *et al.* 2008; Buckling and Brockhurst 2008; Vigneux *et al.* 2008; Inglis *et al.* 2009; Köhler *et al.* 2009). However, relatively little attention has been given to how the expression of one trait influences selection on another (Lehmann *et al.* 2007; Harrison and Buckling 2009; Brown and Taylor 2010). This is crucial to understanding the evolution of microbial social behaviours, because multiple social traits are typically expressed simultaneously (Williams *et al.* 2007; Harrison and Buckling 2009). Here, we determine how natural selection on two common and important microbial social traits (spite and public goods cooperation) is altered by their interaction, and how this affects virulence caused by a microbial pathogen.

Spiteful behaviours result in a fitness cost for both the actor and recipient, and appear to be widespread in microbes in the form of the suicidal (or metabolically costly) production of anti-competitor toxins (Riley and Wertz 2002; Hawlena *et al.* 2010). Spiteful behaviours can be favoured by kin selection when non-relatives are preferentially affected (negative relatedness between actor and recipient). (Hamilton 1964; Hamilton 1970; Hamilton 1972; Gardner and West 2004b; Gardner *et al.* 2004; Brown *et al.* 2006; Gardner and West 2006; Brown *et al.* 2009), and the cost to the recipient is high relative to the cost

to the actor. Differential effects of the toxins on non-kin versus kin is achieved by microbes via linkage between toxin and immunity genes, rendering all individuals with the same toxin gene resistant to the toxin; an example of a 'green beard' (Hurst 1991; Gardner and West 2004b; Gardner *et al.* 2004; Massey *et al.* 2004; Gardner *et al.* 2007; Greig and Travisano 2008; Vigneux *et al.* 2008; Wloch-Salamon *et al.* 2008; Inglis *et al.* 2009; Gardner and West 2010). Both theory and data suggest that selection for spite is maximised when the spiteful lineage is at an intermediate frequency in the population. At low frequencies, competitors affected by spite will on average be only marginally less related than competitors who aren't targeted; and at high frequencies, only a few competitors will be affected by the spiteful behaviour, hence relative costs of spite will be high.

Social environments that influence selection for spiteful behaviours are also likely to affect selection on indiscriminate altruism. Indiscriminate altruism (individually costly behaviours that benefit all others in the vicinity) is extremely common in microbes and includes the public goods production of extracellular enzymes and nutrient-scavenging molecules (Buckling and Brockhurst 2008). Maintaining cooperation in a population relies on individuals interacting with their relatives, which are more likely to possess the same cooperation gene (kin selection), due either to kin discrimination or population viscosity (Hamilton 1963; Smith 1964). Otherwise "social cheats", individuals that don't pay the cost of the cooperative behaviour but reap all the rewards, are able to invade and displace the cooperators. Note that spiteful behaviours can also be viewed as a form of indirect, discriminating altruism, in that unaffected individuals with the spite allele benefit from the behaviour through removal of competitors (Lehmann *et al.* 2006).

In this study we take a joint theoretical and empirical approach, using the pathogenic bacterium *Pseudomonas aeruginosa*, to determine how the relationship between selection for spite and the frequency of the spiteful lineage is altered by the expression, and exploitation, of a public good (extracellular iron-scavenging siderophores). Crucially, the operation of different social behaviours is likely to have an important impact on disease virulence. In the case of spite, virulence is attenuated as infecting microbial populations kill each other (Massey *et al.* 2004; Vigneux *et al.* 2008; Inglis *et al.* 2009), whereas, if growth is primarily affected by the production of altruistic public goods, cooperation will result in high virulence (Harrison *et al.* 2006). We, therefore, also investigate the impact of interactions between spite and cooperation on *P. aeruginosa* virulence in insect hosts.

4.2 MATERIALS AND METHODS

4.2.1 Bacterial Strains

P. aeruginosa strain PAO1 was used as the spiteful cooperator as it is a known producer of pyocin S2 and pyoverdine type 1 (Smith *et al.* 1992; Meyer 2000; Denayer *et al.* 2007; Inglis *et al.* 2009). PAO 1150-2, a transposon, knock-out mutant of *psy2*, that still produces pyoverdine type 1 was used as an isogenic control. Serotype O:9, which is known to be sensitive to pyocin S2, was evolved for 14 generations in iron limited conditions to generate susceptible cheats . Six phenotypically white colonies that exhibited a reduction in iron chelating compounds of more than 60% (as confirmed by a CAS assay) were chosen to compete against PAO1 (Schwyn and Neilands 1987; Smith *et al.* 1992; Harrison *et al.* 2006). Pyocin sensitivity of the six O:9 siderophore 'cheats' was confirmed using a simple plate assay as described by Fyfe *et al.* ((Fyfe *et al.* 1984).

4.2.2 Competition Assays

Overnight cultures of all strains were grown in 30 ml glass universals containing 6ml of King's medium B (KB), shaking at 0.65g and 37°C for 18 hours and then diluted to an OD_{600nm} of 1.8 to ensure similar numbers of bacteria per millilitre. These cultures were subsequently grown on agar plates to determine the number of bacteria inoculated at the beginning of the experiment, using colony forming units (CFUs) as an approximate measure. 30ml glass universals containing 6ml of KB broth were inoculated with a total of 10^4 cells with different starting frequencies of the individual strains. Populations were subjected to iron-limited conditions by the addition of 70 μg/ml human apotransferrin (Sigma), a natural iron chelator, and 20 mM sodium bicarbonate, necessary for iron

chelator activity (Meyer *et al.* 1996). PAO1 was competed against each of the six O:9 isolates exhibiting reduced siderophore production at starting frequencies of: 99.9%, 99%, 90%, 50%, 10%, 1%, and 0.1%. This exact design was replicated in the PAO 1150-2 and O:9 competition. Cultures were propagated in a shaking incubator at 0.65g and 37°C and sampled after 96 hours, allowing time for effect of the bacteriocin to be observed. To see if predictions made in a previous study (Inglis *et al.* 2009) still held under conditions of iron limitation, the competition assays were also performed between PAO1/PAO 1150-2 and the ancestral O:9 strain, as described above.

We calculated the relative growth PAO1 to O:9 and PAO 1150-2 to O:9 at the different starting frequencies. This was done by plating the various treatments on KB agar plates and counting the number of CFUs for each strain. All strains were easily distinguishable from one another because of unique colony morphology and size. At the more extreme frequencies antibiotic plates were required to give better resolution of colony counts, which was possible due to the different antibiotic resistance profiles of the assorted strains (PAO1 resistant to streptomycin 1250 μg/ml, O:9 resistant to rifampicin 312.5 μg/ml, and PAO 1150-2 resistant to tetracycline 312.5 μg/ml). Relative Malthusian parameters (m) were used to estimate at what frequency bacteriocin production is favoured in PAO1 relative to 1150-2 using the common competitor O:9, where relative (m) = ($m_{PAO1/1150\text{-}2}$), and (m) refers to ln(final density/starting density) (Lenski *et al.* 1991). All frequencies were replicated six times (one for each O:9 isolate).

4.2.3 *In Vivo* Virulence Assays:

Virulence assays were performed as previously described in an earlier study (Miyata *et al.* 2003; Harrison *et al.* 2006; Inglis *et al.* 2009). Briefly, overnight cultures of PAO1, the six O:9 isolates, and PAO1150-2 were diluted in minimal salt solution. Fifth instar waxmoth (*Galleria mellonella*) larvae (Livefood UK; http://www.livefood.co.uk) were randomly allocated to be inoculated with 10^4 CFUs of PAO1/O:9 and PAO 1150-2/O:9 mixtures. The starting frequencies of the bacterial combinations consisted of 99%, 50%, and 1% PAO1 and PAO1150-2 to O:9. Larvae were swabbed with 70% ethanol to prevent contamination of the injection site and injected into the abdomen using Terumo 1ml disposal syringes and BD Microlance 30G ½" needles. The injection volume was 50µl in all cases. Twenty larvae were assigned to each treatment, and a further 20 larvae were injected with 50µl of minimal salt solution as negative controls. Larvae were then incubated at 37°C and monitored for death at 30 minute intervals between 10 and 18 hours post-inoculation. Larvae were scored as dead if they failed to respond to mechanical stimulation of the head.

Overall density of the different bacterial strains within the caterpillar hosts was also measured. Caterpillars were inoculated as previously described (Harrison *et al.* 2006; Inglis *et al.* 2009) and incubated for 8 hours at 37°C. Larvae were then weighed, dipped in 70% ethanol to kill surface contaminants, and homogenised in 500µl minimal salt solution using a plastic pestle. Homogenates were centrifuged at 3000 rpm for 3 minutes to pellet the solid, and aliquots of diluted homogenate plated onto KB agar. Agar plates were supplemented with 15µg/ml ampicillin to select against growth of native larval-gut bacteria (this concentration of ampicillin does not affect the growth of *P. aeruginosa*). Plates were

incubated overnight at 37°C and subsequently scored for CFUs. All statistical analyses were performed in R (2.9.2).

4.3 RESULTS

A simple mathematical model was analysed to derive invasion conditions (into a structured population) for individuals exhibiting a range of different social traits. Mutant individuals can vary (relative to the resident, whose parameter values are fixed at zero) in their investments into cooperation, y and/or into spite, s (see Appendix). Figure 4.1a illustrates the classic invasion conditions for a spiteful individual ($s>0$, $y=0$) and a cooperative individual ($s=0$, $y>0$), as a function of the scale of competition (from purely local, $a = 1$, to purely global, $a = 0$) and local frequency, p. In keeping with existing theoretical and empirical findings, we see that spite is most favoured under conditions of high local competition and intermediate local frequencies (red area, Figure 4.1a, (Gardner and West 2004b; Gardner *et al.* 2004; Inglis *et al.* 2009)), whereas cooperation is most favoured under conditions of low local competition and high local frequencies (blue area, Figure 4.1b, (Frank 1998; Griffin *et al.* 2004)). Note that if we assume the individual trait to be vanishingly rare across the global population, and locally present at frequency p, then p is the coefficient of relatedness measured with respect to the global population (Frank 1998; Inglis *et al.* 2009).

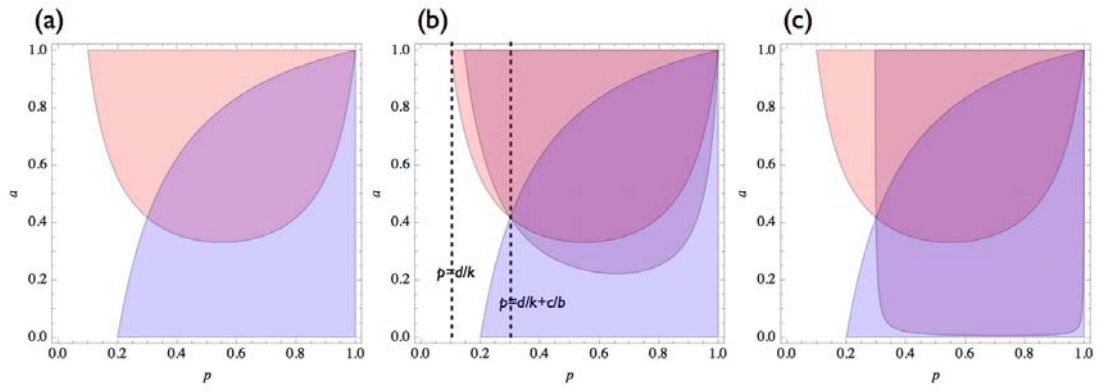

Figure 4.1: Mutant invasion conditions as a function of local competition *a*, and local frequency, *p*. Regions favouring investment in spite only ($s>0$, $y=0$), cooperation only ($s=0$, $y>0$) and spite given cooperation ($s>0$, $y>0$) are shaded in red, blue and purple, respectively. (a), Red region favours investment in spite, given cooperative investment $y = 0$. (b), Purple region favours investment in spite, given cooperative investment $y = 1$. (c), Purple region favours investment in spite, given cooperative investment $y = 100$. Models are derived in the appendix. Parameters are $c = 0.4$, $d = 0.2$, $k = 2$, $b = 2$.

We now ask, what is the effect of increasing cooperation y on the value of investments into spite, s? Any additional investments into cooperation (with costs c and shared benefits b) act to change the relative costs and benefits of investments into spite in a systematic way. Specifically, we find that the benefit/cost ratio of investments into spite (where the individual spite cost is d and the negative effect on the recipient is k) is modified by the factor $\frac{1+y(bp-c)}{1-yb(1-p)}$ in these "spiteful cooperators" (see appendix). The consequence of these changes to relative costs and benefits is that the ecological parameter space where additional investments in spite are favoured shifts towards higher values of p and more global investment (purple shading, Figures 4.1b,c). Specifically, we find that for sufficiently high values of y, further investments in spite are favoured whenever $p > d/k + c/b$. In contrast, when p is low to intermediate ($d/k < p < d/k + c/b$), sufficient y will reduce the value of spite (details in Appendix). Together, these effects entail a shift of spiteful investments towards higher local frequencies.

The analysis, summarized in Figure 4.1, assumes that investments in spite and/or cooperation have negligible consequences for the local frequency p of the focal strain (weak selection). By relaxing this assumption we can see further reasons to expect a strengthening of selection for spite at higher frequencies. Specifically, if we assume that cooperation reduces the local frequency p (due to cheater exploitation), then when p is initially high (whenever $p > (d+k)/2k$, see appendix), any reduction in p towards intermediate frequencies will generate an increased reward from spiteful investments.

Next, we ask what are the implications for pathogen virulence of the coupling of cooperative and spiteful traits in a single lineage? Looking at each trait in isolation, we recover established results (Figure 4.2, Appendix), specifically that the virulence of a cooperative individual is maximized when it is locally dominant (Brown *et al.* 2002; West and Buckling 2003; Harrison *et al.* 2006), whereas the virulence of a spiteful individual is minimized when it is at intermediate frequencies (Gardner *et al.* 2004; Inglis *et al.* 2009). Turning to the spiteful cooperators, we find that virulence will follow a hybrid path with increasing local frequency p (purple line, fig 2), with the intermediate minima (characteristic of spiteful interactions) lost whenever $y > s(d+k)/(b-c)$.

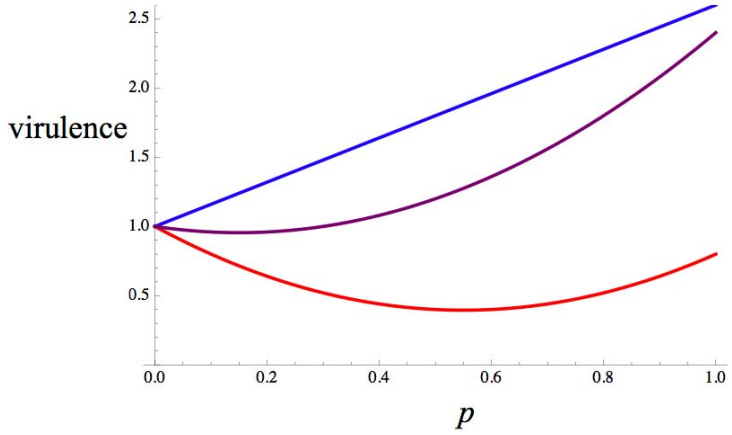

Figure 4 **:y of focal strain.** For all lines, the resident strain displays neither social trait, and has virulence = 1. Blue line, a focal cooperative strain (y = 1). Red line, a focal spiteful strain (s = 1). Purple line, a focal cooperative and spiteful strain (y = s = 1). Other parameters, b =2, c = 0.4, d = 0.2, k = 2.

Our model predicts that competing against public good 'cheats' should boost the relative fitness of invading spiteful versus non-spiteful genotypes at higher frequencies. To empirically test this hypothesis, we determined the relative fitness of spiteful versus isogenic, non-spiteful (sensitive) genotypes under a range of starting frequencies and cooperator-cheat combinations. All strains were isogenic, apart from the differences in spiteful and cooperative traits. Specifically, we manipulated densities of both a bacteriocin and siderophore producing strain (PAO1, a "spiteful cooperator" as it produces the pyocin S2 and pyoverdine) and six "sensitive cheats" (independent spontaneous mutants showing reduced siderophore production, derived from pyocin S2-sensitive *P. aeruginosa* O:9) to create a range of different starting frequencies with respect to the spiteful cooperator (between 0 and 1). For comparison, we established the same range of starting frequencies for an isogenic mutant (PAO1150-2), that does not produce a bacteriocin (pyocin S2) that affects the sensitive strains, but still produces siderophores (and so can be considered a "non-spiteful cooperator").

We calculated the Malthusian parameters of the spiteful and non-spiteful cooperator under the different scenarios (Lenski *et al.* 1991). Our model (like others) predicts that cooperative behaviours are never favoured under entirely local competition, thus we calculated the relative fitness of the spiteful cooperator by dividing its Malthusian parameter by that of the non-spiteful cooperator; this assumes that the different lineages are competing globally to some extent. We first determined how the relative benefit of spite changed with frequency when competing against sensitive genotypes that were also producing wildtype levels of siderophore in iron-limited media (i.e. no differential investment in cooperation, $y = 0$). This experiment replicates our previous study (Inglis *et al.* 2009), except here the media is iron-limited. Consistent with this previous work and theoretical predictions, the benefit of spite was maximised at intermediate frequencies. (linear term $F_{1, 30} = 3.91$, $p>0.056$; quadratic term $F_{1, 29} = 6.12$, $p<0.019$) (Figure 4.3). Note that fitness of the spiteful genotype peaks at a lower frequency of the spiteful lineage in this study, because in previous work fitness was measured relative to the sensitive competitor. Results from the current study are almost identical to previous work in non iron-limited media if we measure fitness relative to the sensitive competitor (data not shown).

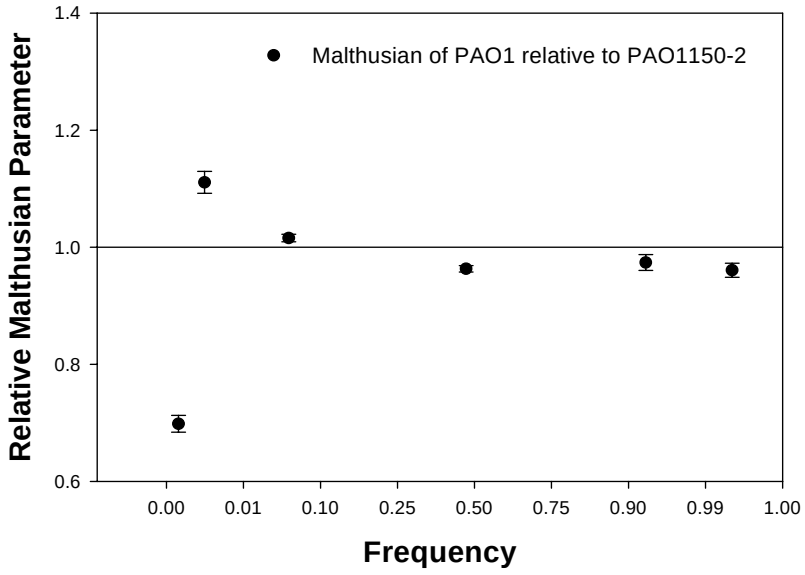

Figure 4.3: Relative Malthusian parameters of spiteful compared to non-spiteful bacteria when all strains produce siderophores in iron limited media (in model terms, $s > 0$, $y = 0$). Spiteful bacteriocin production is favoured at intermediate starting frequencies as previously described in non-iron limited media.

Relative fitness of the spiteful cooperators (compared with the non-spiteful cooperators), when competed with sensitive cheats (now, y>0), also shows a unimodal relationship with frequency of the spiteful genotype (linear term $F_{1, 37} = 15.15$, $p<0.001$; quadratic term $F_{1, 36} = 5.16$, $p<0.029$) (Figure 4.4). However, unlike the relative fitness where cooperation is held constant between competitors (Figure 4.3), the spiteful behaviour is now favoured across a wider range of higher starting frequencies (a clear peak shift to the right), as predicted by our model. A single polynomial model was fitted for the whole data set, and a significant interaction between the two different treatments (iron limited control and sensitive cheats) and cubic term was observed ($F_{1, 67} = 8.64$, $p<0.005$), indicating that the two lines are in fact different.

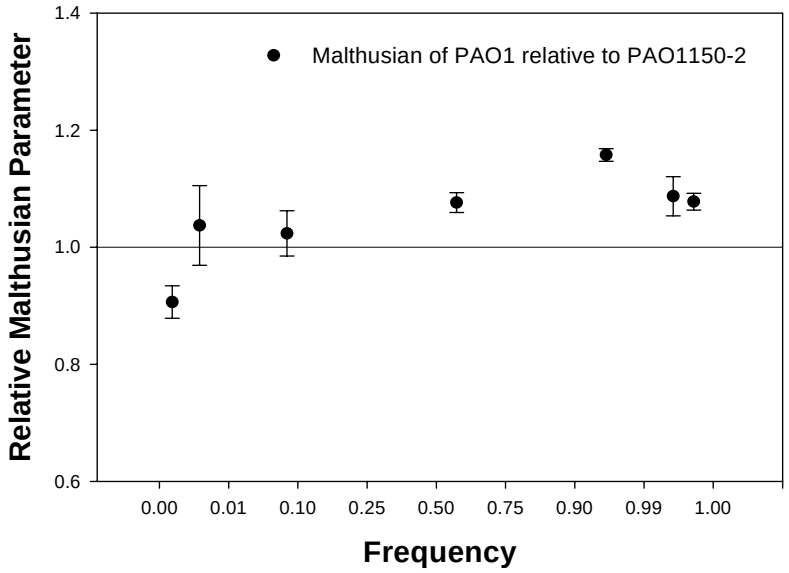

Figure 4.4: Relative Malthusian parameters of spiteful compared to non-spiteful cooperating bacteria when in competition with public goods cheats (i.e. bacteria that do not produce siderophores)(in model terms, $s > 0$, $y > 0$). Spiteful bacteriocin production is now favoured across a wider ranger of higher starting frequencies.

We next determined the relationship between the starting frequency of the spiteful genotype and virulence (as measured by time to death in caterpillar hosts) when spiteful cooperators are in competition with sensitive cheats. We manipulated starting populations to give high (99%), intermediate (50%), and low (1%) frequencies of the spiteful cooperator relative to the sensitive cheat. This design was also replicated in the non-spiteful cooperator and sensitive cheat competitions. We found that virulence was most greatly attenuated at intermediate and low frequencies of spiteful cooperators in competition with sensitive cheats (frequency was highly significant in the survival analysis $p<0.0001$ with 50 d.f. and intermediate and high frequencies differed significantly from the low frequency, $p<0.004$ and $p<0.001$ respectively) (Figure 4.5). The highest virulence was observed at high frequencies of non-spiteful cooperators (as previously described by Harrison *et al.* (Harrison *et al.* 2006)), and no difference was seen between intermediate and low

frequencies (frequency was again highly significant in explaining survival, $p<0.0001$ with 50 d.f., but there was no significant difference between intermediate and low frequencies $p>0.53$ with 51 d.f.) (Figure 4.5). These results are qualitatively consistent with our theoretical predictions, in that virulence is most attenuated when spiteful bacteria compete against intermediate and high frequencies of cheats, and is maximal when there is a high frequency of non-spiteful cooperators.

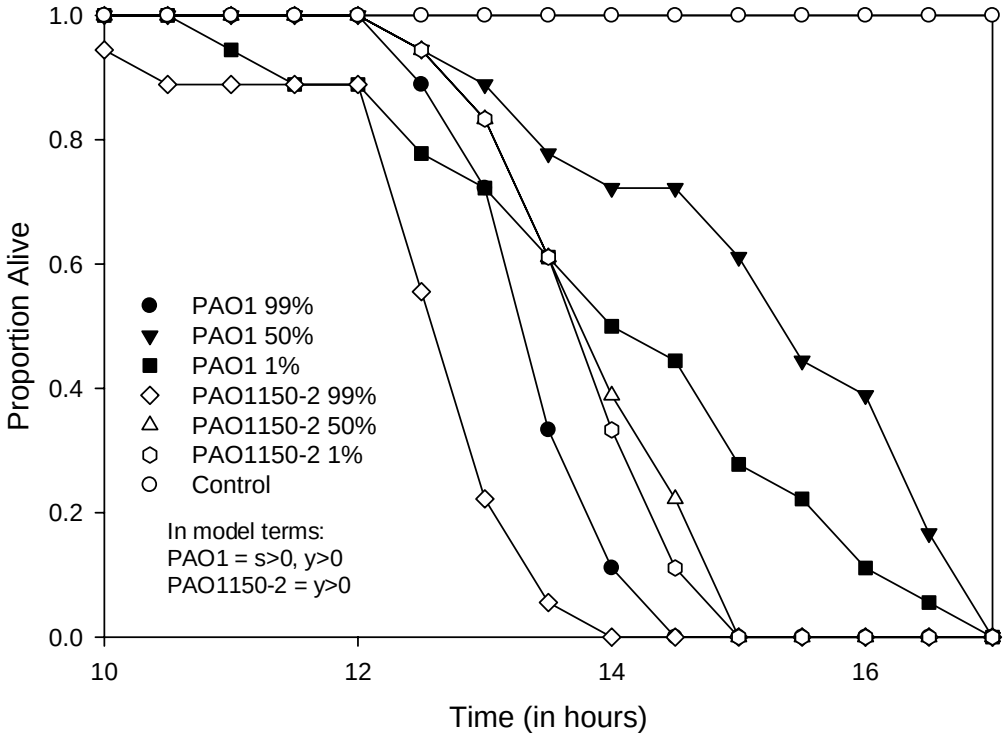

Figure 4.5: Time to death of caterpillars infected with different starting frequencies of spiteful and non-spiteful cooperators. Lowest virulence (as measured by time to death) was observed in the low and intermediate spiteful cooperator treatments. The highest virulence is found in the high starting frequency of non-spiteful cooperator, with similar virulence occurring in the intermediate and low non-spiteful cooperator treatments and high starting frequencies of spiteful cooperators.

The proposed mechanism in our model for the varying virulence seen between treatments is population growth rate. To further corroborate these results we measured bacterial densities before death in the insects, finding that bacterial density corresponds closely to our virulence data and our mathematical model. Both cooperative strains

(spiteful/non-spiteful) displayed a positive curvilinear relationship between starting frequency and bacterial density when in competition with sensitive cheats (linear term $F_{1, 95} = 43.15$, $p<0.0001$; quadratic term $F_{1, 93} = 4.65$, $p<0.034$), with non-spiteful cooperators reaching overall higher densities ($F_{1, 94} = 75.56$, $p<0.0001$) (Figure 4.6).

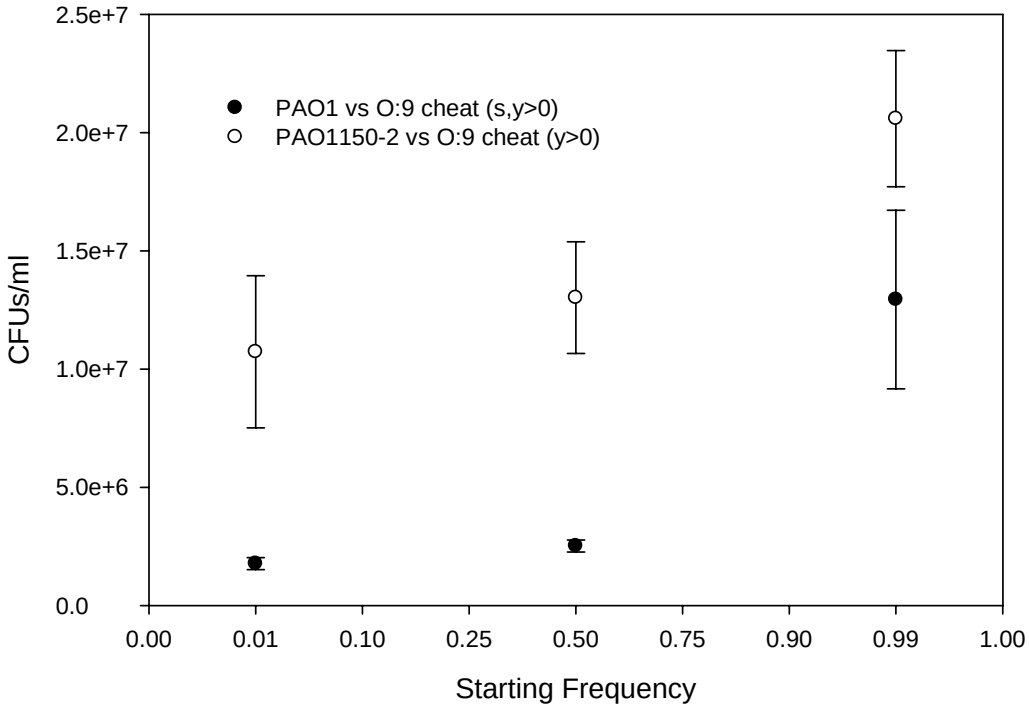

Figure 4.6: Bacterial density affected by starting frequency of spiteful and non-spiteful cooperators. Lowest densities were observed in low and intermediate spiteful cooperator treatments. High starting frequencies of spiteful cooperators reached similar densities to that of low and intermediate non-spiteful cooperators. The highest density was observed in the high non-spiteful cooperator frequency.

4.4 DISCUSSION

In this study, we investigated how selection for spiteful behaviours (bacteriocin production) is altered by simultaneous public goods cooperation (the production of iron-scavenging siderophores), and the resulting impact on the severity of infections, in the opportunistic pathogen, *Pseudomonas aeruginosa*. We establish experimentally that the unimodal relationship between the fitness of spiteful, toxin-producing bacteria and their frequency (Figure 4.3) is altered when the spiteful lineage is also more cooperative than its competitor strain, resulting in a systematic shift in peak fitness towards higher frequencies (Figure 4.4). Furthermore, we demonstrate that the virulence of infections resulting from competition between spiteful (or non-spiteful) and sensitive bacteria is significantly altered depending on the starting frequencies of the genotypes, and whether the sensitive bacteria are public goods cheats or cooperators (Figure 4.5,4.6). Notably, virulence is most attenuated when spiteful bacteria compete against intermediate and high frequencies of cheats. All these results are qualitatively consistent with the predictions from our mathematical model (Figure 4.1, 4.2, Appendix).

Our model gives us some very simple insights as to why spite is more beneficial at high frequencies when competing with public goods cheats. First, the model shows that simultaneous investments in cooperation can change the benefit/cost ratio of investments into spite, with the result that selection for spite is systematically enhanced when spiteful cooperators are at high frequencies. Second, if we additionally allow for cheats to initially increase locally in frequency (due to their competitive advantage with respect to the public good trait), then investments in spite can be further favoured if the initial frequency of the spiteful cooperators is sufficiently high. The net result of both forces is to shift the maximal value of spite to higher frequencies, when coupled with cooperation.

Note that while our experimental results highlight how public goods cooperation enhances the value of spite, our theory also illustrates that the converse can also hold true – spite can enhance the value of public goods investment by increasing the local frequency of producers (Appendix, equation 10). Together, these results outline a potential mechanistic synergy between spiteful and cooperative traits, which suggests that the long-term joint evolutionary dynamics will be more complex than predicted by analysis of either trait alone (Brown and Taylor 2010).

In our experiments, we do not investigate how levels of spite evolve, but rather investigate the fitness of a spiteful relative to a non-spiteful genotype. However, the results can be interpreted in an analogous way: public goods cheats can increase in frequency, resulting in a spiteful genotype having the greatest fitness at higher starting frequencies than it would if competing against a public goods cooperator. There may of course be other mechanisms operating that help to explain our results that are not captured in our model: for example, if these traits are expressed at different times. However, it is not necessary to invoke such specific biological details to explain the qualitative results.

In accordance with out theoretical results, we find the greatest reduction in virulence under conditions where spiteful cooperators were in competition with sensitive cheats at low and intermediate starting frequencies. These conditions allow for the maximum amount of conflict between the strains: cheats are using iron-scavenging pyoverdine reducing the growth rate of the spiteful cooperators (Harrison *et al.* 2006; Buckling and Brockhurst 2008) while cooperators are reducing the growth of the cheats by producing bacteriocins (Massey *et al.* 2004; Inglis *et al.* 2009). Unsurprisingly the highest virulence occurs under conditions of high public goods cooperation where the strain does

not produce the costly pyocin (Harrison *et al.* 2006). Both factors allow for faster absolute growth within the caterpillar and, therefore, greater host exploitation and virulence.

In the current study, cheating genotypes are always susceptible to pyocins, and hence spite can be considered as a form of punishment in this experimental context; an association noted in some recent theoretical studies (Nakamaru and Iwasa 2006; Lehmann *et al.* 2007). The concept of strong reciprocity – the punishment of non-cooperators by cooperators – is suggested to be an important process in the evolution of cooperation (e.g. Bowles and Gintis 2004). Our study suggests that punishment can be favoured over non-punishment when at intermediate to high frequencies in structured populations, but cannot invade from rare. This result is broadly consistent with recent theory under conditions where cooperation and punishment are not in linkage; an assumption appropriate for our experimental results, given that we are measuring fitness of cooperating punishers relative to cooperating non-punishers. By contrast, punishment can theoretically invade from rare when these traits are in linkage. It is interesting to note that there is an association between pyocin and pyoverdine outside of this experimental context. Specifically, *P. aeruginosa* pyocin S2 is taken up through the primary pyoverdine receptor, FvpA type 1 (Denayer *et al.* 2007). Genotypes that evolve to exploit a strain's pyoverdine type (of which there are a number of alleles (Tüimmler and Cornelis 2005)) may, therefore, become vulnerable to the competitor's pyocin.

Here we have shown that spiteful behaviours, or more specifically bacteriocin (pyocin) production is critically affected by simultaneous investments in another social trait, in this case altruistic cooperation (pyoverdine production). We have shown that this, in turn, can have important repercussions when considering virulence in *P. aeruginosa*

infections. Both pyocin (spite) and pyoverdine (cooperation) are likely to be of importance in clinical settings, especially cystic fibrosis, where pyocin producing and pyoverdine cheating strains are commonly found during the course of an infection (Govan 1986; De Vos *et al.* 2001). This study may, therefore, have practical implications in terms of manipulating the competitive arena (through some type of directed therapy) so that spiteful cooperators are in competition with sensitive public goods cheats, thereby reducing overall virulence.

4.5 APPENDIX

We base our approach on Frank's (Frank 1998) model of evolution under local competition, where fitness w is defined by the fecundity of a focal strain g_f, measured relative to the focal strain's competitors – a fraction a of which are found locally (with average fecundity g_l) and a fraction (1-a) of which are found globally (with average fecundity g_g), i.e.

$$w = \frac{g_f}{a g_l + (1-a) g_g}. \qquad [1]$$

Next we need to define focal, local and global growth rates as a function of independent investments by the focal mutant strain in cooperation (y) and spite (s), in a population of residents that do not invest in either trait. Note that our analyses allow for mutants that differentially invest in spite only ($s > 0, y = 0$), cooperation only ($s = 0 , y > 0$) or both ($s > 0, y > 0$). Let cooperation cost (per unit of additional investment) be c and benefit be b. Let spite cost be d and spite effect be k. We assume that $b > c$ and $k > d$. Let the proportion of the focal strain in its local patch be p. Therefore we have

$$g_f = 1 - ds - cy + pby \qquad [2a]$$

$$g_l = p(g_f) + (1-p)(1 - pks + pby) \qquad [2b]$$

$$g_g = 1 \qquad [2c]$$

Together, these assumptions yield a neighbour-modulated fitness function

$$w(s,y) = \frac{1 - ds + y(pb - c)}{1 + ap(y(b-c) - s(d + k(1-p)))}. \qquad [3]$$

Selection on separate investments in cooperation and spite

We begin by considering the growth of a rare mutant deviant in one strategy only, invading a resident population. First, consider a mutant spite strain, the marginal value of increasing investments in spite is captured by the inclusive fitness effect

$$H_s = \frac{dw}{ds}\Big|_{s=0,y=0} = akp(1-p) - d(1-ap) \qquad [4]$$

The condition for increasing spite to be favoured is then $H_s > 0$ (red regions in figure 1), which simplifies to

$$d < \frac{akp(1-p)}{1-ap} \quad . \qquad [5]$$

This is an established result (Hamilton 1970; Hamilton 1996; Gardner *et al.* 2004), which can be readily expressed in terms of Hamilton's rule, $RB>C$ with negative 'benefits' and negative relatedness to the victims of the spiteful act ($C_s = d$, $B_s = -(1-p)k$, $R_s = -ap/(1-ap)$). Next, we can take the same approach to investigate selection on mutants differing only in their cooperative investments. The marginal value of increasing investments in helping is captured by the inclusive fitness effect

$$H_y = \frac{dw}{dy}\Big|_{s=0,y=0} = (1-a)bp - c(1-ap)\,. \qquad [6]$$

Simplifying $H_y > 0$, we find the condition for increasing coop to be favoured is

$$c < \frac{b(1-a)p}{1-ap}, \qquad [7]$$

illustrated by the blue shading in figure 4.1. This again is a classic result (Frank 1998), which can be expressed in terms of Hamilton's rule, $RB>C$, with $C_y = c$, $B_y = b$ and $R_y = (1\text{-}a)p/(1\text{-}ap)$, which is again a measure of relatedness relative to competitors (Queller 1994), but now relatedness to clone mates, relative to competitors.

Selection on spite, given simultaneous investments in cooperation

Next we ask whether mutant investments in cooperation ($y>0$) will help or hinder investments in mutant spite. The marginal value of spite, given non-zero levels of mutant cooperation is given by

$$H_{s|y} = \frac{dw}{ds}\Big|_{s=0} = \frac{akp(1-p)[1+y(pb-c)] - d(1-ap)[1-yb(1-p)]}{[1+yap(b-c)]^2} \qquad [8]$$

As y tends to zero, all terms in square brackets tend to one, and equation 8 converges on equation 4. Simplifying $H_{s|y} > 0$, we find that spite is now favoured when

$$d < \frac{akp(1-p)}{1-ap}\left(\frac{1+y(bp-c)}{1-yb(1-p)}\right), \qquad [9]$$

as illustrated by the purple regions in Figures 4.1b,c. From equations [9] and [5] we can see that given non-zero mutant investments into cooperation y, the benefit/cost ratio R_sB_s/C_s of investments into spite is modified by the factor $\frac{1+y(bp-c)}{1-yb(1-p)}$. To identify whether this modifying effect of y enhances selection for spite, we look for parameter regions where $H_{s|y}$ is more permissive of spite evolution than H_s. Specifically we look for conditions where both inequalities $H_{s|y} > 0$ and $H_s < 0$ are met. Some algebra shows this to be the case when $p > d/k + c/b$, and y is sufficiently large (specifically, $y > \frac{akp(1-p)-d(1-ap)}{ap(1-p)(ck+b(d-kp))}$). In contrast, when p is low to intermediate ($d/k < p < d/k + c/b$), sufficient y will reduce the marginal value of spite (Figures 4.1b,c).

Interactions mediated by changes in local frequency, p.

One general assumption of our model approach that is worth further examination is the assumption of weak selection, i.e. the social traits themselves do not immediately modify the population structure, represented by the local frequency of the focal strain, p. However, it is well understood that investments in spite can significantly increase the local frequency of the focal lineage (due to death of competitors), whereas investments in cooperation can decrease the local frequency (due to competition from cheats). To explore the consequences of changing p on the value of investment in cooperation, consider

$$\frac{dH_y}{dp} = b(1-a) + ca\,. \qquad [10]$$

From equation 10 we see that increasing spite (by increasing p) can have a positive effect on the value of investments in cooperation. Conversely,

$$\frac{dH_s}{dp} = (a(d + k(1-2p)). \qquad [11]$$

Here we see that increasing cooperation (if decreases p below $(d+k)/2k$) can enhance selection for spite.

Virulence

We now return to the simple fecundity equations [2] to ask what are the general implications for host virulence of varying proportions p of an (s,y) mutant lineage competing within a focal host with a wildtype lineage. We make the simplifying assumption that host virulence v (additional host mortality) is directly proportional to the average fecundity of pathogens within a focal host g_l (see equation 2b). This assumption is supported by our virulence assay which measured bacterial density (Figure 4.6). Now we ask, what is the effect on virulence of increasing the proportion of the focal mutant strain?

$$\frac{dv}{dp} \propto y(b-c) - s(d + k - 2kp) \qquad [12]$$

Thus, in the absence of any spiteful trait (s = 0), we see that increasing the local presence of a cooperative lineage will always increase virulence (Brown *et al.* 2002; West and Buckling 2003; Harrison *et al.* 2006). Conversely, in the absence of any cooperative trait (y

= 0), we see that increasing the local presence of a spiteful lineage competing with a non-spiteful lineage will decrease virulence if $p < (d+k)/2k$, and then increase virulence thereafter. Given the cost of spite is less than the magnitude of its maximal effect (ie $d < k$), this result implies that virulence will be minimised for intermediate local frequencies of a spiteful lineage (Gardner and West 2004b; Inglis *et al.* 2009). Finally, for the case of a double *s*, *y* mutant, we can see from equation [12] that increasing the local presence of the mutant strain will increase virulence whenever

$$p > \frac{s(d+k) - y(b-c)}{2ks}.$$

Therefore the intermediate minima that are characteristic of spite-mediate virulence (Gardner *et al.* 2004; Inglis *et al.* 2009) are lost whenever $y(b\text{-}c) > s(d\text{-}c)$. Figure 4.2 illustrates the virulence v as a function of p, under these three distinct scenarios.

CHAPTER 5

THE ROLE OF "SOAKING" IN SPITEFUL TOXIN PRODUCTION IN *PSEUDOMONAS AERUGINOSA*

SUMMARY

Spiteful bacteriocin production represents an important class of social behavior in bacteria. Previous studies have indentified several ecological conditions that can favour the production of bacteriocins. However, bacteriocin production normally entails the production of antidote complex which can neutralize the toxin, thereby only targeting unrelated individuals. This absorption and neutralization of the bacteriocin has the potential to alter the conditions under which spiteful toxin production is favoured. In this study we investigate how the "soaking" of one's own toxin is affected by starting frequency, by using mutant toxin producers that do not express the toxin receptor. We find that in the absence of self-soaking, bacteriocin production is more beneficial both when the producers are at high and low starting frequencies in the population. We argue that this feature of bacteriocin production might be a general phenomenon of toxin production and will have important evolutionary consequences, especially in microbial communities where several different species may be able to "soak" toxins.

5.1 INTRODUCTION

Social interactions in microbes have been shown to be both widely prevalent and an important factor when considering microbial ecology and evolution (West *et al.* 2007). Recently, there has been a proliferation of interest in interactions that can be considered spiteful, where an actor pays a cost to harm a recipient (Gardner *et al.* 2004; Massey *et al.* 2004; Brown *et al.* 2006; Vigneux *et al.* 2008; Inglis *et al.* 2009; Hawlena *et al.* 2010). The production of antibacterial toxins such as bacteriocins, widespread amongst bacteria (Riley and Wertz 2002), can be considered a spiteful trait. For a behaviour to be considered spiteful it has to fulfil the criteria as set out by Hamilton's rule (RB>C), or the relatedness (R) between the actor and recipient and the benefit (B) experienced by the recipient has to be greater than the cost (C) to the actor (Hamilton 1963; Hamilton 1970; Hamilton 1996). In the case of bacteriocin production, spite can be observed if the cost (C) of bacteriocin production (either metabolically and/or if cell lysis is required for release) is less than the negative benefit, or harm, (-B) experienced by the recipient and if the actor is negatively related to the recipient (-R). Here relatedness refers to the similarity between the actor and the recipient compared to the population as a whole. In this context spite can also be thought of as indirect altruism where the benefit of the spiteful action is shared among individuals which also possess the spiteful allele (Gardner and West 2006; Lehmann *et al.* 2006; Gardner *et al.* 2007).

Recent theory and experiments suggest that bacteriocin production is favoured when the spiteful lineage is at intermediate frequencies within the local populations (Gardner *et al.* 2004; Inglis *et al.* 2009). This is because when spiteful individuals are at a low

frequency in the population the benefits created by the spiteful behaviour, such as reducing competition and "freeing-up" resources will be shared by the sensitive non-spiteful individuals as much as those exhibiting the spiteful trait. Similarly at high frequencies of the spiteful lineage there will be a reduction in the benefit of the spiteful trait, as there are few competitors to harm and, therefore, fewer resources to gain. Only at intermediate frequencies is spite beneficial as it is able to harm competitors and exploit the resulting unspent resources.

Despite qualitative consistency between the theoretical and empirical studies, a potentially important feature of bacteriocins may alter the frequency dependent fitness of spiteful lineages: Bacteriocins can often be absorbed by producing cells which are unaffected by the toxin, as they possess an immunity protein found in gene clusters with the production gene or genes (Michel-Briand and Baysse 2002; Riley and Wertz 2002). The overall effect of the bacteriocins will, therefore, be reduced as the bacteriocinogenic line increases in both density and total proportion of the population. This "self-soaking" of toxins might alter the frequency-dependent fitness of bacteriocin producers, and be important in explaining the persistence of sensitive strains even when they are rare in the population, especially if only a small percentage of the bacteriocinogenic strain are responsible for toxin production as it will be more likely that the toxin will be neutralized by concomitant cells. To explore the role of "self-soaking" in the evolution of bacteriocin production, we determine how the absence of the bacteriocin receptor affects the frequency-dependent fitness of bacteriocin-producing *Pseudomonas aeruginosa*.

5.2 MATERIALS AND METHODS

5.2.1 Bacterial Strains

Pseudomonas aeruginosa strain PW5036 was employed as the bacteriocin producing "non-soaker" in competition with the bacteriocin sensitive strain serotype O:9. PW5036 is isogenic to the wildtype PAO1 in every respect except for having its FpvA receptor rendered non-functional through transposon mutagensis in the *FpvA* gene (Jacobs *et al.* 2003). FpvA is the primary receptor for type I pyoverdine and the pyocin S2 produced by PAO1 (Denayer *et al.* 2007). Bacteriocin production and sensitivity were confirmed using a simple plate assay where the production of relevant bacteriocin is determined by overlaying bacteria mixed in semi-solid agar on plates that have been spotted with bacteria of another strain, as described by Fyfe *et al.* (Fyfe *et al.* 1984). If the strain inoculated on the plate produces bacteriocin that kills the strain mixed with semi-solid agar, a halo shaped zone of clearing can be observed in the bacterial lawn after incubating at 37°C for 18 hours. The absence of a clear halo indicates that either the overlaid strain is insensitive to the bacteriocin producer or the inoculated strain does not produce any bacteriocin.

5.2.2 Competition Assays

Overnight cultures of each strain were grown shaking at 0.65g and 37°C for 18 hours and then diluted to an OD_{600nm} of 1.8 to ensure similar numbers of bacteria per millilitre. These cultures were subsequently grown on agar plates to determine the number of bacteria present using colony forming units (CFUs) as an approximate measure. 30ml glass universals containing 6ml of Kings Media B broth were inoculated with a total of 10^4

cells with different starting frequencies of the individual strains. PW5036 and O:9 were competed against each other at starting frequencies of, 99%, 90%, 50%, 10%, 1%, and 0.1%. Cultures were propagated in a shaking incubator at 0.65g and 37°C and sampled at 96 hours, allowing time for effect of the bacteriocin to be observed. All frequencies were replicated 6 times.

At 96h we calculated the relative growth of the producer to sensitive and non-producer to sensitive at the different starting frequencies. This was done by plating the various treatments on KB agar plates and counting the number of CFUs for each strain. The strains were easily distinguishable from one another because of unique colony morphology and size. At the more extreme frequencies antibiotic plates were required to give better resolution of colony counts, and this was possible due to the different antibiotic resistance profiles of the strains (O:9 is resistant to rifampicin 312.5 µg/ml). Selection coefficients (*S*) were used to estimate at what frequency bacteriocin production is favoured in PW5036 relative to the wildtype PAO1 using the common competitor O:9, where $S = (m_{PAO1/1150\text{-}2} - m_{O:9})/ m_{O:9}$, and ($m$) refers to ln(final density/starting density) (Lenski *et al.* 1991).

To control for intrinsic growth rate differences between PW5036 and the wildtype PAO1, they were also competed against each other directly for 96h and selection coefficients were calculated. These were then used to scale the performance of PW5036 in competition with serotype O:9. All statistical analyses were performed in R (version 2.9.2).

5.3 RESULTS

In our experiment we manipulated the densities of both a bacteriocin producing "non-soaker" (PW5036 deficient in its bacteriocin, S2, receptor, FpvA, but isogenic to wildtype PAO1 in every other respect) (Jacobs *et al.* 2003) and a sensitive competitor (serotype O:9) to create a range of different starting frequencies with respect to the producing "non-soaker" (between 0 and 1). Selection coefficients were used to estimate the fitness of the producing "non-soaker" and sensitive strains (Lenski *et al.* 1991). The frequency-dependent fitness of this strain was then compared to that of its isogenic wildtype ancestor, PAO1. To control for intrinsic differences in growth rate between PAO1 and PW5036, a competition experiment between both strains was conducted and selection coefficients were scaled appropriately.

Both show a unimodal relationship, as previously described (Inglis *et al.* 2009), with the bacteriocin producing strain (PW5036) (Figure 5.1), peaking at intermediate values (linear $F_{1,63} = 29.49$, $p < 0.0001$, quadratic $F_{1,62} = 4.98$, $p<0.029$). However, when compared to the wildtype, the non-soaker peaks at higher starting frequencies and reaches overall higher levels of selection coefficients (significant difference observed between intercepts $F_{1,61} = 35.01$, $p<0.0001$, and shape of quadratic captured by interaction term $F_{1,60} = 5.16$, $p<0.027$). Pairwise comparisons between the two strains also show significant differences at starting frequencies of 0.1% ($p<0.0001$), 1% ($p<0.003$), 90% ($p<0.0001$), and 99% ($p<0.002$) after a sequential Bonferroni correction was applied (Holm 1979).

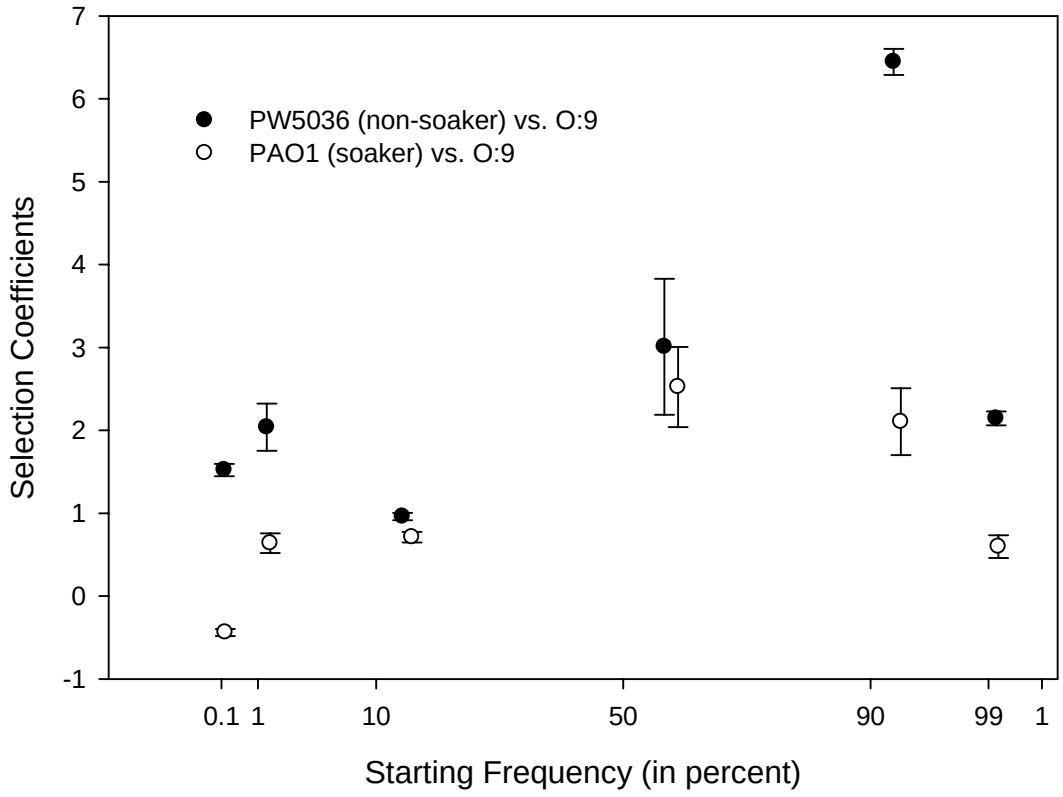

Figure 5.1: The effect of "soaking" on bacteriocin production. PW5036 (an isogenic knockout mutant that no longer produces the receptor for bacteriocin S2) displays a much higher fitness at high starting frequencies when compared against the wildtype PAO1.

5.4 DISCUSSION

The costs of soaking are clearly most pronounced at both high and low frequencies. At high frequencies, this is entirely as expected because more wildtype cells will inevitably lead to more soaking. The low frequency effect, however, is more surprising, but probably reflects the fact that the non-soaking strain (PW5036) has a slight growth rate advantage compared to the wildtype, as it does not pay the metabolic cost of producing the FpvA receptor. This difference disappears at intermediate frequencies as the cost of soaking is reduced, because under these conditions there is little room for further increases in fitness anyway.

As well as investigating the effect of soaking on frequency-dependent fitness, we were interested if soaking could contribute to the observed ability of sensitive cells to persist when at initially low starting frequencies (Inglis *et al.* 2009). Whereas approximately 10^5 cells/ml survived competition with the wildtype (when the sensitive strain (O:9) is at starting frequencies of 1% and 10%), none of the sensitive competitor strain (O:9) was detectable when competed with our non-soaking strain at these frequencies (with a detection threshold of approximately 200 CFUs per ml). Note that the resulting selection coefficients for these two treatments are a minimum and assume 200 cells per ml of the competitor. Soaking may, therefore, contribute to the maintenance of sensitive cells in both clinical and natural populations, and may contribute to the intransitive dynamics of producing, resistant, and sensitive cells (Kerr *et al.* 2002).

It is also interesting to note that under conditions where the producing strain is dominant in the population one would expect the loss of the bacteriocin receptor, as strains without it would be able to outcompete the wildtype as they no longer pay the costs of

producing and expressing this receptor and are more effective at killing competitors. However, in this system FpvA is also responsible for the uptake of pyoverdine type I (Denayer *et al.* 2007), an important iron scavenging molecule, so its loss would be unlikely to occur in iron limited environments (such as the cystic fibrosis lung).

This phenomenon is likely to be an important aspect of the evolution of microbial toxin production in general, particularly in cases where the toxin can be neutralized by the production of an immunity compound. Many toxins target receptors that have important fitness consequences for competitors, which may often be shared by toxin producing lineage. Moreover, this soaking effect also suggests another important role of community context in driving evolutionary dynamics. It is not clear of the extent to which bacteriocins can bind to receptors for other species (whether they have a toxin effect as well), but cases have been identified where interspecies inhibitions occur (Michel-Briand and Baysse 2002; Riley and Wertz 2002). Finally, the increase in the short-term efficacy of receptor-less mutants, as well as their inevitable long term cost through their inability to use siderophores, suggests a potential role in the biocontrol of clinical and agricultural infections.

CHAPTER 6

THE EVOLUTION OF BACTERIOCIN RESISTANCE

SUMMARY

The evolution of resistance is an important aspect of the ecology of bacteriocin production that has received little attention. Previous experimental studies have demonstrated that there is a large fitness benefit to producing pyocin S2 by *Pseudomonas aeruginosa*, but this will also create a strong selection pressure for sensitive strains to become resistant. Resistance normally results from the expression of an immunity protein, but it is likely that alteration of the bacteriocin receptor could also provide a resistance mechanism. However, many natural strains of *P. aeruginosa* are sensitive, and this suggests that non-immunity protein resistance may be costly. A likely reason for this cost is that the S2 receptor also binds iron-scavenging pyoverdine and alteration may therefore reduce iron uptake. We, therefore, hypothesise that resistance would be more likely to evolve under conditions where the requirement for pyoverdine is reduced (where iron is more available). Consistent with this view, we found that resistance to pyocin S2 appears to evolve faster and more often in populations where iron is less limited.

6.1 INTRODUCTION

The evolution of resistance to antimicrobial compounds is a widely documented phenomenon (Palumbi 2001; Perron *et al.* 2006). However, surprisingly little is known about any possible resistance mechanisms to a prolific form of antimicrobial compound produced by bacteria, bacteriocins (Riley and Wertz 2002), beyond the presence of immunity proteins that are often coded by genes in linkage with the bacteriocin gene. Resistance to bacteriocins has been shown to play an important role in natural environments (Hawlena *et al.* 2010) and in our understanding of community structure and intransitive interactions (Kerr *et al.* 2002). Other studies have also confirmed that many environmental isolates are sensitive to bacteriocins (Pirnay *et al.* 2005). The presence of sensitive strains is indicative that either resistance is hard to evolve, because novel genes are required, or that there is a large cost associated with resistance, as commonly observed for antibiotics (MacLean *et al.* 2010). Despite the importance of bacteriocin resistance, to date there is no experimental evidence on how such resistance can evolve.

In this study some preliminary data is presented on the evolution of resistance using the bacterium *Pseudomonas aeruginosa* which produces a large repertoire of bacteriocins. By co-culturing a bacteriocin producing strain (PAO1 which produces pyocin S2) and sensitive strain (O:9 which is sensitive to pyocin S2) under two common environmental conditions (iron and iron limitation), we demonstrate that resistance to bacteriocins can evolve but is dependent on the environment. We propose that resistance is harder to evolve in iron limited conditions, as sensitive bacteria express more receptors for the bacteriocin in this environment. This will make it more costly to evolve resistance as a simple change in

the receptor will also affect its ability to bind pyoverdine (an iron chelating compound), required for growth when the environment is iron limited.

6.2 MATERIALS AND METHODS

6.2.1 Bacterial Strains

Pseudomonas aeruginosa strain PAO1, was employed as the bacteriocin producer and serotype O:9 as the bacteriocin sensitive competitor. PAO1 is a known producer of pyocin S2, whereas serotype O:9 is sensitive to S2 pyocins (Denayer *et al.* 2007). Bacteriocin production, sensitivity, and insensitivity were confirmed using a simple plate assay where the production of relevant bacteriocin is determined by overlaying bacteria mixed in semi-solid agar on plates that have been spotted with bacteria of another strain, as described by Fyfe *et al.* (Fyfe *et al.* 1984). If the strain inoculated on the plate produces bacteriocin that kills the strain mixed with semi-solid agar, a halo shaped zone of clearing can be observed in the bacterial lawn after incubating at 37°C for 18 hours. The absence of a clear halo indicates that either the overlaid strain is insensitive to the bacteriocin producer or the inoculated strain does not produce any bacteriocin.

6.2.2 Transfer Regime

Overnight cultures for both strains were grown shaking at 0.65g and 37°C for 18 hours and then diluted to an OD_{600nm} of 1.8 to ensure similar numbers of bacteria per millilitre. 30ml glass universals containing 6ml of Casamino acids medium (CAA; 5 g Casamino acids, 1.18 g $K_2HPO_4.3H_2O$, 0.25 g $MgSO_4.7H_2O$, per litre) were inoculated with a total of 10^4 overnight culture cells of 90% serotype O:9 and 10% PAO1. To create an iron limited environment half of the tubes (12 in total) were also supplemented with 20mM sodium bicarbonate and 100µg/ml apo-transferrin (Sigma), a natural iron-chelator. Inoculated tubes were subsequently grown for 96 hours shaking at 0.65g in a 37°C

incubator. After 96 hours individual cultures were serially diluted and plated on rifampicin 312.5 μg/ml agar plates (incubated for 18 hours at 37°C) to select for serotype O:9 (Inglis *et al.* 2009). Overnight cultures of O:9 were initiated from these plates. To limit co-evolution between O:9 and PAO1, new overnights of PAO1 were initiated from frozen stocks at each transfer. These overnight cultures where then used to inoculated new tubes of CAA medium as described, and this selection procedure was repeated for a further 8 transfers. Colony forming units (CFUs) for O:9 were recorded at each transfer.

6.3 RESULTS

In this experiment, bacteria sensitive to the pyocin S2 (serotype O:9) where co-cultured with pyocin producing bacteria PAO1, under two different environmental conditions: freely available iron and iron limitation. The final density of O:9 was measured at each transfer to test for the evolution of resistance to S2 pyocin. Although, it is true that density is not strictly a measure of resistance, they can be correlated with more resistant genotypes being able to reach higher densities in the population. It is also worth noting that the manner in which the experiment was conducted also selects for resistance and increased growth in a correlated fashion (as fast growers, reaching high densities will be represented at greater frequencies in the next transfer), making final density an adequate, preliminary measure of resistance.

A mixed effects model was fitted to our data, and we observed that resistance in both environments increased over time ($F_{(1,190)} = 109.24$, $P < 0.0001$). However, there was a significant difference between the two different environments ($F_{(1,22)} = 42.97$, $P < 0.0001$) and an interaction between the number of transfers and the environments ($F_{(1,190)} = 13.46$, $P < 0.0001$)(Figure 6.1). Under conditions of non-iron limitation O:9 was able to reach much higher final densities, which increased more quickly over time. Whereas, under iron limited conditions there was a limited overall increase in density, as fewer cultures were able to evolve any resistance. More extinction events (where no O:9 bacteria could be extracted) also occurred in the iron limited environment. To investigate any differences that mutation supply rate, through reduced population size, might play in explaining our results, we grew O:9 by itself in both iron and iron limited conditions. We observed a two-fold increase in population density when iron was freely available (Figure 6.2).

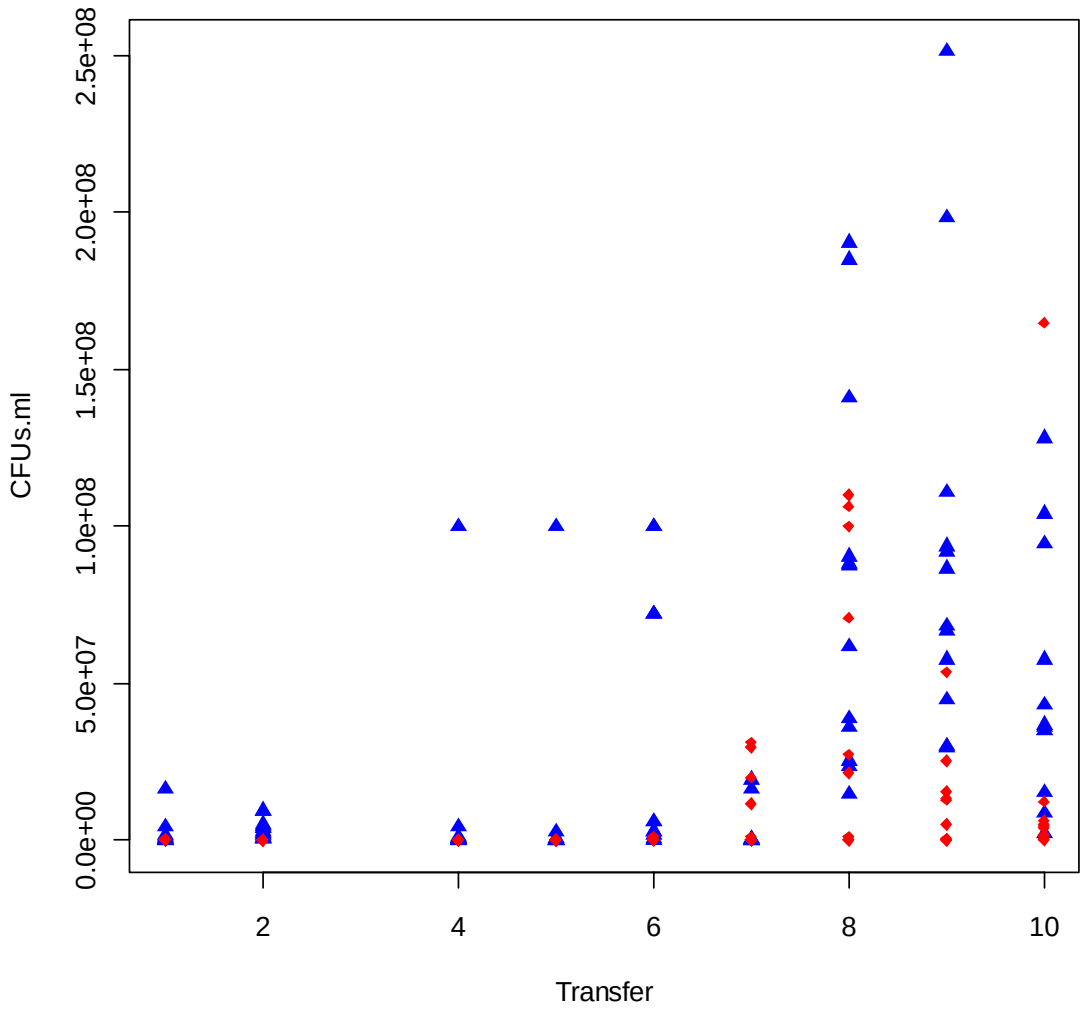

Figure 6.1: The final density of serotype O:9 at each transfer. Iron limited environments are represented by the red diamonds and non-iron limited are represented by blue triangles. Resistance increases over time under both conditions, but more culture become resistant under conditions of freely available iron.

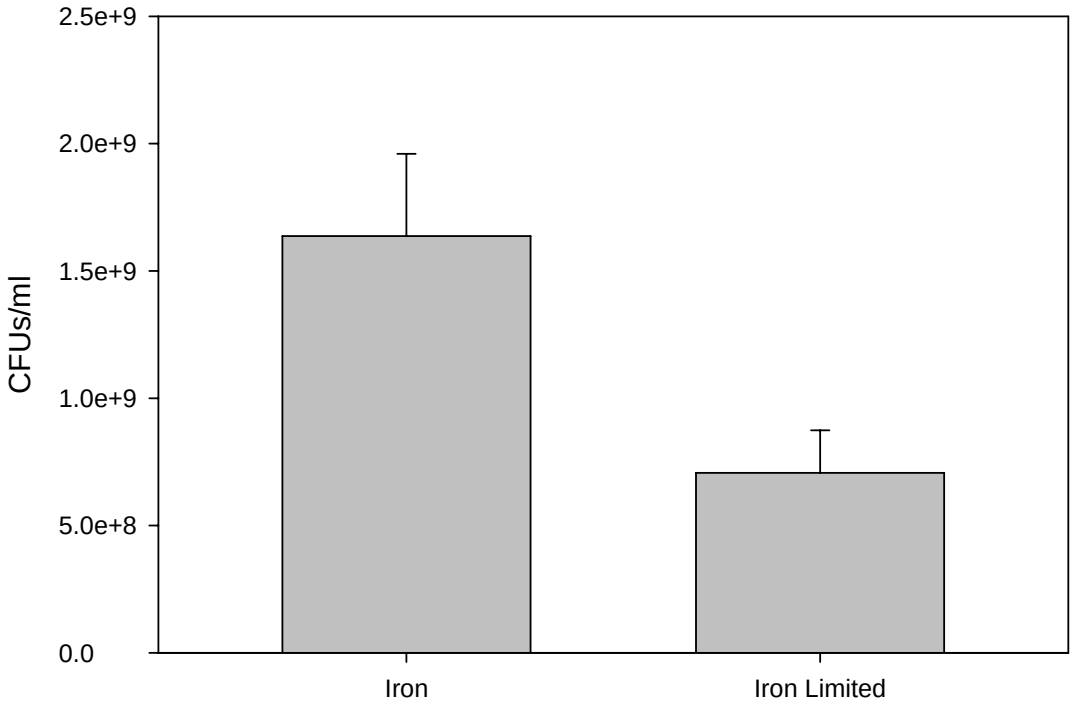

Figure 6.2: Density of serotype O:9 when grown under conditions of iron and iron limitation. A two fold increase in density is visible when O:9 is grown under conditions where iron is freely available. This means that mutation supply rate is unlikely to explain our results.

6.4 DISCUSSION

In this study we find that resistance to the bacteriocin S2 is able to evolve under both iron limited and non-iron limited environments. However, under conditions where iron is freely available (i.e. there is less requirement for pyoverdine) resistance evolves both more frequently and quickly. One possible explanation for this observed difference may be due to the dual function of the receptor responsible for iron uptake (Denayer *et al.* 2007). Under conditions of iron limitation *P. aeruginosa* produces iron chelating compounds such as pyoverdine (Meyer 2000). The receptor FpvA, involved in translocating the pyoverdine-iron complex, is also constitutively expressed during iron limitation and has been shown to absorb pyocin S2 (Denayer *et al.* 2007). This may explain why under conditions of iron limitation the effect of pyocin S2 is increased (Ohkawa *et al.* 1980).

One obvious mechanism for resistance would be the loss of the FpvA receptor, as this would prevent the pyocin from entering the cell. In environments where iron is easily accessible this may be a likely form of resistance, as *P. aeruginosa* also produces a second pyoverdine receptor FpvB with a much lower affinity, but it does not bind the pyocin. Moreover, *P. aeruginosa* is able to produce other siderophores such pyochelin (Ratledge and Dover 2000). However, in environments where iron is scarce and limits growth, cells will suffer a large fitness cost in terms of growth rate if they no longer express the FpvA receptor. Iron limitation also decreases overall density (Figure 6.2), and this may have some impact on the rate at which resistance mutations occur. Although a decrease in mutation rate can play an important role, it is probably not that important in this context as the difference in resistance between populations is much larger than their difference in population size.

These results and interpretation remain preliminary. We have not explicitly measured resistance (although density in the presence of the antimicrobial is a good surrogate measure) and have not determined the resistance mechanism. Further research will measure resistance directly, sequence the *FpvA* gene in all resistant clones to determine if resistance in both environments evolves in a similar fashion, and determine if alteration of FpvA (assuming this is the resistance mechanism) is more costly when there is a stronger requirement for the use of the FpvA iron-uptake system.

There is currently much interest in the use of bacteriocins for medical applications (Gillor *et al.* 2005; Kirkup Jr. 2006). Although it is not clear how many bacteriocins are translocated via the iron-uptake system, our findings suggest that in iron limited environments (e.g. some infections; note that in cystic fibrosis patients there can be more free iron present (Harrison 2007)) bacteriocins will have synergistic benefit of increased effectiveness and decreased evolutionary resistance. However, our results also tell the cautionary tale of resistance evolving even under iron limitation, suggesting that bacteriocins, though a promising source of new antimicrobials, are no silver bullet.

CHAPTER 7

THE ROLE OF PUNISHMENT IN PROMOTING ALTRUISTIC COOPERATION

SUMMARY

Punishing defectors has been suggested as potent force in facilitating the evolution of altruistic cooperation. However, for this to be a successful strategy the punishment and cooperative genes must be in linkage. In this study we mimic the effects of recombination between the punishment and cooperative traits, in the bacterium *Pseudomonas aeruginosa*, to examine how this affects the ability of an initially rare cooperative punisher to invade a population of public goods cheats. We find that cooperative punishers are able to invade, whereas when the linkage between the two traits is broken they cannot. This suggests that punishment could in principle play an important role in maintaining the high levels of cooperation observed in natural populations of bacteria, although evidence is currently indirect.

7.1 INTRODUCTION

Explaining the emergence of altruistic (i.e. individually costly) indiscriminate cooperation remains a fundamental problem for evolutionary biology. Kin selection is one of the key explanations, where cooperation is favoured because it primarily benefits other individuals who share the same genes (Hamilton 1963; Hamilton 1964; Hamilton 1972; Hamilton 1996). However, for kin selection to operate, relatedness must be sufficiently high, and these conditions will be difficult to meet when cooperators are invading a population of defectors. The existence of mechanisms that repress competition, for example, punishment of cheating individuals, will also by definition aid the invasion of cooperation. However, the punishing strategy itself must invade, and to do this it must be either directly beneficial or indirectly beneficial (Gardner and West 2004a; Lehmann *et al.* 2007), with the latter again requiring sufficiently high relatedness (Frank 1995).

It has long been suggested that the invasion of cooperation may be facilitated if cooperators are also punishers, a behaviour described as strong reciprocity (Frank 1995; Frank 1996b; Gintis 2000; Fehr and Fischbacher 2003; Gintis *et al.* 2003; Bowles and Gintis 2004; Gardner and West 2004a; Lehmann *et al.* 2006; Nakamaru and Iwasa 2006; Lehmann *et al.* 2007; West *et al.* 2007). Recent analyses suggests that this may simply be because it is easier for a punishing allele to invade a patch (i.e. single locally competing population) of defectors than it is for a cooperation allele, hence the cooperation allele can simply hitchhike to high frequencies with the punishing allele (Gardner and West 2004a; Lehmann *et al.* 2007). However, where the punishing behaviour incurs a direct fitness cost to the actor, the conditions for the invasion of strong reciprocity requires tight linkage between the cooperating and punishment alleles (Lehmann *et al.* 2007). In the absence of

linkage, neither costly punishing or costly helping can invade without each other: punishing defectors will harm themselves and their kin as they are now all cheating, and non-punishing cooperators cannot invade a population of cheats. Here, we test the importance of linkage between helping and punishing for the invasion of strong reciprocity in experimental populations of bacteria.

Bacteria have previously been shown to engage in a number of different social behaviours, such as the cooperative production of public goods (West and Buckling 2003; Griffin *et al.* 2004) (e.g. iron chelating compounds such as siderophores) and the spiteful killing of closely related strains through production of anti-competitor toxins, such as bacteriocins (Gardner *et al.* 2004; Inglis *et al.* 2009). In this study we use the bacterium *Pseudomonas aeruginosa* which is able to produce a variety of siderophores and bacteriocins, which in itself does not constitute punishment (Meyer 2000; Michel-Briand and Baysse 2002). However, both pyocin S2 and pyoverdine type I (which are the focus of this study) are taken into the cell via the same surface receptor FpvA (Denayer *et al.* 2007). This reduces the potential for unrelated bacteria or bacteria that do not engage in the production of the public goods to benefit, as only cells with both the receptor and immunity compound will be able to successfully utilise the public goods. The bacteria in this system can, therefore, be considered to be cooperative punishers (although we do not suggest that S2 pyocins have evolved primarily for this purpose), because they are able to harm non-cooperative individuals by producing a costly toxin. We also mimic the products of recombination (thereby breaking the linkage between punishment and cooperation) by mixing punishing cooperators with non-punishing cooperators. Although, it is true that recombination could also yield punishing defectors, these are not included in this study, as

by definition individuals that kill themselves and their kin would only exist transiently in a population and so be of little evolutionary consequence (Lehmann *et al.* 2007).

In this study we are using the term "punishment" to describe the production and action of bacteriocins which in previous chapters we have referred to as spite. The distinction between spiteful and punishing behaviours is dependent on their context. A behaviour can be considered punishment when it affects cheats only (as we have created in this experiment), whereas spite affects competitors in general. This context dependent nature of spite and punishment has been considered in previous theorietical papers (Nakamaru and Iwasa 2006; Lehmann *et al.* 2007).

7.2 MATERIAL AND METHODS

7.2.1 Bacterial Strains

Pseudomonas aeruginosa strain PAO1, was employed as a strong reciprocator (policing cooperator) as it is a known producer of pyocin S2 (punishing behaviour) and pyoverdine type I (cooperative behaviour). PAO1150-2, a transposon knock-out mutant of the *psy2* gene involved in S2 production (not spiteful) but still produces pyoverdine type I (cooperative behaviour), was employed as a non-policing cooperator. These strains were competed against six clones of serotype O:9 (cheats) which are sensitive to S2 pyocins and show a reduction in iron chelating ability by over 60%. Strains were grown in 30 ml glass universals containing 6 ml of King's medium B (KB), shaking at 0.65g and 37°C and were subsequently diluted to equal densities to start the experiment.

7.2.2 Experimental Design

In this experiment we set out to test the ability of cooperators to invade a population of cheats. To facilitate this we performed three different treatments: 1) a cooperative strain (PAO1150-2) invading a population of cheats (O:9), 2) a cooperative strain that also produces a punishing toxin (PAO1) invading a population of cheats (O:9), 3) a cooperative strain (PAO1150-2) and a cooperative strain that produces a punishing toxin (PAO1) invading a population of cheats (O:9). Each treatment was replicated in six metapopulations that contain nine individual subpopulations. Every subpopulation was grown in a tube of KB broth which was subjected to iron-limited conditions by the addition of 100 μg/ml human apotransferrin (Sigma), a natural iron chelator, and 20 mM sodium bicarbonate, necessary for iron chelator activity (Meyer *et al.* 1996)..

At the start of the experiment every subpopulation was inoculated with approximately 10,000 cells of O:9. One subpopulation within each metapopulation was inoculated by 1% of the invading strain (circa 100 cells) of either PAO1150-2 (treatment 1), PAO1 (treatment 2), PAO1150-2 and PAO1 (treatment 3). Cultures were then grown at 37°C for 96 hours in an orbital incubator, shaking at 0.65g. Every tube, within a metapopulation, was then subsequently mixed together by adding equal volumes of culture. This mixture (one for each metapopulation, six for each treatment) was diluted and then transferred to nine fresh tubes of KB, with approximately 10,000 cells being transferred each time. This selection procedure was repeated for a further eight transfers. At every round of selection we scored the frequencies of the strains (based on their colony morphology and different antibiotic resistance profiles) by growing them on agar plates and counting colony forming units.

7.3 RESULTS

To investigate the role of linkage between cooperative and punishment traits in facilitating the invasion of cooperators, we created eighteen experimental metapopulations of bacteria (serotype O:9) that do not produce pyoverdine type I but are able to use that produced by other bacteria. We subsequently inoculated these metapopulations with cooperative punishers (PAO1 which produces both pyoverdine type 1 and bacteriocin S2), solely cooperators (PAO1150-2 which only produces pyoverdine type 1), and both cooperators and cooperative punishers (PAO1 and PAO1150-2 to break the linkage between the cooperative and punishment traits) (six metapopulations for each treatment). These populations underwent nine transfers of growth and the relative proportions for each strain were calculated at each transfer (Figure 7.1).

Of the eighteen metapopulations almost all became extinct, bar three populations of the cooperative punishers (Figure 6.1). A Kruskal-Wallis test was applied to the data, after confirming similar distributions of the data between the treatments. There was a highly significant difference between the three treatments with an H of 9.66 and $p < 0.009$. To test for differences between pairs of treatments a Wilcoxon rank-sum test was carried out, employing a sequential Bonferroni correction (Holm 1979). Significant differences were observed between the invasion of cooperative punishers (PAO1) compared to normal cooperators (PAO1150-2)($\chi^2 = 7.16$, $p < 0.008$, and α of 0.018) and when the cooperative and punishment traits were not in linkage (PAO1 and PAO1150-2) ($\chi^2 = 5.57$, $p < 0.019$, and α of 0.025)(Figure 7.1). However, no difference was detected between the cooperators (PAO1150-2) and un-linked traits (PAO1 and PAO1150-2)($\chi^2 = 0.02$, $p > 0.59$, and α of 0.05)(Figure 7.1).

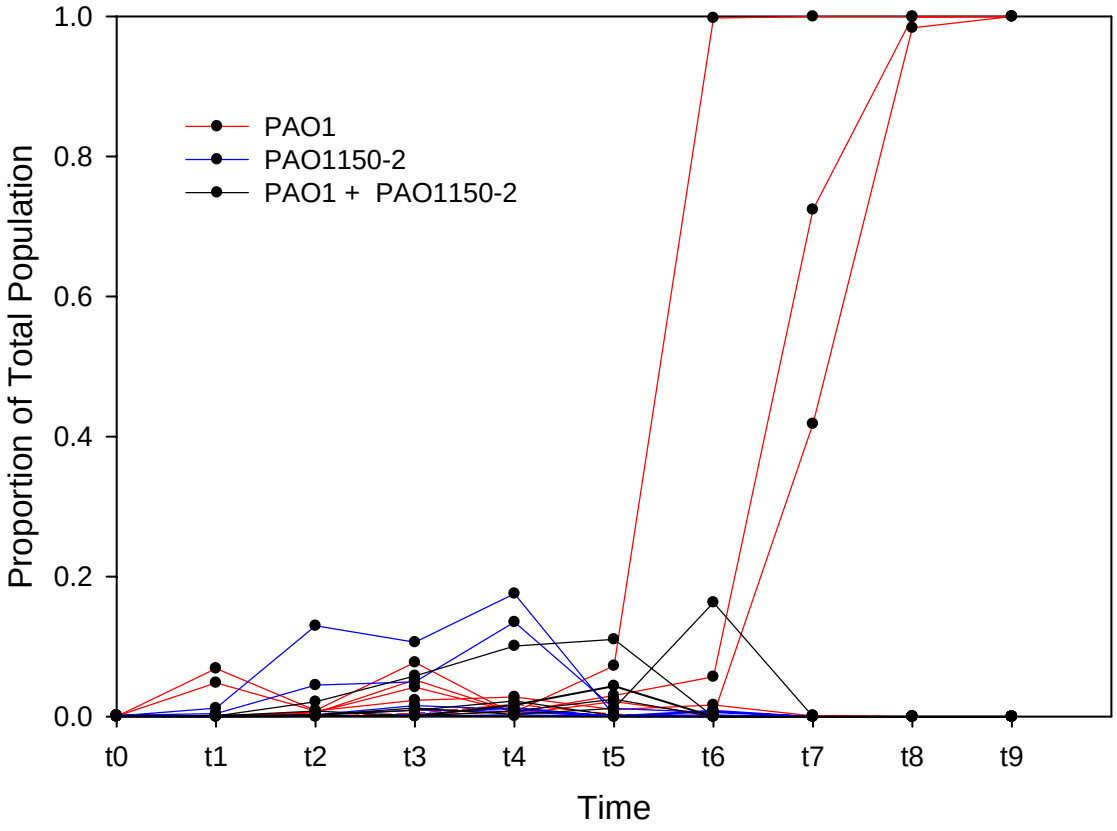

Figure 7.1: Invasion of cooperating punishers in a population of public goods cheats when initially rare. Only when cooperation and punishment are in linkage is the strain (PAO1) able to invade. Solely cooperating genotypes (PAO1150-2) are unable to invade, and when the linkage is broken (through a process such are recombination) the strain is no longer able to invade (PAO1 and PAO1150-2).

6.4 DISCUSSION

In this study we found that cooperative punishers were able to invade a population of cheats when initially rare in the population. Through the action of the bacteriocin they are able to locally increase in frequency by selectively killing sensitive cheats. Patches that contain the highest frequencies of cooperative punishers will reach the highest densities (Griffin *et al.* 2004), thereby contributing higher numbers of individuals to the metapopulation. The toxin will confer little benefit when the cooperative punishers invade new patches in the metapopulation at low frequencies (Chapter 2, (Inglis *et al.* 2009)), as the sensitive cheats are more likely to benefit from reduction of competition and greater access to resources. However, if the number of bacteriocin producing punishers inoculated into a patch is above a threshold, they will then be able to increase in frequency.

When the solely cooperative strain was competed it showed an initial increase in frequency but was unable to reach a sufficiently high proportion to invade a single patch and spread throughout the population, finally going extinct. The short term advantage of the cooperator genotype results from patches containing cooperators reaching higher densities than pure cheat patches, and hence contributing more to the metapopulation. This dynamic was mirrored when linkage was broken between cooperation and punishment traits. In this treatment the effects of recombination were created by combining the two different genotypes so half of the population had lost the ability to produce the toxin. This population was presumably unable to invade, as the solely cooperative genotype (which does not pay the cost of toxin production) is able to outcompete the cooperative punishers (Chapter 3) and also gains any benefit conferred by the toxin. This leads to a scenario where although an initial increase is possible due to action of the toxin, the population

eventually crashes as the cooperative punishers are outcompeted by the cooperators, who are then outcompeted by the cheats.

The initial increase in frequency of the pure cooperators demonstrates that the selective advantage of the cooperative punishers is not purely driven by the bacteriocin allele. This is consistent with our previous work that shows that fitness of a spiteful lineage is significantly altered by the operation of social conflict with respect to siderophore production. Moreover, 3-way competition experiments between bacteriocin producing, resistant and sensitive strains of *Escherichia coli* typically report coexistence of the three types in metapopulations (unlike here, where the sensitive type dominates) (Kerr *et al.* 2002; Czárán and Hoekstra 2003; Kirkup and Riley 2004), further suggesting the additional effect of the cooperative trait. That said, we have also previously demonstrated that the wildtype strain PAO1 is able to invade from low starting frequencies (1%; the frequency used here within a single tube in the metapopulation) against the pyoverdine-producing ancestors of the cheats (serotype O:9) used in this study (Inglis *et al.* 2009), and can also be favoured under conditions of global competition. Hence, even though siderophore production is important in determining the invasion of cooperative punishers, bacteriocins are having a greater impact on their fitness.

Although recombination occurs in many bacteria, genetic linkage is likely to be the norm (Vos and Didelot 2009). This high degree of linkage may help to explain why the maintenance of cooperative genes is common in bacteria. However, toxins such as bacteriocins are often located on plasmids (Riley and Wertz 2002), and so it easy to envisage scenarios where this linkage can be broken. It is also interesting to note that the

bacteriocin S2 is found on the chromosome which might suggest that plasmid borne versus chromosomal toxins may perform different ecological functions.

This punishing allele can also be viewed as a greenbeard. Greenbeards are not just important when considering cooperation, as they can also be used to direct harmful behaviours (Gardner and West 2010). This is also the case here, as only individuals expressing the immunity compound (which is closely linked to the toxin) remain unharmed. Although it was originally assumed that greenbeards were unlikely to be occur in nature because they could be invaded by "falsebeards" (individuals that display the tag but do not engage in the behaviour) (Dawkins 1976), this assumption seems incorrect as examples of greenbeards have been identified (Keller and Ross 1998; Queller *et al.* 2003). In this sense, punishment could be important in aiding the maintenance of greenbeards, punishing cheating falsebeards that might arise in the population. However, this seems unlikely in this system, as even though there is a biological association between the cooperation and punishment traits through a common receptor, mutations are still possible that halt pyoverdine production but do not affect the receptor or S2 immunity protein (Cornelis 2008). This suggests that S2 pyocin has not evolved as a means to punish cheats evolving from within clonal lineages. However, the shared receptor is likely to prevent other (genetically distant) lineages evolving to exploit the focal lineage's pyoverdine, because this exploitation would then make the strain potentially susceptible to pyocins produced by the focal strain. Moreover, this results in a situation where toxin production may act to promote the diversity of the greenbeards for cooperation (if a lineage produces the receptor but does not produce the immunity compound, there would a strong selection pressure to alter to the receptor). This would result in a frequency dependent mechanism, whereby

common receptors (greenbeards) would be the most common targets for bacteriocins; analogous to host resistance against bacteria (Hamilton *et al.* 1990)

In this study, we have shown that linkage between punishment and cooperation can favour the invasion of cooperation alleles. The primary (though not the sole) mechanism is simply hitch-hiking of the pyoverdine allele with the bacteriocin allele, but the pyoverdine alleles do, however, have some selective advantage in this metapopulation context. The system we have used is primarily a model for punishing, rather than evolved for this purpose, but punishing defectors could certainly confer an evolutionary advantage under conditions of intermediate relatedness (Frank 1998; El Mouden and Gardner 2008). Some tentative examples of punishing systems (specifically evolved for this reason) can be found in *P. aeruginosa* (Goodwin *et al.* 1972), *Bacillus subtilis* (González-Pastor *et al.* 2003), and *Streptococcus pneumoniae* (Cornejo *et al.* 2009), where self-killing toxins are produced and may act as a type of cheater control (Travisano and Velicer 2004).

CHAPTER 8

DISCUSSION AND CONCLUSION

This body of work has largely been concerned with elucidating ecological conditions that favour the production of spiteful bacteriocins and how this can be understood in terms of bacterial virulence. Prior to these experiments, our understanding of spite in bacteria was limited to theoretical predictions. As a thesis these studies provide some of the first experimental evidence illustrating how bacteriocin production closely follows many of the mathematical predictions made about spiteful behaviour. It also confirms the importance of considering sociality when considering interactions between bacteria.

I began this thesis by introducing the topic of social evolution and how it relates to kin selection and spite. I then reviewed previous examples of behaviours that have been identified as spiteful, finally arguing that bacteriocin production can be considered to be spiteful. The experimental system is discussed with reference to previous experiments that have studied bacteriocins in an evolutionary and ecological setting. This section is concluded with a discussion of the molecular mechanisms that are involved in the production of bacteriocins in the study organism *Pseudomonas aeruginosa*.

Chapter 2 forms the experimental foundation for the whole thesis as it required the development of a novel system to test and control the effects of bacteriocin production. More importantly, it provides the first experimental evidence that spiteful bacteriocin production is favoured (as predicted by mathematical models) when it is at intermediate frequencies in the population. These intermediate frequencies of bacteriocin producers also correspond to a reduction in virulence in our caterpillar model system (also predicted by mathematical models). This model gives a simple insight into the mechanism at work in both these cases. When the spiteful lineage is at low frequencies in the population, the

reduction in competition resulting from the production of bacteriocin will be experienced to a greater extent by the sensitive strain, whereas when the spiteful lineage is frequent there is very little relative benefit to be gained by killing a rare competitor. Only at intermediate frequencies does bacteriocin production confer the largest fitness benefit by reducing competition and increasing resource availability. This also consistent with a reduction in virulence, as overall bacterial growth will be reduced when the spiteful strain is engaged in microbial genocide.

I then proceed to consider how the scale of competition can affect bacteriocin production. Scale of competition has previously been shown to be important in facilitating cooperation and spite. This study is again performed in conjunction with predictions made by simple mathematical models. I find, experimentally, that bacteriocin production is favoured under conditions of low relatedness and both local and global competition as this maximises the interaction between producing and sensitive individuals.

Experiments so far have only considered the spiteful trait in isolation, but natural populations of bacteria are likely to be engaged in several, simultaneous interactions that can be considered social. To understand how these types of behaviours can interact, we investigated how cooperation can affect the previously established relationship between starting frequency and spite. Following on from mathematical models, we find that when in competition with public goods cheats, spite and cooperation act synergistically to favour these spiteful cooperators when at higher frequencies in the population. I suggest that this is due to the cheating strain's ability to usurp the excess iron-bound siderophores when at low starting frequencies, thereby increasing their overall frequency in the population. This in turn increases the benefit of being spiteful, as there are more sensitive individuals to kill.

This synergism also affects bacterial virulence, and we now find that greatest reduction in virulence when spiteful cooperators are at low and intermediate starting frequencies. In this environment cheats reduce the growth potential of co-operators though their exploitation of the public goods, while spiteful cooperators actively decrease the density of the cheats through the action of their toxin. These two processes combine to reduce the overall density of bacteria.

One facet of bacteriocin production which has yet to be explored is the ability of producing bacteria to absorb and neutralise their own toxin (I refer to this as "soaking"). This may explain why we observe a dip in fitness when bacteriocin producers are initially common in the population as the action of their toxin is reduced. Although we do find that when an isogenic, knockout mutant that no longer absorbs its own toxin is competed with the sensitive strain, it is much more efficient at killing the sensitive strain when at high starting frequencies, these are still qualitatively similar our previously described results (Chapter 2). This type of soaking interaction may have important implications in natural microbial communities where many bacteria may be able to "soak" a number of different toxins.

The evolution of resistance is another aspect of the ecology of bacteriocin production that I examine in more detail. The previous chapters have demonstrated that there is a large fitness benefit to producing pyocin S2, but this will also create a strong selection pressure for sensitive strains to become resistant. I find that resistance to the pyocin S2 can evolve but it is dependent on the environment. When conditions are iron limited, resistance evolve less often and more slowly. I suggest that this is due cost of resistance being increased as the receptor for S2 also binds pyoverdine (an iron chelating

molecule important for bacterial growth when iron is scarce). These results remain tentative, and further work is required to confirm the mechanisms involved.

Finally I consider how spiteful bacteriocin production in this system can be thought of as a method to punish individuals that do not engage in public goods cooperation. As previously mentioned, both the pyocin S2 and pyoverdine type I are translocated into the cell via the same cell surface receptor FpvA. Punishment is often invoked to explain how cooperation can evolve, but must be closely linked to cooperative for this mechanism to be effective. This entails that only individuals that produce the immunity compound (closely linked to S2 production) are able to benefit from the public good. I specifically test how this relationship is altered after linkage between the cooperative and spiteful trait is broken. I find that cooperative punishers are able to invade population of public goods cheats when initially rare, but when between the traits is broken they are no longer able to invade. Toxin production may, therefore, be an important component in enforcing cooperation in microbial populations.

The work presented in my thesis suggests several possible avenues for further investigation. Experiments, so far, have only considered a single bacteriocin type interacting with a single sensitive strain. However, under natural environmental conditions multiple bacteriocins will be present in the environment. Although resistance can evolve to single bacteriocin, it is unclear whether a single strain will be able to evolve resistance to all bacteriocins present, due to the high costs of multiple resistance. This may in part explain why so many different bacteriocin types occur naturally and represents an interesting area to explore. Bacteriocin resistance in general, remains an important topic of research, especially as these types of compounds are currently undergoing medical trials.

Moreover, there is considerable diversity in siderophores, probably as a result of selection to avoid exploitation by cheats; analogous to frequency-dependent selection of host resistance against parasites (Hamilton *et al.* 1990). How the diversity within these two system interact is unclear, and requires further investigation

Chapter 4 also highlighted the importance of considering situations where multiple social traits are involved. Further experimentation could include relatively simple studies examining traits such as spiteful bacteriocin production in cooperative biofilm formation, or more involved studies could be undertaken where several traits are followed. However, it is likely that many traits will only be important under certain environmental conditions so growing bacteria in more realistic environments such as soil microcosms becomes extremely important. Conversely strains could be isolated from infected individuals or grown in mice models to ascertain social traits involved in virulence.

I have also considered only bacteriocins as a functionally similar class of toxins, but this is a simplification that may miss much interesting biological detail. For example, S type pyocins are able to disperse much greater distances that R and F types. R and F have also been shown to have a much broader killing spectrum. This suggests that these toxins may play extremely different ecological roles. Understanding these differences may give us further insight into why bacteria engage in the production toxins.

In conclusion, though, there is a large scope for further work, I hope I have convinced the reader that spiteful bacteriocin production represents an important social interaction, and that we now understand how a range of different ecological conditions can affect its production and consequences such as disease virulence.

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APPENDIX

Spite and virulence in the bacterium *Pseudomonas aeruginosa*

R. Fredrik Inglis[a,1], Andy Gardner[b], Pierre Cornelis[c], and Angus Buckling[a]

[a]Department of Zoology, University of Oxford, South Parks Road, Oxford OX1 3PS, United Kingdom; [b]Institute of Evolutionary Biology, University of Edinburgh, West Mains Road, Edinburgh EH9 3JT, United Kingdom; and [c]Department of Molecular and Cellular Interactions, Microbial Interactions Group, Flanders Institute for Biotechnology and Vrije Universiteit Brussel, Pleinlaan 2, 1050 Brussels, Belgium

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Social interactions within populations of pathogenic microbes may play an important role in determining disease virulence. One such ubiquitous interaction is the production of anticompetitor toxins; an example of a spiteful behavior, because it results in direct fitness costs to both the actor and recipient. Following from predictions made by mathematical models, we carried out experiments using the bacterium *Pseudomonas aeruginosa* to test under what social conditions toxin (bacteriocin) production is favored and how this in turn affects virulence in the larvae of the wax moth *Galleria mellonella*. Consistent with theory, we found that the growth of bacteriocin producers relative to sensitive non-producers is maximized when toxin producers are at intermediate frequencies in the population. Furthermore, growth rate and virulence in caterpillars was minimized when bacteriocin producers have the greatest relative growth advantage. These results suggest that spiteful interactions may play an important role in the population dynamics and virulence of natural bacterial infections.

allelopathy | bacteriocins | disease | kin selection | microbial evolution

In recent years there has been a growing interest in understanding the evolution of social behaviors in microbes (1). The evolution of cooperation (behaviors that benefit the recipient) has received considerable theoretical and empirical attention, whereas the evolution of spite (behaviors that harm both the actor and recipient) has been relatively neglected. Conditions that favor the evolution of spite can be understood in terms of selection maximizing an individual's inclusive fitness (transmission of one's own genes and of one's own genes in other individuals). Spiteful behaviors can, therefore, theoretically evolve when they target individuals that are less likely to share the same genes as the actor than an average member of the competing population. That is, the relatedness between the recipient and the actor is negative (2–8).

Spiteful behaviors found in nature are surprisingly common, and one well-documented example is the production of bacteriocins. Bacteriocins are extracellular antimicrobial compounds produced by almost all bacteria (9). They can be considered spiteful, because they are costly to produce and because they kill susceptible cells via a range of mechanisms, including enzyme inhibition and the breakdown of DNA and cell membranes. The costs of production can be suicide (in *Escherichia coli*, for example, cell lysis is required to release the bacteriocins), but even where cell death is not required there will be an inevitable metabolic cost that is likely to be greater than the direct fitness benefits. Bacteriocins are highly diffusible; hence, the producing cell is unlikely to experience the benefit of killing a competitor (9, 10). Crucial for the evolutionary maintenance of bacteriocin production is that bacteriocins specifically target nonrelated individuals while doing no harm to the bacteriocinogenic cells, usually due to immunity factors that are genetically linked to the toxin (9). Note that relatedness in this context specifically refers to similarity at the bacteriocin loci between interacting individuals rather than average similarity across the whole genome. In this sense, bacteriocins can be viewed as spiteful green beards, whereby the same gene complex is capable of directing spite toward individuals that do not have the gene complex for the spiteful behavior (11).

A number of theoretical and empirical studies identify ecological conditions that favor the maintenance of spite (12–18). Assuming that individuals possess mechanisms to distinguish between related and unrelated individuals (2), spiteful behaviors are predicted to evolve to maximal levels when the frequency of individuals with the same spiteful trait makes up some intermediate frequency of the population (16). If the spiteful group is at a high frequency in the interacting population, spite will be less favored because the reduction in the competition resulting from the spiteful action will be small compared to the costs of being spiteful. Conversely, if the spiteful group is at a low frequency in the interacting population, the few individuals that are targeted will be on average no less related than the individuals that are not targeted. Hence, relatedness will be zero or weakly negative. This result leads to the prediction that spite will be most favored when the spiteful genotype is at an intermediate frequency in the interacting population. Note that in a previous paper (16) we refer to frequency of a particular genotype within the interacting population as "kinship." This term has a different meaning to relatedness, which refers to similarity between actor and recipient relative to the competing population as a whole.

An explicit test of the predicted unimodal relationship between spite and the frequency of spiteful genotypes has yet to be carried out. Existing empirical studies are, however, consistent with this prediction. Specifically, it has been shown in vitro that toxin producers can invade sensitive populations only when they are above a threshold starting frequency in both *E. coli* and the yeast *Saccharomyces cerevisiae* (12, 17).

Understanding how the genetic population structure of microbial pathogens affects production of bacteriocins has important applied implications, most notably in terms of the amount of harm infections cause their hosts (virulence). Attenuated virulence is predicted to coincide with maximal levels of spite (16), because under these conditions the growth rate of the infecting population will be lowest, as a result of increased killing and investment into the spiteful behaviors.

Here we use the opportunistic human pathogen *Pseudomonas aeruginosa* and a caterpillar model to explicitly test the predictions that (*i*) bacteriocin production is most favored when the spiteful genotype is at intermediate frequencies in the interacting population and (*ii*) that this results in minimal in vivo population growth rate and virulence. We also extend our previous evolutionary mathematical models to confirm that the qualitative predictions still hold in the ecological context of this experimental system.

Author contributions: R.F.I., A.G., and A.B. designed research; R.F.I. and A.G. performed research; P.C. contributed new reagents/analytic tools; R.F.I. analyzed data; and R.F.I., A.G., P.C., and A.B. wrote the paper.

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[1]To whom the correspondence should be addressed. E-mail: robert.inglis@zoo.ox.ac.uk.

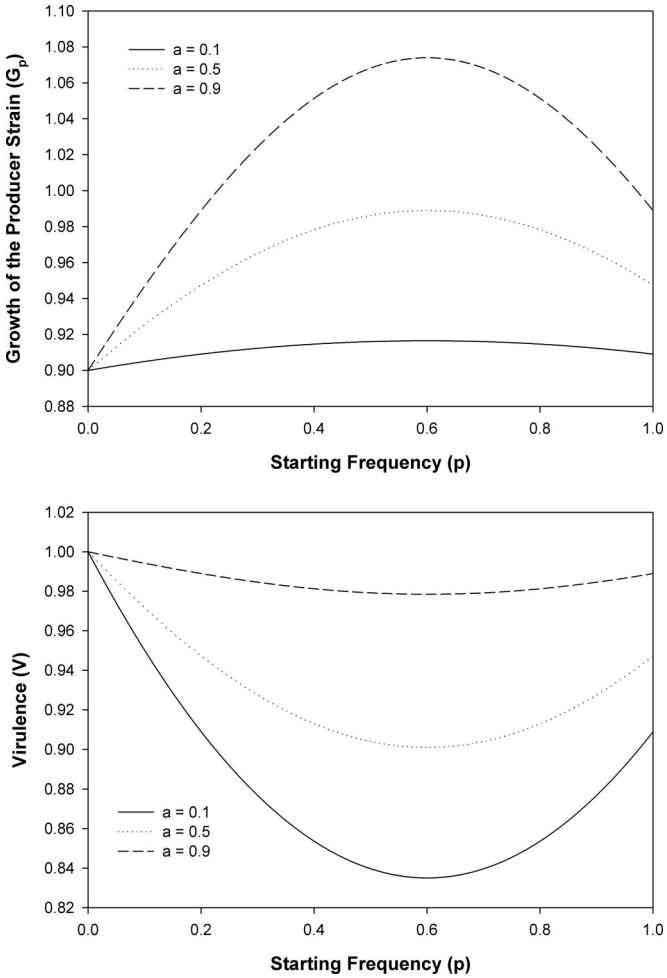

Fig. 1. Modeling the relative growth and virulence of a bacteriocin producer. Output from our mathematical models across a range of resource competition (*a*), when $c = 0.1$ and $k = 0.5$, showing that producers growth (*Upper*) is maximized at intermediate frequencies and at these frequencies virulence (*Lower*) is attenuated.

Results

A simple mathematical model was developed to describe the ecological conditions that favor bacteriocin production. In this model, a focal lineage of bacteriocin-producing bacteria compete with sensitive bacteria and remain insensitive to the bacteriocins of its sensitive competitors. Under these conditions (which are described in more detail in *Materials and Methods*), bacteriocin production provides the greatest advantage when producers are at intermediate frequency in the population and virulence is reduced at these frequencies. Examples of the growth rate of the producing strain at different frequencies and extent of local competition are shown in Fig. 1. Note that same qualitative relationships hold if the *y* axis displays the selection coefficient of the producing strain (assuming a minimum susceptible strain growth rate that is greater than zero, as observed in the experiment).

In our in vitro experiments we manipulated densities of both a bacteriocin-producing strain (PAO1, producer of pyocin S2) and a sensitive competitor (*P. aeruginosa* O:9) to create a range of different starting frequencies with respect to the producer (between 0 and 1) (19). As a control, we established the same range of starting frequencies for an isogenic mutant (PAO1150-2) of PAO1 that did not produce a bacteriocin that could affect the sensitive strain. Selection coefficients were used

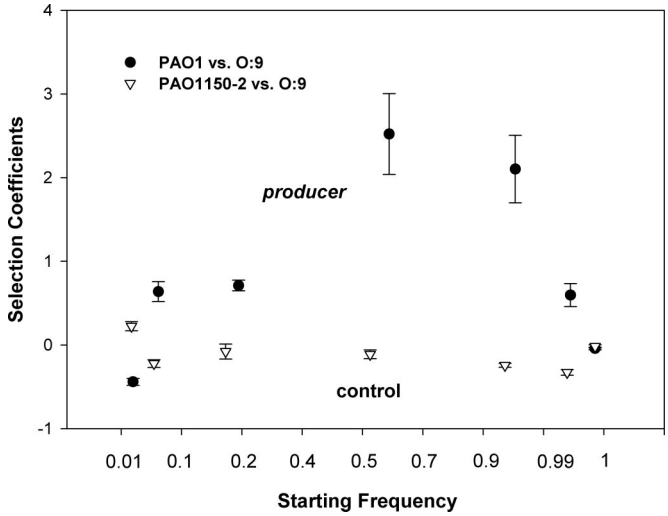

Fig. 2. Relative growth of bacteriocin producer. Relative growth rate of PAO1 vs. O:9. (producer, black circle) compared to relative growth rate of PAO1150-2 vs. O:9 (control, white triangle) along a range of different starting frequencies used to manipulate relatedness. PAO1 vs. O:9 shows a distinct peak in relative growth at intermediate frequencies (linear term, $F_{1,32} = 20.76$, $P < 0.001$; quadratic term, $F_{1,31} = 29.64$, $P < 0.001$).

to estimate the fitness of the producing and isogenic nonproducing strain relative to the sensitive strain. As predicted by the mathematical model, the fitness of the producing strains showed a unimodal relationship with starting frequency (Fig. 2), peaking at intermediate values (linear term, $F_{1,32} = 20.76$, $P < 0.001$; quadratic term, $F_{1,31} = 29.64$, $P < 0.001$). By contrast, the isogenic nonproducing strain showed a weakly negative relationship with starting frequency (Fig. 2), with a slope of -0.0975 (linear term, $F_{1,34} = 11.34$, $P < 0.002$; quadratic term, $F_{1,33} = 0.13$, $P > 0.721$). The ratio of the selection coefficients of the producing and isogenic nonproducing strain also displayed a significant unimodal relationship (linear term, $F_{1,32} = 23.44$, $P < 0.001$; quadratic term, $F_{1,31} = 25.85$, $P < 0.001$).

The effect of bacteriocin production on growth of the producer strain depends on the amount of time the bacteria spend competing. Specifically, only a weak relationship is observed between relative growth and starting frequencies before 96 h of growth, probably because of the time it takes for PAO1 to reach sufficient densities for pyocin to have an effect on O:9. Allowing bacteria to grow for longer periods of time does not qualitatively change the results but only makes detection of both PAO1 and O:9 harder at treatments with very high and low producer frequencies. As the overall numbers of bacteria decrease, there is an increasing build up of waste metabolites and depletion of resources. This dynamic is consistent with our model and is captured by the intensity of local competition ("*a*" parameter).

We next determined the relationship between virulence (as measured by time to death of infected caterpillars) and frequency of the producing strain. We manipulated the infecting bacterial populations to give high (99%), intermediate (50%), and low (1%) frequencies of the producing strain relative to the susceptible strain. Consistent with the above theory (see also ref. 16), intermediate frequencies resulted in much longer time to death than the high and low frequencies (linear term, $F_{1,58} = 52.47$, $P < 0.001$; quadratic term, $F_{1,57} = 55.85$, $P < 0.001$) (Fig. 3*A*). The proposed mechanism behind this reduction in virulence at intermediate frequencies is reduced growth rate of the population as a whole, resulting from the high mortality rates of the susceptible strain. Consistent with this view, we found that bacterial density before the death of the insects showed an

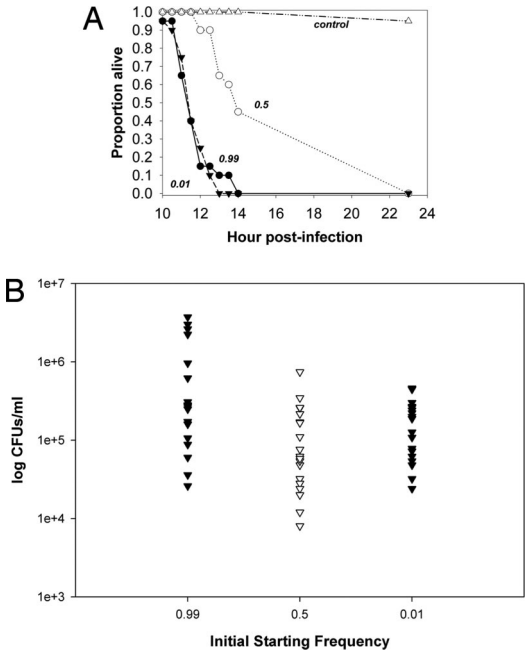

Fig. 3. Virulence and density affected by frequency of bacteriocin producer. (*A*) Time to death of caterpillars inoculated with PAO1/O:9 mixtures. Initial starting frequencies of PAO1 are indicated on the graph and correspond to the adjacent line. At the intermediate starting frequency death is significantly delayed (linear term, $F_{1,58} = 52.47$, $P < 0.001$; quadratic term, $F_{1,57} = 55.85$, $P < 0.001$). (*B*) The average total bacterial density of PAO1 and O:9 is indicated for the 3 different starting frequencies of the bacteriocin producer. A significant reduction in overall density occurs after 8 h of growth in the intermediate frequency treatment of PAO1 vs. O:9, where bacteriocin producers and sensitive non-producers are inoculated at initially near equal densities.

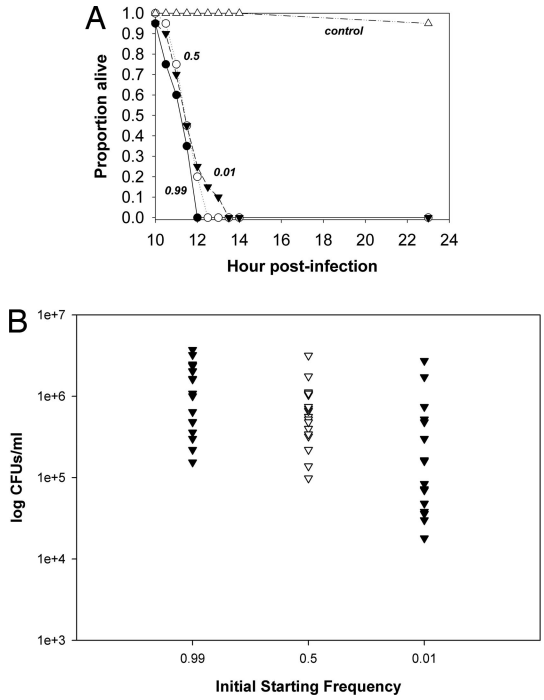

Fig. 4. Virulence and density are unaffected by frequency when bacteriocins are not produced. (*A*) Time to death of caterpillars inoculated with PAO1150-2/O:9 mixtures. Initial starting frequencies of PAO1150-2 are again indicated on the graph and correspond to the adjacent line, but in this case there is no significant difference delay in time to death (linear term, $F_{1,58} = 1.28$, $P > 0.263$; quadratic term, $F_{1,57} = 0.65$, $P < 0.422$) (*B*) The average total bacterial density PAO1150-2 and O:9 is indicated for the 3 different starting frequencies of the bacteriocin-negative mutant. There is no significant difference in overall density after 8 h of growth in the caterpillars between the different starting frequencies.

inverse unimodal relationship with frequency of the bacteriocin producer, such that density was lower for the 50% treatment (linear term, $F_{1,59} = 4.5$, $P < 0.038$; quadratic term, $F_{1,58} = 7.99$, $P < 0.007$) (Fig. 3*B*). Note that when the nonproducing strain was competed with the susceptible strain, there was no significant difference in virulence between the high, intermediate, and low starting frequency treatments ($P > 0.25$ for both linear and quadratic terms) (Fig. 4*A*), and only a linear relationship exists between density and starting frequency of the non-producer (linear term, $F_{1,58} = 16.24$, $P < 0.001$; quadratic term, $F_{1,57} = 2.66$, $P > 0.108$) (Fig. 4*B*).

Discussion

In this study, we show that a unimodal relationship exists between the growth of spiteful, toxin-producing bacteria when competing with susceptible strains and their starting frequency. Furthermore, we demonstrate that conditions that favor spiteful behaviors result in minimal virulence in caterpillar hosts, as a result of reduced population growth rate. Finally, we show theoretically that this unimodal relationship between the fitness of bacteriocin producers holds in both ecological and evolutionary contexts.

It is necessary to emphasize that in our experiments the producer differs from the sensitive strain in other ways than pyocin production and susceptibility, because the strains are not isogenic. This fact, however, does not alter our interpretation of the data, because the non-producer, which is isogenic to the producer, shows only a weak negative relationship between its growth and frequency, probably because of the slightly different resource uses of the different strains (20). Furthermore, virulence at intermediate frequencies is attenuated in our producer strain, whereas there is no difference in any treatment with the non-producer.

Our model suggests some very simple mechanisms to explain our results. When the producer is at low frequency, the benefits of reducing competition and "freeing-up" resources will be shared by the sensitive strain as much as producing strains; thus, there is little net benefit to the producing strain (relatedness is only weakly negative). Similarly, bacteriocin production has less benefit at high frequencies, because there are few competitors to kill, and hence there are fewer resources to be gained from costly bacteriocin production. Only at intermediate frequencies will bacteriocin production confer the greatest fitness advantage by killing competitors and thereby "freeing-up" resources.

The impact of spatial structure on the fitness of bacteriocin producers can also be understood in terms of the frequency of the producer in a competitive arena. In Chao and Levin's (12) experiments using *E. coli*, spatial structure was manipulated to give 2 scenarios: mass habitat and structured habitats. In mass habitats there was a frequency-dependent relationship to the success of bacteriocin production, where bacteriocin producers were only able to invade if relatively common. In structured habitats, however, bacteriocin producers are able to invade even when at low starting frequencies. Spatial structure makes individuals interact locally and through stochastic processes can result in higher local frequencies of the producer. These conditions lead to a situation in which the producer is at a high enough frequency and relatedness is sufficiently negative to allow bacteriocin producers to dominate. In our experiment we used homogeneously mixed environments similar to the "mass habitats" of Chao and Levin (12), but by considering a range of different relatedness structures we were able to show that frequency, facilitated by habitat structure, is driving this dynamic.

Consistent with theoretical results, virulence was greatly attenuated when mixing bacteriocin producers with sensitive bacteria. This result is consistent with recent studies showing that (*i*) a mixture of one bacteriocin-producing strain and sensitive strains of *Photorhabdus* and *Xenorhabdus* spp. resulted in lower virulence in caterpillars than the respective single strain infections (21) and (*ii*) mixing of *Xenorrhabdus nematophila* and its symbiotically associated nematode reduced virulence and increased susceptibility to bacteriocins (22). Here, we clarify these results by demonstrating that mixed infections show reduced virulence but only when the bacteriocin producers are at an intermediate frequency in the infecting population. Furthermore, we determine that attenuation of virulence at intermediate frequencies is almost certainly explained (as predicted) by a reduced growth rate of the infecting population as a whole, resulting from the high mortality rate of the susceptible strain. Density may not be the only important factor in determining virulence because intrinsic genetic differences between the various strains may also have a notable effect.

The specific shape (inverse unimodal) of the relationship between virulence and strain frequency of the infecting population is likely to depend entirely on the spiteful interactions (23). When other types of social interactions are more important than spite in determining the outcome of competition, different relationships are predicted. First, a monotonic negative relationship is predicted when bacteria are simply competing for resources because high diversity results in greater resource competition, leading to rapid host exploitation and increased virulence (24). Second, a positive relationship between virulence and diversity is predicted when bacteria need to cooperate to grow, because cooperation is most likely to be favored when diversity is low (25, 26). What remains to be investigated, both theoretically and empirically, is how the relationship between virulence and strain frequency is affected when multiple social interactions are important to the outcome of competition.

This study has provided experimental evidence of how strain frequency and, in turn, relatedness affects bacteriocin production. Note, however, that we have specifically measured local fitness and not global fitness under conditions that favor bacteriocins. We have also ignored any evolution of nonproducing resistance types (that would lead to a "rock–paper–scissors" interaction) (14, 15), because we are only competing producing versus sensitive or nonproducing versus sensitive strains. However, nonproducing resistance in competition with producing resistance represents a form of social cheating, and thus we can apply this kin selection framework to understand this problem in the future.

Here we have shown that spiteful behaviors, or more specifically, bacteriocin (pyocin) production is crucially affected by the frequency in the population of a given strain. We have also shown that pyocin production can have a major impact on the virulence of *P. aeruginosa* infections. The study may ultimately have practical applications in terms of manipulating the competitive arena such that toxin producers are favored and therefore reduce virulence. Pyocin production in *P. aeruginosa* is also likely to be important in a clinical setting, especially in diseases such as cystic fibrosis, where pyocin-producing strains are commonly found (27) and different strains are often outcompeted as the disease progresses.

Materials and Methods

Model. ***Bacteriocins.*** We consider 2 strains of bacteria growing under resource competition, with the focal strain making a relative investment c into bacteriocin production and the competitor strain making no such investment. We assume that the focal strain is immune to its bacteriocin, but a proportion pk of cells of the competitor strain is killed, where p is the proportion of the focal strain in the local medium.

The ''per capita'' growth of the focal (producing) strain (the growth scaled to that of a nonproducer strain in pure culture) is given by

$$G_{\mathrm{P}} = \frac{1-c}{1-a(pc+(1-p)pk)}, \qquad [1]$$

where a is the extent of local competition for resources (e.g., the degree of soft selection), and the growth of the competitor (nonproducing) strain is

$$G_{\mathrm{N}} = \frac{1-pk}{1-a(pc+(1-p)pk)}. \qquad [2]$$

The total growth is given by

$$G_{\mathrm{T}} = \frac{1-(pc+(1-p)pk)}{1-a(pc+(1-p)pk)}. \qquad [3]$$

Thus, in the extreme of complete local competition ($a = 1$), the total growth is fixed at $G_{\mathrm{T}} = 1$.

The growth of the focal (producing) strain is independent of its local frequency p in the absence of resource competition ($a = 0$), and is given by $G_{\mathrm{P}} = 1 - c$. Here, the bacteriocin producer always exhibits lower growth than a pure culture of the nonproducing strain (i.e., $1 - c < 1$). In the presence of local competition for resources ($a > 0$), the growth of the producing strain is dependent on its local frequency; the derivative

$$\frac{dG_{\mathrm{P}}}{dp} = \frac{a(1-c)(c+k-2pk)}{(1-a(pc+(1-p)pk))^2} \qquad [4]$$

takes the same sign as $c + k - 2pk$, i.e., $dG_{\mathrm{P}}/dp > 0$ when $p < (c + k)/2k$ and $dG_{\mathrm{P}}/dp < 0$ when $p > (c + k)/2k$. Thus, the growth of the producing strain is a monotonically increasing function of its frequency if $c > k$, and a unimodal-shaped function of its frequency if $c < k$. In particular, the growth of the producing strain is $G_{\mathrm{P}} \to 1 - c$ as $p \to 0$, and $G_{\mathrm{P}} \to (1 - c)/(1 - ac)$ as $p \to 1$. Note that $(1 - c)/(1 - ac) < 1$ so, if $c > k$, the growth of the producing strain is always less than that achieved by a pure culture of the nonproducing strain. If $c < k$ then growth of the producing strain is maximized at the $p^* = (c + k)/2k$, and here it is given by $G_{\mathrm{P}} = 4(1 - c)k/(4k - a(c + k)^2)$, which exceeds the growth of the nonproducing strain in pure culture if $a > 4ck/(c + k)^2$. Note that c (the cost to the producer) must be $< k$ (the maximum cost experienced by the recipient) for pyocin production to be maintained by natural selection.

Assume that the above growth is occurring in a single subpopulation of a much larger structured population in which the producing strain is vanishingly rare and that the focal subpopulation is representative of all of the subpopulations in which the producing strain is located. Then the local frequency (p) of the producing strain is equivalent to the kin selection coefficient of relatedness (r) describing the genetic similarity of cells of the producing strain to the other cells growing in its locality. The producing strain is expected to invade from rarity if its growth is greater than the average in the whole metapopulation (nonproducing strain in pure culture), i.e., when $G_{\mathrm{P}} > 1$. This yields the condition

$$\frac{1-c}{1-a(pc+(1-p)pk)} > 1, \qquad [5]$$

which may be reexpressed as

$$\left(-\frac{ap}{1-ap}\right)(-(1-p)k) > c \qquad [6]$$

which is of the form $RB > C$, where $R = -ap/(1 - ap)$ is Queller's (28) form of relatedness (genetic similarity of social partners relative to competitors), and is equivalent to equation A2 in Gardner, West, and Buckling (16).

Virulence. Now consider that each subpopulation represents a single host individual carrying a bacterial infection. Assume that the virulence of the bacterial infection is proportional to its growth, i.e.,

$$V = bG_{\mathrm{T}}. \qquad [7]$$

Under the extreme of complete resource competition ($a = 1$), bacterial growth is $G_{\mathrm{T}} = 1$ and virulence is fixed at $V = b$. With less intense resource competition ($a < 1$), virulence is dependent on the frequency of the producing strain within the infection; the derivative

$$\frac{dV}{dp} = -b\,\frac{(1-a)(c+k-2pk)}{(1-a(pc+(1-p)pk))^2} \qquad [8]$$

has the opposite sign of $c + k - 2pk$, i.e., $dV/dp < 0$ when $p < (c + k)/2k$ and $dV/dp > 0$ when $p > (c + k)/2k$. The sign of dV/dp is always opposite of that of dG_P/dp, and so virulence is monotonically decreasing with the frequency of the producing strain when $c > k$ and is a U-shaped function of the frequency of the producing strain when $c < k$. In particular, virulence is maximized in the absence of bacteriocin production ($p = 0$), and is minimized when the producing strain is at the intermediate frequency $p^* = (c + k)/2k$.

Bacterial strains. *P. aeruginosa* strain PAO1 was used as the bacteriocin producer, and serotype O:9, as the bacteriocin-sensitive competitor PAO1, is a known producer of pyocin S2, whereas serotype O:9 is sensitive to S2 pyocins (19, 29). PAO1150-2, a transposon bacteriocin-knockout mutant of *psy2*, acted as a nonproducing, isogenic control strain (30). Bacteriocin production in *P. aeruginosa* can involve lysis, but it is not clear whether it is essential for the release of the soluble pyocins that are the focus of this study (10). Bacteriocin production, sensitivity, and insensitivity were confirmed by using a simple plate assay where the production of relevant bacteriocin is determined by overlaying bacteria mixed in semisolid agar on plates that have been spotted with bacteria of another strain, as described by Fyfe et al. (31). If the strain inoculated on the plate produces bacteriocin that kills the strain mixed with semisolid agar, a halo-shaped zone of clearing can be observed in the bacterial lawn after incubating at 37 °C for 18 h. The absence of a clear halo indicates that either the overlaid strain is insensitive to the bacteriocin producer or the inoculated strain does not produce any bacteriocin.

Competition Assays. Overnight cultures of each strain were grown with shaking at 0.65 × *g* at 37 °C for 18 h and then diluted to an OD_{600} of 1.8 to ensure similar numbers of bacteria per milliliter. These cultures were subsequently grown on agar plates to determine the number of bacteria present, with colony forming units (CFUs) as an approximate measure. Thirty-milliliter glass universals containing 6 ml of Kings Media B broth were inoculated with a total of 10^4 cells with different starting frequencies of the individual strains. PAO1 and O:9 where competed against each other at starting frequencies of 99%, 90%, 50%, 10%, 1%, and 0.1%. This exact design was replicated in the PAO 1150-2 and O:9 competition. Cultures were propagated in a shaking incubator at 0.65 × *g* at 37 °C and sampled at 48 and 96 h, allowing time for the effect of the bacteriocin to be observed.

At each time point (48 h and 96 h), we calculated the relative growth of the producer to sensitive and non-producer to sensitive at the different starting frequencies. This was done by plating the various treatments on KB agar plates and counting the number of CFUs for each strain. All strains were easily distinguishable from one another because of unique colony morphology and size. At the more extreme frequencies, antibiotic plates were required to give better resolution of colony counts, and this was possible due to the different antibiotic resistance profiles of the assorted strains (PAO1 resistant to 1,250 μg/ml streptomycin; O:9 resistant to 312.5 μg/ml rifampicin; and PAO 1150-2 resistant to 312.5 μg/ml tetracycline). Selection coefficients (*S*) were used to estimate at what frequency bacteriocin production is favored in PAO1 relative to 1150-2 using the common competitor O:9, where $s = (m_j - m_j)/m_j$, and *m* refers to ln(final density/starting density) of strain *j* (in this case either PAO1 or 1150-2) and strain *j* (O:9) (32). All frequencies were replicated 6 times, and statistical analyses were performed in Minitab 15. Selection coefficients were preferable to simply using growth rates (*m*) to control for between-tube variation.

In Vivo Virulence Bioassay. Virulence assays were performed as described by Harrison et al. (33). Briefly, overnight cultures of PAO1, O:9, and PAO1150-2 were diluted in minimal salt solution. Fifth-instar waxmoth (*Galleria mellonella*) larvae (Livefood UK) were randomly allocated to be inoculated with 10^4 CFUs of PAO1/O:9 and PAO 1150-2/O:9 mixtures. The starting frequencies of the bacterial combinations consisted of 99%, 50%, and 1% PAO1 and PAO1150-2 to O:9. Larvae were swabbed with 70% ethanol to prevent contamination of the injection site and were injected into the abdomen with Terumo 1-ml disposal syringes and BD Microlance 30-gauge 1/2 needles. The injection volume was 50 μl in all cases. Twenty larvae were assigned to each treatment, and a further 20 larvae were injected with 50 μl of minimal salt solution as negative controls. Larvae were then incubated at 37 °C and monitored for death at 30-min intervals between 10 and 14 h and again at 24 h after inoculation. Larvae were scored as dead if they failed to respond to mechanical stimulation of the head.

Overall density of the different bacterial strains within the caterpillar hosts was also measured. Caterpillars were inoculated as previously described and incubated for 8 h at 37 °C. Larvae were then weighed, dipped in 70% ethanol to kill surface contaminants, and homogenized with a plastic pestle in 500 μl of minimal salt solution. Homogenates were centrifuged at 455 × *g* for 3 min to pellet the solid, and aliquots of diluted homogenate were plated onto KB agar. Agar plates were supplemented with 15 μg/ml ampicillin to select against growth of native larval-gut bacteria (this concentration of ampicillin does not affect the growth of *P. aeruginosa*). Plates were incubated overnight at 37 °C and subsequently scored for CFUs.

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