# Understanding the Evolutionary Ecology of Dispersal

An experimental approach using the bacterium *Pseudomonas aeruginosa*

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# Contents

# Statement of Authorship

The work presented in Chapter 2 was published as part of Taylor and Buckling (2010), The American Naturalist **176**:83-89 (included as Appendix 3). I carried out and analysed all experimental work with guidance and helpful advice in experimental design, statistical analysis and editorial suggestions from Angus Buckling.

Chapter 4 is about to be submitted to the journal PNAS. This work was done in collaboration with Antonio Rodrigues, Andy Gardner and Angus Buckling. Antonio Rodrigues and I co-authored this paper. I designed the experimental methods, collected the data and analysed the results. Antonio Rodrigues designed and analysed the theoretical model, with input from Andy Gardner. I wrote experimental details in the manuscript and Antonio Rodrigues wrote the modelling details. All authors edited and agreed upon the final manuscript before submission.

Chapter 5 was published as part of Koskella et al. (2011), ISME J (in press; included as Appendix 4), myself and Britt Koskella co-authored this paper. Bacterial and phage species used in the experiment were collected by Britt Koskella, and used for a previous experiment (Koskella et al. 2011). Preliminary work showing the correlational trends between growth and resistance in *Pseudomonas* spp. and *Erwinia* spp. was performed by a Masters student, Jennifer Bates. Experimental work and analysis in experimental evolution study was shared between myself and Britt Koskella. Angus Buckling offered helpful advice and comments in data analysis and manuscript editing.

Chapter 7 was published as part of Taylor and Buckling (2011), Evolution (in press; included as Appendix 5). I carried out and analysed all experimental work with guidance and helpful advice in data analysis and manuscript editing from Angus Buckling.

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# Abstract

For the thesis entitled: **"Understanding the Evolutionary Ecology of Dispersal"**

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Understanding dispersal is a central aim of evolutionary ecology. Theoretical analyses of dispersal have been crucial in identifying key variables which contribute to its evolution and maintenance, but the supporting empirical data remains elusive. Microbes offer a powerful model system on which ecological and evolutionary theory can be experimentally tested with controlled and replicated experiments, and with the convenient malleability of selective pressures and bacterial genomics. *Pseudomonas aeruginosa* is an ubiquitous, opportunistic pathogen that is able to induce acute or chronic infections in a broad array of hosts. As well as *in vivo* environments, *P. aeruginosa* can be found in a range of ecological habitats, from solid to aqueous, and as such requires a variety of dispersal mechanisms (including swimming, gliding on a surfactant and 'crawling') for effective colonisation and infectivity. In this thesis, I present a collection of papers which outline empirical ecological and evolutionary experiments to identify the abiotic and biotic forces that shape the evolution of these different dispersal mechanisms, with particular focus on the theoretically important role of kin competition and the structure of the abiotic environment.

# Chapter 1: General Introduction

***"Dispersal is one of the most important, yet least understood, features of ecology, population biology, and evolution." (Weins 2001)***

Throughout the natural world, dispersal strategies have dramatic evolutionary consequences from the population to the species level (Clobert et al. 2001). Because of its importance and ubiquity, dispersal has received increasing attention from biologists across disciplines (Figure 1.1). However, fundamental questions remain unanswered: is it best 'to stay' or 'to go'; and how do particular dispersal strategies evolve?

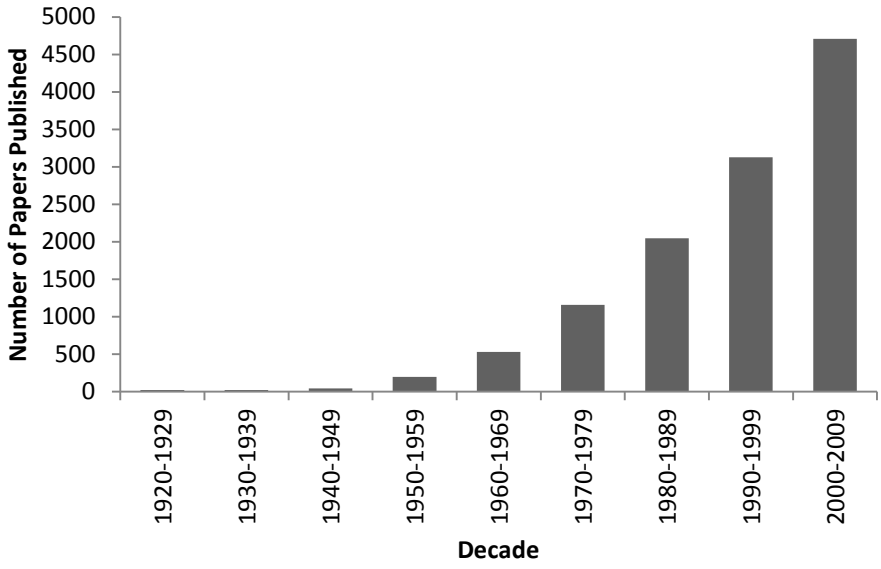


**Figure 1.1: Number of Papers Dedicated to the Topic of 'Dispersal' Over the Decades**

Using ISI Web of Knowledge[SM] (www.isiknowledge.com) inputting the search fields: Dispersal 'Topic'; Dispersal 'Title'; and each decade shown in 'Year Published'.

With so many researchers tackling the topic of dispersal there is now a somewhat large and confusing vocabulary surrounding it. Definitions of commonly used terms are

given in the glossary below (Box 1.1). For clarification purposes, those glossary terms used in this chapter, will be indicated **as so**.

In this chapter, I will highlight research in the evolution and ecology of dispersal that has been key to the development of the ideas and predictions tested in the following chapters. I will begin with a brief overview of dispersal, discussing current theories into its ultimate causes, and then examine the benefits and limitations of different research approaches. Finally, I will consider the characteristics of *Pseudomonas aeruginosa* that make it an ideal model organism for the study of dispersal, and also address the applications and limitations of the system.

## What is dispersal and why does it matter?

**Dispersal** describes the permanent relocation of organisms away from an origin, and promotes the incorporation of genes from one population into another (a process known as **gene flow**). It therefore influences the persistence and distribution of a population as well as its ability to track favourable habitats in variable ecosystems – a crucial consideration in a time of increasing global habitat fragmentation and climate change. Gene flow has the potential to increase genetic variation and significantly alter allele frequencies among populations. As such, this key evolutionary process has consequences for speciation (Barton 1979), inbreeding depression (Richards 2000), cooperation and sociality (Johnstone and Cant 2008), and many life-history traits (Bohonak 1999).

There is tremendous variation in dispersal strategies, not only between different organisms but also between individuals of the same species, with some dispersal traits being gender or age dependent. For example: pollen and seeds have adapted different and

unique dispersal methods (Howe and Smallwood 1982); many mammalian and bird species show sex-biased dispersal (Pusey 1987); in particular, vertebrates show fundamental differences between natal and **breeding dispersal** (Greenwood and Harvey 1982); and, with organisms which undergo different life-history morphologies (such as many insects) dispersal strategies can vary dramatically between larval and adult forms (Harrison 1980). To add to the complexity, dispersal is not necessarily a fixed trait but can act as a plastic response in reaction to different environmental conditions (Murren et al. 2001). In order to understand the abundant and complex processes which shape the evolution of dispersal one must understand (1) the costs and benefits directly influencing the selective direction of dispersal, and (2) the ecological subtleties underlying it (a brief schematic overview of the selective pressures influencing dispersal is given in Figure 1.2).

In addition, to understand the maintenance and stability of dispersal strategies, it is important to consider feedbacks between evolutionary and ecological processes associated with dispersal, as population dynamics and the evolution of dispersal are highly interdependent. Specifically, demographic features of the population will determine dispersal rate, and dispersal rate will alter population demography (Ronce 2007). For example, consider a hypothetical group of cooperative breeders. Within this group relatedness will determine the helping effort (input) of the individual helping the breeder (for example, the Seychelles warbler; Komdeur 1994). High relatedness selects for cooperation, which is achieved with a low dispersal rate (Koenig et al. 1992), but a low dispersal rate will increase the effects of kin competition (*i.e.* related individuals will be competing for the same limited resources; Lambin et al. 2001), and kin competition will select to increase dispersal rate. This feedback between the ecology and evolution of dispersal will result in balancing selection, with the ideal dispersal rate being a

compromise between the two processes. In other words, the magnitude of relatedness affects the rate of dispersal, and in turn, the rate of dispersal affects the magnitude of relatedness. Other biotic factors such as resource availability, environmental stochasticity and breeding opportunities will influence this balance, resulting in a dynamic and highly complex dispersal pattern.

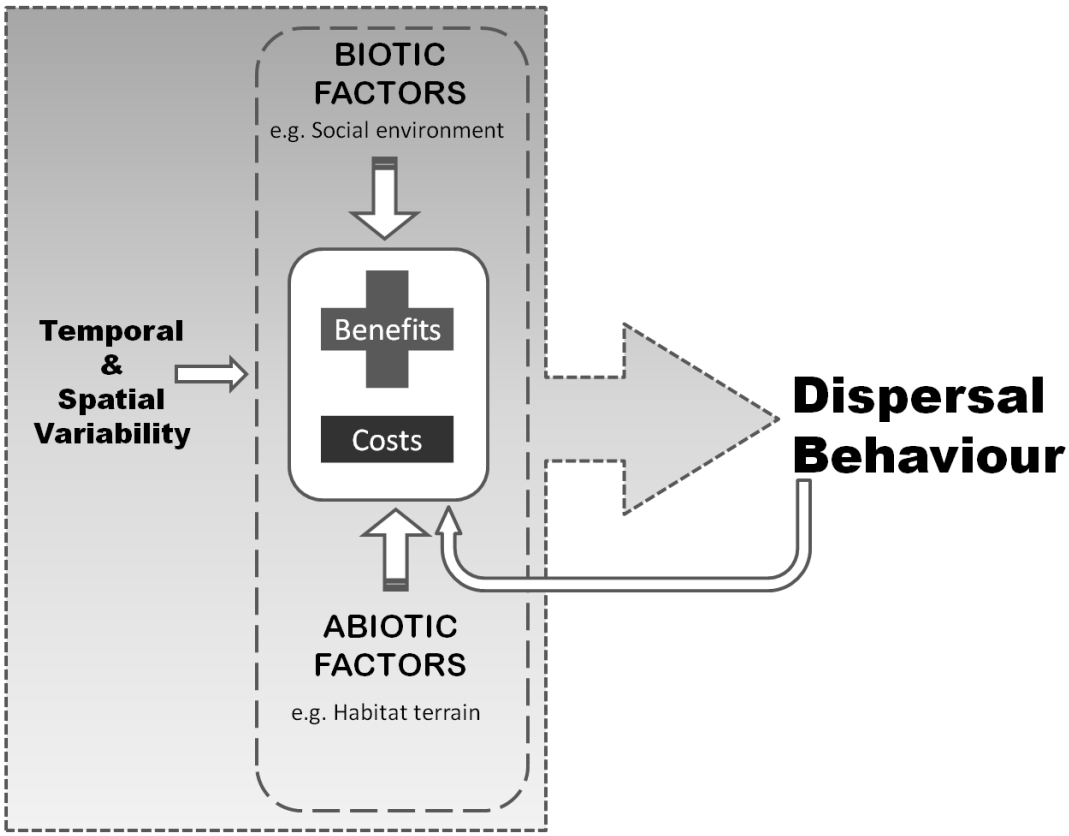


**Figure 1.2: A brief overview of the evolution of dispersal behaviour**

The evolution of dispersal will be mediated by the costs and benefits associated with it, which will determine whether dispersal will be selected for or against. In turn, these costs and benefits will change depending on biotic and abiotic factors. Moreover, the relative influence of these factors and the stability of the trait will depend on temporal and spatial stability of the environment. Dispersal behaviour will feedback onto the costs and benefits of dispersal, based on population dynamics under the current dispersal strategy. The costs and benefits of dispersal will directly determine the evolutionary direction of dispersal; the other factors are ecological processes which will influence the evolutionary trajectory.

## Major determinants for the evolution of dispersal

Before we consider '*how'* an individual disperses, we must ask '*why'* an individual might disperse. If an individual is in a suitable habitat, why risk moving to an area of unknown habitat suitability and potential competition with locally adapted rivals? Despite evident risks, nearly all species disperse and many have acquired elaborate adaptations for doing so. For example: slime moulds normally exist as solitary amoeba, however under conditions of starvation thousands will aggregate together and form a differentiated, multi-cellular organism known as a 'slug'. This slug enables efficient migration towards the soil surface where it metamorphoses into a stalk which holds up a fruiting body of spores facilitating more efficient dispersal (Kuzdzal-Fick et al. 2007; Crespi 2001); Ballistic fruits, like members of the Violaceae, exhibit explosive seed dispersal, where valves in the seed pod walls exert large pressure on the seeds until the pod breaks open with enough force to propel the seeds several meters from the parental plant (Beattie and Lyons 1975; Howe and Smallwood 1982); And certain species of fungi are known to mimic floral anatomy in order to trick pollinating insects to collect fungal spores and disperse them. For example, the violet-spored smut fungus (*Ustilago violacea*) replaces the host plant's pollen with purple fungal spores, tricking pollinators into assisting dispersal and sexual reproduction of the fungus (Roy 1994; Brown and Hovmoller 2002).

Both biotic and abiotic factors have been proposed as the causes of dispersal (Johnson and Gaines 1990). These can be formalised into three commonly applied theories:

*(i)* **Kin selection theory** (Hamilton and May 1977):

**Global competition** occurs between mobile individuals dispersed throughout a large area, whereas **local competition** is restricted to among individuals exploiting the same resources within a finite area; when limited dispersal results in relatives aggregated together and competing for the same resources, then local competition becomes **kin competition** (Lambin et al. 2001).

The selective force of kin competition on the evolution of dispersal attracted strong attention after a seminal paper by Hamilton and May (1977). This theoretical research suggested that within stable environments, even when migrant mortality is high and the environment offers no empty sites for colonisation, it is still advantageous for more than half the population to disperse away from their natal site. Specifically, this distribution organises competitive interactions between unlike genotypes, and thus, increases **indirect fitness** because costly competitive interactions will not be directed towards kin members.

This has become a key theoretical argument for the evolution of dispersal in stable environments, but the empirical evidence is elusive. There have been a few cases described in vertebrates (Cote et al. 2007) which suggest that dispersal caused by the avoidance of kin competition is more prevalent between parents and offspring than between siblings. But contradictory to this prediction, there are more studies which suggest that frequent kin interaction actually encourages philopatry more than it increases dispersal rates (Lambin et al. 2001).

*In Chapters 2 and 3 I provide: firstly, an explicit test of kin competition driving dispersal patterns, and find support for Hamilton and May's early model; and secondly, an experimental evolutionary test, which (under the experimental*

*conditions used) the availability of resources and not the relatedness of populations appeared to dictate the evolution of dispersal.*

Theoretical investigation has been crucial in identifying factors which are important for the evolution of dispersal, but due to the complex nature of dispersal evolution, these factors tend to be treated in isolation, and as such this can limit applicability. However, Gandon and Michalakis (2001) focused on three commonly cited causes for dispersal: (1) avoidance of kin competition; (2) population extinctions, and; (3) avoidance of inbreeding. They investigated the effects of these three hypotheses on their own and in duets. In each simple case (without the influence of the other) they found kin competition, population extinction and inbreeding to be positively correlated with dispersal rate. However, when each was combined with kin competition, they found a higher cost of dispersal did not necessarily select against dispersal (see also Comins et al. 1980). This is because higher costs of dispersal resulted in decreased immigration rates and therefore increased relatedness within each patch – the result was higher kin competition, which consequently increased the benefit of dispersal. It is a sign of progress that models are now beginning to incorporate multiple potential causes for the evolution of dispersal into a single model, because it allows us to see interactions which might emerge from a combination of different factors acting on the evolution of dispersal – and as the example above demonstrates, this can sometimes lead to unexpected results.

*In Chapter 4, I discuss the simultaneous action of two opposing forces on dispersal – kin competition and cooperation. The results support the findings of Gandon and Michalakis (2001) and find high costs of dispersal can select for higher dispersal*

*rates. In addition, it shows that dispersal is increasingly favoured with patch availability and relatedness, but that the presence of social cheats can dramatically alter these patterns, depending on their representation within the environment. Moreover, it exemplifies the value of multi-predictor models and the power of combining theoretical models with empirical data.*

*(ii)* **Habitat selection theory** (Stamps 2001):

Departure and settlement 'decisions' are required when an individual disperses. Habitat selection theory includes all behaviours during relocation between patches which influence the fitness of an individual entering or leaving a patch – *e.g.* departure due to overcrowding or increased **inbreeding**, and settlement encouraged by conspecific and heterospecific attraction (Stamps 2001).

Foundations for this theory were established four decades ago by Fretwell and Lucas (1969) in a concept called the '**ideal free distribution**' (IFD), which predicts individuals will distribute themselves across habitats based on fitness parameters (habitat quality) such that they are equally fit across patches. Therefore, the distribution of individuals within a population should provide information regarding resource quantity and quality among and between patches as a function of habitat features and density of competitors (Guthrie and Moorhead 2002).

The IFD assumes an individual has full perception of habitat suitability prior to departure or settlement. Danchin et al. (2001) suggested **public information** (that is, cues regarding the fitness of conspecifics) to be most valuable in 'deciding' whether to leave or enter a patch because of its close association with fitness. Simulation assays suggested that individuals using public information (such as comparable patch quality and conspecific success) to make settlement/departure decisions

performed better than those adopting alternative strategies (such as random settlement, philopatry and absence/presence of conspecifics) under intermediate patch stochasticty (equivalent to what is expected in nature; Cote and Clobert 2007).

Intraspecific competitive interactions have long been suggested as an important force in determining the evolution of dispersal. However, selective pressures caused by interspecific competition (via processes such as predation, parasitism, disease, and mutualisms) will also undoubtedly hold consequences for dispersal. These pressures are less well studied perhaps due to the diversity and complex nature of such interactions. It is now the task of current theory and ecological research to include multi-level complexity and to provide and examine more realistic models.

*In Chapters 5 and 6 I consider how phage might influence patterns of dispersal both in artificial and natural environments. In an artificial environment (Chapter 6), phage presence led to an increase in motility which conferred resistance to the parasite thus indirectly encouraging dispersal within the environment. However, those bacteria isolated from natural settings (Chapter 5), did not show a response to sympatric phage imposed selection on motility. The results suggest that the large amount of variability observed in the environment is likely dependent on other, stronger selective pressures, yet to be identified.*

*(iii)* **Stochastic Environment Theory** (Kuno 1981):

Metapopulations are many interconnected populations which persist in a balance between local extinction and colonisation (Harrison 1991). Extinction within a patch is obviously costly for the directly affected populations, but it also provides

the opportunity for dispersal and colonisation events. Any environment is subject to change, and when habitat quality varies across both space and time, so will fitness (McPeek and Holt 1992). Local patch extinction becomes more of a threat in stochastic environments (Comins et al. 1980; Levin et al. 1984; Olivieri et al. 1995; Roff 1975; Van Valen 1971) and a population must either adapt to change, or face extinction (Levin et al. 1984). However, by dispersing and colonising numerous patches, the potential for a species extinction event is reduced and genetic diversity can be increased (Hall and Colegrave 2007; Hastings 1980).

The risk of local extinction is a popular route of investigation, both via theoretical and empirical methods, intuitively suggesting that increased risks of local extinction will increase the rate of dispersal (the **local extinction hypothesis**; Holt and McPeek 1996). Temporal and spatial variability in habitat quality is an extension of the local extinction hypothesis, but it emphasises the antagonistic nature of the two factors – spatial variability selects against dispersal due to increased risks of settling in an unsuitable patch (Hastings 1983), whereas temporal variability selects for dispersal because over time the likelihood of your patch changing to an unsuitable environment increases (Travis and Dytham 1999).

A number of models have tried to tackle the effects of variable environments on dispersal (Johnson and Gaines 1990). Hastings (1983) and Holt (1985) found that spatial variation alone was not sufficient to select for increased rates of dispersal because passive diffusion took individuals from sources (high quality patches) to sinks (poor quality patches) more often than in the reverse, since the more favourable habitats tended to have more occupants in the first place.

Empirical evidence for these trends is limited, but support has been found for the theoretical effects of stochastic environments on dispersal rate. Gilbert and Singer

(1973) examined variation in dispersal rates of two populations of the butterfly species *Euphydryas editha,* and found a population that was regularly disturbed by logging activities had a significantly higher dispersal rate. These differences were found to be genetically based and most likely due to long-term selection pressures associated with the ecological pressures on each of the populations. Southwood (1962) reported that insects which colonise "temporary habitats" (such as dung, carrion, fungi and plant debris) tended to have higher migratory (which under his definition can be equated to dispersal) rates than those which live in "permanent habitats" (for example, rivers, lakes or perennial plants, which have longer generation times than the insects). His extensive meta-analysis provides key support in an overwhelmingly theoretical topic, and there is a necessity for more recent studies to adopt this approach.

Multiple causes of the evolution of dispersal have led to multiple approaches to its research. Competition, habitat, and heterogeneity of the environment will all contribute towards the evolution of dispersal; however it is only when we can understand each in sufficient detail in isolation that we can begin to bring these ideas together. This requires careful comparisons and contrasts between overlapping theories in order to differentiate between them, and importantly collaborative empirical data which might allow us to disentangle this disconnected and sometimes muddled field.

**Box 1.1 Quick Reference Glossary**

**Breeding dispersal:** movement between two successive breeding grounds

**Dispersal:** a process which permanently relocates organisms away from their origin

**Emigrate:** to disperse or migrate away from ones natal patch

**Gene flow:** homogenises geographically separated populations (increases diversity within populations); genes are able to exchange between one or more populations

**Global Competition:** competition at the level of individuals across a whole population (over a large spatial scale)

**Ideal Free Distribution (IFD):** describes a theory which predicts individuals will distribute themselves across habitats based on fitness parameters (habitat quality), such that they are equally fit across patches

**Immigrate:** to settle in a patch away from ones natal patch

**Inbreeding:** breeding between related individuals. Can lead to expression of deleterious and recessive traits via increased homozygosity

**Indirect Fitness:** fitness profit gained from facilitating the reproductive potential of non-descendant relatives (e.g. siblings)

**Kin Competition:** competition for resources between related individuals

**Local Competition:** competition at the level of individuals interacting within a population (over a small spatial scale)

**Local Extinction Hypothesis:** increasingly stochastic environments result in an increasing rate of local population extinctions

**Migration:** periodical, directional or seasonal movement

**Natal dispersal:** the relocation of an individual away from its birthplace

**Philopatry:** to stay in ones natal patch (non-dispersal)

**Public Information:** individual assessment cues regarding the fitness of conspecifics

## Studying Dispersal – an Empirical vs. a Theoretical Approach

There are a number of problems associated with both empirical and theoretical approaches to research on the evolutionary processes involved in dispersal. Among them,

empirical studies are often constrained by the difficulties associated with collecting dispersal data over large or complex species ranges. There are four processes which influence population growth: (1 & 2) birth and immigration (which increase population size); and, (3 & 4) death and emigration (which decrease population size). Unfortunately, estimating these rates using observational data has proven difficult as emigration rates are frequently confounded with mortality, especially when the study areas do not (or cannot) include maximal dispersal distances of individuals within a population (Koenig et al. 2000). Genetic techniques coupled with observational data are becoming a more favourable ecological tool, as it can provide information on gene flow without individual tracking (Waser and Strobeck 1998). But gene flow estimates only provide information regarding those migrants which have successfully established and reproduced, missing key information concerning all the immigrants who arrived and were outcompeted. In addition, as with all empirical approaches on natural populations it can only provide correlational data based on observation and not the causal evolutionary progression.

Theoretical analysis of dispersal has been crucial in locating key components which contribute to its evolution and maintenance, but without empirical synergy the applicability of these models is often narrow, and unfounded acceptance can mean the biological relevance of such models can be overlooked for too long.

## Using Microbes to Study Dispersal

Identifying mechanisms that directly influence dispersal has proved a difficult task, as one must be able to tease apart the genetic, behavioural and physiological components within a phenotypic context. Microbes offer a powerful model system with which dispersal ecology and evolutionary theory can be experimentally tested using controlled and easily replicated experiments, in real time and with the convenient

malleability of selective pressures and bacterial genomics. A brief summary of the major advantages of microbial experimental evolution are given in Box 1.2 (adapted from Elena and Lenski 2003).

**Box 1.2 | The Advantages of Using Microbes in Evolutionary Ecology**

- Fast generation times allow experiments to be conducted in real time
- Cultures can be stored in suspended animation which allows ancestral strains to be catalogued and revived
- Asexual reproduction facilitates linkage between genetic markers and experimental replicability
- Both the biotic and abiotic environment are easily manipulated
- There are numerous microbes which are important to humans – in ecology, medicine, industry and agriculture. Making the dynamics and mechanisms which drive microbial evolution important and widely applicable.
- Experimental evolution studies allow causality of evolutionary processes to be addressed

## Microbial-Specific Insights into Motility

Although microbes offer a versatile model system on which to explore general predictions of the evolution of dispersal, it is also interesting to consider dispersal and motility for the sake of understanding microbial evolutionary ecology. Microbes are essential to every ecosystem on the planet and understanding why microbes move is an interesting question – but one that has been rarely asked. The understanding of the microbial cell's motility machinery is fairly extensive (Bardy 2003), although the vast natural variation about what is considered "normal for the species" requires constant re-evaluation. However, the evolutionary questions addressing: why microbes move; what

selective pressure change the way things move; and the changeability of motility mechanisms, offer new and exciting avenues for research.

*In Chapter 7 I consider constraints within the bacterial motility mechanism and find that trade-offs between swimming and twitching might limit the available distribution of specialised strains and may also hold consequences for a pathogenic lifestyle.*

## The Role of Motility as a Virulence Factor

Motility plays a vital role in host-parasite interactions, both in the colonisation of a host and in the establishment and persistence of an infection (Josenhans and Suerbaum 2002). Chemotaxis and motility are intimately linked (Faguy and Jarrell 1999), enabling bacteria to pursue beneficial resources, such as nutrients and colonisation sites, and defect away from potentially harmful products such as toxins (Wei et al. 2001). There are numerous studies that provide evidence that non-motile isogenic pathogenic bacteria have a significantly reduced efficacy in colonisation, infectivity, or both (for review see Ottemann and Miller 1997). Specific studies related to *P. aeruginosa* have indicated the importance of motility in colonisation of a host, and the initiation of an infection. For example, Feldman et al. (1998) found isogenic flagellin mutants were unable to spread invasively in a newborn mouse model of pneumonia, whereas the wild type caused 30% mortality (see also, Drake and Montie 1988). It is therefore intriguing to consider whether motility could be an appropriate target for drug and vaccine therapies. This is a relatively unexplored area, but research by Tsutsui et al. (2000) looking at the role of motility in the ulcer-causing bacterium *Helicobacter pylori* found proton pump inhibitors (PPI) frequently used for the treatment of *H. pylori* ulcers specifically targeted the bacterium's

motility and not its cell growth. In addition, flagellar preparations of *P. aeruginosa* were developed as a vaccine formula for cystic fibrosis patients (Doring et al. 2007; Holder and Naglich 1986). Mouse trials proved effective (Holder and Naglich 1986), and human phase III clinical trials also produced high and long-lasting serum antiflagella IgG titers (Doring et al. 2007). Unfortunately, due to lack of funding these studies are no longer ongoing, but they provide a starting block for future research and development. The exact mechanisms eliciting this response are not completely understood, but initial exposure to *P. aeruginosa* flagella may equip host antibodies to prevent bacterial adhesion to host cells, reduce host cell reactions leading to inflammation, block mobility and invasiveness of the pathogen, or increase efficacy of phagocytic activity of leukocytes (Doring et al. 2007; Feldman et al. 1998; Holder and Naglich 1986). However, caution must be taken to target specific pathogens when using motility-targeted treatments, as unspecified application could cause damage to important commensals.

## *Pseudomonas aeruginosa* – a Model Organism

The microbial species *Pseudomonas aeruginosa* is a common laboratory pathogen which is easily cultured and well enumerated for a number of traits (it has been extensively studied due to its medical significance to immunocompromised individuals and its role as the leading cause of nosocomial (hospital-borne) infections (Tümmler et al. 1991). Extensive genetic (and more recently genomic) analysis has meant that key genes associated with motility and social traits can be easily identified and manipulated. *P. aeruginosa* can be found in a wide variety of ecological habitats, and as such requires a range of motility mechanisms (which inevitably affect dispersal) to enable successful colonisation and infectivity (this allows the influence of abiotic factors to be tested). Finally, *P. aeruginosa* is a common plant and animal pathogen which allows host-

pathogen interactions to be explored, with potential application to medicine and agriculture.

## *Pseudomonas aeruginosa* – Modes of Motility

*P. aeruginosa* has three modes of motility: twitching, swimming and swarming, regulated via two surface motility organelles: a single polar flagella and polar type IV pili. In addition it is able to secrete a lipid-based biosurfactant called rhamnolipids from surface pores, allowing for increased movement across a surface (Murray and Kazmierczak 2008; Figure 1.3). These mechanisms used individually or in conjunction enable *P. aeruginosa* to inhabit and move between various environments. The bacterium switches between these modes of motility depending on environmental and nutritional triggers (Mata-Sandoval et al. 2001). For example, the viscosity of the environment will determine which bacterial motility mechanism is most efficient, with more liquid environments favouring swimming and more viscose favouring twitching (Rashid and Kornberg 2000); in addition, the type of carbon source will also determine swarming efficiency on agar, with bacteria readily swarming when grown with succinate or glutamate, but exhibiting poor swarming ability on glucose (Shrout et al. 2006).

Twitching motility (so called due to the characteristic jerky motion) is independent of flagella and is induced on hard, moist surfaces. It is a common mode of bacterial motility also found in many other gram-negative Proteobacteria (e.g. *Acinetobacter calcoaceticus* (Henrichsen 1983), *Neisseria gonorrhoeae* (Wolfgang et al. 1998), and *Myxococcus xanthus* (where it is referred to as “gliding motility”; Spormann 1999)). The motive force is caused by the extension and retraction of polar type IV pili which interact and grip the surface, pulling the cell forward during retraction (Mattick 2002). Twitching motility is an important virulence factor as its functionality is required

in complex social behaviours, such as biofilm formation, that often underlies successful infection of host tissue (Mattick 2002). Type IV pili are also important for a number of other vital bacterial functions such as immune escape, DNA uptake, biofilm formation, microcolony formation, secretion, phage transduction and signal transduction (Craig and Li 2008).

Swimming motility is induced in aqueous environments and is entirely mediated by the flagella. *P. aeruginosa* typically show monotrichous (singular) flagellation, though natural variation is common (Leifson 1951). The flagella mechanism comprises of a thin helical filament, driven by a transmembrane proton-gradient powered motor (Bai et al. 2007). In addition to motility, flagella are coupled with chemotaxis (the ability to respond to chemical stimuli; Köhler et al. 2000). This allows the bacteria to sense its environment and move appropriately depending on the signal (Wei et al. 2011). Reduced functionality results in decreased virulence and colonisation rate (Drake and Montie 1988; for review see, Josenhans and Suerbaum 2002). The flagellum is also a common target for phage invasion, which suggests that motility-resistance trade-offs, may be common in shaping microbial evolution (Mahenthiralingam et al. 1994; Wolkin and Pate 1986; Chapter 5).

Swarming motility is functional on semi-solid surfaces under the conditions of nitrogen limitation or in response to certain amino acids. Swarming is a coordinated response which combines flagella-mediated motility with the production of rhamnolipids (Caiazza et al. 2005; Köhler et al. 2000). From the point of inoculation, bacteria migrate outwards in defined tendrils, sensing and responding to signals from other groups of cells (Caiazza et al. 2005). *P. aeruginosa* can also spread on semi-solid surfaces in the absence of motility organelles in a behaviour regulated by many of the factors known to influence swarming motility (Murray and Kazmierczak 2008). This motility is called sliding (a term

coined by Henrichsen 1972) and it is thought to aid colonisation under conditions where flagella expression is down-regulated (such as in the human airways; Jyot et al. 2007).

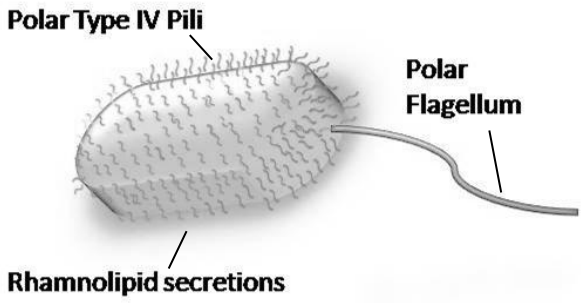


**Figure 1.3: Motility Structures of *Pseudomonas aeruginosa***

A schematic diagram of a *P.aeruginosa* cell highlighting the traits which enable the cell to move efficiently on a variety of substrates. The polar type IV pili aid translocation over hard surfaces, the flagella is used in aqueous environments and the rhamnolipid secretions are used in conjunction with flagella-mediated propulsion to 'slide' over semi-solid surfaces.

## Concluding Comment

Dispersal behaviour spans across taxa (from prokaryotes to humans) and across disciplines (including ecology, evolution, epidemiology and conservation). Understanding the diversity, maintenance and evolution of dispersal is vital to biology. In a time of changing climate conditions, research is crucial into understanding how encouraged or disrupted dispersal will influence diversity and persistence of a species. Similarly, in the face of ever increasing problems associated with antibiotic resistance, microbial-specific motility research may hold key advancements for new drug therapies. It is only by using an interdisciplinary approach including pragmatic field data, controlled and inventive experimental design, and realistic theoretical models, that major advancements can be made in understanding the evolution and ecology of dispersal.

# Chapter 2: Competition and Dispersal in *Pseudomonas aeruginosa*[1]


## Abstract

Dispersal plays a crucial role in a range of evolutionary and ecological processes, hence there is strong motivation to understand its evolution. One key prediction is that the relative benefits of dispersal should be greater when dispersing away from close relatives, because in this case dispersal has the additional benefit of alleviating competition with individuals who share the same dispersal alleles. I tested this prediction for the first time using experimental populations of the opportunistic pathogen, *Pseudomonas aeruginosa*. I measured the fitness of isogenic genotypes which differed only in their dispersal behaviours in both clonal and mixed populations. Consistent with theory, the benefit of dispersal was much higher in clonal populations, and this benefit decreased with increasing growth rate costs associated with dispersal.


[1] *Note: This is a modified version of the published manuscript, Taylor, T.B., and A. Buckling. 2010. Competition and Dispersal in Pseudomonas aeruginosa. The American Naturalist 176:83-89. DOI: 10.1086/652995*

# Introduction

Dispersal plays a crucial role in a range of evolutionary and ecological processes, hence there is strong motivation to understand its evolution (Bullock et al. 2002; Clobert et al. 2001; Clobert et al. 2004; Dieckmann et al. 1999; Gadgil 1971). Theoretical work suggests that dispersal is likely to be favoured by selection when the environment varies in time (McPeek and Holt 1992; Van Valen 1971), and if it reduces inbreeding depression (Bengtsson 1978) and kin competition (Hamilton and May 1977). Selection for dispersal will in turn be opposed if it is associated with any costs, such as increased mortality or reduced reproduction (Rousset and Gandon 2002). Here, I carry out an experimental study using bacteria to simultaneously test for the first time the qualitative predictions that competition with individuals who share the same dispersal alleles (Comins et al. 1980; Gandon and Michalakis 1999; Hamilton and May 1977; Taylor and Frank 1996) should increase the benefit of dispersal, whereas decreased mean growth rates away from the home patch should decrease the benefit.

Evidence for the importance of kin competition in determining dispersal patterns has been found in both experimental and field studies and in a wide range of taxa including mammals (Bollinger et al. 1993), reptiles (Cote et al. 2007), insects (Kasuya 2000) and birds (Strickland 1991). I explicitly investigated the importance of kin competition and dispersal costs on the evolution of dispersal in the opportunistic pathogen, *P. aeruginosa*. This bacterium possesses a range of motility mechanisms presumably to cope with the wide range of environments in which it inhabits. *P. aeruginosa* expresses two surface organelles which aid motility: a single polar flagellum and retractable polar type IV pili. In addition it is also able to secrete a lipid based biosurfactant called rhamnolipids (Mattick 2002). This enables the bacterium to twitch

(using type IV pili as ‘grappling hooks’; Henrichsen 1972), swim (using flagella; Bai et al. 2007) or swarm (comparable to gliding, using rhamnolipids coupled with flagella; Caiazza et al. 2005; Köhler et al. 2000) depending on the environment. I used isogenic mutants of *P. aeruginosa* strain PAO1 which showed high (disperser) or low (non-disperser) swimming dispersal. These phenotypes were achieved by using transposon mutants defective in type IV pili function. The dispersers were unable to express type IV pili (PilA mutant) and the non-dispersers constitutively expressed them (PilU mutant). Within a semi-solid substrate these pili cause drag and reduced the efficiency of flagella-mediated swimming motility, resulting in a smaller colonisation area.

I manipulated the degree of kin competition by either inoculating strains as clonal or mixed populations into home sites; the former being the high kin competition treatment. Note that non-kin only differ from each other with respect to their dispersal strategy; as is assumed in recent theoretical studies (e.g. Gandon and Michalakis 1999; Gandon and Rousset 2002). The relative fitness of dispersers versus non-dispersers was determined by calculating the ratio of the total number of each cell type in both the home site and the surrounding area after replication and dispersal. This measure of fitness for the clonal and mixed populations therefore indicates how dispersal strategies would likely evolve in metapopulations under conditions of high and low relatedness, respectively. These simple competition assays were carried out with variable costs of dispersal; the latter manipulated by reducing nutrient availability away from the home patch.

# Experimental Procedures

## *Strain Details and Growth Conditions*

Two transposon mutants defective in type IV pili and generated from a wild-type strain of *P. aeruginosa* (PAO1) were used: a PilU (non-disperser) mutant which is able to express but unable to retract pili (hyperpiliated), and a PilA (disperser) mutant which is absent of pili.

Cultures for plate colonisation were grown overnight in a homogeneous (shaken) environment (0.9 g) at 37 $^{\circ}$C, in 30ml universal vials containing 6 ml fresh medium (King's B: 20 g/l protease peptone, 10 g/l glycerol, 1.5 g/l potassium phosphate and 1.5 g/l magnesium sulphate heptahydrate).

## *Treatment Conditions*

Overnight cultures (shaken at 0.9 g at 37 $^{\circ}$C) were vortexed thoroughly, and $10^7$ cells of dispersers, non-dispersers or a 1:1 mixture of the two were pipetted into 25 ml semi-solid King's B Medium agar (0.6% wt/vol) plates (which were briefly dried in a flow hood for 20 minutes) just below the surface line so the point of colonisation could be located. Plates were left in a humid incubator for 42 hours at 37 $^{\circ}$C. Within the mixed treatment each genotype was at half the cell density compared to clonal treatments. In order to understand the effects of density on dispersal a range of dilutions (1 in 2, 1 in 10, 1 in $10^3$ and 1 in $10^6$) were also used to inoculate soft agar plates using the same method described above (distance travelled from inoculation site was determined as the radius calculated from total colony area). Finally, to ensure any growth inhibition of the disperser within mixed treatments was only due to the effects of competition, a soft agar

plate inoculated with a 1:1 mixed treatment was left for 138 hours at 37 °C. Control conditions where dispersal behaviour was not under selection were created by growing bacteria at 37 °C in 6 ml liquid KB shaken at 0.9 g; and in 0.6% wt/vol KB agar where the bacteria were distributed evenly throughout the plate. Six replicates of disperser, non-disperser and mixed treatments were set up, in three experimental blocks.

### *Data Collection*

The area covered by the bacteria was calculated (using *AnalyzingDigitalImages* software: http://mvh.sr.unh.edu/software/software.htm) and 9 samples were taken from each plate to calculate cell densities. These samples were taken using a 1 ml pipette (Finnpippette) at 5 mm intervals from the point of colonisation along the radius line of the colony. Samples were placed in an eppendorf containing 1 ml M9 solution (12.8 g/l $Na_2HPO_4$, 3 g/l $KHPO_4$, 0.5 g/l NaCl, 1 g/l $NH_4Cl$). The pipette tip was washed thoroughly in the solution and the eppendorf was subsequently vortexed. Bacteria were then plated onto KB agar at an appropriate dilution. Plates were incubated overnight and the number of colony forming units (CFUs) counted. Morphological differences between disperser and non-disperser CFUs were sufficient to allow visual differentiation between strains (with the former being noticeably larger and having less defined colony edges). At the very edge of the non-disperser colonies, the morphology of the CFUs increased in size, although they could still be readily distinguished from the dispersers. Total cell numbers on the plate were estimated by scaling up the CFU counts from the area of the pipette tip. The relative fitness of dispersers versus non-dispersers was determined by calculating the ratio of the total number of each cell type in both the home site and in the surrounding agar after 48 hours replication and dispersal. For the clonal populations, this

ratio was calculated between pairs of plates (from the same experimental block), whereas for the mixed population the ratio was calculated within each plate.

### *Imposing a Cost to Dispersal*

25 ml semi-solid King's B Medium agar (0.6% wt/vol) was allowed to dry for approximately 10 minutes, after which 0%, 25%, 50%, 75% or 100% of the agar was cut away (leaving a high nutrient zone of 1.0 cm diameter around the inoculation site to ensure only those dispersing were incurring a cost) and replaced with 0.6% wt/vol agar that did not contain any nutrients (6 g/l agar and water). Plates were dried for a further 10 minutes and inoculated with $10^7$ of clonal or mixed cells. Plates were left in a humid incubator for 42 hours at 37 ºC. Sampling techniques were as described above, except 17 samples were taken from each plate (one from the central colonisation point and 8 more at regular 5mm intervals from the area of high nutrient and no nutrient agar). One replicate of disperser, non-disperser and the mixed treatments were set up in each of the five cost groups.

## Results and Discussion

I measured the relative fitness of isogenic dispersing and non-dispersing genotypes of *P. aeruginosa* under conditions that allow swimming dispersal, in both clonal and mixed populations. Dispersing genotypes grew in the area in which they were initially inoculated, as well as dispersing away and replicating outside this area. By contrast, non-dispersing mutants were largely limited to growth within the initial inoculation area (Figure 2.1A; GLM: the effect of genotype on dispersal area, $F_{1,6} = 128.48$, $P < 0.0001$). Increased dispersal had a clear fitness advantage in clonal populations, with cell density

counts of the dispersing genotype after 42 hours growth and dispersal approximately sixteen-fold higher than that of the non-dispersing genotype (Figure 2.1A & C: Wilcoxon test, $P$ = 0.036). The advantage of dispersal was presumably that the initial site of inoculation deteriorated in quality through time as a result of resource depletion and the build-up of toxins. This is consistent with general predictions that temporal variation (in this case local environmental deterioration) favours dispersal (Friedenberg 2003; McPeek and Holt 1992; Van Valen 1971). By contrast, when both dispersers and non-dispersers were co-inoculated into the centre of the agar plate, the dispersers only achieved a two-fold higher final density relative to the non-dispersers, (Figure 2.1B & C: Wilcoxon, $P$ = 0.059). The greater advantage to the dispersing genotype in clonal versus mixed populations (Mann-Whitney, $P$ = 0.0051) suggests that the advantage of dispersal will be greatest under conditions of high within-patch relatedness in metapopulations, as predicted by theory (Gandon and Michalakis 1999; Hamilton and May 1977; Rousset and Gandon 2002; Taylor and Frank 1996).

The fitness of dispersing genotypes is predicted to increase with increasing kin competition because dispersal reduces competition within the home patch (Gandon and Michalakis 1999; Hamilton and May 1977; Rousset and Gandon 2002). Consistent with this prediction, I found that the non-disperser had a much stronger competitive effect than the disperser within the home patch: the density of the dispersing genotype was reduced by more than three-fold within the inoculation site by the presence of the non-dispersing genotype (t = 2.38, $P$ = 0.038), whereas the density of the non-dispersing genotype was not altered by the presence of the dispersing genotype (t=1.08, $P$ = 0.3; t-test comparing relative density reduction caused by the competing genotype of dispersing versus non-dispersing genotype: t = 2.86, $P$ = 0.017). It was necessary to address the possibility that reduced competitiveness of the disperser in the inoculation site was not in fact the result

of dispersal, but instead was because the disperser was an intrinsically worse competitor than the non-disperser. To resolve this I measured relative growth rates of the genotypes in environments where dispersal ability was expected to have little or no impact on fitness. First, I competed the disperser and non-disperser in shaken tubes, where swimming dispersal would be irrelevant compared to mechanical dispersal. Second, I competed the genotypes where bacteria were evenly inoculated throughout soft agar, hence moving from one colonised 'patch' would simply result in entering another. I found no difference in relative growth rate between the disperser and non-disperser under these conditions (in shaken liquid: Wilcoxon; $P$ = 0.933; and evenly distributed throughout soft agar: Wilcoxon; $P$ = 0.14).

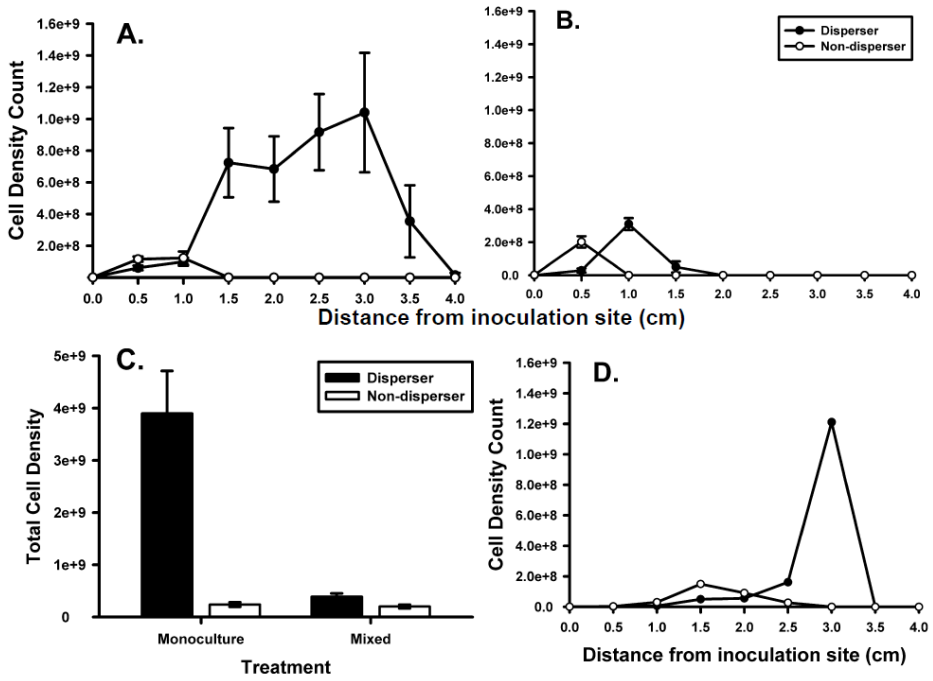


**Figure 2.1: Average Density of Disperser and Non-disperser between Treatments (±SEM)**

Monocultures of dispersers, non-dispersers (**A.**) and a 1:1 mixture (**B.**) were grown up in KB agar (0.6% w/v) for 42 hours. 9 samples were taken along the radius of the colony and the total cell density over the plate estimated (**C.**). Mixtures were also left for 138 hours (**D.**).

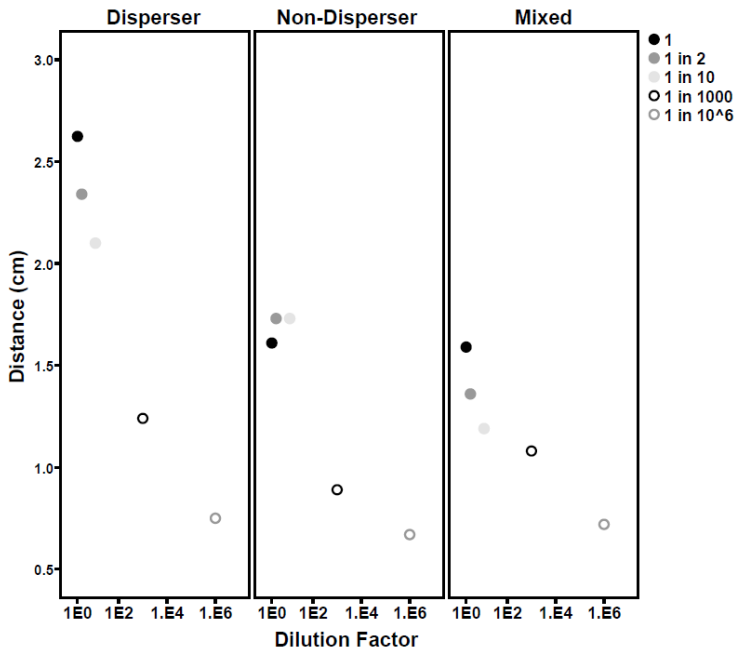


**Figure 2.2: Dispersal Distance Depends on Cell Number**

A number of dilutions (1 in $10^0$, 1 in 2, 1 in $10^1$, 1 in $10^3$ and 1 in $10^6$) of each treatment group were used to inoculate KB agar plates (0.6% wt/vol). After 42 hours the distance travelled from the inoculation site was measured. The results clearly show that fewer inoculating cells reduce subsequent dispersal distance.

A potentially confounding feature of our experimental design is that the disperser colonised a significantly smaller distance in the presence versus the absence of the non-disperser (Figure 2.1B; t = 5.52, $P = 0.003$). Despite this, the dispersers always dispersed beyond the range of the non-dispersers in mixed populations ($F_{1,10} = 121.0$, $P < 0.0005$) and showed a recovery of cell number beyond the range of the non-dispersers when left for 138 (rather than 42) hours (Figure 2.1D). Reductions in dispersal distance in the mixed populations might have arisen if non-dispersers physically prevented dispersal of the dispersers, in which case the mixed treatment might have altered not only the strength of kin competition, but also dispersal rates of individual bacterium. However, colonisation distance of bacteria is determined by both growth and dispersal (Henrichsen

1972); hence these data are also consistent with a reduction in density of the disperser. To distinguish between these possibilities (physical prevention of dispersal and reduced density), I determined how colonisation distance changed as a function of inoculation density. Consistent with the latter hypothesis, colonisation distance decreased with decreasing density in both clonal and mixed populations (Figure 2.2; Main effect of density: $F_{1,13} = 8.65$, $P = 0.011$). It is of particular significance that colonisation distance decreased with density in mixed populations: if reductions in dispersal resulted from physical interference of the disperser by the non-disperser, colonisation distance should have increased with lower inoculation densities.

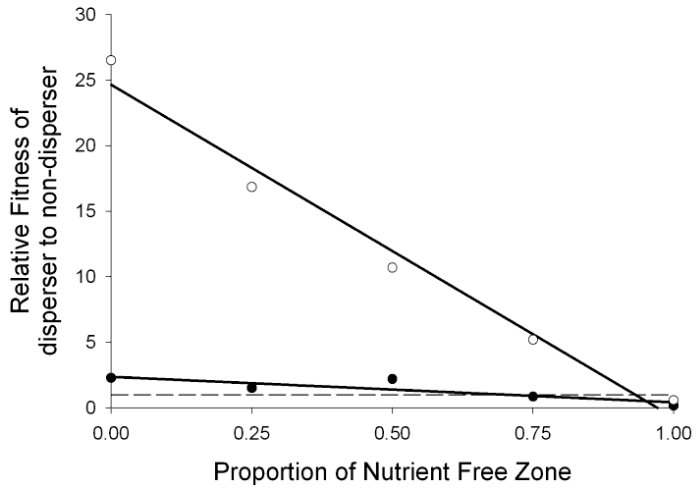


**Figure 2.3: Imposing a Cost to Dispersal**

A cost to dispersal was imposed by introducing nutrient free areas over 0%, 25%, 50%, 75% or 100% of the agar plates. The results showed the relative fitness of the disperser to the non-disperser in a clonal population (○) to be consistently greater (except when 100% is a nutrient free area), however in a mixed population (●) the relative fitness of the disperser falls below that of non-disperser when 71% of the environment was devoid of nutrients and unable to support bacterial growth. The dashed line indicates a relative fitness of 1, i.e. when the fitness of the disperser and the non-disperser are equivalent.

Within natural populations there are likely to be costs associated with dispersal (Ronce 2007), either via direct resource consumption (for example, in the form of energy expenditure during travel (Weber and Stilianakis 2007), or in the manufacture of necessary motility structures (Zera and Denno 1997)), increased risk of mortality (Hanski et al. 2000), or reduced fitness due to an unsuitable destination habitat (McPeek and Holt 1992). I wanted to introduce costs of dispersal into our experimental design and observe how it affected the fitness of the dispersal strategies. This was achieved by introducing low-nutrient areas into the environment, on which bacterial growth was severely limited. This area encompassed 0%, 25%, 50%, 75% or 100% of the total area available for colonisation beyond 5 mm from the point of inoculation. The results are shown in Figure 2.3. There was an overall decline in fitness of the disperser with increasing costs in both clonal (Spearman rank correlation: $r = 1$, $P < 0.001$) and mixed ($r = 0.9$, $P = 0.037$) populations. Moreover, the costs of dispersal that resulted in non-dispersers having a selective advantage are greater in clonal compared to mixed populations (treatment by cost interaction: $F_{1,6} = 103.75$, $P < 0.0005$), as predicted by theoretical work (Gandon and Michalakis 1999; Rousset and Gandon 2002). Specifically, the relative fitness of the disperser dropped below that of the non-disperser when greater than 93% and 71% of the environment was unable to support growth, in clonal and mixed populations, respectively.

My experiments inevitably do not capture many of the details developed in theoretical studies, and this may limit the interpretation of the study. First, I only infer evolution within a metapopulation from our single patch competition experiments, hence I have been unable to investigate feedbacks between dispersal, relatedness and variation in colonisation dynamics (Comins et al. 1980; Gandon and Michalakis 1999; Rousset and Gandon 2002). However, I argue that the fitness of different dispersal strategies within clonal and mixed populations is a good proxy of how dispersal strategies would likely

evolve in metapopulations under conditions of high and low relatedness, respectively. Second, dispersal in this experimental context is also dependent on growth. As a result, I cannot unequivocally rule out the possibility that dispersal is also limited by physical inhibition between genotypes, although this seems unlikely given that reducing density (and hence opportunities for physical contact) decreased, rather than increased, colonisation distance in mixed populations. Third, I did not allow the dispersal phenotypes to evolve as a result of mutations that are generated during the experiment, but instead rely on simple competition experiments between two defined, and widely contrasting, strategies. This inevitably results in strong selection operating between genotypes in the experimental set up, whereas most kin selection models of the evolution of dispersal assume, for analytical ease, very weak selection (but see, Gardner et al. 2007). Finally, as shown in Figure 2.1, the results are likely to quantitatively vary if densities are measured after different time points: allowing a longer time to disperse increases the relative fitness of the dispersing genotype in the mixture. However, there is no reason why this should qualitatively affect the results, as longer dispersal time also benefited the dispersing genotype in clonal populations, to the extent that it completely colonised the whole agar plate.

These results may have implications for understanding the evolution of virulence (the amount of host damage caused by the parasite) of *P. aeruginosa* and similar opportunistic pathogens. Dispersal has been linked to virulence both directly – via the colonisation of host tissues (Drake and Montie 1988; Feldman et al. 1998), and indirectly – via the production of biofilms (Jenkins et al. 2005; Josenhans and Suerbaum 2002). Clinical research has identified natural pili variants in the airways of cystic fibrosis sufferers infected with *P. aeruginosa* (Head and Yu 2004; Smith et al. 2006), with the general trend that motility function tends to be lost over time (Mahenthiralingam et al. 1994).

This loss could be due to a survival advantage, such as a greater resistance to phagocytosis (Mahenthiralingam et al. 1994) and bacteriophages (Bradley 1974; Brockhurst et al. 2005), but the social environment may also impose selection for changes in motility.

# Chapter 3: Using Experimental Evolution to Examine the Role of Kin competition in the Evolution of Dispersal


## Abstract

It has been recently shown using microbial populations that dispersal has the potential to alleviate competition between those who share the same dispersal alleles, thus providing evidence that kin competition should promote the evolution of dispersal (see Chapter 2). Here I extend this work, explicitly testing how relatedness of bacterial populations determines the evolution of dispersal. I evolved twelve initially isogenic metapopulations of *Pseudomonas aeruginosa* (9 populations in each of 6 replicate lines) under treatments of high and low relatedness, for a total of fifteen transfers. I did not find evidence in support of my prediction, but instead found that the environment was the most important factor in selecting for dispersal patterns. Although not explicitly tested, it appears that low nutrient diffusion within the chosen experimental environment caused a depletion of resources in the home patch, thus creating a general benefit to dispersal which was independent of the relatedness treatment. More generally, this experiment helps to give insight into potentially important feedbacks between resource-driven and kin-based determinants of dispersal.

# Introduction

Dispersal is a key life-history trait which dictates the resilience and distribution of natural populations (Clobert et al. 2001; Ronce 2007). Theoretical studies have highlighted key benefits of dispersal which are summarised as: (*i*) persistence in temporally heterogenic environments (Levin et al. 1984; McPeek and Holt 1992); (*ii*) inbreeding avoidance (Bengtsson 1978; Gandon 1999); (*iii*) and reduced competition between relatives (kin competition; Gandon 1999; Hamilton and May 1977). However, dispersal is often a costly behaviour in terms of energetic expenditure (Roff and Fairbairn 2001; Weber and Stilianakis 2007) and increased probability of mortality (Hanski et al. 2000), and therefore understanding its maintenance can be difficult. Dispersal can be viewed as an altruistic trait and favoured through kin-selected indirect fitness benefits, because it provides the opportunity to alleviate competition between kin. The importance of kin competition in determining dispersal patterns has been reiterated many times in the theoretical (Comins et al. 1980; Comins 1982; Gandon 1999; Gandon and Michalakis 1999; Leturque and Rousset 2002; Rousset and Gandon 2002; Taylor 1988; Taylor and Frank 1996) and experimental literature (Nakajima and Kurihara 1994; Moore et al. 2006; Wei et al. 2011; Chapter 2). Specifically, dispersal can be maintained in highly related groups even when the environment carries a high cost of dispersal (Hamilton and May 1977; Chapter 2).

In Chapter 2, I setup an experimental system to test the hypothesis that competition with individuals who share the same dispersal alleles should increase the benefit of dispersal (Comins et al. 1980; Gandon and Michalakis 1999; Hamilton and May 1977; Taylor and Frank 1996) and this benefit would decrease asymmetrically between high and low related groups with increasing costs associated with dispersal (Rousset and Gandon

2002). My results were consistent with theory, showing that the benefit of dispersal was much higher in clonal populations and this benefit was maintained over high costs of dispersal, *i.e.* under conditions of high relatedness dispersal is favoured. However, this study had certain limitations: firstly, the effect of relatedness on the evolution of dispersal had to be extrapolated by comparing growth of clonal versus mixed populations; secondly, metapopulation dynamics are inferred from single patch competitions; and thirdly, isogenic mutants were used to calculate fitness parameters, rather than letting phenotypes evolve.

To this end, I continued this research by experimentally evolving multiple populations of initially isogenic bacterial lines under conditions of high and low relatedness; this allowed dispersal phenotypes to evolve as a result of mutations generated during the experiment. As before, I used the common opportunistic pathogen *Pseudomonas aeruginosa*, which has been well enumerated for motility behaviour (from phenotypic to genomic level). I manipulated the degree of kin competition by either inoculating strains as clonal or mixed patches within a 9-patch metapopulation; the former being the high relatedness treatment.

## Methods

### *Selection Regime*

PAOΔ*mutS*, a mutator strain (chosen to accelerate evolutionary change) created via a deletion of the mismatch repair gene *mutS* (Oliver et al. 2004), was used to inoculate initial populations. This strain was not used to gain information regarding the rate of evolutionary change, but rather the direction. Starting culture for initial plate colonisation was grown overnight in shaken (0.9 g) 30 ml glass vials (VWR; Leicestershire, UK) at 37

°C, in 6 ml fresh King's B broth (KB: 20 g/l protease peptone, 10 g/l glycerol, 1.5 g/l potassium phosphate and 1.5 g/l magnesium sulphate heptahydrate). The overnight culture was vortexed, and 2.5 µl pipette onto hard KB agar (1.2% wt/vol). A total of one hundred and eight populations were evolved in one of two treatments: each treatment group contained six replicate metapopulations (with nine individual populations in each), equating to fifty four populations under conditions of high relatedness (all starting populations throughout the lineage were clonal), and fifty four under conditions of low relatedness (all starting populations throughout the lineage were a mixture of nine colonies). I used 140 mm diameter petri dishes (Sterelin; Newport, UK) poured with 40 ml KB agar media, which were briefly dried in a flow hood for 20 minutes. Nine populations were inoculated onto each plate in a three by three grid formation, and six replicate lines were set up for each of the treatments (high and low relatedness). Bacteria were left to grow and disperse for 48 hours, after which all bacteria were washed from the plate with 10 ml M9 buffer (12.8 g/l $Na_2HPO_4$, 3 g/l $KHPO_4$, 0.5 g/l NaCl, 1 g/l $NH_4Cl$), diluted to an appropriate factor to allow individual colony differentiation, and the diluted sample plated onto hard KB agar. These were left to grown overnight at 37 ºC, then nine colonies (each of approximately 3 x $10^8$ cells) were picked using a random number grid with a sterile toothpick, and inoculated into 100 µl of M9 buffer. 2.5 µl (approximately 5 x $10^5$ cells) was used to reinoculate populations on hard agar plates, and these were left to grow and disperse for 48 hours. This procedure was repeated for fifteen transfers (60 days), after which the samples taken directly from the washed plate were frozen at -80 °C in 20% (wt/wt) KB glycerol (for schematic of methodology see Appendix 1; Figure A1.1).

### *Motility Assays*

Frozen evolved samples were streaked across hard KB agar plates, and nine colonies randomly chosen and removed using a sterile tooth pick. These colonies and a frozen stock of the ancestral strain were used to inoculate 1 ml KB broth. Cultures were left to grow overnight in shaken 24-well cell culture clusters (Corning Inc., New York) at 0.9 g and 37 °C. Overnight cultures were vortexed and 2.5 µl inoculated onto hard KB agar (1.2% wt/vol), in each case, the evolved line and ancestor were inoculated onto the same plate to allow direct comparisons to be made under the same environmental conditions. Bacteria were left to grow and disperse for 48 hours, after which area covered by the bacterial colony was measured using ImageJ 1.42q (Abramoff et al. 2004).

### *Growth Rate Assay*

In order to determine the density of the evolved strain relative to the ancestor, frozen evolved samples were streaked across hard KB agar plates, and nine colonies randomly chosen and removed using a sterile tooth pick. These colonies and frozen stocks of a wild type were used to inoculate 1 ml KB broth. The wild type was a marked PAO1 strain resistant to tetracycline used to allow differentiation between the evolved lines and the wild type. This strain was verified against the ancestral strain (PAOΔ*mutS*) used in the study and did not have significantly different growth rate or dispersal areas (data not shown). Cultures were left to grow overnight in shaken 24-well cell culture clusters (Corning Inc., New York) at 0.9 g and 37 °C. Overnight cultures were vortexed and 2.5 µl inoculated onto hard KB agar (1.2% wt/vol). In addition, overnight cultures were diluted to an appropriate factor, and plated out onto hard KB agar to provide starting densities for inoculated populations; these were left overnight and counted. Inoculated bacteria were left to grow and disperse for 48 hours, then 5 ml M9 buffer was added to each plate, and

all bacteria washed off using a disposable plastic spreader. A sample of the wash-off was taken, and diluted to an appropriate factor to allow individual colony differentiation. 5 µl of diluted samples were spotted onto hard KB agar plates and hard KB agar plates supplemented with 60 µg/ml tetracycline, at three different concentrations. Plates were left overnight at 37 °C until colony differentiation could be made, and then cells were counted.

The doubling rate was calculated from this data using the following equation:

$$D = \ln(N_f/N_0) \quad (1)$$

Where D equals number of cell doublings, $N_0$ is the starting cell density and $N_f$ is the final cell density after 48 hours growth and dispersal.

## Results

After fifteen transfers I looked for differences between the treatment groups (low relatedness and high relatedness) in area dispersed over 48 hours, by measuring the area of dispersal relative to the ancestor when the evolved isolates were grown in clonal and mixed populations (Figure 3.1). I found no differences between the treatment groups, suggesting relatedness under the given experimental conditions did not affect the evolution of dispersal (two-sample t-test: in clonal population, $t_{10} = 0.136$, $P = 0.894$; in mixed population, $t_{10} = 0.466$, $P = 0.652$). However, I did find that when evolved bacteria were grown in clonal conditions, in both treatment groups the area of dispersal was larger than that of the ancestor ($t_{11} = 3.133$; $P = 0.010$), but when the same starting colonies were mixed together the area of dispersal was equivalent to that of the ancestor ($t_{11} = 1.085$; $P = 0.301$).

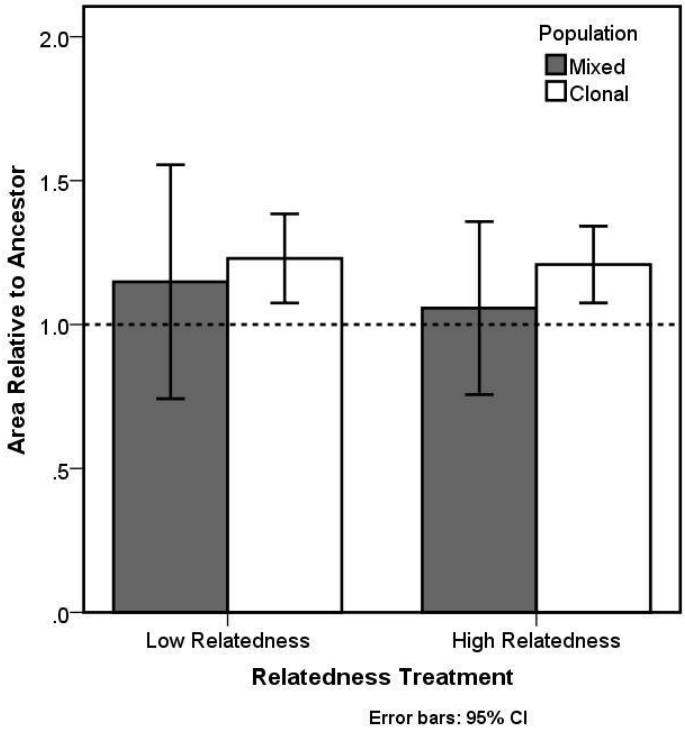


**Figure 3.1: Average area of evolved strains relative to the ancestor**

The results show the average area of dispersal of the evolved strains (6 lines, and 9 replicate colonies in each treatment group – low and high relatedness) relative to the ancestral strain from which all strains were derived (± 95% CI). The dashed line indicates a relative dispersal value of 1, that is, when the area of dispersal was equivalent to that of the ancestor.

Finally, I considered the growth rate on hard agar (calculated by the doubling rate) of evolved strains relative to the ancestor (Figure 3.2). I found that neither of the treatment groups had a doubling rate significantly different from the ancestral strain (one-sample t-test: low relatedness, $t_5 = 2.091$, $P = 0.091$; high relatedness, $t_5 = 0.935$, $P = 0.393$), and the two treatment groups had comparatively the same doubling rates as each other (two-sample t-test: $t_{10} = 0.394$, $P = 0.702$). These results combined suggest that the increase in colony area is not a factor of increased growth rate, but specifically increased motility. It also implies that the environment was imposing strong selection for motility,

and could potentially be masking any effects of relatedness between the two treatment groups.

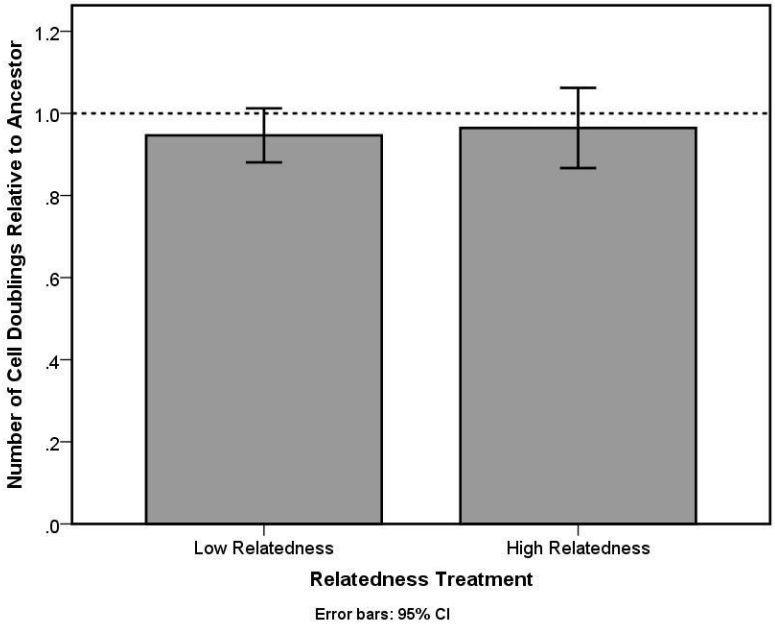


**Figure 3.3: Average number of cell doublings relative to ancestor**

Number of cell doublings of evolved strains relative to ancestor in each treatment group (high relatedness and low relatedness; ± 95% CI). Doublings were calculated via initial and final cell densities. The dashed line indicates a relative growth rate of 1.

## Discussion

The results from this study do not support my prediction that conditions of high relatedness select for higher dispersal, but in fact show an overall increase in dispersal across both treatment groups when cells were grown clonally. This suggests there is a general benefit to dispersal under the current experimental design. However, when evolved clonal populations were mixed together the dispersal range of the colony was reduced, and did not differ from the ancestral strain (Figure 3.1). This pattern is likely to be caused by a reduction in density of the strains which disperse the farthest, therefore reducing the combined dispersal area (as was shown in Chapter 2). The overall increase

in dispersal seen is not due to increased growth rate as both treatment groups showed growth rates equivalent to the ancestor in hard agar (Figure 3.2), and therefore the increased dispersal range is likely to be caused by increased motility on hard agar surfaces, and suggests that growth within the test environment has not changed over evolutionary time. This result is surprising, there appears to have been a response to selection for increased dispersal which one would expect to equate to a higher fitness (*i.e.* a higher growth rate). Although the reason for this is unknown, perhaps other group behaviours which are likely to be up regulated during a coordinated motility response (such as quorum sensing) have the net effect of reducing absolute growth, and as such, the growth rate appears no different from the ancestor.

Although these results are largely non-significant, they provide interesting future directions for research. Firstly, it suggests that the abiotic environment is particularly important in determining dispersal patterns. One potential explanation for the results of this experiment is that competition for resources was driving the pattern of dispersal independent of relatedness treatment. This would support the theoretical prediction that temporal variation (in this case local environmental deterioration) favours dispersal (Friedenberg 2003; McPeek and Holt 1992; Van Valen 1971). Previous work used experimental evolution on different agar types which varied in their viscosity to look for trade-offs between different types of motility in *P. aeruginosa* (Chapter 7). In this study, under hard agar conditions I found an overall increase in dispersal over evolutionary time independent of whether the trait was under artificial selection. I believed this result was due to the lower diffusion rate of resources in more viscous environments, meaning those which were able to reach virgin territory first would have more resources available for growth (also see, Wei et al. 2011). This was supported by the fact that in a liquid agar environment, where resources could easily diffuse, there was a decrease in motility in

those which were not under selection for increased dispersal. This could be tested explicitly using experimental evolution to evolve isogenic strains on similarly viscose environments which vary in the nutrient content to determine whether reduced local resources promote increased dispersal rates. Data which was omitted from Chapter 7 showed that an intermediately viscose environment (soft agar) did not impose any selection for dispersal, and motility was neither increased nor decreased in these selection lines (see Appendix 1; Figure A1.2). In the current experimental design it is possible that selection imposed by the hard agar environment is masking any effects which might be caused by the relatedness treatment. Repeating this design in a selectively neutral environment in terms of dispersal (*i.e.* soft agar) might allow the effects of relatedness on dispersal to be more clearly observed.

Finding explicit evidence for kin competition in laboratory or field based studies has proved difficult, and there are conflicting results as to its importance in different systems. For example, Innocent et al. (2010) explicitly tested whether kin competition determined the evolution of dispersal in the parasitoid wasp *Melittobia australica*, and found it was competition for resources which determined the production of dispersing morphs, not the degree of relatedness between competitors. However in contrast Moore et al. (2006) looked at the closely related fig wasp species, *Platyscapa awekei*, and found both kin competition and resources available in the natal patch to promote dispersal in male pollinating fig wasps. In addition, experiments with common lizards (*Lacerta vivipara*) have combined these factors and found physical condition of individuals, information regarding dispersal success, and relatedness within the population are all import factors in determining dispersal patterns (Cote 2010). Firstly, Cote found that larger individuals were more likely to disperse (because of increased probability of survival of the event); secondly dispersal was more likely to occur when there was steady

immigration into a population and immigrants were in good condition (suggesting connected good quality patches were available); and finally he determined that when relatedness within a population was high, those in good condition would always disperse regardless of information gained from quality of immigrants (because they benefitted indirectly by alleviating kin competition regardless of whether they found a suitable patch to colonise).

This experiment and those highlighted above give insight into potentially important feedbacks between evolutionary and ecological processes. Here, I find evidence that under these experimental conditions abiotic factors appear to play a more important role than biotic factors in dispersal evolution. Although superficially, this appears to contradict my previous work, the results are in fact consistent. Specifically, I previously found that dispersers on average had a higher absolute fitness than non-dispersers regardless of relatedness, but my experimental design allowed me to detect a difference in relative fitness between dispersers and non-dispersers under the different social conditions. The isolates used in Chapter 2 had very large phenotypic disparity in terms of motility which inevitably results in strong selection operating between genotypes, however by allowing dispersal phenotypes to evolve as a result of mutations generated during the experiment selection will be weakened, thus changing the relative importance of other selective factors (such as environment). Although competition experiments are an important tool for evolutionary biologists and have given essential insight into evolutionary processes, they require significant extrapolation to predict the true evolutionary outcome. However, the reason for their usefulness is that experimental evolution studies are notoriously difficult. When allowing a focal trait to evolve *de novo* one will also need to account for unpredicted traits influenced by factors such as drift, pleiotropy, and unaccounted adaptation to the experimental conditions (*e.g.* the nutritional

composition of media). Predicting all the potential influences of evolution and controlling/accounting for it in experimental design can prove problematic, although when carried out successfully, experimental evolution data can be extremely powerful.

# Chapter 4: The Social Evolution of Dispersal with Public Goods Cooperation


## Abstract

Dispersal provides the opportunity for populations to expand and persist. Understanding the selective pressures which are important in shaping the evolution of dispersal requires the qualification and quantification of several factors. Theoretical investigation has been important in identifying such factors and, coupled with experimental results can provide a powerful tool in dispersal research. However, due to the complex nature of dispersal evolution these factors tend to be treated in isolation, and as such can lead to narrow applicability. Often, populations are not groups of randomly interacting individuals, but exist under some kind of social structure. This can lead to asymmetry between interacting individuals – they might be mutually beneficial, benign, or antagonistic. When interactions are more likely to be beneficial, for example between kin engaging in cooperative behaviours, low dispersal is favoured because this increases the probability that interactions will be between kin, and reduce the likelihood of social cheats invading. However, limited dispersal will result in increased competitive interactions resulting in antagonism between kin over shared resources (kin competition), and as such increased dispersal will be favoured. Dispersal patterns will allow a balance between these opposing forces, by reducing kin competition but maintaining high relatedness. Here, we show theoretically that dispersal is increasingly favoured with patch availability and relatedness, but that the presence of social cheats can dramatically alter these patterns. I test these predictions experimentally using genetic variants of *Pseudomonas aeruginosa*, which differ in their dispersal and cooperative behaviour, and find our results consistent with the predictions of our model.

# Introduction

Understanding dispersal is a central aim of evolutionary ecology (Weins 2001). Theoretical work suggests that selection can favour dispersal when the environment varies in time (Van Valen 1971; McPeek and Holt 1992); and if it reduces inbreeding depression (Bengtsson 1978) and kin competition (Hamilton and May 1977). Conversely, dispersal can be opposed by selection if it is associated with any costs, such as increased mortality or reduced reproduction (Rousset and Gandon 2002). The benefit of dispersal by alleviating kin competition highlights dispersal as an altruistic trait (Hamilton and May 1977; Comins et al. 1980; Nakajima and Kurihara 1994; Taylor and Frank 1996; Gandon and Michalakis 1999; Wei et al. 2011; Chapter 2), in that dispersal can be favoured through kin-selected indirect fitness benefits, even when individual costs of dispersal are very high. However, such altruism is only likely to evolve under conditions of high relatedness, *i.e.* when there is a tendency for individuals who share the same 'dispersal alleles' to be associated in space. Dispersal itself is likely to reduce relatedness (Queller 1994; Gandon 1999), which in turn can reduce selection for dispersal.

The change in population structure resulting from dispersal is likely to have particularly important consequences for the evolution of optimal dispersal rates when other social traits have important fitness consequences. High relatedness, as well as selecting for elevated dispersal rates, also selects for other forms of altruistic cooperation, where individuals pay a cost for the benefit of the group as a whole (Hamilton 1964). In contrast to dispersal, where leaving the group is often an altruistic act, many altruistic traits require individuals to stay in groups. There are numerous examples of such altruism across a range of taxa (Ratnieks and Wenseleers 2008), but we focus here on public good production in bacteria. Wild type bacteria produce numerous extracellular molecules,

such as tissue degrading enzymes, iron-scavenging siderophores, and the production of sticky polymers to protect surface growing bacteria (biofilms), that are individually costly but benefit the group as a whole. Such behaviours are readily exploitable by non-producing cheats, hence dispersal might be selected against in public-good producing bacteria if it increases the chance of encountering exploiting cheats. Conversely, selection for dispersal may be beneficial if it allows escape from social exploitation or if it promotes individuals to move from saturated to empty patches. Here, we investigate how dispersal evolution is affected by public goods production and exploitation under different degrees of patch occupancy using a combination of theory and experiments.

## Methods

### *Model*

We derived an expression for the personal fitness of a cooperator exhibiting a rare genetic variant dispersal strategy. We then employed a neighbour-modulated fitness approach to kin selection analysis (Taylor and Frank 1996; Taylor 1996; Frank 1997; Frank 1998; Rousett 2004; Taylor et al. 2007) to identify the evolutionary equilibrium rate of dispersal, which we then checked for evolutionary stability (Taylor 1996; Maynard Smith and Price 1973; Day and Taylor 1998) and convergence stability (Eshel and Motro 1981; Taylor 1996). Full details are given in the Supporting Information (Appendix 2).

### *Strain Details and Growth Conditions*

PilA and PilU (PAO1 mutant's defective in type IV pili) were used as motility variants. A third mutant defective in siderophore production (PAO1Δ*pvdD pchEF*; Ghysels et al. 2004) was used as the "cheat", this strain is unable to grow in isolation in

an iron limited environment, and requires access to a siderophore producing strain in order to grow. Bacterial cultures were grown overnight at 37 °C in 6 ml Casamino acid media (CAA; 5 g/l Casamino acids, 1.18 g/l $K_2HPO_4.3H_2O$, 0.25 g/l $MgSO_4.7H_2O$ ) shaken at 0.9 g. Cells were then pelleted and re-suspended in M9 buffer solution twice to ensure all nutrients were removed from the media.

All re-suspended cultures were made iron limited (by adding 100 µg $ml^{-1}$ of human apo-transferrin and 20 mM $NaHCO_3$; Sigma; Griffin et al. 2004; Meyer 1996). Bacteria were left to grow and disperse for 72 hours.

### *Treatment Conditions*

25 ml of iron limited CAA agar (0.6% w/v agar), was poured into 20 cm diameter petri-dishes and allowed to dry in the laminar flow hood for 20 minutes. One third of the agar plates were supplemented with 250 µl overnight cheat culture. Inoculum was prepared as follows: (*i*) Local: cheats were mixed in an Eppendorf with each of the dispersal variants at 1:10; (*ii*) for Global: pure disperser and non-disperser cultures were used as the inoculum (See Appendix 2; Figure A2.2). 2.5 µl of inoculum was pipetted into the centre of the agar plate.

For controls I replaced the cheat with the PAO1 siderophore-producing wild-type in the Global and Local treatment groups. In addition, I grew the motility variants with the cheats at 37 °C in 6 ml liquid KB shaken at 0.9 g.

### *Data Collection*

Methods were as described in Chapter 2. Samples were taken using a 1 ml pipette (Finnpipette), at regular 5 mm intervals along the radius of the colony. The samples were then washed in M9 (12.8 g/l $Na_2HPO_4$, 3 g/l $KHPO_4$, 0.5 g/l NaCl, 1 g/l $NH_4Cl$), diluted

to an appropriate dilution to allow colony differentiation, and plated in order to count colony forming units (CFUs). The relative fitness of dispersers versus non-dispersers was determined by calculating the ratio of the total number of each cell type across corresponding plates.

Visual differentiation was made between siderophore producing (green) and non-producing colonies (white). The relative fitness of dispersers versus non-dispersers was determined by calculating the ratio of the total number of each cell type across corresponding plates.

## The Model

We develop a theoretical model to investigate the dynamics between public goods cooperation and kin competition in the context of evolving dispersal behaviour. We assume an infinite metapopulation, in which a proportion $\alpha$ of patches contain both cooperator and cheat bacterial cells ("occupied patches"), a proportion $\beta$ of patches are empty ("vacant patches") and a proportion $\gamma = 1- (\alpha+\beta)$ of patches contain only cheats ("cheat patches"). We denote the genetic relatedness among cooperators within patches by $r$. Each cooperator disperses with independent probability $z$ to a random patch, or else remains in their natal patch with probability $1-z$. We assume that cheats do not disperse. Following dispersal, bacteria grow and reproduce, reaching carrying capacities of $aK$ in occupied patches, $(1-a)K$ in vacant patches, and 0 in cheat patches. That is, we allow for occupancy to deteriorate a patch, resulting in a decreased carrying capacity ($a < 1/2$), or alternatively for occupancy to improve a patch, resulting in an increased carrying capacity ($a > 1/2$), and we assume that further growth is impossible on a cheat patch, owing to exploitation by social parasites.

We use these model assumptions to determine the continuously stable strategy (CSS; Eshel 1983; Taylor 1996) for the rate of dispersal, $z^*$ (see Methods and Appendix 2). This is the rate of dispersal that is both evolutionarily stable (Taylor 1996; Maynard Smith and Price 1973; Day and Taylor 1998) and convergence stable (Taylor 1996; Eshel and Motro 1981; Christiansen 1991). An important special case of our model is where we assume the absence of vacant patches ($\beta = 0$) and clonal relatedness among the cooperators in each patch ($r = 1$): this is equivalent to the model of Hamilton & May (1977), and here we recover their key result, $z^* = 1/(1+c)$, where the cost of dispersal is simply the probability of landing upon a cheat patch (i.e. $c = \gamma$). In this classic model, increasingly costly dispersal favours a lower rate of dispersal. However, a surprisingly large rate of dispersal is nonetheless favoured despite even extreme costs (e.g. $z^* \to 1/2$ as $c \to 1$).

More generally, analysis of our model reveals that relatedness ($r$), the proportion of habitable patches that are occupied ($p = \alpha/(\alpha+\beta)$), and the relative carrying capacity of occupied patches ($a$) also mediate the evolution of dispersal. Increasing genetic relatedness of cooperators within patches always increases the CSS rate of dispersal ($dz^*/dr > 0$). This is because the indirect fitness benefits of dispersal, owing to the relaxation of resource competition for one's kin, scale with the relatedness of patch mates. When occupation deteriorates patches ($a \leq 1/2$), then increasing the proportion of habitable patches that are occupied ($p$) always decreases the CSS rate of dispersal ($dz^*/dp < 0$). When occupation improves patches ($a > 1/2$), the CSS rate of dispersal may be either an increasing or a decreasing function of the proportion of habitable patches that are occupied (Figure 4.1). This proportion impacts upon both the direct and indirect fitness consequences of dispersal: the impact upon direct fitness can be positive or negative, depending upon the relative carrying capacity of occupied patches, but the

impact upon indirect fitness is always negative, as a more saturated population (higher $p$) leads to a greater number of immigrants arriving at occupied patches, which reduces the relatedness of the dispersing individual to those cells who will occupy her native patch after dispersal – *i.e.* those cells who stand to benefit from her dispersal. Increasing the relative carrying capacity of occupied patches ($a$) always reduces the CSS level of dispersal ($dz^*/da < 0$). This is because any direct benefit of dispersal owes to the individual finding herself in a patch that is better than the one that she dispersed away from.

Finally, we observe the relationship between the cost of dispersal ($c$) and the CSS rate of dispersal ($z^*$) is qualitatively affected by both the proportion of habitable patches that are occupied ($p$) and relatedness ($r$). Increasing mortality cost of dispersal always reduces the direct fitness effect of dispersal and always increases the indirect fitness effect of dispersal. In Hamilton & May's (1977) classic model, the former effect always outweighs the latter, such that increasing mortality cost always reduces the CSS rate of dispersal. However, this is not true in our more elaborate model. Consequently, whilst the CSS rate of dispersal is sometimes monotonically decreasing with increasing mortality cost for some parameter values, for others it may be a U-shaped function of the mortality cost (see also, Gandon and Michalakis 1999; Ronce 2007).

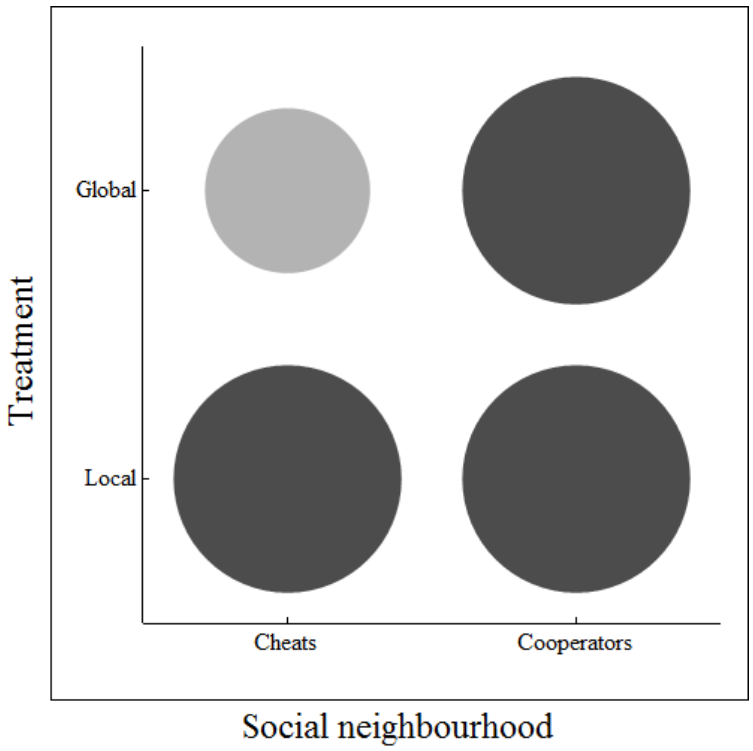


**Figure 4.1: The theoretical effects of treatment and the social neighbourhood on the evolution of dispersal.**

Theoretical results showing the effects of treatment (either Global or Local) and the social neighbourhood (filled with either cheats or cooperators). The area of the circles and the gradient of colour represent the CSS level of the dispersal ($z^*$).

## The Experiment

I used a well-studied bacteria model for social evolution, the opportunistic bacterial pathogen, *Pseudomonas aeruginosa. P. aeruginosa* possess a range of motility mechanisms, which trade-off against each other, in order to move in different environments (Bardy et al. 2003; Chapter 7). I manipulated dispersal behaviour by using genetically modified pili mutants of *P. aeruginosa*. Pili are surface organelles which aid motility in hard environments; PilA mutants are unable to express pili, and PilU mutants are 'hyperpiliated', which is to say they express pili but are unable to retract them. In a semi-solid environment the PilA mutants acted as the 'dispersers' as they were able to

freely move through the substrate; the PilU mutants were the 'non-dispersers', as the permanently extruded pili caused the bacteria to become stuck, restricting movement. Consistent with theoretical results, my previous work using these mutants has shown that conditions of high relatedness generate a relative selective advantage for the disperser (see Chapter 2). *P. aeruginosa* produces numerous public goods, but for simplicity I focus upon a single trait: extracellular iron chelating siderophores (Ratledge and Dover 2000; West and Buckling 2003). Iron is vital for bacterial growth, however most iron in the natural environment exists in the insoluble ferric form, and must be reduced via reactions initiated by siderophore molecules in order to be utilised. Under conditions of iron limitation, clonal populations of siderophore producers reach much higher densities (and lead to more severe infections) than isogenic mutants that do not produce the primary siderophore, pyoverdin (West and Buckling 2003). However, non-producing mutants can exploit the pyoverdin of producers, and hence outcompete them when in direct competition as a result of the metabolic cost of pyoverdin production (Griffin et al. 2004). My experimental design simply involved determining the fitness (growth) of cooperating dispersers and non-dispersers in the presence of cheats or cooperators, with either high or low initial patch occupancy.

In order to experimentally test the relationship, cheats were present in the environment in one of two patterns: "Local", where cheats were distributed only within the inoculation site (in terms of the model, $\alpha$ represents the proportion of patches occupied by cheats and cooperators, which in the experiment is equivalent to the inoculation site, and $\beta$ is the surrounding patches, which under this treatment are empty; therefore $\gamma \approx 0$); or "Global", where cheats were distributed throughout the agar plate (in this case, $\beta$ is zero as there are no unoccupied patches; therefore $\gamma \approx 1$). Following this, I set up four treatment conditions in iron limited agar, which were as follows: (*i*) Disperser

in the presence of Local cheats; (*ii*) Non-disperser in the presence of Local cheats; (*iii*) Disperser in the presence of Global cheats; and, (*iv*) Non-disperser in the presence of Global cheats (see Methods; Appendix 2; Figure A2.2). In addition, a control treatment was set up whereby the disperser and non-disperser were competing with a cooperating strain (PAO1, the wild-type of all mutant strains used in this study), distributed in the same spatial patterns as described above. This allowed us to determine whether the patterns observed were a result of the costs imposed by the social cheats, or due to general competition for site occupation.

In order to understand the effects of cheat distribution on fitness of motility variants, I measured the total cell densities of dispersers and non-dispersers between treatment groups across each plate and then divided the total number of non-dispersers by the total number of dispersers to give a relative fitness value (Figure 4.2). When the motility variants were competing alongside social cheats, I found the treatment group to have a large effect on relative fitness (One-way ANOVA, main effect of treatment: $F_{1, 4}$ = 257.648; $P < 0.001$). But the effect of the treatment of the fitness of the dispersers depended on whether the dispersers were competing with cheats or cooperators (Two-way ANOVA, interaction between Treatment*Competitor; $F_{1, 8}$ = 17.215; $P = 0.003$), such that it is better to disperse when cheats are Localally represented within the environment (One-sample t-test, test value = 1: $t_2$ = 14.28; $P = 0.01$), but better to remain sessile when cheats are Globalally distributed throughout the environment (One-sample t-test, test value = 1: $t_2$ = 374.71; $P < 0.001$). However, when motility variants were competing with cooperators the disperser maintained a fitness advantage across both treatment groups (One-sample t-test, test value = 1: Local, $t_2$ = 7.79; $P = 0.032$; Global, $t_2$ = 41.42; $P = 0.002$). Note, all *P* values have been corrected for multiple comparisons using the Bonferroni correction method.

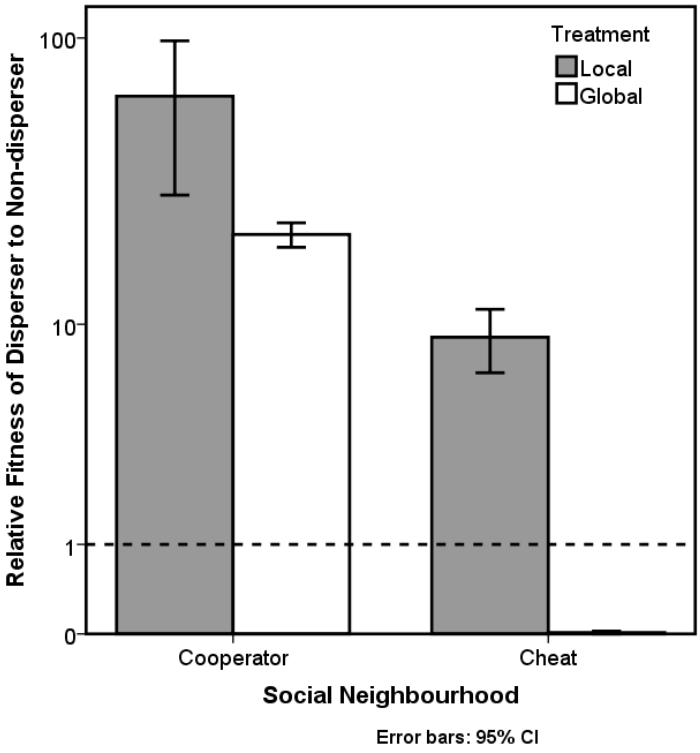


**Figure 4.2: Average fitness of dispersers relative to non-dispersers**

Average fitness of dispersers relative to non-dispersers in environments which vary in competitor type which form the social neighbourhood (motility variants are competing with either cooperators or cheats), and the structure of the social neighbourhood, with competitors distributed throughout the agar (Global) or located only within the colonisation patch (Local). Error bars represent ± 95% CI and the dashed line indicates a relative fitness of 1, that is, when the fitness of the disperser and the non-disperser are equivalent.

## Discussion

The results from this study suggest that patch occupation by social cheats is likely to have strong evolutionary consequences for dispersal patterns, such that when cheats are localised to the home patch, higher dispersal rates will evolve, but when cheats are present throughout available patches, lower dispersal rates will evolve. This is supported by both the theoretical and experimental results from this study.

The key novel result from the model is the interaction between the presence of cheats and patch occupancy on the evolution of dispersal (Figure 4.1). I verified this relationship empirically by determining the fitness (growth of isogenic dispersers and non-dispersing mutants of *P. aeruginosa*) in presence of either siderophore cheat or cooperator competitors and under high and low patch occupancy (Figure 4.2). The model highlights how direct and indirect fitness benefits independently and simultaneously modify the continuous stable dispersal strategy. Under low relatedness direct benefits drive the dispersal patterns, such that patch occupation will determine the fitness of dispersing phenotypes. Under high relatedness indirect benefits drive the dispersal patterns, such that dispersal is favoured under higher values of patch occupation, because dispersal also carries indirect fitness benefits by alleviating kin competition in the patch of origin (Appendix 2; Figure A2.1).

Experimental results satisfy these predictions made by the model. I found that under Local treatment (*i.e.* low patch occupation), the dispersing motility variant reached higher cell densities and thus had higher fitness compared to the non-dispersing variants. However, under Global treatment (*i.e.* high patch occupation) the dispersing variant reached higher cell density when competing with a cooperative strain for site occupation, but lower cell density when competing with a cheating strain for site occupation, such that dispersing phenotypes would be less fit than non-dispersing phenotypes under these conditions. The cell density (and thus fitness) of the non-dispersing phenotype was equivalent across both treatments. This is because the dispersing morph is adopting a more risky strategy, depleting the numbers in the inoculation site and exposing itself to potential unknown costs within the environment (in this case, the cheats). This strategy pays off when the dispersers find themselves in high nutrient, virgin territory beyond the threat of the cheats (as in the Local treatment group), but dispersal is costly when a mine-

field of cheats waits beyond the home site. On the other hand, the non-dispersers will not expose themselves to external threats and will therefore dominate the home site – but not beyond – in all treatment groups.

These results provide a simplified representation of potential dynamics between cooperation and kin competition in the context of evolving dispersal behaviour. However, the question being addressed here is something every dispersing individual must assess – "is the grass really greener on the other side?" *i.e.* will the destination patch provide relatively greater fitness benefits than the home patch? For a cooperative species, leaving a group which you know fairly contributes to a collective action, for an unknown destination where your cooperative behaviours have the tendency to be exploited, has the potential to carry large fitness costs. The decision will be determined by the indirect benefits gained from alleviating kin competition in the cooperative group, and the direct costs associated with the risk of dispersal and potential loss of the cooperative action. Species whose fitness largely depends on cooperative actions, such as the hymenoptera, overcome this conflict by participating in budding dispersal – where groups from a larger colony will disperse together to ensure the founding colony will maintain high relatedness and thus productivity of cooperative behaviours (Gardner and West 2006; Kümmerli et al. 2009; Ross and Keller 1995). But what will determine dispersal patterns in less extreme cases of cooperative species?

There are many examples from bird and mammalian populations that suggest reduced dispersal as a result of direct fitness restraints act as important factors in the evolution of cooperation (Hatchwell 2009). For example, the cooperatively breeding red wolf (*Canis rufus*), will stay and help rear related offspring, improving their survival and thus providing some indirect fitness benefit; however, it appears these individuals gain even larger direct benefits from staying and helping compared to premature dispersal,

despite the fact they are unlikely to have any breeding opportunities (Sparkman et al. 2011); delayed dispersal results in lower mortality, and equivalent time to first breeding event. Similarly, in acorn woodpeckers (*Melanerpes formicivorus*) a larger proportion will stay and help kin members when habitat saturation reduces the chance of successfully establishing in a breeding patch, and likewise, individuals tend to have higher fitness if they delay dispersal until a patch nearby becomes available (Stacey 1979). But interestingly, there are also many examples of reduced dispersal and high kin association between non-social species. For example, in the Towsend's vole (*Microtus townsendii*) females tend to nest nearer to kin, and those nesting in close proximity with kin have higher survival than those nesting near non-kin (Lambin and Krebs 1993). Staying and cooperating within a highly related group, thus increasing the fitness of kin, will provide indirect fitness benefits, however these examples suggest that direct benefits (such as; group foraging and securing resources (Lambin and Krebs 1993), group defence (Sparkman et al. 2011), territory acquisition (Stacey 1979), or the opportunity for subordinates to parent offspring (Richardson et al. 2002) gained from group behaviours are also crucial in determining dispersal patterns. However, as much as group living provides many benefits, it can also be associated with numerous costs, for example; asymmetry between competing kin and non-kin can lead to greater apparent competition between genetically related individuals, this interaction can be created if kin are more likely to be competing for identical resources than non-kin members, or if parasites are more likely to spread through kin groups compared to non-kin groups; (Hamilton 1987; Schmid-Hempel 1998; Cremer et al. 2007). All of these costs which are directly related to being in a highly related group can be treated as kin competition.

Using an experimental evolution and theoretical approach to the study of dispersal complements natural data by providing detailed information regarding the relative

importance of different factors contributing to the evolution of dispersal. This chapter highlights the balance between direct and indirect selective agents driving the evolution of dispersal and how these agents can be influenced by the addition of other necessarily social interactions.

# Chapter 5: Using Experimental Evolution to Explore Natural Patterns between Bacterial Motility and Resistance to Bacteriophages[2]


## Abstract

Resistance of bacteria to phages may be gained by alteration of surface proteins to which phages bind, a mechanism that is likely to be costly as these molecules typically have critical functions such as movement or nutrient uptake. To address this potential trade-off we combine a systematic study of natural bacteria and phage populations with an experimental evolution approach. We compare motility, growth rate, and susceptibility to local phages for 80 bacteria isolated from horse chestnut leaves and, contrary to expectation, find no negative association between resistance to phages and bacterial motility or growth rate. However, because correlational patterns (and their absence) are open to numerous interpretations, we test for any causal association between resistance to phages and bacterial motility using experimental evolution of a subset of bacteria in both the presence and absence of naturally associated phages. Again, we find no clear link between the acquisition of resistance and bacterial motility, suggesting that for these natural bacterial populations, phage-mediated selection is unlikely to shape bacterial motility; a key fitness trait for many bacteria in the phyllosphere. The agreement between the observed natural pattern and the experimental evolution results presented here demonstrates the power of this combined approach for testing evolutionary trade-offs.


[2] *Note: This is a modified version of the published manuscript, Koskella, B., T. B. Taylor, et al. 2011. Using experimental evolution to explore natural patterns between bacterial motility and resistance to bacteriophages. ISME J. DOI:10.1038/ismej.2011.47*

# Introduction

Resistance to parasites is typically believed to be associated with fitness costs, such as reduced growth rate or competitive ability. Such costs are thought to play a key role in maintaining polymorphism in host resistance, and hence parasite persistence within populations (Antonovics and Thrall 1994; Burdon and Thrall 2003; Boots and Bowers 2004; Morgan et al. 2005; Morgan et al. 2009), and may also impact on interactions with other species both within and across trophic levels (Clancy and Price 1986; Omacini et al. 2001; Lennon and Martiny 2008; Hall et al. 2009). For example, resistance of bacteria to bacteriophages has been associated with substantial fitness costs (Lenski 1988a; Bohannan et al. 1999), including an increased cost of deleterious mutations (Buckling et al. 2006), and decreased competitive ability (Brockhurst et al. 2005; Lennon et al. 2007; Quance and Travisano 2009). Given the ubiquity of bacteria-phage interactions, and their key role in all ecosystems, these costs are likely to have important ecological consequences (Bohannan and Lenski 2000a; Fuhrman and Schwalbach 2003).

Here, we investigate fitness costs associated with resistance to lytic phages in the bacterial pathogen, *Pseudomonas syringae* using two complementary approaches. First, we carry out a correlational study between resistance to phages and other bacterial fitness traits (growth rate and motility) in natural plant-associated isolates. This approach allows for the direct characterization of phenotypic diversity in natural bacterial and phage populations, but can be difficult to interpret because isolates will inevitably have numerous genetic differences between them in addition to resistance to phages. Next, we test for a causal link between resistance to phages and other fitness traits using a subset of these isolates by experimentally evolving bacteria in the presence and absence of phages. This experimental evolution approach is known to be a powerful tool for studying

evolutionary trade-offs among fitness traits (Ebert 1998; Kassen 2002; Buckling et al. 2009), but may be less helpful in elucidating the importance of these trade-offs in explaining natural diversity. Combining these two approaches allows for a more robust assessment for the role of trade-offs in maintaining phenotypic diversity in natural populations.

Lytic phages have the potential to impose strong selection on host populations, as they are obligate killers. They replicate by injecting their viral DNA into a host bacterium, hijacking the host replication machinery to propagate, and then bursting the host cell in order to release their viral progeny (Lenski 1988b). Infection begins with the binding of molecules on the phage tail fibre to a bacterial cell surface receptor (Lindberg 1973) and resistance to phages can be gained by loss or change of these receptors, which is likely to impact on other bacterial functions (Whitchurch and Mattick 1994; Brockhurst et al. 2005). For example, phages commonly exploit bacterial surface motility appendages (flagella and pili); flagellatropic phages are known to reversibly bind to helical grooves on the bacterial flagellum and use the rotation of the flagellum to spiral towards the cell surface (Samuel et al. 1999), and similarly, pilus-specific phages will attach to pili and fuse their membrane with that of the bacterial cell during pili retraction (Romantschuk and Bamford 1985; Mattick 2002). A first step towards bacterial resistance may therefore be the loss or alteration of these structures. For example, phage-resistant mutants often show defective flagella that are unable to rotate (Icho and Iino 1978) and abnormal unpiliated or hyperpiliated bacteria may arise to prevent phage attachment (Bradley 1980; Mattick 2002; Brockhurst et al. 2005).

Reduced motility function is likely to have important implications for bacterial fitness in both pathogenic and non-pathogenic bacteria (Drake and Montie 1988; Korber et al. 1994; O'Toole & Kolter 1998a). Immotile mutants of the opportunistic animal

pathogen *Pseudomonas aeruginosa* show reduced infectivity on human hosts and impaired biofilm formation (Drake and Montie 1988; O'Toole and Kolter 1998a). Similarly, epiphytic, non-motile strains of the plant pathogen *Pseudomonas syringae* were found to have reduced fitness and competitive ability compared to more motile strains, especially under conditions of environmental stress (Haefele and Lindow 1987). Indeed, motility is a key component of fitness for bacteria in the plant phyllosphere and is necessary for successful pathogenicity, as bacteria colonising leaf surfaces are better able to invade the leaf interior through the stomata if they are motile (Panopoulos and Schroth 1974; Beattie and Lindow 1999; Melotto et al. 2006).

Despite the predicted link between phage resistance and motility, and some correlative work suggesting a trade-off between the two (Joys 1965; Whitchurch and Mattick 1994), the association has never been systematically investigated. Here, we combine an examination of natural bacteria and phage isolates (from the leaves of horse chestnut trees) with experimental evolution to address this relationship. Contrary to our expectations, while natural populations of Pseudomonads show a positive relationship between bacterial motility and resistance to phages, subsequent experimental work suggests that this relationship is not clearly causal.

## Materials and Methods

### *Relationship between Resistance and Motility in Natural Populations*

To investigate the natural variation in resistance to phage and motility, we examined 80 natural isolates, from either the surface or the interior of horse chestnut leaves collected around Oxfordshire, United Kingdom, that were part of a larger sampling design from a previous experiment (Koskella et al. 2011). The study included a reciprocal cross-

inoculation of culturable bacteria and communities of phages isolated from each of 32 leaves. Bacteria were isolated from either the leaf surface, using buffer from leaf washes, or leaf interior, using homogenates from surface-sterilized leaves. Washes/homogenates were plated on 1.2% King's medium B (KB) broth (10 g/l glycerol, 20 g/l proteose peptone #3 (Becton Dickinson UK Ltd), 12 g/l agar, 1.5 g/l $K_2HPO_4.3H_20$, 1.5 g/l $MgSO_4.7H_20$) and, after 48 hours of growth, colonies were picked at random based on proximity to a randomly chosen spot on the plate. Phages from the interior and surface of each leaf were separated from bacteria by chloroform treatment of the buffer solutions. This allowed us to generate an inoculum that was representative of the natural phage community as it did not require passaging through a bacterial host. Using a cross-inoculation design, 7 µl of each phage inoculum was spotted in a grid formation onto a lawn, grown in soft KB agar (0.6% wt/vol), of each bacterial isolate. Phage plaque formation within the spot was compared with bacterial growth across the lawn, allowing us to define each bacterial isolate as either susceptible or resistant to the local, sympatric phage population (*i.e.*, to quantify susceptibility to phages from the same leaf). We then randomly chose 40 bacterial isolates from each category (susceptible or resistant) to further characterise and measure motility and growth rate. Of the 80 isolates examined, only 6 pairs showed strong phenotypic and genotypic similarity, and each of these pairs were combined in our statistical analyses.

Bacteria typically exhibit three types of motility: swimming, swarming, which are primarily flagella-dependent, and twitching, which is dependent on type IV pili (Mattick 2002; Harshey 2003). To examine these motility mechanisms, we measured dispersal capability of each of the bacterial isolates under different agar environments. For each motility assay, bacterial isolates were first grown overnight from freezer stocks in KB broth at 28 $^{\circ}$C. A small amount of each culture was then used to inoculate the centre of 90

$cm^2$ petri dishes containing 25 ml of KB medium with the appropriate concentration of agar, dried briefly before use. Twitching motility was assessed on KB medium solidified with 1.2% (wt/vol) agar and estimated using the bacterial movement between the interface of the petri dish and agar surface, while swarming and swimming motility assays were performed on KB medium containing 0.6% and 0.3% (wt/vol) agar, respectively (Rashid and Kornberg 2000), and estimated by area dispersed through the agar. The area of dispersal was measured after either 24 hours (for swimming) or 48 hours (for swarming and twitching) of incubation at 24 ˚C by demarcating the area covered, photographing the plate with a measurement standard, and analysing the area digitally using ImageJ 1.41o (Abramoff et al. 2004). All area data were square root transformed and three replicate assays were run for each bacterial isolate.

### In vitro *Growth Rate and Density Assays*

We measured the growth rate and final bacterial density for each of the 80 natural bacterial isolates. For growth rate assays, KB cultures were grown overnight at 28 ˚C and diluted by a factor of 1:100. Then, 10 µl of each dilution was added to a 96-well microplate containing 90 µl of KB per well and optical density at 600 nm was measured every 45 minutes at an incubation temperature of 24 ˚C with 5 second shaking prior to read for 24 hours using a microplate spectrophotometer (BioTek Powerwave XS, Northstar Scientific Ltd., Bedfordshire, UK). The period of exponential growth occurred between 4 and 12 hours, during which time $V_{max}$ (measured as milli-optical density units per minute (mOD/ min)), the maximal rate of change in optical density during log growth, was calculated. For density assays, cultures incubated for 24 hours were diluted by a factor of 1:1 (in order to bring them within a range of measurements for which the readings were most accurate) and measured for optical density at 600 nm.

## *Characterisation of Isolates*

The identity of each bacterial isolate, to the genus level, was determined by sequencing an 800 bp region of the 16S rRNA subunit using the forward primer 27F (Lane 1991) and reverse primer 907R (Muyzer et al. 1998). The reaction contained 1 U *Taq* DNA polymerase (Invitrogen, Paisley, UK), 1X *Taq* Buffer, 3 mM $MgCl_2$, 0.2 μM dNTP's, 0.2 pm of each primer, and 0.5 μl of a 1:10 dilution of an overnight KB culture. PCR amplification was performed at 95 ºC for 4 minutes, 29 cycles of 95 ºC for 45 seconds, 52 ºC for 1 minute, and 72 °C for 2 minutes, with a final elongation at 72 ºC for 10 minutes. The product was then sequenced by Geneservice (Oxford, UK) using the reverse primer. These sequence data have been submitted to the GenBank database under accession numbers HQ529384-HQ529465. Each bacterial isolate was assigned to the genus level, using the NCBI database, based on highest sequence similarity; all but five of the isolates had over a 97% similarity to a previously characterized isolate, with the other five being between 93 and 95% similar and all isolates had an e value of 0. Isolates were not assigned to the species level due to the highly conserved nature of the sequenced 16S rRNA region.

## *Selection Experiment*

To specifically examine how the acquisition of phage resistance might alter motility function we performed an experimental evolution study using a random subset of the natural isolates. Because of both the observed positive correlation between motility and resistance to phages and the epidemiological significance of the species (Hirano and Upper 2000; Webber et al. 2008; Green et al. 2009), we chose to focus exclusively on 10 bacterial isolates from the leaf interior that had >99% sequence similarity to known isolates of *Pseudomonas syringae.* Importantly, these isolates were all sampled from

separate leaves to decrease the probability of pseudo-replication. To experimentally examine the relationship between swarming motility and phages in the environment, we performed 10 serial transfers (approximately 10-16 bacterial generations per transfer) of each bacterial isolate into fresh soft agar (0.6% wt/vol) that contained either high concentrations, low concentrations or no phages (Figure 5.1). To initiate the experiment, overnight cultures from a single bacterial colony were grown in KB broth, which was then pipetted directly onto the centre of a 144 $cm^2$ square petri-dish (Fisherbrand, Leicestershire, UK) containing 40 ml soft KB agar. Plates were left in a humid incubator at 24 $^{\circ}$C for 24 hours, after which samples were taken for the next transfer under one of two selection regimes: positive (termed 'dispersal' treatment) or neutral (termed 'random' treatment) selection for dispersal (Figure 5.1). For the dispersal selection lines, we took six samples, equally spaced apart, from the outer edge of the colony range. For the random selection bacterial lines we took six samples from throughout the colony, as chosen by a random number grid. Each sample was taken by stabbing a sterile 1 ml pipette (Finnpippette, Northumberland, UK) through the agar to the bottom of the plate and then transferring the agar stab to 1 ml of M9 solution (1 mM thiamine hydrochloride, 0.4% glycerol, 0.2% casamino acids, 2 mM $MgSO_4$, 0.1 mM $CaCl_2$). The pipette tip was washed thoroughly in the solution and, after vortexing, 2.5 µl of the solution was used to inoculate fresh agar plates, as described above. This was repeated every 24 hours for ten transfers. Plates were poured with fresh media at each transfer so as not to confound age of agar plate with dispersal ability.

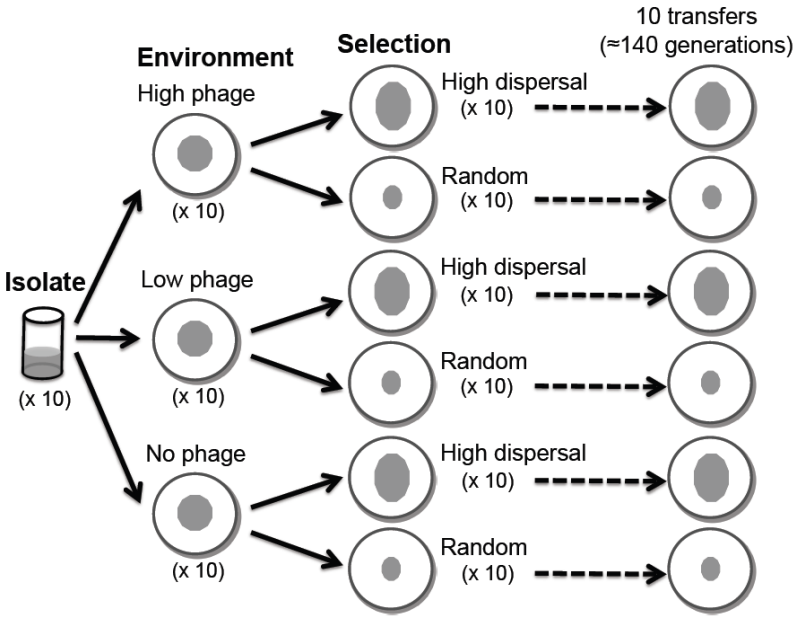


**Figure 5.1: Schematic showing setup of experimental treatment groups**

Ten natural isolates were experimentally evolved over ten transfers in one of three phage environments: high phage concentration (40 µl phage inoculum /40 ml 0.6 % agar), low phage concentration (4 µl phage inoculum /40 ml 0.6 % agar) or no phage treatment (where no phage was added to 0.6 % agar). In addition, each line was evolved under one of two selection regimes: positive (termed 'dispersal' treatment) or neutral (termed 'random' treatment) selection for dispersal. In total, we had 60 selection lines evolved over approximately 140 generations of selection.

The phage inoculum was generated by isolating 16 individual phage types (*i.e.*, independent plaques) from 16 different horse chestnut leaves used in the previous cross-inoculation (Koskella et al. 2011). Each phage isolate was passaged once through one of two previously characterized strains of *P. syringae pv. aesculi* (*P.s. pv aesculi* 6617 and 6623; Green et al. 2010), in order to amplify numbers of phage particles, and separated from the bacteria using chloroform. The 16 phage isolates were mixed to produce a stock inoculum, representing a subset of the naturally occurring phage community in the leaf environment, and stored at 4 ºC. This design allowed us to hold the phage environment

relatively constant while the bacteria evolved in response. To create a homogeneous selective environment, phage inoculum was vortexed into the soft agar prior to solidifying, when the agar reached about 40 ºC. After ten transfers, each line was assayed for growth rate and motility in a phage-free common garden (*i.e.*, all treatments were grown under the same laboratory environment), and under three agar concentrations. Bacterial densities ($OD_{600}$) of overnight cultures of each evolved and ancestral strain were also measured in both the presence and absence of phages.

### *Statistical Analyses Comparing Bacterial Dispersal and Susceptibility to Phages*

Analyses and figures were produced on PASW Statistics 18 (SPSS; part of IBM UK ltd, Middlesex, UK). We first used a two-way analysis of variance to compare the area dispersed (square-root transformed) across bacterial isolates of different genera and susceptibility to phages. In addition, independent samples t-tests were run to compare susceptibility to phages and dispersal within the *Pseudomonas* and *Erwinia* isolates from both the leaf surface and leaf interior. For the experimental evolution results, we examined the initially susceptible and initially resistant isolates separately, due to the dramatic differences in means and variance between them, and we included bacterial strain as a random factor in each model. At the end of the experiment, we examined the area dispersed within a common garden (in the absence of phages) at the end of the experiment using a separate two-way analysis of variance for: (1) the 30 susceptible lines; and, (2) the 30 resistant lines, and compared population growth parameters to dispersal using Pearson correlation coefficients. In all cases, the area dispersed was square root transformed.

# Results

## *Relationship between Resistance, Growth and Motility in Natural Populations*

Sequencing of the chosen bacterial isolates revealed that the surface of the leaf was primarily dominated by *Erwinia*-like species (>85%), while the leaf interior community was comprised of both *Erwinia*-like (50%) and *Pseudomonas*-like species (47%). We excluded bacterial isolates from other genera, including *Rhanella* and *Pantoea,* from subsequent analyses due to low replication. Overall, 44% ($N/N_{TOT}$ = 24/54) of the *Erwinia*-like isolates and 61% ($N/N_{TOT}$ = 14/23) of *Pseudomonas*-like isolates were susceptible to sympatric phages, *i.e.* those collected directly from the same leaf as the bacteria being tested. We chose to focus specifically on susceptibility to sympatric phages, using the leaf homogenate as an inoculum, because this measure more accurately reflects local selection pressures and did not require amplification through a bacterial host.

The relationship between swarming motility and resistance to sympatric phages significantly differed across bacterial genera (Figure 5.2a; interaction effect for genus x phage susceptibility: $F_{1,38}$ = 5.99, $P$ = 0.020). Specifically, there was no difference in dispersal capability between resistant and susceptible strains of the *Erwinia*-like isolates from the leaf surface or interior (Figure 5.2a). However, for the *Pseudomonas*-like isolates from the leaf interior (surface isolates were excluded due to low sample size) resistant isolates showed a higher swarming motility range than the susceptible isolates (Figure 5.2a; $t_{17}$ = 2.241, $P$ = 0.039) but did not show a difference for swimming or twitching motilities (swimming: $t_{17}$ = 1.143, $P$ = 0.271; twitching: $t_{17}$ = 1.219, $P$ = 0.099).

There was no difference in growth rate, measured as $V_{max}$ (mOD/ min) between resistant and susceptible *Erwinia*-like isolates from the surface or the interior ($P > 0.05$), but for *Pseudomonas*-like isolates from the leaf interior, resistant isolates had a higher growth rate than susceptible isolates (Figure 5.2b; $t_{17} = 3.234$, $P = 0.005$). Importantly, the dispersal area during swimming and swarming motility was significantly correlated with growth rate for both the *Pseudomonas*-like isolates (Pearson correlation, swimming: $r = 0.517$, $P = 0.012$; swarming: $r = 0.669$, $P < 0.001$) and the *Erwinia*-like isolates (swimming; $r = 0.317$, $P = 0.019$; swarming: $r = 0.359$, $P = 0.008$). However, twitching dispersal was not correlated with growth rate for either *Pseudomonas*-like isolates ($r = 0.310$, $P = 0.150$) or *Erwinia*-like isolates ($r = -0.100$, $P = 0.473$).

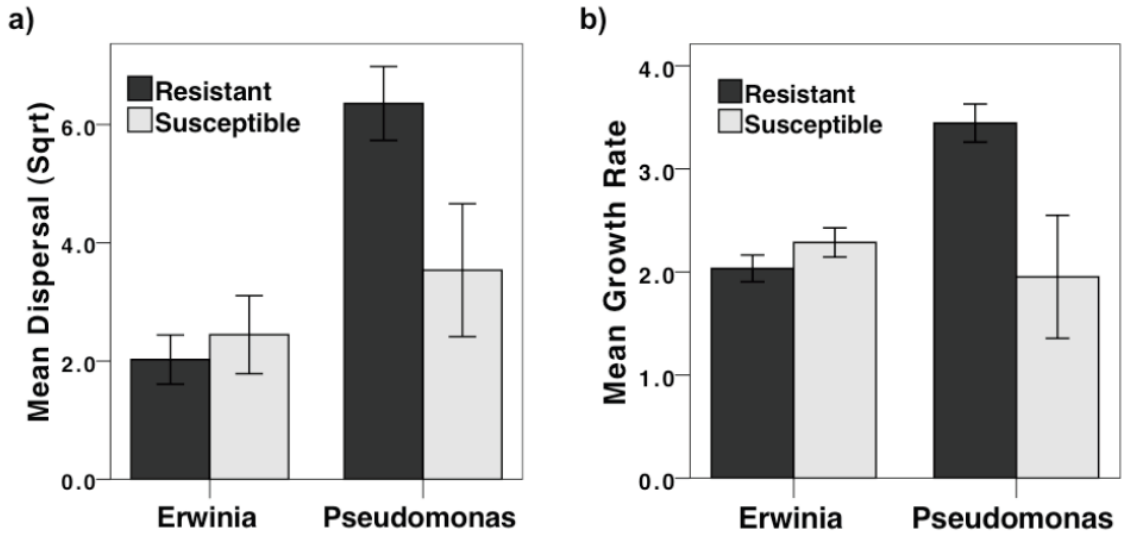


**Figure 5.2: Graph to show dispersal and growth rate between most common isolates**

Relationship between dispersal (a) and growth rate (b) for the 40 *Pseudomonas*-like and *Erwinia*-like isolates collected from the leaf interior. Resistance (dark grey) or susceptibility (light grey) to local phage is compared to determine whether there exists a cost to resistance with regard to bacterial motility or growth. Dispersal was measured as area covered ($cm^2$) over 48 hours on soft agar (0.6%). Values were square root transformed to correct for non-normality. Error bars represent ±1 SEM.

### *Selection for Bacterial Resistance to Phages and/or Motility*

Of the ten Pseudomonad isolates chosen for experimental evolution, five were initially susceptible and five were initially resistant to the phage inoculum. Of the initially susceptible strains, all were susceptible to at least half of the 16 phage isolates used in the inoculum (mean susceptibility of 70.0% ±19.7 SD). Of the five initially resistant bacterial isolates, resistance was complete across all 16 phage isolates (susceptibility of 0%). At the start of the experiment the presence of phage had a significant negative effect on motility for the initially susceptible strains ($F_{1,24} = 7.223$, $P = 0.013$), but had no effect on the initially resistant strains ($F_{1,24} = 1.624$, $P = 0.215$). After ten transfers of experimental evolution in the presence of phages (at both high and low concentration and across random and dispersal selection regimes), each of the five bacterial isolates that were initially susceptible to phages had evolved resistance to most or all of the 16 phage isolates used in the inoculum (Figure 5.3). For these isolates there was no longer an effect of phages in the environment on motility ($F_{1,24} = 0.400$, $P = 0.533$). These strains did not evolve increased resistance in the absence of phages (GLM for mean proportion of infective phages, effect of Time: $F_{1,18} = 16.56$, $P = 0.002$; Phage treatment: $F_{2,18} = 4.173$, $P = 0.032$; Time x Phage treatment interaction: $F_{2,18} = 4.173$, $P = 0.032$). The bacterial isolates that were initially resistant remained resistant throughout the duration of the experiment. Although we did not directly allow for coevolution between bacteria and phages (Bohannan and Lenski 2000a; Buckling et al. 2009), some degree of coevolution may have occurred between phages that were passively collected along with bacteria at each transfer; creating an additional benefit to dispersing away from the inoculation site, as coevolved phages are likely to have increased infectivity to the evolving bacteria (Bohannan and Lenski 2000a; Brockhurst et al. 2005). However, all resistance assays

were run using ancestral phages, as these represented the selection pressure throughout the experimental environment.

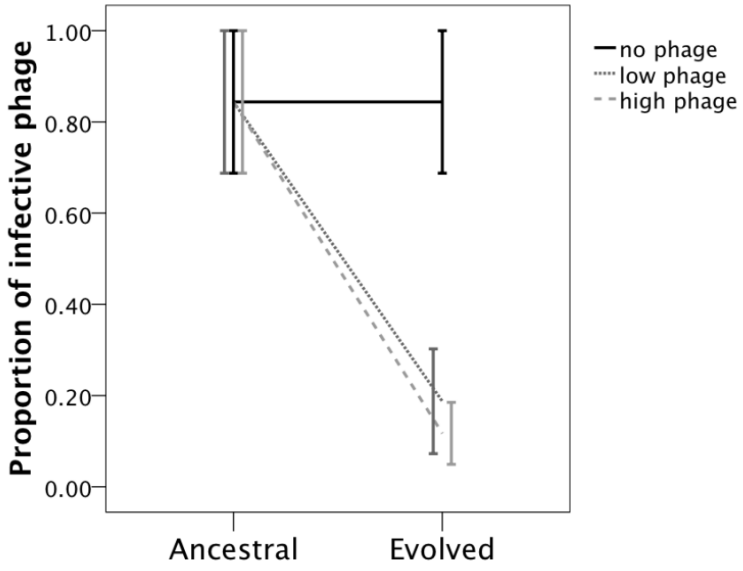


**Figure 5.3: Change in bacterial susceptibility to phage over the course of the experiment**

Evolution of resistance for the five initially susceptible isolates over ten serial transfers in either the presence (dashed lines) or absence (solid line) of phage. Proportion of infective phage represents susceptibility to each of the 16 phage clones used in the experimental evolution inoculum. Error bars represent ±1 SEM.

To confirm our measures of phage resistance, we also measured growth rates of ancestral and evolved lines in the presence of phages. For those strains that were initially resistant, we found no difference in population density in either the presence or absence of phages (GLM with arcsinsqrt transformed density, interaction effect of Time x Phage presence: $F_{1,\ 42} = 0.206$, $P = 0.652$). However, for those strains that were initially susceptible and evolved resistance to phages over the course of the experiment, the ancestral bacterial lines had a significantly lower density than evolved bacterial lines in the presence of phages, but not in the absence of phages (Time x Phage presence: $F_{1,\ 42} = 5.977$, $P = 0.019$), indicating that phages were initially reducing population size of

susceptible bacteria but that population size was not affected by phages once resistance had evolved.

We measured area dispersed and growth rate of all isolates at the start of the experiment in the absence of phages and found that the initially resistant bacterial isolates had a higher mean swarming dispersal (mean area dispersed 56.45 $cm^2$ ± 7.05 SD in 0.6% agar) than the susceptible isolates (3.14 $cm^2$ ± 3.36 SD) and that initially resistant isolates had a higher growth rate (mean Vmax 3.49 ± 0.36 SD) than susceptible isolates (1.55 ± 0.79 SD). These results were consistent with the findings from the full sample of natural bacterial isolates.

After the ten serial transfers of experimental evolution we again assayed motility in a phage-free environment to examine the effect of both phage and dispersal selection regime on the evolution of dispersal, without the confounding ecological effects of phages (Figure 5.4). The initially susceptible strains did not show a response to selection for increased dispersal ($F_{1,20}$ = 0.333, $P$ = 0.571) and there was no evidence for a direct effect of phage treatment on dispersal ($F_{2,20}$ = 0.337, $P$ = 0.718) nor for any interaction between phage and selection ($F_{2,20}$ = 0.080, $P$ = 0.923), suggesting a lack of a causal link between phage resistance and motility (Figure 5.4a). However, the initially resistant strains were able to respond to selection for increased dispersal ($F_{1,20}$ = 10.505, $P$ = 0.004), regardless of phage treatment (main effect of Phage: $F_{2,20}$ = 1.995, $P$ = 0.162, interaction between Phage and Selection: $F_{2,20}$ = 0.514, $P$ = 0.606, Figure 5.4b). Finally, there were few correlated changes in swimming or twitching motility resulting from either the dispersal or phage selection regimes: the only significant effect, after controlling for multiple tests, was increased swimming motility under positive selection

for dispersal (compared with random) in the initially resistant lines (Figure 5.4b; main effect of selection: $F_{1,20} = 8.268$, $P = 0.009$).

We also investigated how the selection regimes affected growth rate of each population, with the specific focus on whether there were costs associated with the acquisition of resistance. We found no evidence that dispersal selection regime or phage-imposed selection affected population growth rate for either initially resistant or initially susceptible bacteria ($P > 0.10$ for all treatments). Importantly, although we found a correlation between growth rate and dispersal for the initially resistant strains (Pearson $r = 0.390$, $P = 0.033$) there was no correlation between growth rate and dispersal for the initially susceptible lines ($r = 0.000$, $P = 0.999$), demonstrating that dispersal estimates were not simply a function of population growth. This result was qualitatively the same when comparing population density and dispersal.

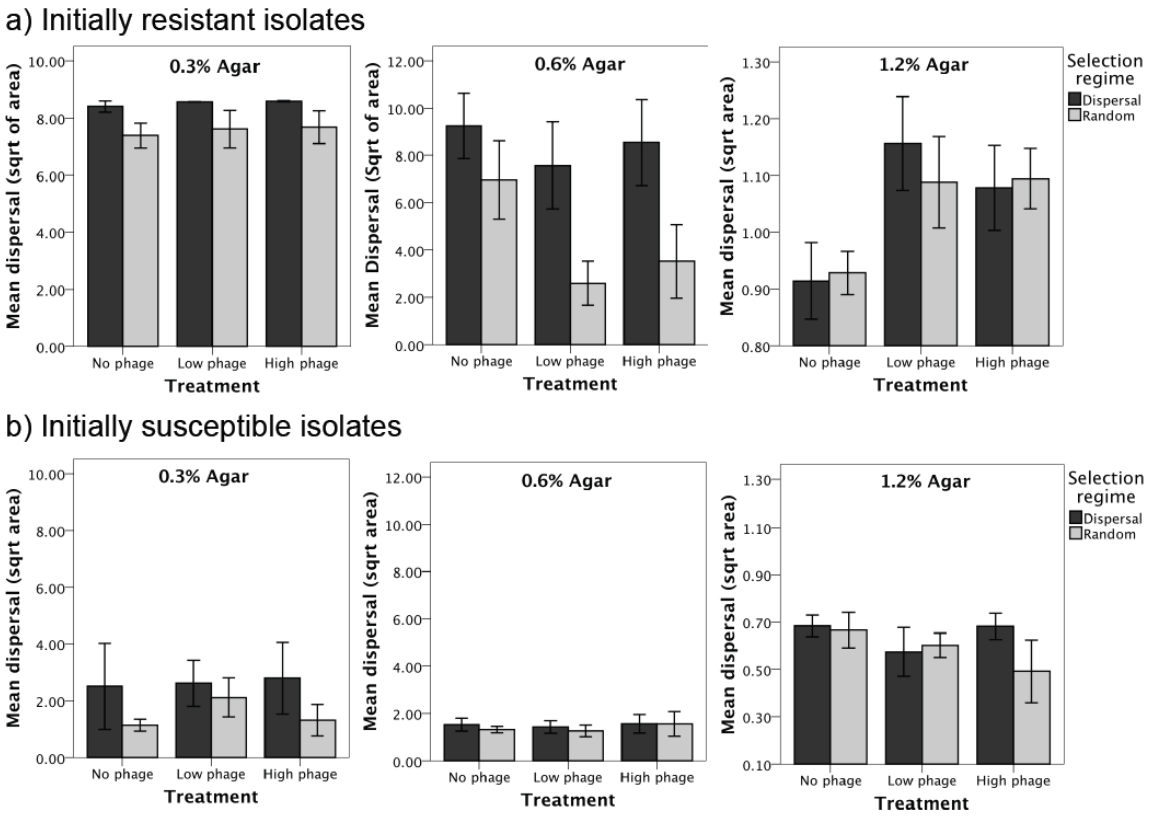


**Figure 5.4: Graph to show motility of evolved strains across different agar concentrations**

Results from common garden experiment run at the end of the selection experiment (*i.e.*, transfer 10) for initially resistant (a) and initially susceptible (b) isolates. Measured on 0.3% agar (swimming; left panel), 0.6% agar (swarming; middle panel); and 1.2% agar (twitching; right panel). These experiments were run in the absence of phage in the environment to examine differences between the lines that are not resulting from interactions with or ecological feedback from phage. "Treatment" therefore represents the phage environment of each line during the course of experimental evolution and not within the common garden experiment. Area dispersed is square root transformed and error bars represent ±1 SEM.

# Discussion

We combined an examination of natural phenotypic variation with experimental evolution to investigate the potential link between resistance to phages and bacterial fitness (growth rate and motility). We found that, contrary to expectation, natural bacterial isolates that were resistant to local phages had neither reduced motility nor

reduced growth rates relative to those that were susceptible. Generally, there was no relationship between bacterial motility or growth rate and resistance to phages from the local environment (as present in the leaf homogenate) for *Erwinia*-like isolates and a positive correlation for *Pseudomonas*-like isolates under favourable, laboratory conditions (Figure 5.2). This result is in line with previous work from marine Cyanobacteria showing that costs of resistance to phage are not ubiquitous and instead, are highly dependent on the virus and bacteria strain being examined and on whether strains are in direct competition (Lennon et al. 2007). Importantly, we could not rule out the possibility that more motile and resistant strains represented different species or pathovars than less motile and susceptible strains. Therefore to investigate any causal links between resistance to phages and bacterial motility, we experimentally evolved 10 *Pseudomonas*-like bacterial lines in either the presence or absence of phages, and under either positive or random selection for dispersal.

After ten serial transfers of experimental evolution we found that bacteria that were initially susceptible to phages had evolved resistance in the presence, but not the absence, of phages (Figure 5.3). Moreover, at the start of the experiment we saw decreased motility in the presence of phages for those strains that were susceptible to infection, but not for those that were initially resistant. This result adds to a growing body of evidence that parasites can have a direct impact on host demography (Fellous et al. 2010; Bradley et al. 2005; Cameron et al. 1993). In addition, we found that bacterial motility decreased over the course of the experiment for most bacterial isolates (regardless of phage treatment and even under selection for increased dispersal), suggesting a cost to motility such that flagella and/or pili function is reduced under favourable laboratory conditions. Importantly, the fact that the initially resistant bacteria showed reduced motility but no loss of resistance to phages in the control (no-phage) treatments, suggests that there is no

negative correlation between resistance and either growth rate or motility in the laboratory. This overall reduction in motility is initially surprising given that half the lines were selected for increased dispersal and suggests that the imposed selection regime was relatively weak, as only the initially resistant, and not the initially susceptible, lines showed increased motility under selection for high dispersal (Figure 5.4). This result warrants further study as we cannot provide a clear mechanistic explanation, and understanding whether phages directly hinder the response of susceptible bacteria to selection for increased motility is of key interest. In addition, under the no-phage, common garden conditions at the end of the experiment, we found no evidence that phage-imposed selection for resistance in the initially susceptible lines was associated with a change in either the motility trait under selection (swarming), or twitching and swimming motility (Figure 5.4). Finally, there was no evidence of growth costs associated with the acquisition of resistance (although the strains were never put in direct competition). These data suggest no causal relationship, either positive or negative, between the acquisition of phage resistance and dispersal ability.

A number of other studies, like ours, have found no clear cost in terms of population growth associated with resistance to phages (Lenski 1988a; Lythgoe and Chao 2003; Meyer et al. 2010). However, the lack of an observed pattern is somewhat surprising given the predicted association of many phages with flagella or pili number and function (Icho and Iino 1978; Bradley 1980; Mattick 2002). There are a number of possible explanations. First, phages that use motility organelles (*i.e.*, pili and flagella) as attachment sites may be relatively uncommon in the natural leaf environment and hence do not impose very strong selection against bacterial motility. This interpretation may have been influenced by our phage isolation method; chloroform treatment is known to destroy primarily lipid-based phages (Leers 1969), and if a correlation exists between

phage-targeted bacterial receptors and phage coat composition we could have missed an effect of phage-mediated selection. Second, observed dispersal behaviours may be influenced by traits other than motility organelles, such as cell size, chemotaxis or quorum sensing (Harshey 2003), which are not altered by the acquisition of phage resistance; a possibility that would indicate bacterial motility can evolve independently of phage-mediated selection. Third, it is plausible that motility-associated costs may only be visible under stressful environmental conditions. For example, previous experimental work has shown that costs of resistance to phages in both *Escherichia coli* B (Bohannan and Lenski 2000b) and *Pseudomonas fluorescens* (Lopez-Pascua and Buckling 2008) are increased in nutrient-poor environments. Fourth, it is possible that any costs were rapidly compensated by second site mutations, as is commonly observed with antibiotic resistance (MacLean et al. 2010). However, such rapid compensation would suggest that costs of phage resistance are relatively unimportant in natural populations.

Overall, our results do not support a causal link between the evolution of resistance to phages and bacterial motility in natural populations, although we did find a surprising positive association between phage resistance and bacterial motility for Pseudomonads in the phyllosphere. This of course does not rule out an important role of phage-imposed selection on the evolution of motility, as costs associated with resistance are likely to be contingent upon genetic background, local environment and the precise measures of fitness, but does suggest that the effect of phage-imposed selection is unpredictable and is likely to depend on the natural phage community and bacterial environment. This is an important consideration for phage therapy of pathogenic bacteria, where virulence is often associated with motility traits and any association between phage resistance and bacterial motility could have important consequences over coevolutionary time (Josenhans and Suerbaum 2002). Understanding these potential costs will be key as phage therapy

becomes a more common method for controlling pathogenic bacterial populations (Goodridge 2004; Levin and Bull 2004). More generally, these results add to a growing body of work across a range of taxa that points to the wide range of host fitness traits correlated with pathogen resistance (Boots and Begon 1993; Ferdig et al. 1993; Fellowes et al. 1998; Langand et al. 1998; Zhong et al. 2005; Buckling et al. 2006; Morgan et al. 2009; Williams et al. 1999; Yourth et al. 2002; Lythgoe and Chao 2003; Sanders et al. 2005). This method of combining natural observations with laboratory selection experiments shows promise in furthering our understanding of the importance of phages, and other selective pressures, as drivers of bacterial evolution and diversity in natural environments.

# Chapter 6: Bacterial Motility Confers Fitness Advantage in the Presence of Phages


## Abstract

Dispersal provides the opportunity to escape harm and colonise new patches, enabling populations to expand and persist. However, the benefits of dispersal associated with escaping harm will be dependent on the structure of the environment and the likelihood of escape. Bacteriophages are a strong and common threat for bacteria in natural environments and offer a good model system on which to explore parasite-mediated selection on host dispersal. In order to understand how bacteriophages can influence the optimal dispersal strategy of a bacterial host I used two transposon mutants of the opportunistic bacteria, *Pseudomonas aeruginosa* which varied in their motility (a disperser and a non-disperser), and the lytic phage ΦKZ. The phage was distributed either: in the central point of colony inoculation only, thus offering an escape route for the bacteria; or, present throughout the agar. I found dispersal to be a favourable trait under both phage conditions, with dispersers maintaining a relatively higher fitness over non-dispersers. This study helps to highlight the importance of structure in determining responses to bacteria-phage interactions, and the crucial role parasites can play on the evolution of motility.

# Introduction

Dispersal provides a number of potential benefits including; reduced kin competition (Hamilton and May 1977; Chapter 2), reduced inbreeding (Bengtsson 1978), and increased probability of survival in temporally heterogeneous environments (McPeek and Holt 1992; Van Valen 1971). Dispersal will be selected against if there are associated high costs, such as reduced reproduction or increased mortality (Rousset and Gandon 2002). One potential cost comes from the probability of encountering harm imposed by natural enemies. However, dispersal does not only have the potential to increase encounters, but also offers the opportunity for escape in heterogeneous environments (Augspurger 1983; Brown et al. 1995). Examples of the overwhelming benefit of escaping your enemies are well documented throughout modern history in numerous instances of introductions of invasive species to non-native environments (Torchin et al. 2003).

In this chapter, I explore the dynamics of parasite (bacteriophage) imposed selection on the evolution of dispersal of the pathogenic host bacterium, *Pseudomonas aeruginosa*, where phages are both localised and distributed around the environment. Phages are ubiquitous and will act as strong natural selective agents for bacteria. Moreover, understanding the selective pressures which shape bacterial motility (which in this experimental design is equivalent to dispersal) is particularly important for pathogenic species, as motility traits have been identified as important virulence factors (Drake and Montie 1988; Josenhans and Suerbaum 2002). Phage infection begins with the attachment of the phage to a specific surface receptor on the bacterial host. The phage injects its DNA into the host cell, which then hijacks the host's replication machinery in

order to propagate new phage particles. The bacterial cell then lyses, releasing the viral progeny into the environment (Lenski 1988b).

Phages can affect selection for bacterial dispersal in numerous ways. Phages are known to exploit motility surface structures (flagella and pili), and experiments have shown that pilus-specific and flagellatropic phages can gain resistance through alteration of these structures, resulting in reduced motility (Bradley and Altizer 2005; Brockhurst et al. 2005; Icho and Iino 1978; Mattick 2002). To investigate the ecological generality of this association, we (Chapter 5) used experimental evolution combined with an observational survey to look at the relationship between bacterial motility and phage resistance in natural populations of *Pseudomonas* spp. and a community of phages from horse chestnut trees. Surprisingly, there was no association between bacterial motility and phages, in spite of phages being present throughout the experimental environment and even a suggestion that the opposite could in fact be true – survey data suggested natural phages might have been imposing selection for motility in natural populations (Chapter 5). This suggests that dispersal (or correlates of dispersal – such as motility) might be advantageous in the presence of phages. Plausible mechanisms are that: (*i*) it is harder for phages to transmit within populations of motile bacteria, because their local densities might be lower; (*ii*) phage attachment could be more difficult on a motile host, and thus motility might reduce the rate at which phage can successfully bind. Here I explicitly test how phages which do not target motility organelles impose selection for dispersal in a bacterial host.

I use the phage ΦKZ which targets the bacterial cell wall (lipopolysaccharide) of *P. aeruginosa* (Ceyssens and Lavigne 2010), but importantly does not target motility organelles (*i.e.* pili or flagella). *P. aeruginosa* is an opportunistic pathogen of clinical interest which inhabits a wide range of environments – from solid to aqueous. In order to

cope with a variety of substrates, the bacterium uses versatile motility mechanisms (swimming, swarming, and twitching) which enables it to move across most substrates. Natural isolates have been found to maintain this variability suggesting motility is likely to be under strong selection in many different habitats (Head and Yu 2004). Within the experimental setup, phages act as an environmental threat either localised to a central patch, or omnipresent throughout the environment. I manipulated dispersal behaviour by using genetically modified pili (surface organelles which aid motility in hard environments) mutants of *P. aeruginosa*; PilA mutants were unable to express pili, and PilU mutants were 'hyperpiliated', *i.e.* they expressed pili but were unable to retract them. In semi-solid agar the PilA mutants acted as the 'dispersers' as they were able to freely move through the substrate; the PilU mutants were the 'non-dispersers', as the permanently extruded pili caused the bacteria to become stuck, restricting movement. I hypothesised that dispersing bacteria should have a clear advantage in both the Local conditions, where they could readily escape phage, and the Global conditions, where increased motility might limit the ability of phages to successfully bind and transmit.

## Methods

### *Strain Details and Phage Amplification*

Two transposon mutants defective in type IV pili and generated from a wild-type strain of *P. aeruginosa* (PAO1) were used: a PilU (non-disperser) mutant which is able to express but unable to retract pili (hyperpiliated), and a PilA (disperser) mutant which is absent of pili. Cultures were grown overnight in a homogeneous (shaken) environment (0.9 g) at 37 $^{\circ}$C, in 30 ml universal vials containing 6 ml fresh medium (King's B (KB):

20 g/l protease peptone, 10 g/l glycerol, 1.5 g/l potassium phosphate and 1.5 g/l magnesium sulphate heptahydrate).

The phage ΦKZ was chosen because it targets the bacterial LPS cell membrane (Ceyssens and Lavigne 2010) and not motility structures (such as pili), which is important in the context of this experiment because I wanted the motility profile and not the presence or absence of motility structures to determine the fitness of the genotypes. An amplified phage stock solution of ΦKZ was created via passaging through an ancestral host, followed by chloroform treatment and centrifugation, to burst and separate bacterial cells, respectively (Buckling and Rainey 2002). The phage stock solution containing approximately $2.2x10^{10}$ phage particles/ml was stored at 4 $^{\circ}$C. Phage was spotted onto a bacterial lawn to test the infectivity to each of the motility variants, and both types were found to be sensitive.

### *Treatment Conditions*

To test the optimal dispersal strategy of *P. aeruginosa* in the presence of ΦKZ I measured the fitness of the two motility variants (disperser and non-disperser), under two phage treatment conditions – Local, whereby the phages were local only to the inoculation site; and Global, whereby the phages were present throughout the environment. 25 ml of semi-solid KB agar (0.6% wt/vol agar), was poured into 20 cm diameter petri-dishes and allowed to dry in the laminar flow hood for 20 minutes. Agar plates being used for the Global treatment group were supplemented with 1000 µl phage stock solution. Inoculum for starting bacterial populations under Local treatment were created via a 1:1 mix of ΦKZ phage stock and each of the dispersal variants (mixed immediately prior to inoculation onto agar plate). For Global treatment group, pure

disperser and non-disperser cultures were used as the inoculum. 2.5 µl of inoculum was pipetted into the centre of the agar plate. There were 4 replicates in each treatment group.

As controls I grew dispersers and non-dispersers with phage ΦKZ in both KB liquid broth (where both motility variants should have entirely unrestricted movement), and hard KB agar (1.2 % wt/vol) (where both motility variants should have entirely restricted movement). In addition I grew the motility variants in semi-solid KB agar in the absence of any phages. In order to account for any differences in dispersal distance due to growth rate I measured the period of exponential growth, $V_{max}$ (the rate of change of optical density during log growth), for the disperser and non-disperser and found no significant difference between the strains (data not shown).

### *Data Collection and Statistical Analysis*

Methods were as in Chapter 2. Samples were taken using a 1 ml pipette (Finnpipette), at regular 5 mm intervals along the radius of the colony. The samples were then washed in M9 (12.8 g/l $Na_2HPO_4$, 3 g/l $KHPO_4$, 0.5 g/l NaCl, 1 g/l $NH_4Cl$), diluted to an appropriate dilution to allow colony differentiation, and plated in order to count colony forming units (CFUs). The relative fitness of dispersers versus non-dispersers was determined by calculating the ratio of the total number of each cell type across corresponding plates (disperser and non-disperser replicates were paired randomly).

Analyses and figures were produced on PASW Statistics 18 (SPSS). Significance of treatment on cell density was analysed parametrically using general linear models (GLMs). Terms used in the model are defined as: “Cell Density” [response variable], the total mean CFUs; “Treatment” [explanatory variable, factor with two levels], the phage treatment (Global or Local); and, “Strain” [explanatory variable, factor with two levels],

the motility variant (disperser or non-disperser). T-tests were performed between strains within treatment groups to test for differences in cell density.

## Results

I first established how motility behaviour was affected by phages. The average area dispersed (±1 SD) of the motility variants in soft agar (KB 0.6 % wt/vol agar) in the absence of the phages given 48 hours for growth and dispersal was 8.82 $cm^2$ (± 1.66) for the disperser, and 1.73 $cm^2$ (± 0.12) for the non-disperser (Figure 6.1c; t = 5.56, $P$ = 0.029). Under both Global and Local treatments the disperser covered a larger area than the non-disperser, with apparent directional movement (chemotaxis) out towards the edge of the plate under the Global treatment (Figure 6.1a & b; Figure 6.2a; GLM: Strain as main-effect; $F_{1,12}$ = 421.42, $P < 0.0001$), and interestingly, the disperser covered a larger area under the Global treatment compared to the Local treatment, whereas the non-disperser covered an equivalent area across both (GLM: Strain*Treatment interaction; $F_{1,12}$ = 76.92, $P < 0.0001$). These results suggest phages are not qualitatively affecting motility behaviour, *i.e.* dispersers always disperse more. Note that when strains were grown in hard agar however (and thus essentially immobilised), in the presence of phages there was no difference in area dispersed, suggesting that the motility type expressed by the dispersers on soft agar is likely to be a flagella-dependent (Figure 6.1d; Figure 6.2b; t = 2.035, $P$ = 0.112).

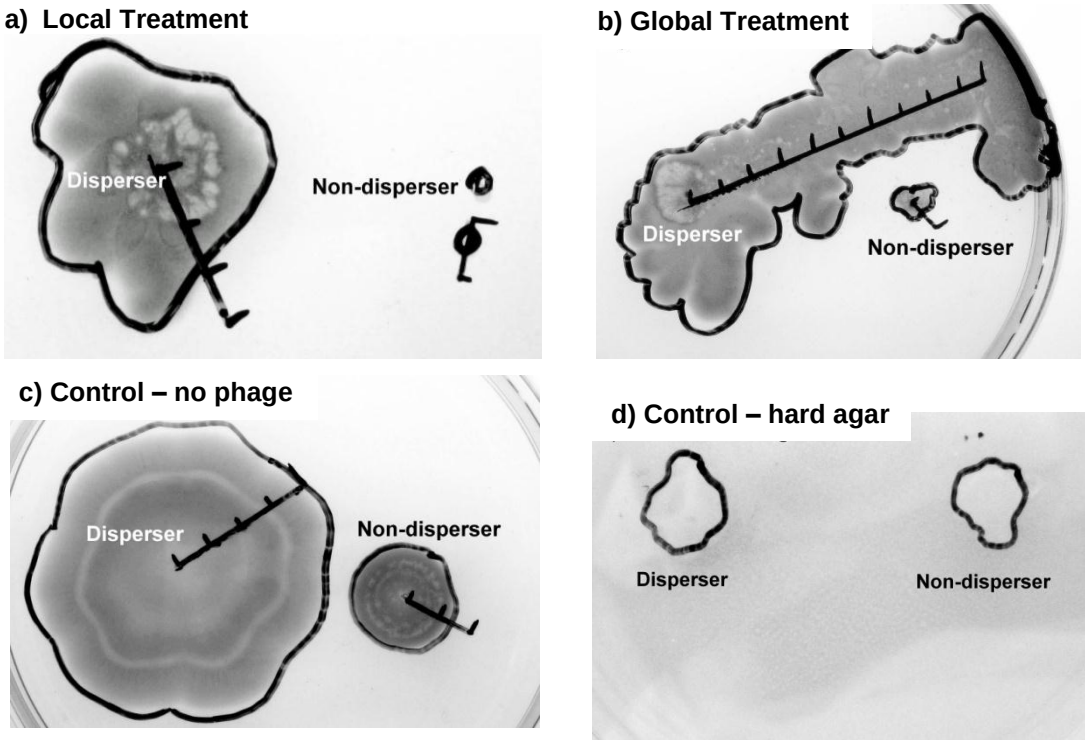


**Figure 6.1a – d: Photographs of agar plates showing colony morphology across treatment groups**

Under Local treatment the disperser moves beyond the range of the phages and moves outward in a fairly regular pattern (a). The growth of the non-disperser is dramatically reduced by the presence of the phages; under Global treatment the disperser adopts a different motility type, moving in a directional manner towards the edge of the agar plate, the non-disperser suffers a reduction in growth in the presence of phages, and does not appear to show altered motility phenotype (b); when no phages are present in the environment, in soft agar the motility variants have different dispersal ranges with the disperser covering a larger distance than the non-disperser (c); when phages are preset in the environment, in hard agar the motility variants do not have different dispersal ranges and cover similar distances (d). Note, colonies have been drawn around in pen to allow colony shape to be recognised more easily.

I then wanted to determine if motility conferred a selective advantage in the presence of phages, under conditions where bacteria could physically escape, and when phages were equally distributed throughout the environment. I compared densities after 48 hours growth and dispersal of high and low dispersing bacteria in the absence of phages, when phages were present in the inoculation site (Local) and when phages were

present everywhere (Global). The relative fitness of dispersers (*i.e*. the ratio of density of dispersers to non-dispersers) was influenced by presence of phages (Figure 6.3) (ANOVA: $F_{2,8}$ = 12.383, *P* = 0.003), with no differences between relative fitness values in the phage absent and Global treatments (*P* = 0.087), but dispersers having a fitness advantage under Local versus Global treatments (*P* = 0.049), and Local versus phage absent (*P* = 0.002). The non-dispersers suffer a large reduction in density in the presence of phages under both treatment conditions (Global: t = 930.37, *P* < 0.001; Local: t = 1269.50, *P* < 0.001), whereas the dispersers only suffer a slight reduction in density under the Global treatment (t = 20.05, *P* = 0.002), but do not incur a density reduction under the Local treatment (t = 0.064, *P* = 0.995).

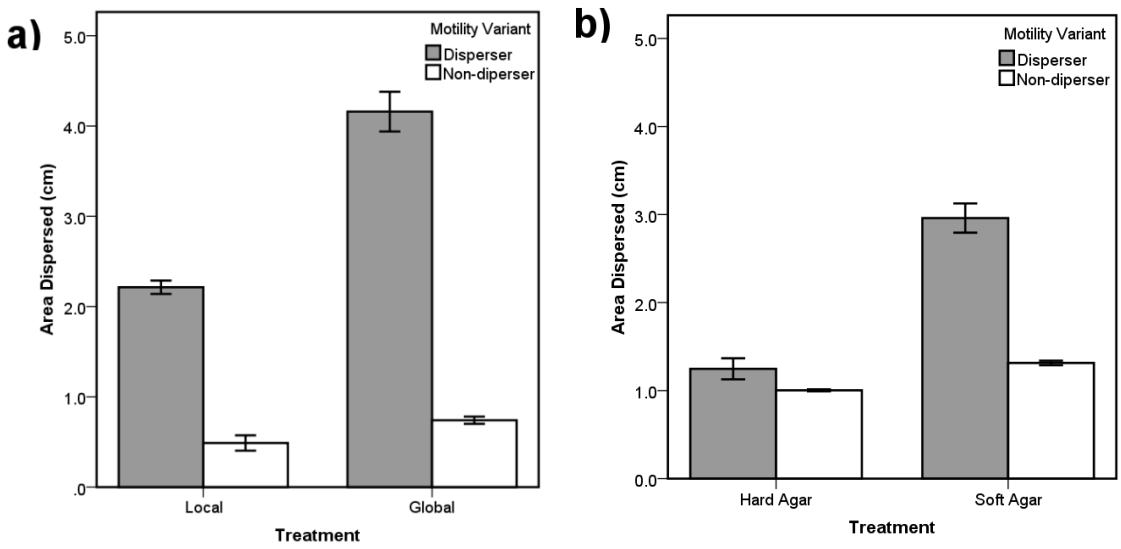


**Figure 6.2a & b: Mean dispersal area between phage treatments.**

Bars show the mean area dispersed of the disperser (grey bars) and non-disperser (white bars) when phages were located only within the inoculation site (Local treatment), and when they were present throughout the agar (Global treatment) (a). In addition, graph shows area dispersed when motility variants were grown in hard agar and soft agar in the absence of phages (b). All areas are square root transformed and error bars depict ±1 SE.

I next considered whether reduced density in the presence of phages of the non-disperser is simply the result of increased susceptibility. To test this I measured density of bacterial cells exposed to phages when swimming motility was unlikely to be important; in hard agar (where the cells are immobilised) and shaken liquid (which creates a homogeneous environment that the cells do not need motility to move though). After correcting for multiple comparisons (using the Bonferroni correction), in both cases there were no differences between total cell density of motility variants (t-test: in liquid broth, t = 2.371, $P = 0.077$; in hard agar t = 3.40, $P = 0.126$; data not shown). This suggests that the fitness differences noted above are only true when there is a disparity in motility, but not when they are equally motile.

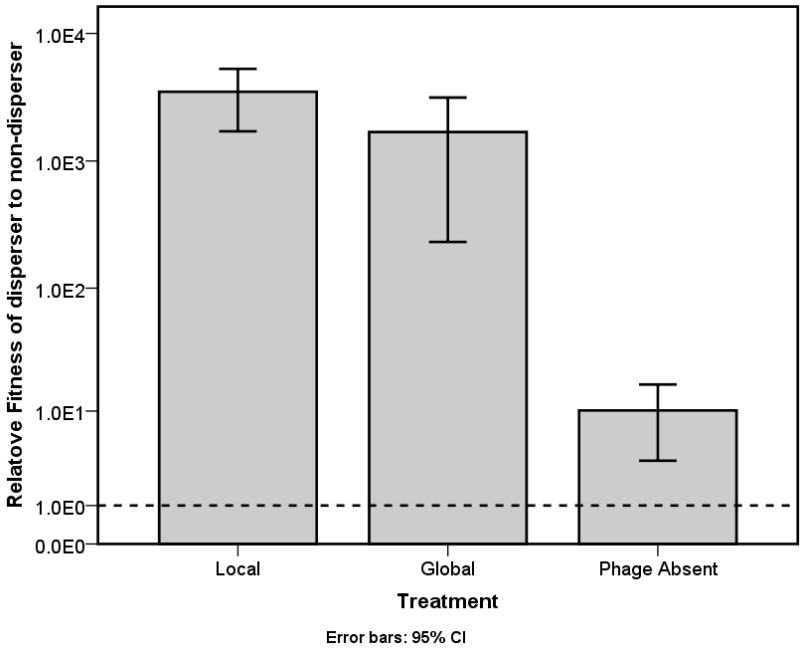


**Figure 6.3: Relative fitness of the disperser to the non-disperser across phage treatments.**

Bars show relative fitness of disperser to non-disperser in soft agar treatments: Local, phages localised to inoculation site; Global, phages present throughout the agar; and Phage absent, phages absent from the agar. The relative fitness of dispersers versus non-dispersers was determined by calculating the ratio of the total number of each cell type. The dashed line indicates a relative fitness of 1, that is, when the fitness of the disperser and the non-disperser are equivalent. Relative fitness is presented on a log scale, errors bars depict ± 95% CI.

# Discussion

My results suggest that within a semi-solid environment, motile bacteria have a fitness advantage over non-motile bacteria in the presence of phages which bind to LPS surface receptors. It has been hypothesised that the increased motility adopted by many bacteria in stressful environments (termed negative chemotaxis), facilitates the cell by: (*i*) increasing contact with more satisfactory conditions either by allowing cells to reach more favourable areas; or, (*ii*) by increasing the circulation of medium around the cell, thus making better use of available resources (Smith and Doetsch 1969). I confirm that the ability to move away from phages is advantageous. However, I also show that even when there are no favourable areas to reach, and when increased contact of medium to LPS could potentially have a negative effect on the bacterial cell (presumably by increasing phage encounter rate), there is still an advantage to initiating a motility response.

The precise mechanism behind this effect is unknown. I can rule out intrinsic differences in susceptibility to phages, because when motility was prevented there were no differences between motile and non-motile mutants. One possible mechanism is that close association between cells might increase the infectivity of the phage, presumably via increased transmission efficiency; if more motile bacteria are able to increase distances between bacterial cells, this might indirectly offer increased resistance to phages. It is also possible that the rhamnolipid secretions which enable the cell to move efficiently on soft agar surfaces acts as some form of barrier to the phages. Studies have found that alginate production is a common effective defence against phage attack (although these cells tend to be immotile; Martin 1973), and extracellular polysaccharide material (slime) allows better survival for *Staphylococcus epidermidis* in rat peritoneal

macrophages (Baldassarri et al. 2001). In addition, it is plausible that motile bacteria could provide a "moving target" for the phages which might reduce the efficiency of attachment.

Increased bacterial motility on semi-solid agar plates has also been reported to confer antibiotic resistance to cells which were found to be sensitive in a planktonic state (Lai et al. 2009; Overhage et al. 2008). In these studies the mechanism is also not clearly understood, but has been hypothesised to require the initiation of a coordinated multicellular response (such as is required in swarming and biofilm formation). The particular motility type described in these studies is swarming, which is primarily flagella dependent, but requires pili for characteristic tendril formation. Because the disperser in this study does not express any pili it is unable to form defined tendrils (Murray and Kazmierczak 2008). However, the directional motion of tendrils and the directional motility exhibited by the disperser are comparable, and it is perhaps this unified motion which is an important aspect of the resistance profile to both phages and antibiotics. Biofilms also allow community structure, and research has found that bacteria which form biofilms can also withstand antimicrobial agents at concentrations thousands of times higher than their lethal dose in a planktonic state (Stewart and Costerton 2001). Although the mechanisms involved in biofilm resistance are not well understood, it is likely that multiple interacting mechanisms are important, such as physical prevention via the exopolysaccharide matrix, reduced metabolism and growth rate (Drenkard 2003; Mah and O'Toole 2001; Stewart and Costerton 2001), and the existence of persister cells (Brooun et al. 2000; Stewart and Costerton 2001). Biofilm formation and swarming have been reported to show linked phenotypic characteristics, and therefore might convey similar life-history traits in contrasting environments (Verstraeten et al. 2008).

Another important point to address is the potential implications for *P. aeruginosa*'s pathogenic life-history. ΦKZ is known to infect *P. aeruginosa* strains pathogenic to humans and is therefore attractive for phage therapy research. Phage therapy uses phages as an antibacterial agent for the treatment of pathogenic bacterial infections, and although it is not currently used in western medical practises, its potential applications are an area of intensive current research (Skurnik and Strauch 2006). There is a large amount of literature which identifies motility as an important virulence factor (Drake and Montie 1988; Josenhans and Suerbaum 2002), and it is therefore important to determine whether behavioural responses to phages, such as increased motility, will have correlated consequences for virulence factors, and importantly, to understand what these responses are likely to be in an environment similar to within host conditions.

To date, the majority of research looking at mechanisms of resistance tends to focus on interactions in planktonic cells, but this does not account for the large variability of habitats in which microbes are found (Bohannan and Lenski 2000a). Structured communities are likely to hold different consequences and allow different responses to potential threats compared to well-mixed, free-floating populations. This study suggests that the optimal dispersal strategy for *P. aeruginosa* in the presence of LPS phages is to always disperse, even if there is no escape of the phages. This result is only apparent when the bacteria are observed in a structured environment which facilitates motility on a semi-solid surface. Although the mechanisms are not well understood, this highlights the importance of appreciating bacteria-phage associations in environments relevant to their application, and indicates warnings for the potential negative implications this might have for phage therapy. More generally, this work highlights the crucial role parasites can play in the evolution of motility.

# Chapter 7: Selection Experiments Reveal Trade-offs between Swimming and Twitching Motilities in *Pseudomonas aeruginosa*[3]


## Abstract

Bacteria possess a range of mechanisms to move in different environments, and these mechanisms have important direct and correlated impacts on the virulence of opportunistic pathogens. Bacteria use two surface organelles to facilitate motility: a single polar flagellum, and type IV pili, enabling swimming in aqueous habitats and twitching along hard surfaces, respectively. Here, I address whether there are trade-offs between these motility mechanisms, and hence whether different environments could select for altered motility. I experimentally evolved initially isogenic *Pseudomonas aeruginosa* under conditions which favoured the different types of motility, and found evidence for a trade-off mediated by antagonistic pleiotropy between swimming and twitching. Moreover, changes in motility resulted in correlated changes in other behaviours, including biofilm formation and growth within an insect host. This suggests environmental origins of a particular motile opportunistic pathogen could predictably influence motility and virulence.


[3] *Note: This is a modified version of the published manuscript, Taylor, T.B. & A. Buckling. 2011. Selection experiments reveal trade-offs between swimming and twitching motilities in Pseudomonas aeruginosa. Evolution (in press). DOI: 10.1111/j.1558-5646.2011.01376.x*

# Introduction

Motility, in a variety of forms, is common within the microbial world. Bacterial motility is presumably a crucial component of fitness in most habitats because it enables cells to move towards sources of nutrition (Wei et al. 2011), move away from potential causes of harm (such as toxins and predation; Chet and Mitchell 1976), and avoid competition with clone mates (Nakajima and Kurihara 1994; see Chapter 2). It is also particularly important in host colonisation success of opportunistic pathogens (Drake and Montie 1988), and indirectly influences other virulence-related characteristics under strong selection, such as biofilm development (Klausen et al. 2003) and bacteriophage attachment (Schade et al. 1967). Crucially, natural populations of motile bacteria show a large amount of variation in motility even over small spatial scales (Vos and Velicer 2008), suggesting that different habitats favour different motility strategies (Fux et al. 2005). Understanding the selective forces that act on motility is likely to be important in microbial evolutionary ecology in general, as well as the evolution of virulence of bacterial pathogens. There is a large literature describing associations between virulence and motility (Drake and Montie 1988; Josenhans and Suerbaum 2002; Krukonis and DiRita 2003), but we know little about why different motility strategies evolve. Interactions between host and pathogen might play a key role in driving motility and virulence evolution, but in the case of opportunistic pathogens, such as *Pseudomonas aeruginosa*, selection imposed within their natural environment is likely to be crucial in pre-determining a genotype's propensity to cause severe infections (Pallen and Wren 2007; Friman et al. 2011).

Bacteria have a range of mechanisms that allow efficient motility under diverse environmental conditions. *P. aeruginosa*, arguably the most studied bacteria in terms of

motility, expresses two surface organelles which aid motility: a single polar flagellum and retractable polar type IV pili. In addition it is also able to secrete a lipid based biosurfactant, rhamnolipids (Mattick 2002). This enables the bacterium to adopt pilus-based twitching on hard surfaces (Henrichsen 1972), flagella-mediated swimming in aqueous environments (Bai et al. 2007), and a coupled pili-flagella action combined with rhamnolipid production known as swarming (comparable to gliding) on semi-soft surfaces (Caiazza et al. 2005; Köhler et al. 2000). The evolution of bacterial motility could be greatly affected by the existence of trade-offs between different motility mechanisms (Stearns 1989), such that an increase in one form of motility necessarily results in a reduction of another (antagonistic pleiotropy; Futuyma and Moreno 1988). The aim of this study is to explore whether such trade-offs exist, and the evolutionary consequences if they do.

There is some evidence to suggest a trade-off between swimming and twitching, although the question has not been extensively addressed. Pili have been shown to be unable to withstand shear forces (Touhami et al. 2006), and this is hypothesised to be important in explaining expression patterns in the highly motile predatory bacteria, *Bdellovibrio bacteriovorus. B. bacteriovorus* only extrude pili during prey interactions because, it is thought, fast swimming speeds would be enough to break the pilus fibres from the cell (Evans et al. 2007). Moreover, type IV pili-absent mutants show higher swimming motility in soft agar environments (and vice versa, hyperpiliated mutants show reduced motility), suggesting extended pili cause drag and thus inhibit swimming motility (see Chapter 2). The possibility of increased twitching motility in the absence of flagella has not been investigated, but data are consistent: flagella deficient mutants of *P. aeruginosa* retain the ability to twitch (Shrout et al. 2006), and flagella expression is metabolically costly (Soutourina and Bertin 2003). Therefore, one would expect flagella

expression to be lost if it is costly to maintain and not required for motility in the selective environment.

To investigate whether a trade-off exists between motility traits in *P. aeruginosa* I selected for high motility (bacteria that had dispersed the furthest distance on agar plates) in initially isogenic populations under conditions which favoured either swimming or twitching motilities. Unlike investigating a possible trade-off using natural isolates, this experimental evolution approach removes the confounding effects of differing genetic backgrounds. A correlated reduction in one type of motility as the other increased would be suggestive of a trade-off mediated by antagonistic pleiotropy, although decay in an unselected trait may simply arise through genetic drift (Kawecki 1994; Buckling et al. 2007). I therefore set up control lines, where populations were grown in one environment or the other, but there was no selection for high motility: bacteria were selected from random places on agar plates. These control lines would also capture any adaptation to the different agar conditions which might have a correlated effect on motility. Motility was inferred by measuring the dispersal range of the colony over an agar plate after 24 hours. Finally, evolved populations were measured for correlated changes in a range of other potentially important traits, growth rate *in vitro* and *in vivo*, and biofilm formation, which can be linked to motility mechanisms.

## Materials and Methods

### *Selection Regime*

All lines started with the same genetic background, and changes measured against the ancestral population, therefore differences in motility and virulence are comparable across treatment groups. PAOΔ*mutS*, a mutator strain created via a deletion of the

mismatch repair gene *mutS* (Oliver et al. 2004), was used to inoculate twenty four starting populations. This strain was not used to gain information regarding the rate, but rather the direction of evolutionary change, and allowed these changes to be observed more quickly. Starting culture for initial plate colonisation was grown overnight in shaken (0.9 g) 30 ml glass vials (VWR) at 37 °C, in 6 ml fresh King's B broth (KB: 20 g/l protease peptone, 10 g/l glycerol, 1.5 g/l potassium phosphate and 1.5 g/l magnesium sulphate heptahydrate). Overnight cultures were vortexed, diluted and plated to obtain starting densities, and 2.5 µl pipetted into the agar just below the surface. A total of twenty four populations were evolved in one of two environments: twelve in hard KB agar (1.2% wt/vol), which promoted twitching motility; and twelve in liquid KB agar (0.3% wt/vol), which promoted swimming motility. A liquid agar environment was assumed to favour swimming because flagella absent mutants have been shown to be relatively immobile under these conditions (O'Toole and Kolter 1998a), subsequently, a hard agar environment was assumed to favour twitching, because likewise, pili absent mutants are relatively immobile (Semmler et al. 1999). All 90 mm diameter plates (Sterelin) were poured with 25 ml of KB agar media and were briefly dried in a flow hood for 20 minutes. Samples were taken daily (approximately $3x10^8$ cells) from each of the agar plates using a 1 ml pipette (Finnpipette; for methods see Chapter 2). After 24 hours, in most cases, bacteria grown in liquid agar had reached the edge of the plate. The location from which the sample was taken depended on the selection treatment to which it was assigned. Half the populations were under positive selection for motility where a sample was taken from the edge of the colony; the other half was under neutral dispersal selection for motility where a sample was taken randomly from the colony using a random number grid. This experimental design resulted in six replicate lines within each test group. Lines were evolved under neutral dispersal selection to capture evolutionary

change resulting from adaptation to the different agar conditions, which could have correlated effects on motility, as well as providing insight into the mechanistic basis of the trade-off.

Samples were then placed in a microfuge tube containing 1 ml M9 solution (12.8 g/l $Na_2HPO_4$, 3 g/l $KHPO_4$, 0.5 g/l NaCl, 1 g/l $NH_4Cl$). The pipette tip was washed thoroughly in the solution and the microfuge tube was subsequently vortexed. 2.5 µl was re-inoculated onto a new plate (approximately 8 x $10^5$ cells), incubated overnight at 37 $^{\circ}$C, and the procedure repeated for fifteen transfers, after which the samples taken directly from the plate were frozen at -80 $^{\circ}$C in 20% (wt/wt) KB glycerol.

### *Motility Assays*

Frozen evolved samples and the ancestral strain were used to inoculate 6 ml KB broth. Cultures were left to grow overnight in shaken 30 ml glass vials (0.9 g) at 37 $^{\circ}$C. Overnight cultures were vortexed and 2.5 µl inoculated onto both hard KB agar (1.2% wt/vol) and liquid KB agar (0.3% wt/vol) in order to observe differences in motility between the two environments. I used large 140 mm diameter petri dishes (Sterelin) in order to try and capture the maximal distance range of the evolved strains. Area covered by the bacterial colony was measured using ImageJ 1.42q (Abramoff et al. 2004).

### In vitro *Growth Assays*

To determine the fitness of each individual isolate, and the extent to which growth rate could account for differences between dispersal ranges, growth rate was assayed across ancestral and evolved isolates. Overnight cultures were grown in 6 mL KB at 37 $^{\circ}$C and diluted in M9 by a factor of 1:100. 10 µl was added to a 96-well flat-bottomed plate containing 90 µl of KB per well. Optical density at 600 nm ($OD_{600}$) was measured

every 45 minutes for 20 hours using a microplate spectrophotometer (BioTek Powerwave XS) at an incubation temperature of 37 ˚C. During the period of exponential growth $V_{max}$ (the rate of change of optical density during log growth) was calculated.

### *Biofilm Production*

In order to determine the effect of evolutionary environment on biofilm production ancestral and evolved isolates were grown overnight in 3 ml Luria Broth (LB: 10 g/l Tryptone, 5 g/l Yeast extract, 5 g/l NaCl) shaken 30 ml glass vials at 37 $^{\circ}$C. $OD_{600}$ was measured and cells diluted to 0.05 OD in 3 ml fresh LB and grown for 2 hours at 37 $^{\circ}$C in a rotator shaker (0.9 g) to allow cells to reach exponential growth phase. Cultures were diluted by 1 in 2 with $dH_2O$ and then to 0.025 $OD_{600}$ in 10 ml fresh Tryptone Broth (TB: 10 g/l Tryptone). Into a 96-well flat-bottomed plate I added 150 µl of each cell culture and left in a humid container at room temperature for 24 hours to allow biofilm formation. The plate was washed well in water, and 150 µl of 0.1% crystal violet (CV) solution added. After mixing, the CV solution was removed and 200 µl of 33% acetic acid solution added to each well. Finally, 25 µl of dissolved stain was added to 175 µl $dH_2O$ and $OD_{595}$ was measured and recorded: a higher OD reading corresponds to a larger biofilm layer. Methods adapted from O'Toole and Kolter (1998b), and Fletcher (1977), by Jiricny et al. (in prep.). This method maximises biofilm production allowing the phenotype to be observed under optimal conditions.

### In vivo *Growth Assays*

Wax moth larvae, *Galleria mellonella*, have been previously shown to be a good model host for the study of *P. aeruginosa* infections (Jander et al. 2000). Methods were as in Harrison et al. (2006). *G. mellonella*, were obtained from Livefood UK

(www.livefoods.co.uk) and were in their fifth instar at time of inoculation. They were stored in the fridge prior to injection. Overnight KB cultures of ancestral and evolved strains were diluted to 1 in $10^4$ in 0.8% NaCl solution. 100 µl plastic syringes were sterilised and 8 larvae randomly assigned to each of the 13 populations (6 populations evolved in hard agar, 6 populations evolved in liquid agar, and ancestral strain), 4 were used for controls (injected with 0.8% NaCl). The fresh weight of each larva was recorded prior to injection and the abdomen swabbed with 70% ethanol to prevent contamination. 10 µl of culture was injected into the haemocoel of each larva. Post infection, the larvae were stored in 24-well plates and incubated at 37 $^{\circ}$C for 8 hours. Larvae were then immersed in 70% ethanol to kill surface microbes and placed individually in microcentrifuge tubes containing 1.0 ml 0.8% NaCl solution. Larvae were homogenised by shaking with a sterile ceramic bead (FastPrep®-24, MP Biomedicals; speed 5.0 m/s for 45 s). Thereafter samples were centrifuged at 1000 rpm for 3 minutes in order to pellet hosts' tissues. 20 µl of the homogenate was extracted and diluted to 1 in $10^4$ using 0.8% NaCl. Diluted homogenate was spotted onto KB agar plates supplemented with 15 µg/ml of ampicillin, and incubated overnight at 37 $^{\circ}$C. The concentration of ampicillin used restricts the growth of natural gut fauna, without impairing *P. aeruginosa* growth (Harrison et al. 2006).

### *Microscopy*

Pictures were taken in order to visualise differences in colony morphology due to modifications to surface motility organelles, using a Leica DM IL LED, with a Leica EC3 digital camera attachment.

### *Statistical Analysis*

Analyses and figures were produced on PASW Statistics 18 (SPSS). Significance of treatment on motility was analysed using general linear mixed models (GLMMs) (Crawley 2007). Terms used in the model are defined as: (square root) "Dispersal area" [response variable], the area of the bacterial colony after 24 hours growth and dispersal relative to the ancestral strain (calculated as area/ancestral area); "Evolutionary environment" [explanatory variable, factor with two levels], the agar type which the strains were evolved in (hard or liquid); "Test environment" [explanatory variable, factor with two levels], the agar type which the strain was grown in during cross inoculation experiment (hard or liquid); "Selection" [explanatory variable, factor with two levels], the selective regime assigned to the strain (positive or neutral selection for high motility); and, "Replicate line" [nested random factor, within "Selection" and "Evolutionary Environment"]. Validity of models were determined by distribution and homogeneity of residuals. Where replicates were measured only once (*i.e.* when looking at correlated effects of selection on non-motility phenotypes), General Linear Models (GLMs) (Crawley 2007) using the same factors as above were used. Note that significance levels calculated when multiple tests were carried out were adjusted using sequential Bonferroni correction (Rice 1989).

## Results

### *The Trade-off between Twitching and Swimming*

To determine whether selection had an effect on motility, evolved strains were cross inoculated into both the agar type that they were evolved in and the contrasting agar type (*i.e.* hard and liquid agar). In all cases dispersal range was measured relative to the

ancestral strain from which they were derived and taken as an estimate of motility (Figure 7.1). The average initial area (±1 SD) of the parental non-evolved strain after 24 hours growth and dispersal within each environment was: hard agar, 1.7 $cm^2$ (± 0.65); and liquid agar, >185 $cm^2$. After selection, I found large differences between the dispersal ranges of strains from different evolutionary environments, and these differences were dependent on the agar they were grown in, as well as the selection type they were under (GLMM, three-way interaction between "Evolutionary Environment", "Test Environment", and "Selection"; $F_{1,20}$ = 34.010, $P < 0.001$). For lines under positive selection for dispersal, I found that motility was relatively greater in the environment in which they were selected (GLMM, interaction between evolutionary and test environments: $F_{1,10} = 320.522$, $P < 0.001$); and strong evidence for a trade-off in the lines under positive selection for dispersal, in that lines showed significant increases (relative to the ancestral strain) in their selective environment, but also showed significant decreases in their non-selective environment (one-sampled t-tests when test environment = evolutionary environment, and test environment ≠ evolutionary environment, respectively: under positive selection, $t_{11} = 4.443$, $P = 0.004$, and $t_{11} = 3.644$, $P = 0.012$).

By contrast, there was no tendency for greater dispersal in the evolutionary environment for strains under neutral selection (GLMM, interaction between evolutionary and test environments: $F_{1,10} = 1.520$, $P = 0.250$), demonstrating that selection for motility rather than differences in the agar *per se* were responsible for the observed changes in lines under positive selection. However considered independently, there was an overall increase in motility in lines under neutral selection evolved and tested on hard agar ($t_5 = 5.097$, $P = 0.004$), and an overall decrease in motility in those evolved and tested on liquid agar under neutral selection ($t_5 = 3.657$, $P = 0.015$).

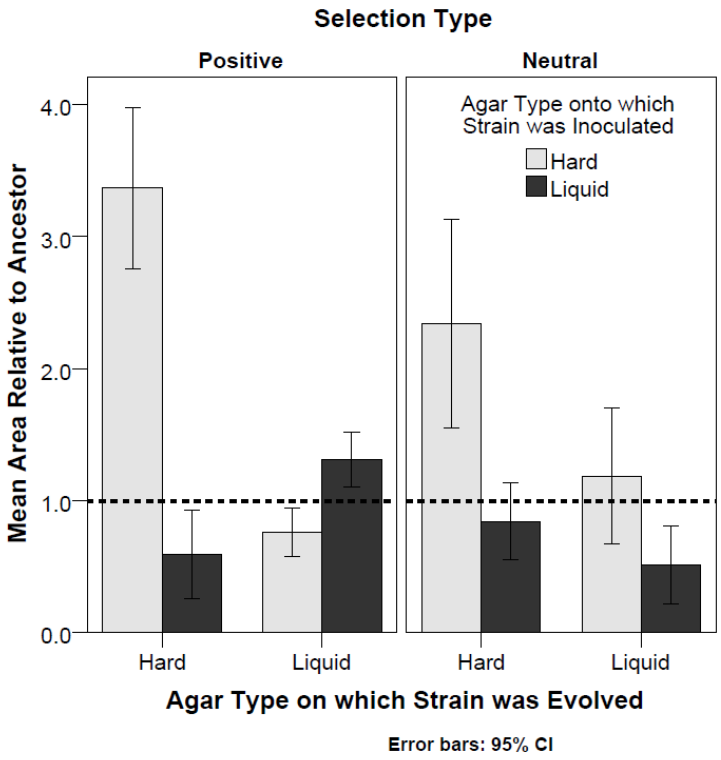


**Figure 7.1: Cross inoculation of evolved strains between evolutionary environments**

The results show the average area of dispersal of the evolved strains (6 lines in each treatment group) relative to the ancestral strain from which all strains were derived (± 95% CI). The dashed line indicates a relative dispersal value of 1, that is, when the area of dispersal was equivalent to that of the ancestor.

This observed trade-off may have resulted from antagonistic pleiotropy (a causal negative genetic correlation between traits), or via the build-up of deleterious mutation in the unselected environment, that are neutral in the selective environment (mutation accumulation), or both. If mutation accumulation was important in driving the trade-off, I would expect to see evidence of mutation accumulation in the neutral selection lines, given that neutral mutations in the evolutionary environment (and hence deleterious mutations in the alternative environment) should be expected to fix regardless of selection for increased motility. This was not the case: the only reduction in motility relative to the

ancestor occurred in lines both evolved and measured in liquid agar. These data strongly point to antagonistic pleiotropy driving the swimming-twitching motility trade-off. It is important to acknowledge that within this experimental system growth and dispersal are intrinsically linked (bacteria which divide faster will have larger dispersal ranges). Here, I assume equal mutation accumulation (which will be a function of elapsed generations) across selective environments. However, this assumption is not necessarily correct, as selecting from a random location (as is the case under neutral selection) would lead to fewer generations, and thus less time for mutations to accumulate, than under positive selection (where cells are transferred from the colony periphery). Crucially, the lines under neutral selection underwent sufficient generations for evolutionary change to be detected, but this evolutionary change was not consistent with mutation accumulation. The observed increase in motility on hard agar in lines under neutral selection for motility in this environment also highlights the role different environments can play in shaping selection.

### *Growth:* in vitro

Motility is a metabolically costly activity (Ottemann and Miller 1997), and swimming in particular has been documented as an energetic mode of motility (Nandini et al. 2003). I looked for growth differences, both *in vitro* and *in vivo*, between strains evolved in the different environments to see whether selection on motility influenced growth patterns. Under *in vitro* conditions I found both evolutionary environment (GLM: $F_{1,24} = 15.199$, $P = 0.001$) and selection (GLM: $F_{1,24} = 5.955$, $P = 0.024$) to determine growth rate. However, positive selection for motility reduced the overall growth rate compared to the ancestral strain across both environments (Figure 7.2; $t_{11} = 5.557$, $P < 0.001$), and in particular, those evolved in a liquid environment showed lower growth

rates (one-sample t-test, liquid evolutionary environment: mean difference = 0.331, $t_{11}$ = 5.028, $P < 0.001$; hard evolutionary environment: mean difference = - 0.061, $t_{11}$ = 1.689, $P = 0.199$). Those evolved in hard agar under neutral selection for motility had a growth rate equivalent to the ancestor ($t_5 = 0.836$, $P = 0.441$). In addition, there was a large amount of variation in growth rate between strains which were evolved under neutral selection for motility in a liquid environment (mean = 0.756, SD = 0.289). Further investigation of all lines revealed a negative correlation between swimming motility and growth rate (data not shown, correlation of growth rate versus dispersal, $\rho = -0.860$, $P = 0.028$).

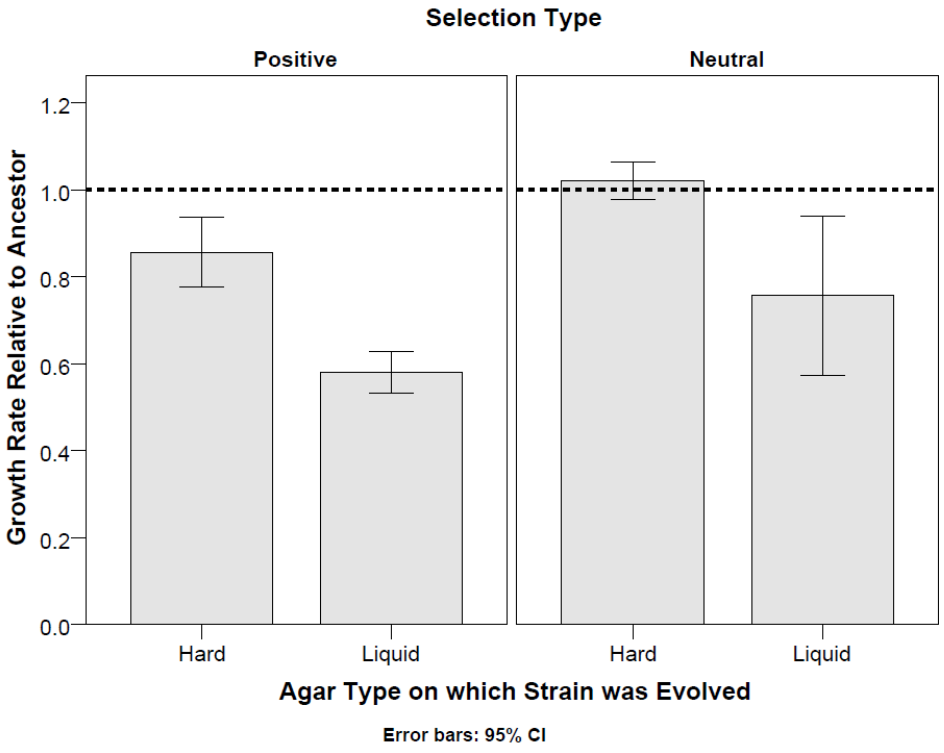


**Figure 7.2: Growth rate *in vitro***

The results show mean average growth rate of evolved strains *in vitro* relative to the ancestral strain (± 95% CI). The dashed line indicates a relative growth rate equivalent to that of the ancestor.

*Biofilm Formation*

I expected to see a link between pili dependent traits and pili use during motility. I chose to examine biofilm formation, a known pili dependent behaviour (Chiang and Burrows 2003). Across all treatments there was a reduction in biofilm formation relative to ancestral behaviour (Figure 7.3; $t_{22} = 6.527$, $P < 0.001$). However, the evolutionary environment had only a slight effect on biofilm production (GLM, main effect of evolutionary environment: $F_{1,19} = 4.204$, $P = 0.054$), as did the selection type (GLM, main effect of selection type: $F_{1,19} = 5.105$, $P = 0.036$). Importantly, under positive selection for motility there was a significant effect of evolutionary environment on biofilm formation, with efficient swimmers showing less ability to form stable biofilms ($F_{1,9} = 8.106$, $P = 0.019$), but no effect was detected under neutral selection ($F_{1,10} = 0.388$, $P = 0.547$). Note, difference in degrees of freedom is due to the exclusion of one replicate evolved on hard agar, due to loss of biofilm caused by biofilm disruption.

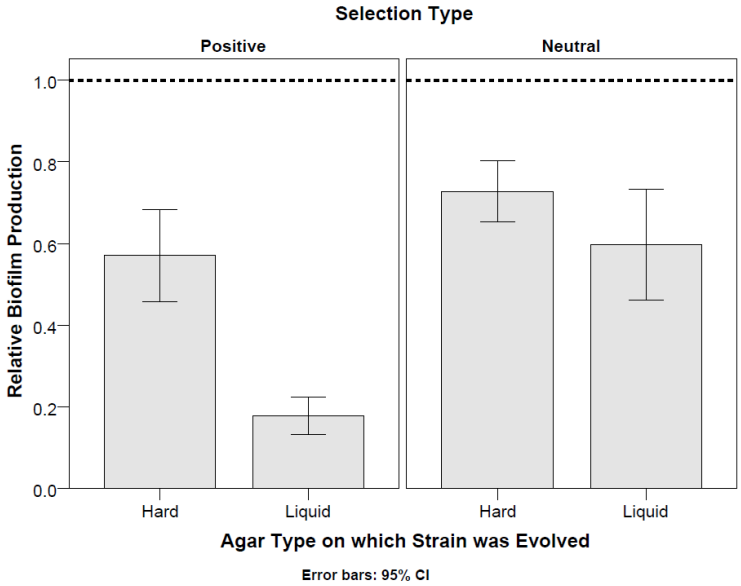


**Figure 7.3: Biofilm production of evolved strains.**

Results show the average biofilm production of 6 isolates evolved in each experimental environment, relative to the ancestral strain from which all evolved strains were derived (± 95% CI). The dashed line indicates a relative level of biofilm production equivalent to that of the ancestor.

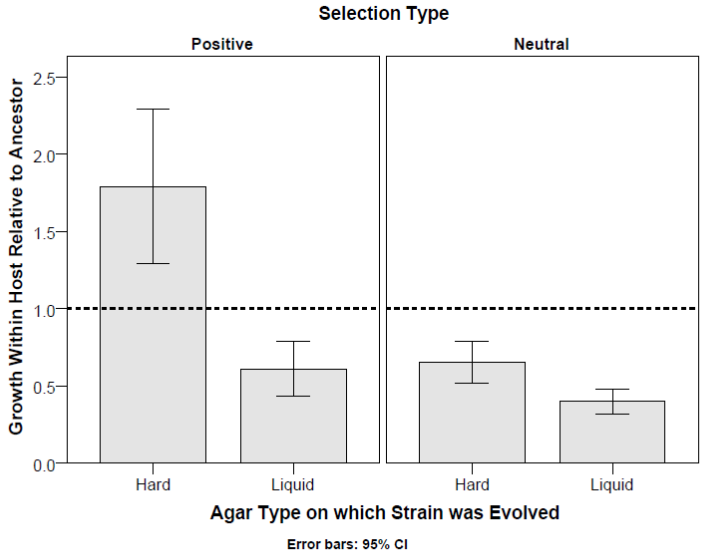


**Figure 7.4: Growth Rate *In vivo***

The results show average growth rate of evolved strains within a host (*G. mellonella*) relative to the ancestral strain. The dashed line indicates a relative growth rate equivalent to that of the ancestor.

### *Growth:* in vivo

Interestingly, growth rates within the *G. mellonella* host do not mirror growth rates *in vitro* (Pearson's correlation: r = 0.37, *P* = 0.086). I found evolutionary environment to be a strong determinant for growth *in vivo* (Figure 7.4; GLM, main effect of evolutionary environment: $F_{1,20}$ = 5.876, *P* = 0.025), as was the selection type (GLM, main effect of selection: $F_{1,20}$ = 5.086, *P* = 0.035). Within a host, strains under positive selection and evolved in a hard agar environment showed higher growth than those evolved in liquid agar ($t_{22}$ = 2.886, *P* = 0.009). Specifically, those evolved on a hard agar environment grew within the host to a density equivalent to the ancestor ($t_{11}$ = 2.013, *P* = 0.069), but those evolved on liquid agar grew significantly less ($t_{11}$ = 2.835, *P* = 0.016). Under neutral selection there were no differences in growth within a host between those strains evolved in a hard or liquid agar environment ($t_{22}$ = 1.741, *P* = 0.096). However, both had lower growth rates than the ancestral strain (one-sample t-test: on hard agar, $t_{11}$ = 2.697, *P* = 0.021; on liquid agar, $t_{11}$ = 8.319, *P* < 0.001).

### *Observational Morphology Differences*

There was a dramatic change in colony morphology over the course of the experiment (Figure 7.5). Bacterial changes in physiology and morphology can be caused both directly, by physical contact to the growth surface, and indirectly, via cell to cell signalling which provides information regarding cell proximity (Harshey 2003). On hard agar, the edge of the colony became less defined, and magnification revealed elaborate colony edges (Figure 7.5c), presumably caused by increased production of extracellular products (e.g. rhamnolipids). On liquid agar, these strains had comparably smaller colonies and magnification showed large waves which suggest periods of density dependent motility (Figure 7.5d; Harshey 2003). Those evolved on liquid agar, showed

small, compact colonies with smooth edges when grown on hard agar (Figure 7.5e). However, on liquid agar, colonies were much larger and under magnification cells appeared to be moving in a directional manner, with small areas of active cells at the edges suggesting apparent coordinated motility (Figure 7.5f).

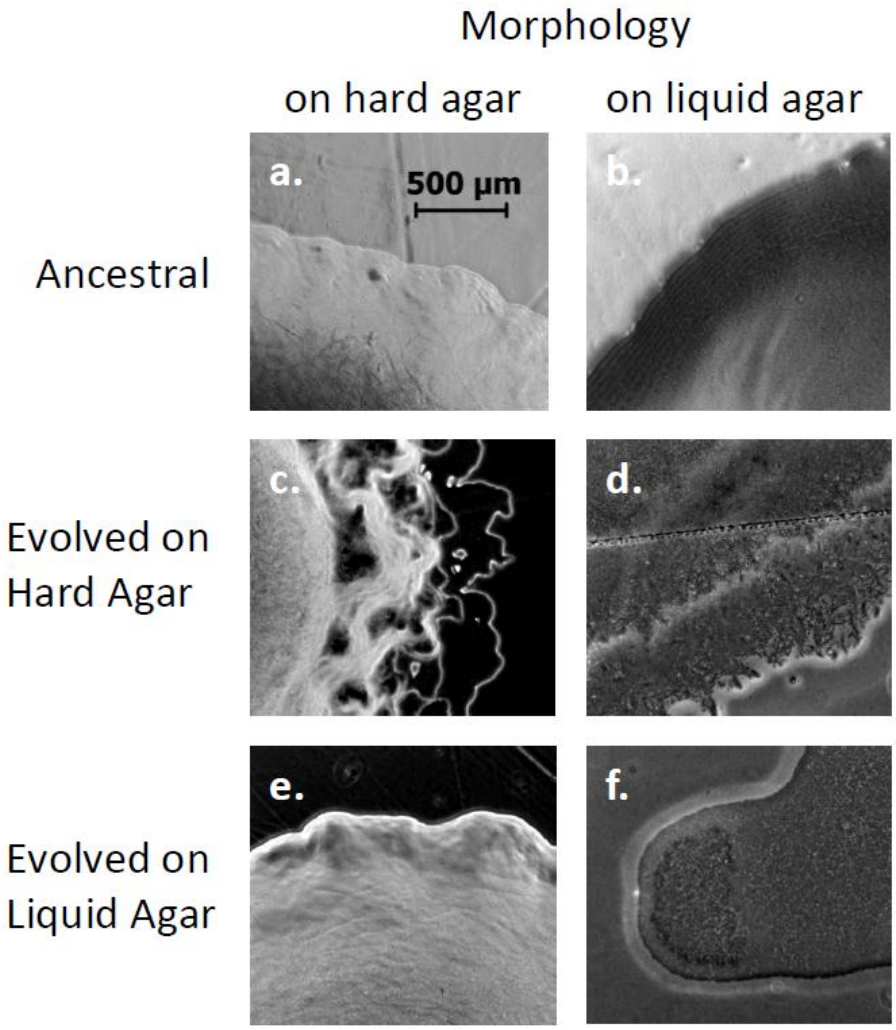


**Figure 7.5: Photographs of agar plates showing colony edge morphology**

Photographs taken with Leica DM IL LED, with a Leica EC3 digital camera attachment. The top row shows the morphology of ancestral strains as seen on hard and liquid agar, respectively (a and b); the second row shows strains which were evolved on a hard agar environment, as seen when grown on hard agar (c), and liquid agar (d); the third row shows strains which evolved in liquid agar, as seen when grown on hard agar (e), and liquid agar (f).

# Discussion

I investigated trade-offs between motility mechanisms in *P. aeruginosa* by experimentally selecting lines for increased dispersal in environments which favoured swimming and twitching. I found evidence for a trade-off between the motility mechanisms: strains that were selected for increased motility in a hard agar environment lost swimming motility relative to the ancestral strain; and conversely, those that were evolved in a liquid agar environment were poor at twitching (Figure 7.1). I found no evidence that the environment *per se* was responsible for this pattern, because lines evolved in the same environments but with no selection for increased motility, did not display this trade-off. I suggest this trade-off largely arises because of antagonistic pleiotropy, whereby improved performance in swimming necessarily reduces twitching performance. It is possible that the build-up of mutations which are neutral in the selective environment but deleterious in the alternative environment (mutation accumulation; Kawecki 1994) contributed to this pattern. However, the absence of consistent reduction in motility in the unselected environment when bacteria underwent neutral selection for motility suggests that this is unlikely.

To determine whether the differences observed in dispersal range could be explained to some extent by differences in growth rate, and whether changes in motility might carry metabolic costs, I tested the growth rate ($V_{max}$) of evolved relative to ancestral strains (Figure 7.2). Surprisingly, *in vitro* maximal growth rate decreased in all lines relative to the ancestor, presumably because maximal growth rate is not an important determinant of fitness under these experimental conditions. This suggests that growth rate *per se* cannot explain patterns of dispersal. However, I did find that growth rate was most reduced in lines evolved in liquid, and within the liquid evolved lines there

was a negative correlation between growth rate and swimming dispersal: bacteria were efficient swimmers and slow growers, or poor swimmers and fast growers.

In order to understand how a trade-off between swimming and twitching might indirectly influence other important life history traits, aside from motility, I looked at two essential bacterial functions: (*i*) biofilm formation (a trait vital for pathogenicity, antibiotic resistance, biofouling, and waste water treatment (Xavier and Foster 2007; Kolter and Greenberg 2006; Costerton et al. 1995)); and, (*ii*) growth within a host (in order to determine how these changes might particularly affect a pathogenic lifestyle). First, type IV pili, which are used for twitching motility, mediate cell interactions between surfaces and other cells, and hence also influence biofilm formation (Strom and Lory 1993; Chiang and Burrows 2003; Jenkins et al. 2005; Barken et al. 2008). Surprisingly, I found reduced biofilm formation relative to the ancestor in all of the evolved lines (Figure 7.3), possibly because flagella, which mediate swimming motility, are also important factors in biofilm formation (Verstraeten et al. 2008). However, when only evolved lines were considered, there was, as expected, a positive correlation between twitching motility and biofilm formation, with greater loss of biofilm formation found in evolved lines which had reduced twitching motility. Second, both flagella and pili have proved to be important in establishing an infection (Tang et al. 1995; Feldman et al. 1998), it was therefore unclear how selection for different swimming motilities would affect *in vivo* growth. I assayed bacterial growth rate (an indicator of bacterial virulence; Harrison et al. 2006; Inglis et al. 2009; Racey et al. 2009) over eight hours of infection of the wax moth larvae (*G. melonella*) and found that both neutral selection treatments, and selection for swimming motility, reduced *in vivo* growth relative to the ancestor, whereas growth increased by more than 50% relative to the ancestor in lines selected for increased twitching motility. This variation was not directly correlated with growth rates *in vitro.*

These data suggest that twitching motility may positively correlate with virulence, a result consistent with previous studies showing greatly reduced virulence of type IV pili mutants which are unable to twitch (Comolli et al. 1999; Jenkins et al. 2005; Lyczak et al. 2000).

The data suggests a link between environmental selection pressures and virulence of *P. aeruginosa*: a potentially important finding given that a majority of cystic fibrosis patients are believed to be typically colonised by strains of environmental origin (Kiewitz and Tümmler 2000; Mathee et al. 2008; Römling et al. 1997; Römling et al. 1994; Spencer et al. 2003). Consistent with this view, numerous studies have investigated differences between environmental and clinical strains, and found a large amount of conservation of genes across the isolates (Head and Yu 2004; Mathee et al. 2008; Römling et al. 1994; Römling et al. 1997; Spencer et al. 2003; Wolfgang et al. 2003). This suggests that selection for maintenance of such traits does exist in environmental reservoirs (Wolfgang et al. 2003), however, environmental isolates tended to be lumped together, and the possible link between motility phenotype and the specific environmental origin has not been investigated.

I also found the agar environment to be a determinant of motility, independent of selective treatment. Motility increased in all strains evolved in a hard agar environment, regardless of whether I imposed positive or neutral selection for motility. This is likely to be due to the distribution and diffusibility of nutrients within the environment. The viscosity of the environment will determine the diffusion of nutrients (and microbial excreted products) (Allison 2005), therefore within a highly viscous patch, when nutrients are used up they will take longer to be replenished. This will increase competition for resources, and an individual will gain a fitness benefit if it is able to escape competition by moving to unoccupied patches (Wei et al. 2011). This benefit will be greater in largely

clonal populations (as is the case here) due to the indirect fitness benefits gained from alleviating competition between kin (Hamilton and May 1977; Rousset and Gandon 2002; Nakajima and Kurihara 1994; Chapter 2). In contrast, I found swimming motility to increase only under positive selection for high motility, and decrease in the absence of selection, regardless of environment. There are two potential causes of this, if swimming is essentially a neutral trait in these environments it might decay by random drift (Hall & Colegrave 2008), alternatively, if it is costly to maintain and perform, the bacteria might gain a selective advantage by loss of function (Velicer et al. 1998).

This study shows that the abiotic environment plays a crucial role in the evolution of bacterial motility, and that fitness in specific environments may be limited by trade-offs between motility traits and linked phenotypic characters. Despite the simplicity of the experimental environments, these trade-offs are likely to hold in natural settings. Indeed, trade-offs may well be stronger in more stressful natural environments, in the presence of lower nutrients, greater competition and natural enemies. This trade-off has the potential to limit the ubiquity of particular strains, diversify populations, and alter within-host behaviour.

# Chapter 8: General Discussion

The motivation of this thesis was to study the evolutionary ecology of dispersal in microbes. Chapter 1 gave a general overview of dispersal and highlighted the vastness of the topic and the interdisciplinary work that has led to a large amount of disconnected literature. Chapter 2 focused on kin competition as a driver for the evolution of dispersal, and for the first time, a paper written in 1977 by Hamilton and May was explicitly tested experimentally using a simple microbial system, providing experimental support that kin competition can drive the evolution of dispersal. This methodology was the basis for the chapters that followed and provided a simple technique to track the dispersal of colonies over time in a homogenous environment, allowing the relative fitness of dispersal variants to be established. In order to further understand this result I looked at how relatedness of populations shaped the evolution of dispersal using experimental evolution (Chapter 3). Surprisingly, I found little evidence that relatedness affected the evolution of dispersal under the experimental conditions used – rather, it was the availability of resources that most likely determined dispersal patterns.

Chapter 4 began to integrate multiple theoretical predictions into one experimental design, investigating how two opposing social traits – kin competition (which selects for dispersal) and cooperation (which selects against dispersal) – interact to determine dispersal patterns. With a collaborative, theoretical and experimental approach we were able to create a complete and dynamic idea of how conflict and cooperation might trade-off against each other (with the distribution of cheats and the relatedness of dispersing populations being key factors) to determine dispersal patterns.

Chapter 5 then expanded the project beyond the laboratory by applying the experimental methods to natural populations. Natural populations of bacteria and phage

from horse chestnut trees were sampled and amplified. These samples were used as ancestral strains for experimental evolution, looking at how natural phage populations might be responsible for determining bacterial dispersal patterns. Again, our results did not match our original predictions, and we found phage to have little effect (and perhaps a suggestion that phage had a positive effect) on the evolution of dispersal. This highlights the importance of understanding which selective pressures are present in the natural environment rather than simply inferring natural patterns based on the most interesting laboratory experiments. Chapter 6 focused specifically on how phages influence bacterial dispersal using a phage known to target non-motility structures. Here, I found support for the survey results from the natural dataset, with evidence that the presence of a lytic phage increases dispersal in a bacterial host, with increased motility acting as a resistance strategy. Moreover, this result was generated using a different bacterial species (with a different life history and habitat), suggesting that this pattern might be considered robust. By understanding this mechanism through additional experimental and simulation data, there is potential for this pattern to be generalised further.

Finally, as the results from these experiments accumulated, I began to suspect that the environment itself was determining dispersal patterns. To gain a better understanding of how the model organism's life-history traits were dictated by the environment I set up an experimental evolution study (Chapter 7), that shows trade-offs between swimming and twitching motilities in *P. aeruginosa*. This, along with other studies in this thesis, highlights the importance of conducting experiments in environments that are comparatively relevant to the environment to which you are applying your results. The work also provides valuable data regarding general microbial motilities, and broadens our understanding of how the environment might limit their diversification.

These chapters offer new insight into "when to stay, and when to go", and the newly explored method for dispersal research has many potential avenues for future work. Specifically, there are two exciting and quite different directions in which I would like to continue this research to further extend its applicability: (*i*) looking for natural determinants of motility and dispersal patterns; and, (*ii*) extending the work to parasite transmission through dispersal, both of which are discussed below.

It is the simplicity of microbial model systems which gives them the power to address fundamental evolutionary questions; however the applicability of experimental evolution to complex natural patterns is debated in the literature (Carpenter 1996; Carpenter 1999; Drenner and Mazumder 1999; Huston 1999). Combining data from natural communities and lab based experiments, as in Chapter 5, eliminates a large amount of ecological noise whilst still exploring patterns using natural standing variation, and allows causality to be determined in the absence of potentially conflicting factors. It is true that in the absence of these interacting factors, the evolutionary trajectory may be altered; however understanding the individual contribution of natural selective pressures to a particular trait will vastly improve our chance of understanding a system as a whole. For example, bacteria do not occur in isolated clonal groups naturally, but are part of larger interacting communities. Understanding how community structure influences motility and dispersal patterns is likely to give insight not only into microbial life-history traits, but also into more general community driven patterns of dispersal.

How might the diversity of natural communities select for dispersal? There have been ecological studies correlating the effects of dispersal patterns on community diversity (Cadotte 2006; Klanderud and Totland 2007; Zobel and Kalamees 2005), but it does not appear to have been approached from the other direction. More diverse communities might select for increased dispersal, due to increased interspecific

competition increasing the benefits of colonising new patches (Brown and Gordon 1970; Pulliam 2000). However, this expectation would depend on the degree of niche specialisation (and hence inter- versus intra-specific competition) and the probability of finding vacant patches. Moreover, communities might evolve towards more mutualistic interactions through time, which could in turn, select against dispersal (Leigh 2010).

An important aspect of this future work will be to understand how the stability of communities can influence dispersal patterns. Natural communities are found at varying degrees of environmental and community stability (Bell et al. 2005; Fierer et al. 2003). For example in a stagnant pool of water, stability of environmental conditions will differ between the surface and the bottom of the pool. At the surface the community will be more susceptible to changes in temperature, light (UV), nutrients, etc., whereas those which dwell at the anaerobic depths may be less affected by these environmental variations. By using experimental evolution and treating naturally isolated communities to temporally homogeneous or heterogeneous environments and community diversity, we could gain insight into whether environmental stability and community diversity can impact factors such as competition, community structure and the expression of important life-history genes, all of which are likely to contribute to motility and dispersal patterns of microbes.

A second important area that can be addressed using the techniques developed in this thesis is the evolution of parasite transmission as a function of dispersal. Specifically, can optimal parasite transmission be thought of in the same way as dispersal? For example, malaria has two distinct life history phases; a reproductive asexual phase (merozoite), and a sexual dispersing phase (gametocytes) (Miller et al. 2002). This results in a trade-off between reproduction within a host and transmission to new hosts (Alizon et al. 2009; Bell et al. 2006). Thought of in terms of a kin competition argument, a parasite

should invest more in dispersal when infected clonally, because competition within the host would be between kin; however when hosts are infected with multiple strains, investing highly in dispersal will come at the cost of reduced competitiveness within the host, which could result in the clearance of the parasite by the host's immune system. Another avenue which follows similar principles is targeted strain specific immune system responses. Many successful infections require parasites to participate in cooperatative actions (such as enzyme secretions, biofilm formation, siderophore production etc.), providing a benefit to kin aggregation. However, if kin aggregation alerts the immune system to the presence of the parasite, this will have detrimental consequences for the entire group. Thus, increased dispersal (transmission) could be selected for under conditions of high relatedness, because dispersing from the host will provide indirect benefits in terms of immune evasion by fellow kin. Ultimately, in these examples the explicit question is the same as is asked throughout my thesis, "should I stay, or should I go?" In addition, experimental systems looking at parasite transmission dynamics lends itself well to dispersal research because patches can be thought of discretely as the infected and susceptible hosts.

The work presented in this thesis provides key advancements in the field of dispersal research and exposes new and exciting possibilities for further research. It offers a good model system, and a better understanding of motility systems in different evolutionary and environmental habitats. It offers new experimental designs that have been applied to many evolutionary questions and under different experimental conditions, with the results giving valuable insights into natural populations, offering "real world" applicability.

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# Appendices

## *Appendix 1: Supplementary Figures for Chapter 3*

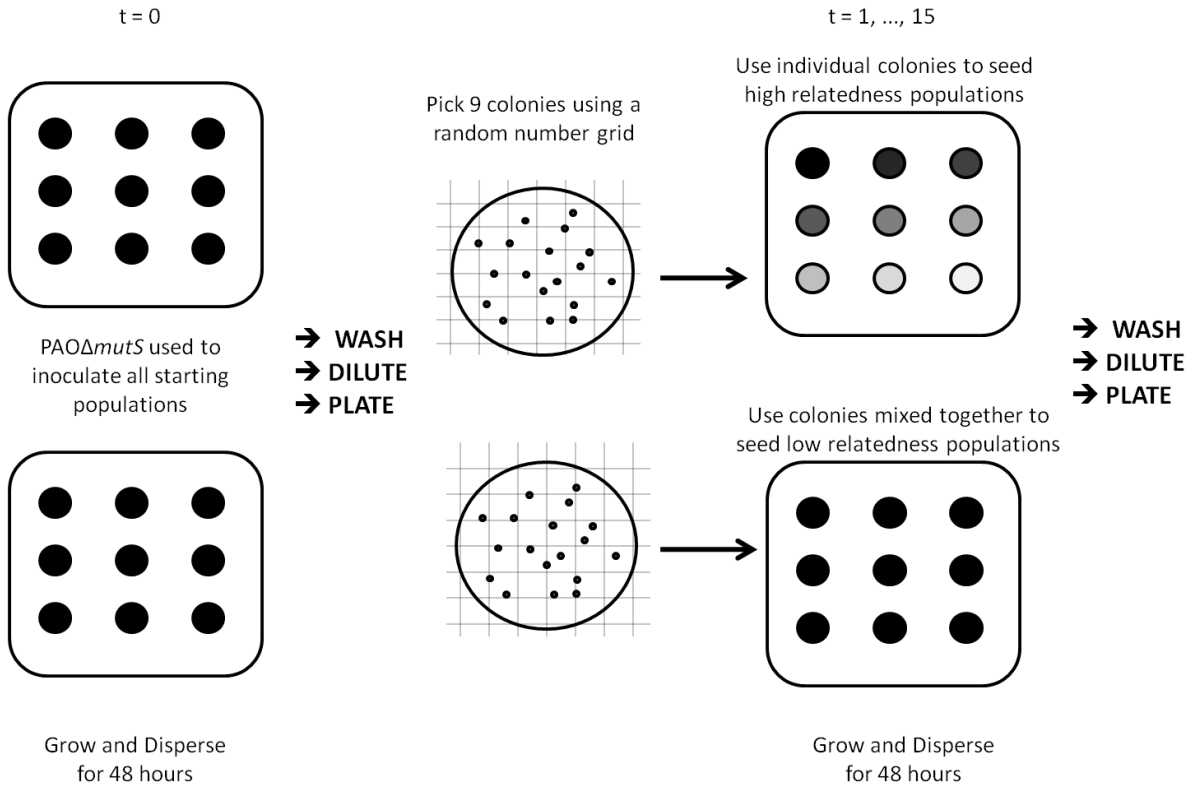


**Figure A1.1:** Schematic diagram of methodology for the preparation evolutionary lines. White squares and circles represent agar plates. Methods are as described in Chapter 3.

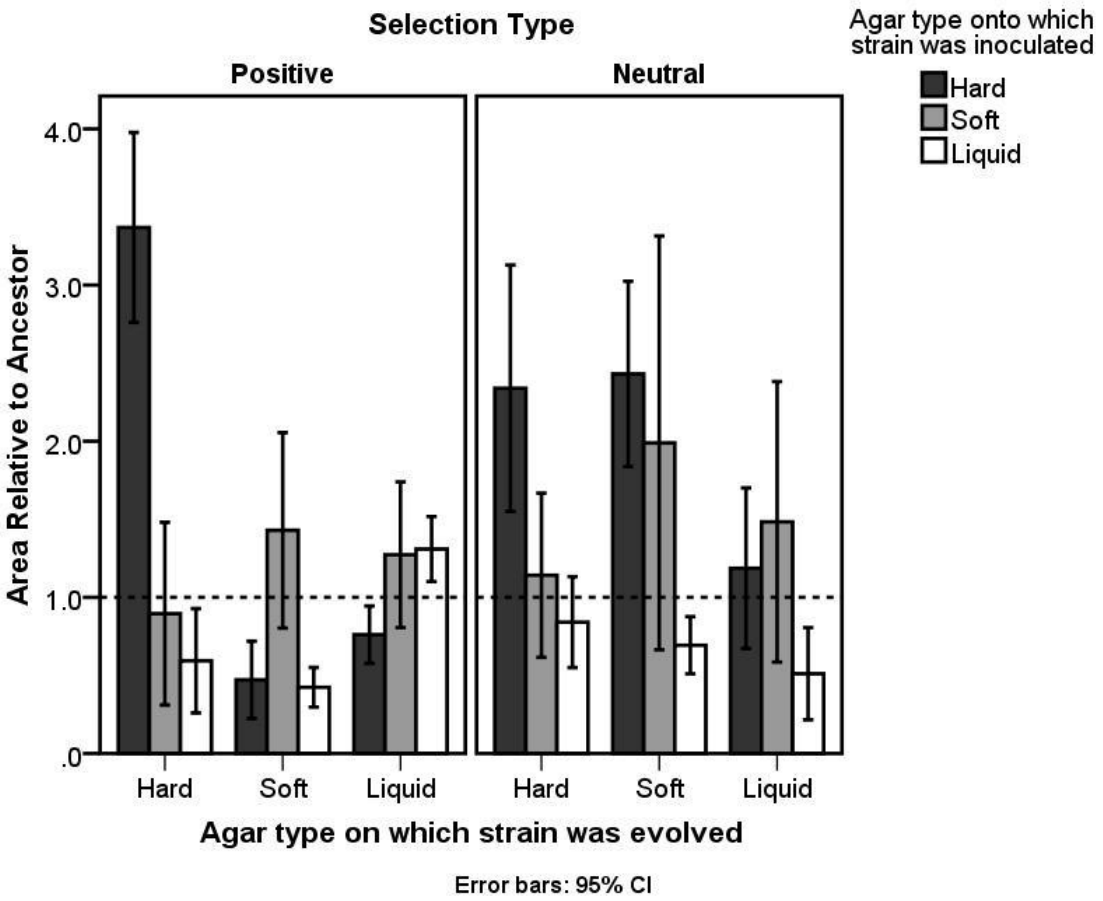


**Figure A1.2**: Data from Taylor and Buckling, 2011 (Chapter 7). Soft agar data was not included in the published manuscript. Graph shows strains which had evolved in hard, soft or liquid agar, cross inoculated between all evolutionary environments. The results show the average area of dispersal of the evolved strains (6 lines in each treatment group) relative to the ancestral strain from which all strains were derived (± 95% CI). The dashed line indicates a relative dispersal value of 1, that is, when the area of dispersal was equivalent to that of the ancestor. Notice, that all lines evolved in soft agar were not significantly different from the ancestral strain regardless of selection treatment. Therefore, it appears the environment does not independently select for or against dispersal.

## *Appendix 2: Supplementary Information for Chapter 4 (extended model by A. Rodrigues)*

An individual's Darwinian fitness is her expected number of surviving descendants, relative to the average of her class. Following the assumptions outlined in the main text, the Darwinian fitness of a cooperative bacterium with dispersal strategy (i.e. probability of dispersal) $x$, in a patch where the average dispersal strategy among cooperators is $y$, and in a population where the average dispersal strategy among cooperators is $z$, is given by:

$$w = (1-x)\frac{aK}{1-y+p(1-c)z} + x(1-c)\left(p\frac{aK}{1-z+p(1-c)z} + (1-p)\frac{(1-a)K}{p(1-c)z}\right), \qquad \text{(A3.1)}$$

where $p = \alpha/(\alpha+\beta)$ is the proportion of habitable patches that are occupied rather than vacant, and $c = \gamma$ is the cost of dispersal (i.e. the probability of landing on a cheat patch, and achieving no reproductive success as a result).

We use a neighbour-modulated fitness approach to analyse how kin selection acts upon dispersal (Taylor 1996; Taylor and Frank 1996; Frank 1997; Frank 1998; Rousset 2004; Taylor et al. 2007). The condition for natural selection to favour an increase in dispersal is $dw/dx = \partial w/\partial x + r\,\partial w/\partial y > 0$, where $r = dy/dx$ is the kin-selection coefficient of relatedness and all derivatives are evaluated at $x = y = z$. This condition is Hamilton's rule, $-C + rB > 0$ (Taylor and Frank 1996; Frank 1998; Hamilton 1963; Hamilton 1964; Hamilton 1970). The candidate CSS probability of dispersal is found by solving $dw/dx|_{x=y=z=z^*} = 0$ (upon the assumption that $0 < z^* < 1$), and this is given by:

$$z^* = \frac{((1-a)(2-p)+(2a-1)p)t - rap}{2((2a-1)pt^2+(1-a)t^2 - rap)} - \frac{\sqrt{(2(1-a)(1-p)t+ap(t-r))^2 - 4(1-a)(1-p)((2a-1)pt^2+(1-a)t^2 - rap)}}{2((2a-1)pt^2+(1-a)t^2 - rap)}, \quad \text{(A3.2)}$$

where $t = 1-(1-c)p$. This can then be checked for evolutionarily stability (Taylor 1996; Maynard Smith and Price 1973; Day and Taylor 1998) and convergence stability (Taylor 1996; Eshel and Motro 1981; Christiansen 1991). Note that the classical model of Hamilton & May (1977) is recovered as a special case of our model. This is done by assuming that all habitable patches are occupied ($p = 1$) and that there is clonal relatedness among co-operators within patches ($r = 1$), prior to dispersal. In this scenario, we recover the usual result: $z^*=1/(1+c)$. Hamilton & May (1977) emphasised that the evolution of dispersal involves a tension between the direct fitness cost ($C > 0$) for the individual disperser and the indirect fitness benefit ($rB > 0$) that she receives as a consequence of the improved reproductive success of her genetical relatives.

In Hamilton & May's (1977) model, an individual who survives dispersal and lands upon a habitable patch is (on average) no better off than if she had stayed in her natal patch. Hence, dispersal is always associated with a direct fitness cost owing to the mortality cost of dispersal ($C = c > 0$). But, by dispersing, the individual frees up breeding opportunities for her non-dispersing relatives, leading to an indirect fitness benefit ($rB = (1-z)/(1-cz) > 0$). Note that this benefit increases with the mortality cost of dispersal ($\partial(rB)/\partial c>0$), as a lower rate of disperser survival increases the extent to which the disperser's patch-mates – rather than immigrants arriving at her natal patch – benefit from her sacrifice. However, in Hamilton & May's (1977) model, the impact of increased mortality cost on direct

fitness is always greater than its impact on indirect fitness, so the net effect of increasing the mortality cost is to reduce the convergence stable level of dispersal ($dz^*/dc < 0$).

In contrast, in our generalisation of Hamilton & May's (1977) model, there is a more complicated relationship between the mortality cost ($c$) and the convergence stable level of dispersal ($z^*$). Owing to the presence of unoccupied habitable patches ($p < 1$), individuals who survive dispersal and land on a habitable patch find themselves – on average – better off than if they had stayed in their natal patch. This reduces the direct fitness cost of dispersal, and may even provide a direct fitness benefit to the disperser. As a consequence, the negative impact of the mortality cost on the direct fitness effect of dispersal ($\partial C/\partial c$) may be less than the positive impact of the mortality cost on the indirect fitness effect of dispersal ($\partial(rB)/\partial c$). This means that, for some parameter values, the convergence stable level of dispersal increases with the mortality cost of dispersal ($dz^*/dc > 0$; see Figure A2.1).

The evolution of dispersal is also mediated by the proportion of habitable patches that are occupied ($p$). Increasing this proportion always decreases the indirect fitness benefit of dispersal (i.e. $\partial(rB)/\partial p = -ar(1-c)z(1-z)/(1-(1-(1-c)p)z)^2 < 0$). However, increasing this proportion may either increase or decrease the direct fitness cost of dispersal (i.e. $\partial C/\partial p = (1-2a)(1-c) + (1-a)(1-p)(1-z)/(p^2 z) + (1-a)(1-z)/(p z)$). If $a \leq ½$, then this impact on the direct fitness cost is guaranteed to be positive, and hence for all $a \leq ½$ the CSS rate of dispersal is a monotonically decreasing function of the proportion of habitable patches that are habitable (Figure A2.1). If $a > ½$, then increasing this proportion may increase or decrease the CSS rate of dispersal (Figure A2.1). In addition, the evolution of dispersal is also directly affected by the relative carrying capacity of occupied patches ($0 < a < 1$). If $a < ½$, then occupied patches support a lower carrying capacity than do vacant patches, and if $a > ½$ then the opposite is true. As vacant patches can only be reached by

dispersers, a lower value of $a$ makes vacant patches – and hence dispersal – more attractive, whereas a higher value of $a$ makes vacant patches – and hence dispersal – less attractive. Hence, as the relative carrying capacity of occupied patches increases, the convergence stable rate of dispersal decreases ($dz^*/da < 0$; Figure A2.1).

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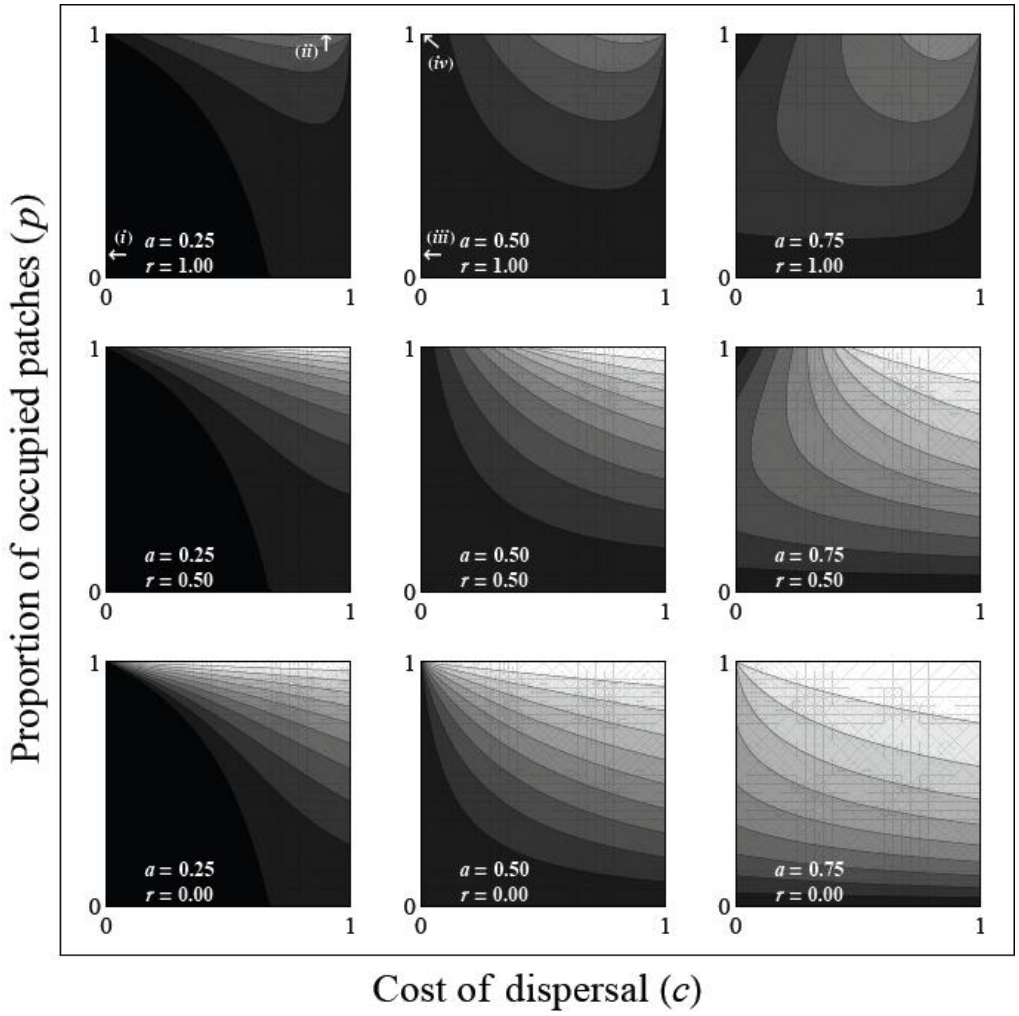


**Figure A2.1:** Contour plots of the CSS probability of dispersal ($z^*$), as a function of the cost of dispersal ($c$; abscissae), the proportion of habitable patches that are occupied ($p$; ordinates), the relative carrying capacity of occupied patches ($a$; columns), and the coefficient of genetic relatedness ($r$; rows). The scale varies from $z^* = 0$ (no dispersal, white), to $z^* = 1$ (full dispersal, black). The CSS probability of dispersal ($z^*$) decreases as the carrying capacity of the home patch ($a$) or the proportion of habitable patches that are occupied ($p$) increases, and decreases as the genetic relatedness within patches ($r$) increases. The relation between the CSS probability of dispersal ($z^*$) and the cost of dispersal ($c$) is more complicated, and is mediated by relatedness ($r$). For relatively low relatedness ($r$ = 0.00, 0.50), the CSS probability of dispersal ($z^*$) decreases monotonically with the cost of dispersal ($c$). For relatively high relatedness ($r$ = 1.00), the CSS probability of dispersal ($z^*$) is a U-shaped function of the cost of dispersal ($c$). The arrows indicate the points in the parameter space that correspond to the four treatments of Figure 4.2, hence: (*i*) local population and cheats as social neighbourhood; (*ii*) global population and cheats as social neighbourhood; (*iii*) local population and cooperators as social neighbourhood; (*iv*) global population and cooperators as social neighbourhood.

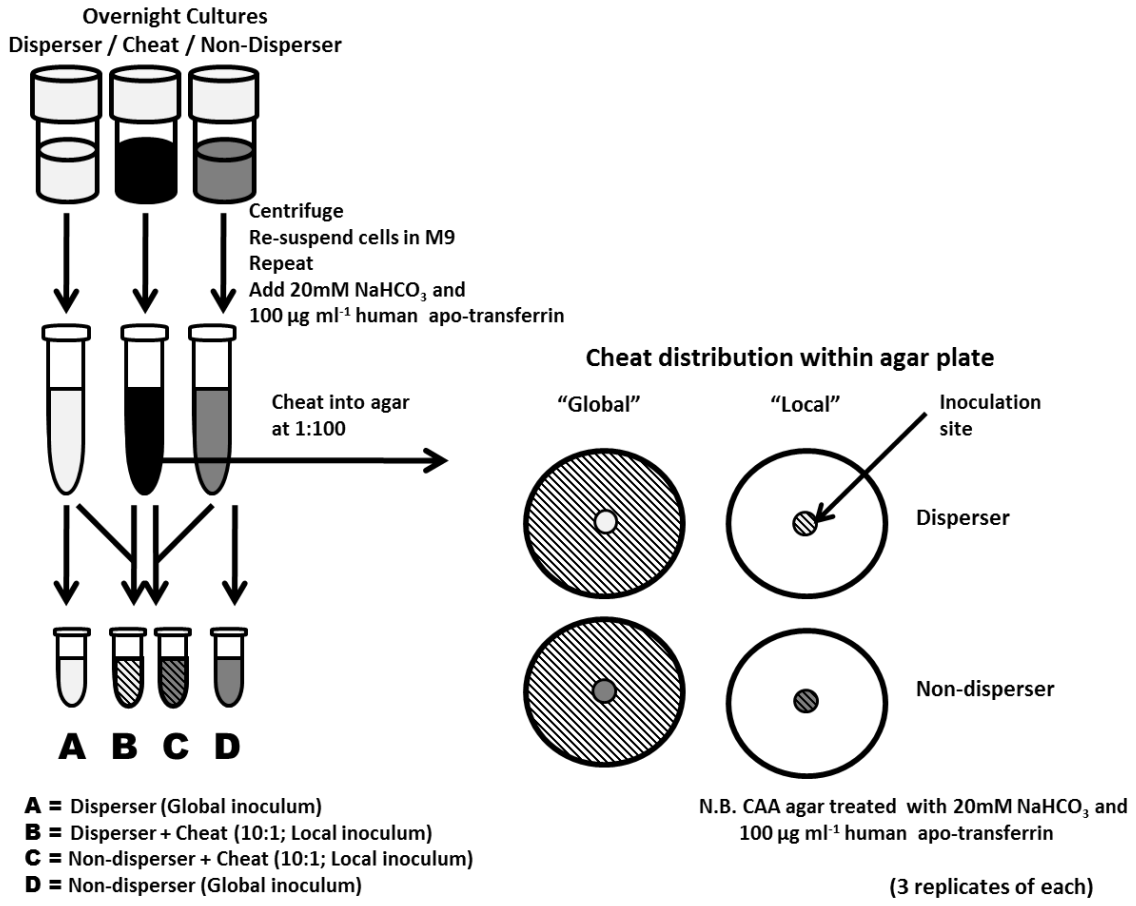


**Figure A2.2:** Schematic diagram of methodology for the preparation of treatment groups. A – D represents prepared inocula, and text in brackets describes which treatment groups inoculum was used in. Large circles are agar plates, where a white background represents agar only, and a striped background represents agar supplemented with cheat bacteria. Smaller circles in the centre of the large circles indicate inoculum used, colours correspond with prepared inocula A – D.