Experimental Evolution with Bacteria in Complex Environments

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Abstract

Experiments with microbes are a powerful tool for addressing general questions in evolutionary ecology. Microbial evolution is also interesting in its own right, and often clinically relevant. I have used experimental evolution of bacteria (*Pseudomonas* spp.) in controlled laboratory environments to investigate the role of environmental heterogeneity in the evolution of phenotypic diversity. Some of my results provide insight on general processes, while others are specific to bacteria. (1) I have shown that variation in resource supply affects the evolution of niche breadth in complex environments containing a range of available resources, leading to a peak in phenotypic diversity at intermediate levels. (2) I have found that resource availability also affects selection against redundant phenotypic characters, which is strongest when resources are scarce. (3) Using experiments with bacteria and their protozoan predators, I have found that selection for predator resistance varies with resource supply during a model adaptive radiation. (4) I have looked at the role of periodic bottlenecks in population size in the evolution of antibiotic-resistant bacteria. My results highlight the importance of biochemical constraints specific to different resistance mutations. (5) Finally, I have shown that bacterial adaptation to novel carbon substrates affects different growth parameters simultaneously, and that the same response is seen in environments that maintain different levels of phenotypic diversity. These findings emphasize the role of environmental heterogeneity in the evolution of phenotypic diversity, but also show how ecological and genetic factors can constrain adaptation to a given niche within a heterogeneous environment.

Declaration

I declare that I composed this thesis myself, and that the work described herein is my own except where explicitly stated below. This work has not been submitted for any degree or professional qualification except as specified.

Details of collaborations:

Chapter 4. Diversification experiments were designed and carried out in Dr. Rees Kassen's laboratory at the University of Ottawa, Canada. I did the experiments and analysed the data. I wrote the manuscript in collaboration with Justin Meyer and Dr. Rees Kassen.

Chapter 5. Dr. Craig MacLean provided the rifampicin-resistant mutants used in this project. I designed the experiment and interpreted the results in collaboration with Dr. Craig MacLean, Dr. Angus Buckling and my supervisor Dr. Nick Colegrave.

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Publications

The following published papers have arisen from this thesis.

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Contents

1. Introduction

1.1. SUMMARY

How does biological diversity evolve, and why is there more diversity in some environments than others? These questions have occupied evolutionary ecologists for over a century. In this thesis I focus on the role of environmental variation in the evolution of phenotypic diversity. I begin by reviewing evidence that patterns of adaptation and diversification reflect the influence of environmental factors. I then introduce the principles of experimental evolution with microbes, which forms the basis of each of the chapters that follow. Finally, I describe some of the key insights gained from experiments with microbes, before outlining the specific questions that are addressed in the remainder of the thesis.

1.2. THE EVOLUTION OF BIOLOGICAL DIVERSITY

Understanding the origin and maintenance of phenotypic diversity is a central aim of ecology and evolution (Wallace 1878; May 1986), and a large body of theory has accumulated on the subject. Broadly, the evolution of diversity is expected to rely on environmental heterogeneity, or the availability of alternative niches to which organisms may adapt (Levins 1979; Schluter 2000). The stable coexistence of different phenotypes also requires that the optimal phenotype varies among different niches (Levene 1953; Futuyma & Moreno 1988). In other words, trade-offs in fitness are required to prevent a generalist phenotype from dominating across the environment (Via & Lande 1985). Despite broad acceptance of a role for environmental heterogeneity in evolutionary diversification (Rosenzweig 1995; Schluter 2000), there is little agreement over why diversity is higher in some environments than others, and why fitness trade-offs are not always apparent.

Variation of the potential for evolutionary diversification is often attributed to the influence of abiotic factors, and the most widely cited of these is primary productivity. There are several alternative hypotheses for how productivity might constrain adaptation and diversification (Rosenzweig 1995), and different theories often make overlapping predictions. In attempting to discriminate between different hypotheses, some progress has been made using comparative methods, where geographic variation in phenotypic diversity or species richness is regressed against environmental variables that approximate primary productivity (Currie & Paquin 1987; Adams & Woodward 1989; Kerr & Packer 1997; Broughton & Gross 2000). Results show that changes in diversity are often correlated with changes in productivity, although the observed relationship varies among taxa and geographic regions (Schall & Pianka 1978; Rajaniemi 2003; Groner & Novoplansky 2003).

A second line of indirect evidence for the role of abiotic factors in evolutionary diversification comes from the fossil record. Past changes in the distribution and

abundance of animal species can sometimes be explained by variation of climatic variables or geological processes such as glaciation (Simpson 1953; Davis *et al*. 2005; Jackson & Erwin 2006). Phylogenetic information obtained from molecular data can be employed in a similar way (Ackerly 2004), mapping biological diversity onto environmental change.

A more direct approach involves experimentally manipulating biological communities and monitoring the resulting phenotypic changes over ecological or evolutionary time. For example, some of the most convincing evidence that phenotypic diversity is related to ecosystem productivity comes from long-term field manipulation studies with plants and associated soil microbes (Tilman *et al*. 2001). However, even when the shape of the productivity-diversity relationship is clear, covariance among explanatory environmental variables can make it difficult to isolate the factors that directly influence diversity (Zak *et al*. 2003; Waldrop *et al*. 2006).

Adaptation and diversification are also affected by biotic factors, such as predation, competition and immigration (Rosenzweig 1995; Schluter 2000). For example, natural enemies that act as predators or parasites can impose strong selection on their prey or hosts (Doebeli & Dieckmann 2000; Chase *et al*. 2002; Abrams 2003). This may lead to an increase in diversity due to the additional ecological opportunity represented by resistant phenotypes, but can also reduce diversity if natural enemies create strong selection for a single resistant phenotype (Vamosi 2005). The potential for natural enemies to influence community structure has been demonstrated in field manipulation studies (Paine 1966; Connell 1970; Nosil & Crespi 2006) and by comparative work (Hairston *et al*. 1999). However, much like primary productivity, the role of predators appears to vary among taxa and environments (Abrams 2000; Chase *et al*. 2002).

Overall, comparative methods and field studies have successfully described patterns of adaptation and diversification, but sometimes fall short of identifying the mechanisms responsible for observed relationships. The inherent complexity of biological systems

makes it difficult to isolate factors that explain ecological and evolutionary processes. Furthermore, the timescale for evolution is typically too long for direct observation. Therefore the best way to understand global patterns of adaptation and diversification may be to study the mechanics of evolution in simple model systems that are more amenable to experimental manipulation.

1.3. EXPERIMENTAL EVOLUTION WITH MICROBES

By using model systems we make simplified versions of nature in which it is easier to study the factors that we expect to be important. Laboratory populations of microorganisms make excellent model systems, and they are now widely employed in ecology and evolution.

The use of microbial model systems today relies on the same basic principles that were used by early experimenters (Dallinger 1878; Atwood *et al*. 1951). Microbes are maintained in controlled conditions and subsequent changes to the phenotypic or genetic characteristics of the population are related to defined properties of the environment. By the late twentieth century, knowledge of microbial physiology was sufficiently advanced for some microbiologists to realise that the genetic mechanisms of evolution could be studied in laboratory populations (Mortlock 1984a). By the early 1990s, the use of chemostats and batch culture systems to grow bacteria for many generations was established as a means of following long-term evolution in real time (Dykhuizen 1990; Lenski *et al.* 1991). Since then microbes have been used to study an increasingly broad range of topics in evolution and ecology (Jessup *et al*. 2004; Buckling *et al*. 2009). The resulting literature has increased our understanding of how selection brings about changes in gene frequencies, and how the outcomes of selection vary with the properties of the environment.

Selection experiments

The focus of experimental evolution with microbes is the use of selection experiments (Bell 2008a), the principal stages of which are as follows. (1) A population of microbes (most commonly bacteria, but algae, viruses and fungi are also used widely), containing either a single clone or a diverse assembly of genotypes, is inoculated into a controlled laboratory environment. This culture is referred to as a microcosm, and can be easily replicated and manipulated for properties such as temperature and nutrient supply. (2) Populations are then maintained for hundreds or thousands of generations of growth. This takes place either by continuous culture in chemostats, where fresh nutrients are constantly added as waste products and dead cells are washed away, or by batch culture, where the population is grown in a closed vessel such as a test tube until the available nutrients are exhausted, at which point a proportion of the population is transferred to a fresh microcosm. (3) At the end of the experiment the genetic or phenotypic characteristics of evolved populations are compared with those of the ancestral population from the start of the experiment, or with populations maintained in different conditions. Any differences among populations are attributed to the effects of evolution during the experiment. Usually the phenotypic property of greatest interest is the ability to reproduce in the experimental conditions (fitness), which can be measured in different ways as described below. Adaptation to the experimental environment is detected by an increase in fitness relative to the ancestral type, and phenotypic diversity within a given population can be measured as the variation in fitness among individual genotypes.

Advantages of microbial systems

Working with microbes has numerous practical advantages. First, their smallness and short generation times make it possible to observe evolution in real time, in large populations, and in workable laboratory conditions (Dykhuizen 1992; Elena & Lenski 2003; Jessup *et al*. 2004). Second, most bacteria can be kept frozen in a non-evolving state, so evolved genotypes and their ancestors can be compared directly. Third, the ecology and genetics of many microbes is well studied, making it possible to infer the

genetic and physiological changes associated with evolution in experimental populations (Harder & Dijkhuizen 1982; Mortlock 1984a; Spiers *et al*. 2002).

The principal difference between microbial experiments in the laboratory and field studies is the capacity to control the environment, and perhaps more importantly to replicate it. A tube of growth media can be replicated more or less exactly, and different replicates can be inoculated with identical bacterial genotypes. Experimental replicates can therefore be made effectively identical except for some critical manipulation of a factor of interest, such as the range of nutrients supplied in the growth media, the presence or absence of a bactericidal virus, or the temperature at which the microcosm is maintained. Laboratory experiments with non-microbes such as *Drosophila* have similar advantages, although microbes reproduce relatively quickly, allowing for comparatively rapid evolution experiments (Bell 2008a).

The simplicity of the microcosm environment has also been cited as its key weakness (Lawton 1995; Carpenter 1996). After all, if we control almost every aspect of the experiment, then surely the population is little more than "a rather inaccurate analogue computer ... using organisms as its moving parts" (Hutchinson 1978). However, what separates microbial experiments from computer simulations is that there are parameters and processes unspecified by the experimenter, most importantly the process of evolution by natural selection. This is illustrated by considering the simplest possible selection experiment, where bacteria are introduced to a novel environment and maintained for some fixed number of generations. We would probably predict that mutations increasing bacterial fitness in the experimental conditions would spread to fixation. However, unless we know the frequency and phenotypic effects of every possible beneficial mutation, we cannot predict the rate or extent of adaptation precisely. Experimental evolution with microbes therefore allows us to test both the logic of our hypotheses and their underlying assumptions (Colegrave & Haydon, unpublished). In the example above, those assumptions concern the occurrence of beneficial mutations.

One final reason for working with microbes is that their evolutionary biology is itself intrinsically interesting, and in some ways distinct from that of larger organisms. Bacteria show vast phylogenetic and ecological diversity, and play a critical role in regulating ecosystem processes (Woese 1987; Pace 1997; Horner-Devine *et al*. 2004). Furthermore, the pathogenicity of many microbes and the options for treating infection can often be better understood in the context of evolution. This is exemplified by the problem of eradicating bacteria that have evolved resistance to antibiotics (Maisnier-Patin & Andersson 2004; Read & Huijben 2009).

Measuring evolution by changes in fitness and diversity

Most selection experiments are concerned with a specific type of evolution: adaptation. During adaptation a population moves toward a phenotype with higher fitness in the present environment (Fisher 1930). Bacteria are certainly capable of adaptation to experimental conditions, but how should we measure the change in fitness during evolution experiments? So far two solutions to this problem have dominated. First, competition assays (Lenski *et al*. 1991), where evolved genotypes or populations are competed directly against their ancestors in a common environment. Second, growth assays in pure culture, where population growth rate or total cell yield in the experimental environment is taken as a measure of adaptedness (Bell 2008b).

The main strength of competition assays is that they incorporate all stages of growth and competition during the growth cycle. However, by essentially measuring several traits at once we gain little information on the relative contributions of different traits to overall fitness. For the long-term evolution experiment with *Escherichia coli* by Lenski and co-workers (Lenski *et al*. 1991; Lenski & Travisano 1994), this has been resolved by detailed study of the physiological differences between ancestral and evolved strains (Vasi *et al*. 1994; Lenski *et al*. 1998). For example, it is now clear that enhanced transport across the cell membrane is a major target of selection in glucose-limited environments, and is associated with simultaneous changes in growth rate, lag time and cell size (Lenski *et al*. 1998). Another potential problem with competition assays is that

they assume transitive increases in fitness during the experiment (de Visser & Rozen 2005; Bell 2008b). If a mutant genotype has higher competitive fitness than the genotype immediately preceding it but lower competitive fitness than its ultimate ancestor, then the selection environment and the assay environment will be different with respect to competitive interactions among cells. Fortunately non-transitive interactions are probably the exception rather than the rule (de Visser & Rozen 2006; Bell 2008b).

Growth assays in pure culture allow us to measure particular parameters, and in practice are often easier to do in large numbers than competition assays. In batch culture experiments the parameter under direct selection is total cell yield, because the genotypes that are transferred will be those that produce the greatest volume of cells over the growth cycle. It therefore seems sensible to measure that parameter directly if possible. However, as I show in Chapter 6, increased yield tends to be associated with changes in other growth parameters, and so we are not much closer to knowing how different traits really contribute to fitness. Furthermore, pure culture assays fail to account for any competitive interactions with other genotypes. Therefore this type of assay is best suited to studying adaptation to novel growth conditions (e.g. Shi & Xia 2003; Barrett *et al*. 2005; Chapter 2), where we are more interested in detecting an increase in fitness in the experimental environment than isolating the physiological mechanisms responsible for it. Growth assays are also useful in experimental settings where *a priori* knowledge of microbial physiology can be used to predict the traits that will be affected. I apply this rationale for rifampicin-resistant *Pseudomonas aeruginosa* with defective RNA polymerase in Chapter 5.

Adaptation is usually measured by changes in fitness at the population level. The fitness of individual genotypes within a single population can also be estimated, providing a measure of genetic variation for fitness. Furthermore, if the fitness of each genotype is measured in a range of niches or environments, then we can detect specialisation to growth in particular conditions. If different genotypes from the same population are

specialised to different environments or niches, then this constitutes phenotypic diversity that can be related back to the properties of the experimental environment. Variation in fitness due to genetic and environmental effects can therefore be used to measure both diversity and niche breadth (Bell 1990, 1991; Chapter 2). In some cases adaptation to a particular niche results in qualitative morphological changes that are easily detected in the laboratory. For example, *Pseudomonas fluorescens* genotypes that are adapted to growth in a biofilm express distinctive colony morphology when grown on agar plates (Rainey & Travisano 1998; Chapter 4). Such differentiation makes it relatively straightforward to estimate functional diversity, even though there may be considerable genetic variance for fitness within a given niche that is not reflected by morphological differences.

Experimental evolution and molecular biology

Hypothesis testing in experimental evolution does not necessarily require identification of the genetic and physiological changes that affect fitness. However, knowledge of the molecular mechanisms that lead to phenotypic evolution can make for more accurate experimental predictions. For example, Notley-McRobb and Ferenci (1999) showed that adaptation of *E. coli* to glucose metabolism in chemostats occurred by mutations that modify the structure of membrane-bound proteins, thereby improving transport into the cell. Because the transport systems for glucose and galactose are similar (Weickert & Adhya 1993), we might predict that adaptation to glucose will lead to a positively correlated response for growth on galactose. That is exactly what Notley-McRobb and Ferenci (1999) observed when they tested glucose-adapted populations for growth on galactose.

The genetic changes that accompany adaptation show some patterns that are consistent across species and environments. One such trend is that bacteria tend to acquire novel functions, such as the ability to metabolise new carbon sources, by modifying existing pathways rather than constructing new ones (Lin *et al.* 1976). In turn, beneficial mutations in microbial evolution experiments usually work by up- or down-regulating

existing genes instead of switching them on or off (Clarke 1984; Mortlock 1984b; Treves *et al*. 1998; Cooper *et al*. 2003; Le Gac *et al*. 2008). This is concurrent with work on non-microbes that suggests a central role for regulatory gene evolution in adaptation (Carroll 2000; Barrier *et al*. 2001; Shapiro *et al*. 2004; Prud'homme *et al*. 2007).

The ideal method for identifying genes involved in adaptation is to compare the DNA sequences of evolved and ancestral genotypes. However, until recently it was only feasible to sequence a subset of the genome from experimental samples of bacteria, either at random (Lenski *et al*. 2003) or by identifying candidate genes expected to be under selection (Notley-McRobb & Ferenci 1999). Today, improved molecular tools are making it easier to identify genetic changes (Ferenci 2008; Le Gac *et al*. 2008), and whole-genome sequencing for replicate populations at the end of selection experiments is within reach. For viruses, whole-genome data has been obtainable for some time (Bull *et al*. 2000). Recent work with bacteriophage even demonstrated the potential to predict the specific mutations that would be fixed during adaptation to particular genetic lesions (Bull & Molineux 2008). Thus, for simple genomes at least, biologists are edging closer to a full understanding of the molecular mechanisms of adaptation. However, the availability of large volumes of genomic data brings with it the new challenge of maintaining some relevance to current evolutionary hypotheses (Buckling *et al*. 2009), rather than simply confirming that phenotypic evolution is caused by genetic changes.

1.4. KEY ADVANCES & UNANSWERED QUESTIONS

Adaptation leads to diversification in some environments but not others. To understand why that is, we first need to explain the mechanics of evolution in simple scenarios. In the following sections I outline some of the key insights from microbial studies into the genetics of adaptation, the evolution of trade-offs, the importance of environmental heterogeneity, and the role of natural enemies in evolution.

The genetics of adaptation

For an isolated population of asexual organisms, adaptation can only proceed if there is a supply of beneficial mutations, and if the effect of those mutations on fitness is strong enough that selection leads to their spread. Understanding the properties of beneficial mutations and the factors that affect their fate is therefore central to the study of adaptation. Experiments with microbes have been used to test fundamental theories on the genetics of adaptation (de Visser & Rozen 2005; Orr 2005). For example, there has been much debate over the fitness effects of mutations that are fixed during adaptation (Orr 2005), specifically whether adaptation will proceed by a series of small genetic changes, or by a few big-effect mutations (Fisher 1930; Kimura 1983; Orr 1998). By observing microbial evolution in real time, the rate of change in fitness per generation can be estimated. Lenski and Travisano (1994) showed by maintaining *E. coli* in batch culture for thousands of generations that the increase in fitness was most rapid during the early stages of adaptation. This supports the notion that adaptation in asexual organisms often proceeds by big-effect mutations initially and smaller-effect mutations later on (Orr 1998; Elena & Lenski 2003; Orr 2005).

The second important property of beneficial mutations is the frequency at which they occur, and this can depend on both population dynamics and genetic constraints. In small populations, beneficial mutations may be so rare that they restrict the potential for adaptation (de Visser *et al*. 1999). In contrast, in very large populations there may be several beneficial mutations present simultaneously, and they can obstruct one another's spread. This process is referred to as clonal interference, and may extend the time it takes for a beneficial mutation to fix, but also means that mutations which are ultimately successful have a relatively large effect on fitness (Gerrish & Lenski 1998; Colegrave 2002; de Visser & Rozen 2006). Thus, even though the copy number of beneficial mutations increases monotonically with population size, the rate of adaptation generally shows a saturating relationship with population size (de Visser & Rozen 2005). In Chapter 5, I investigate the potential for population dynamics to modify the supply of

beneficial mutations during adaptation to the fitness costs of deleterious drug-resistance mutations.

The frequency of beneficial mutations also depends on the genomic rate of mutation. Genotypes with an elevated mutation rate will have a relatively rich supply of beneficial mutations, but will also accumulate more deleterious mutations per generation. Therefore a high mutation rate should be advantageous only when beneficial mutations are in short supply. Several studies with mutator strains of bacteria have shown that the benefits of a high mutation rate do indeed vary among genotypes and environments (Cox & Gibson 1974; Chao & Cox 1983; Giraud *et al*. 2001). For example, de Visser *et al*. (1999) used experimental evolution of *E. coli* to show that an increased mutation rate was beneficial only in populations that were very small or already well adapted to the experimental environment, and were therefore effectively waiting around for new beneficial mutations. Similarly, the constantly changing selection pressure imposed by co-evolution with a bactericidal virus can create selection for elevated mutation rates in *P. fluorescens* (Pal *et al*. 2007). Thus, microbial evolution studies suggest that selection can act on the very mechanisms that produce the genetic variation required for adaptation (Colegrave & Collins 2008).

Trade-offs and the evolution of specialisation

The notion that adaptation to one environment will lead to reduced fitness in another is firmly rooted in evolutionary biology (Roff & Fairbairn 2007). Trade-offs are also central to explanations for the evolution of phenotypic diversity (Levene 1953; Futuyma & Moreno 1988; Stearns 1992). There are three principal mechanisms that might generate such fitness trade-offs during adaptation. The first is antagonistic pleiotropy (Levins 1968), where mutations that have beneficial effects in one environment are deleterious in another. The second is the stochastic accumulation of mutations that are neutral in the present conditions but deleterious in another environment (Whitlock 1996; Kawecki *et al*. 1997). The third is less direct, where adaptation to one environment also

increases fitness at alternative niches but to a lesser extent, so that the correlated response to selection is weaker than the direct response (Fry 1996; Bell 1997).

Trade-offs have been exhaustively studied in experiments with microbes, where they can be detected by measuring the genetic correlation in fitness across different environments or niches. For example, Bell and Reboud (1997) showed clear evidence of a trade-off between growth in light and dark environments for the unicellular green alga *Chlamydomonas rheinhardtii*. Adaptation to growth in either the light or the dark was associated with a reduction in fitness in the other environment. The same principle has been applied in studies with *E. coli* to analyse genetic correlations for growth on different substrates. Results show that beneficial mutations that increase fitness on a particular substrate can either pleiotropically increase or decrease fitness on alternative resources, depending on the similarity of their uptake physiology (Travisano & Lenski 1996; Ostrowski *et al*. 2005).

In some studies it has been possible to show not only that a trade-off exists, but to identify the genetic mechanism behind it. For example, MacLean *et al.* (2004) showed that adaptation of *P. fluorescens* to growth in a biofilm in spatially structured microcosms comes at a cost in the form of impaired carbon catabolism. The same mutation that confers the biofilm-forming phenotype was found to cause the costly catabolic defect. Thus, the cost of adaptation was due to the pleiotropic effects of a mutation that was beneficial at a particular niche (MacLean *et al*. 2004).

One interesting outcome of research into trade-offs is the expectation that they will be environmentally sensitive (van Noordwijk & de Jong 1986; Sgro & Hoffmann 2004). The likelihood of detecting a negative correlation among different traits may depend on environmental factors such as nutrient stress. This prediction has been borne out in studies of trade-offs between competitive ability and predator resistance for *E. coli* (Bohannan & Lenski 2000; Jessup & Bohannan 2008), and between competitive ability and the production of extracellular molecules used in iron uptake by *P. aeruginosa*

(Brockhurst *et al*. 2008). Thus, the expression of an underlying negative genetic correlation between two traits is subject to environmental effects, and in Chapter 3 I demonstrate the potential consequences of this for phenotypic evolution.

Experimental evolution of diversity

The mechanisms by which environmental heterogeneity and fitness trade-offs can influence evolutionary diversification have been studied extensively with microbes (Kassen 2002; Kassen & Rainey 2004). One approach is to present experimental populations with a range of different niches in the form of alternative resources. After a period of selection we can then test for the presence of genotypes that are specialised to different resources. For example, bacteria can diversify in complex environments containing a range of alternative substrates, with different genotypes evolving to exploit particular subsets of the available resources (Friesen *et al.* 2004; MacLean *et al*. 2005; Tyerman *et al*. 2005). Experimentally manipulating the number of alternative resources or niches highlights the importance of environmental heterogeneity. The diversity of *P. fluorescens* phenotypes that evolves in complex batch culture environments is greatest when there are several different substrates available for growth (Barrett *et al*. 2005). In Chapter 2, I show how the evolution of diversity in these conditions also depends on the total concentration of carbon substrates in the growth media.

Alternative niches can also be defined as different patches in a spatially heterogeneous environment. Korona *et al*. (1994) studied the role of spatial structure by comparing bacteria that evolved on agar plates or in liquid culture. More recently, *P. fluorescens* has proven to be an ideal organism for studying diversification in spatially structured environments (e.g. Rainey & Travisano 1998; Kassen *et al*. 2000; MacLean & Bell 2002; Buckling *et al*. 2003). When they are kept in static tubes of liquid growth media, populations that are initially uniform rapidly diversify to a number of specialist genotypes that are adapted to different spatial niches within the environment (Rainey & Travisano 1998). Different phenotypes are easily distinguished by their colony morphologies on agar plates. The short timescale required for this adaptive

diversification, and the ease with which phenotypic diversity can be catalogued, make it an ideal system for studying the mechanism of diversification and the role of environmental factors. For example, Kassen *et al*. (2000) showed that changing the nutrient concentration in the growth media alters the relative productivities of the different niches, leading to variation in diversity with resource supply. In this thesis I study the influence of environmental resource supply during adaptive diversification in both spatially uniform (Chapter 2) and spatially structured (Chapter 4) environments.

Natural enemies and co-evolution

In microbial microcosms, natural enemies come in the form of parasitic viruses (bacteriophage), or predatory grazers that eat bacteria (protists). Studies with *Pseudomonas* spp. in spatially structured microcosms have shown that diversity can be altered by both parasitism (Buckling & Rainey 2002) and predation (Meyer & Kassen 2007). However, any general role for natural enemies is unclear, both in nature and in microbial microcosms (Chase *et al*. 2002; Chesson & Kuang 2008). This is largely because their influence appears to vary with the properties of the environment (Bohannan & Lenski 2000) and with the fitness costs associated with resistance (Holt *et al*. 1994; Leibold 1996). In Chapter 4, I test the hypothesis that selection for predator resistance varies with changing resource supply during adaptive diversification.

Co-evolution, where bacteria and their parasites or predators evolve reciprocally in response to each other's changing phenotypes, can occur in multi-species microcosms that are maintained for many generations. Co-evolution is a major selective force in nature (Thompson 1994) and can help to explain the evolution of virulence (Bull 1994). However, there is no general explanation for the genetic mechanism of co-evolution (Agrawal & Lively 2002). The potential for reciprocal adaptation in microbial microcosms has been recognised for some time (Chao *et al*. 1977; Modi & Adams 1991). Recently, host-parasite co-evolution has been studied explicitly using *P. fluorescens* and the lytic bacteriophage Φ2, with repeated short-term increases in bacterial resistance and phage infectivity indicating adaptation to selection pressures

imposed by the parasite and host respectively (Brockhurst *et al*. 2003, 2007). This system has been used to test the predictions that different theories of co-evolution make for traits such as parasite infectivity and host resistance. For example, Poullain *et al*. (2008) found that co-evolution could increase the ability of phage to infect a range of different host bacteria. The challenge now is to test the generality of co-evolutionary processes that are observed in particular experimental circumstances, and the potential for environmental effects to modify their outcomes (Morgan *et al*. 2005; Forde *et al*. 2008; Lopez-Pascua & Buckling 2008).

In summary, experiments with microbes have increased our understanding of the genetic mechanisms behind the evolution of phenotypic diversity, and emphasized their dependence on environmental effects. This approach is being adopted in increasingly diverse areas of ecology and evolution, many of which relate to the broad themes of adaptation and diversification. Recent research threads include the repeatability of mutational pathways during adaptation (Riley *et al.* 2001; MacLean & Bell 2003), the evolutionary origins of senescence (Ackermann *et al*. 2003, 2007), the evolution of sexual recombination (Colegrave 2002) and sexually selected traits (Rogers & Greig 2009), microbial biogeography (Hughes-Martiny *et al*. 2006), testing social evolution theory (Griffin *et al*. 2004; West *et al*. 2006), adaptation to increasing carbon dioxide levels (Collins & Bell 2004; Collins *et al* 2006), the relationship between diversity and population-level productivity (Hodgson *et al*. 2002), and the evolution of genome size (Nilsson *et al*. 2005).

1.5. CHAPTER SUMMARIES

In this thesis I develop and test hypotheses relating to the role of environmental variation in the evolution of phenotypic diversity.

2. I show that the evolution of diversity in complex environments containing a range of substrates available for growth varies with resource supply rate, with diversity most likely to be maintained at intermediate levels.

3. I present evidence that the fitness cost of a redundant phenotypic character is greatest in low-resource environments, and that this leads to relatively strong selection and rapid decay over evolutionary time in these conditions.

4. I use experiments with bacteria and protozoan predators to show firstly that selection for predator resistance can increase total phenotypic diversity, and secondly that coexistence of resistant and susceptible prey is most likely at intermediate levels of resource supply.

5. I use bacteria with defined drug-resistance mutations to show that periodic bottlenecks in population size can inhibit adaptation to the cost of a deleterious mutation, and that different deleterious mutations impose particular biochemical constraints on evolution.

6. I show that bacteria adapt to novel substrates by changes to several components of fitness simultaneously, and that the same qualitative selection response is seen in populations with different levels of phenotypic diversity.

2. How Does Resource Supply Affect Evolutionary Diversification?[§]

2.1. SUMMARY

The availability of different resources in the environment can affect the outcomes of evolutionary diversification. A unimodal distribution of diversity with resource supply has been widely observed and explained previously in the context of selection acting in a spatially heterogeneous environment. Here, we propose an alternative mechanism to explain the relationship between resource supply and diversification that is based on selection for exploitation of different resources. To test this mechanism we conducted a selection experiment using the bacterium *Pseudomonas fluorescens* in spatially homogeneous environments over a wide range of resource supply rates. Our results show that niche diversification peaks at intermediate levels of resource availability. We suggest that this unimodal relationship is due to evolutionary diversification that is driven by competition for resources but constrained by the ecological opportunity represented by different resource types. These processes may underlie some general patterns of diversity, including latitudinal gradients in species richness and the effects of anthropogenic enrichment of the environment.

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2.2. INTRODUCTION

A central aim in evolutionary biology is to understand which factors explain patterns of adaptation and diversity. For example, diversity is greater in some environments than others, and some species are narrowly specialised while others are generalists (Futuyma & Moreno 1988; Gaston 2000). One factor that is often cited in explaining these patterns is the availability of resources in the environment (Tilman 1982; Abramsky & Rosenzweig 1984; Rosenzweig 1995). Although any general relationship remains unclear (Abrams 1995; Rosenzweig 1995), a unimodal distribution of species richness with resource supply has been observed at a range of spatial scales (Abrams 1995; Rosenzweig 1995). Several mechanisms have been suggested to account for these observations (Rosenzweig 1995) but experimental tests have proved problematic due to the time-scale typically associated with evolutionary diversifications.

Microbial systems offer a way to address these questions experimentally. Microorganisms are small and reproduce rapidly, so evolution can be observed in real time and in workable laboratory conditions (Dykhuizen 1992; Elena & Lenski 2003; Jessup *et al*. 2004). *Pseudomonas fluorescens* is a bacterium that is being used increasingly to study diversity in the laboratory (e.g. Rainey & Travisano 1998; Buckling *et al*. 2003; MacLean & Bell 2003; Brockhurst *et al*. 2004). Populations that are initially uniform will rapidly diversify into a number of specialist genotypes when selected in spatially structured microcosms (static tubes of growth media). The different genotypes that emerge are adapted to different spatial niches within the environment (Rainey & Travisano 1998) and are identified by their different colony morphologies. If spatial structure is removed, by constantly shaking the tubes, diversity is quickly lost because there is no longer a range of distinct niches. Kassen *et al*. (2000, 2004) experimentally manipulated nutrient concentration in static microcosms and found that morphological diversity peaks at intermediate levels of resource supply. This pattern was explained by the effects of nutrient concentration on the relative productivities of alternative spatial niches in the heterogeneous environment (Kassen *et al*. 2000).

However, these results may not provide a general explanation for the link between diversity and resource supply that is widely observed in natural communities. In these experiments the same phenotypes emerge repeatedly and diversification is based on adaptation to spatially discrete niches (Kassen *et al*. 2004). Whilst spatial structure is an important component of natural environments, most species do not occupy spatially distinct niches and instead exploit a subset from a wide range of simultaneously available resources. For example, the diversification in beak morphology among Darwin's finches is explained by parallel variation in the size and shape of food resources (Lack 1947; Grant 1986). Selection based on exploitation of available resources may be a more general feature of evolutionary diversification than the effects of spatial heterogeneity.

If this is the case, then we expect the outcomes of diversification to vary with the availability of different resources, even in a spatially homogeneous environment. Bacteria in spatially uniform conditions can diversify to specialize on particular substrates if a range of different resources is available (Friesen *et al*. 2004; Tyerman *et al*. 2005; Barrett *et al*. 2005; Barrett & Bell 2006). For example, if *P. fluorescens* is selected in a complex environment containing multiple carbon sources, the population diversifies into a number of phenotypes with slightly different niches, as defined by their performance on different carbon sources (Barrett *et al*. 2005; Barrett & Bell 2006). In this chapter we use experimental populations of *P. fluorescens* to examine the role of resource availability in adaptive diversification in spatially homogeneous environments.

We compare the outcomes of long-term evolution in replicate populations selected in environments that differed only in resource supply rate. We used similar complex environments to Barrett *et al.* (2005) but at several different resource supply rates. Following selection, adaptation and phenotypic diversity within each population were quantified from variation in growth rates between individual genotypes on each substrate within the complex environment. By measuring the degree to which bacteria diversify

and adapt to different subsets of available resources (different niches), we can test critically the role of resource supply rate in the evolution of niche breadth and diversity.

2.3. METHODS

Selection experiment

A clonal isolate of *Pseudomonas fluorescens* SBW25 was used to found all selection lines, so that all populations were initially isogenic. The ancestral strain is stored at -80°C in 50% v:v glycerol. Selection was carried out in 28ml glass universal tubes containing liquid media at 28°C under shaken conditions. This constitutes a spatially homogeneous environment. The liquid media in each tube comprised 6ml of M9 salt solution (NH_4Cl 1 gl^{-1}, Na_2HPO_4 6 gl^{-1}, KH_2PO_4 3 gl^{-1}, NaCl 0.5 gl^{-1}) plus an equal concentration (gl^{-1}) of each of four carbon sources. Carbon substrates were selected that have been shown previously to support growth and adaptation of *P. fluorescens* SBW25 (MacLean *et al.* 2004; Barrett *et al*. 2005): acetic acid, glycerol, malic acid and succinic acid. Thus, resource supply rate can easily be manipulated by changing the total concentration of carbon substrates in liquid media. Based on pilot work we chose appropriate concentrations so that population density varied between selection environments. Population density was measured following growth over 48h by optical density (OD) at 600nm using a Jenway 6300 Spectrophotometer. We initiated selection lines in each of six selection environments, each with a specific resource supply rate. Resource supply was increased two-fold between selection environments from the lowest level, so that lines were selected at the following concentrations: 0.009375, 0.01875, 0.0375, 0.075, 0.15, and 0.3 gl^{-1}.

Eight replicate lines were selected at each level of resource supply by transferring 60µl from each 6ml culture to fresh media every 2d for 50 transfers. If populations grow to stationary phase between each transfer and then stop, a 100-fold dilution would allow

approximately 6.7 generations per transfer. However, gram-negative bacteria such as *P. fluorescens* do not stop growing completely (Zambrano *et al*. 1993) or evolving (Finkel & Kolter 1999) once they reach stationary phase and there is some cell turnover without increasing population size (Zambrano & Kolter 1996). This makes the number of generations per transfer difficult to determine exactly. Our selection experiment therefore involved a minimum of 350 generations of growth, with the actual number probably closer to 500. Following selection all lines were frozen at -80°C in 50% v:v glycerol.

Phenotypic assays

Patterns of diversity and specialisation were determined from variation in growth between individual genotypes on each of the different carbon sources. We measured the growth yield of ten genotypes from every population on each of the four substrates. To isolate distinct genotypes, each population was reconditioned overnight in standard growth medium (King's Medium B (KB): proteose peptone 20 gl^{-1}, glycerol 12 gl^{-1}, K_2HPO_4 1.5 gl^{-1}, $MgSO_4.7H_2O$ 1.5 gl^{-1}) at 28°C in shaken conditions. Two replicate populations of the ancestral clone were also reconditioned. Cultures were then diluted and spread on agar plates. 10 colonies were picked at random from each population, constituting 10 genotypes. The growth of each genotype was then assayed twice on all four substrates independently at a concentration of 0.15 gl^{-1}. The same concentration was used in all assays to allow comparisons of growth scores across selection environments.

To ensure that cells in all trials were initially at equivalent physiological states, each genotype was first grown in KB medium for 2d and then starved for 2h by transferring to M9 salt solution (MacLean *et al*. 2004). To begin the assay, 20µl of starved cells (~10^5 viable cells) were then transferred to individual wells of 96-well plates containing 180µl of the appropriate assay media (M9 salt solution plus 0.15 gl^{-1} of one of the four carbon substrates). The number of assays involved meant that it was impractical to carry

out all assays in the tubes used during selection. However, we measured growth in both conditions for a random sample of 10 genotypes in all four substrates and found that growth measured in 96-well plates is strongly correlated with growth measured in tubes ($r^2_{39} = 0.87$, $p < 0.0001$). Plates were then kept at 28°C for 48h, the same as between transfers during selection. Cell number was then estimated by measuring optical density at 650nm using an EMax precision microplate reader (Molecular Devices Corporation). To obtain growth scores, optical densities were corrected by subtracting control well scores (sterile media).

Measurement of diversity

To quantify overall phenotypic diversity within each evolved population we used Euclidean distances to measure phenotypic differences between genotypes (Barrett & Bell 2006). For two genotypes from the same population, the Euclidean distance is the square root of the sum of the squared differences in growth between the two genotypes over all substrates. Biologically, this measures differences in the metabolic profiles of two genotypes. Thus, by taking the mean Euclidean distance over all pairwise combinations of genotypes in a sample, we quantified phenotypic diversity in each population. However, there are different kinds of diversity that can give rise to high Euclidean distances, and since we are interested in niche diversification in particular, we used further analyses to distinguish different types of diversity.

Within-population variation in growth scores was analysed by ANOVA using JMP 5.1 software (SAS Institute). Variance was partitioned to genotype, environment, and genotype-by-environment interaction components. It is the interaction component that we are particularly interested in since this reflects environment-dependent differences between genotypes, and therefore indicates the emergence of different phenotypes adapted to different combinations or ratios of substrates.

Genotype-by-environment interaction was further decomposed into two components, inconsistency and responsiveness, as described by Bell (1990, see also Barrett *et al.*

2005). Inconsistency variance is due to contrasting correlations among genotypes over environments. If we consider that for each genotype the four carbon substrates can be ranked in terms of growth, then inconsistency indicates variation among genotypes in the order of substrate rankings. For example, in a given population one genotype may perform best on glycerol whilst another genotype grows fastest on succinic acid. Thus, high inconsistency suggests that different genotypes within a population are adapted to metabolise different subsets of the available resources. Responsiveness variance is due to differences in environmental variance among genotypes. For example, if some genotypes within a population grow to a similar extent on all substrates whilst other genotypes grow better on some substrates than others, this would generate responsiveness variance. Thus, genotype-by-environment interaction implies different patterns of adaptation between genotypes, and high inconsistency would support our interpretation of a strong interaction component as an indication of niche diversification.

In fitting models, environment (assay substrate) was taken as fixed and genotype as random. Once different types of diversity had been calculated for replicate populations selected in each of the selection environments, we tested for variation between environments with different resource supply rates by one-way ANOVA. Prior to comparison between selection environments, data for Euclidean distances and environmental variance were log transformed to account for heterogeneity of variance; figures 2.1 and 2.2 show back-transformed data.

2.4. RESULTS

Response to selection was positive in all populations

Evolved lines from all selection environments showed increased growth relative to the ancestral strain in each assay substrate (figure 2.1). After more than 350 generations of selection, this suggests considerable adaptation to substrates within complex environments.

By manipulating the total concentration of carbon substrates, we enacted variation in resource supply rate between selection environments so that population density increased with resource supply rate ($F_{5,47}$ = 266.82, p < 0.0001; figure 2.2a) and saturated at high levels, so that further increases in resource input did not increase population density.

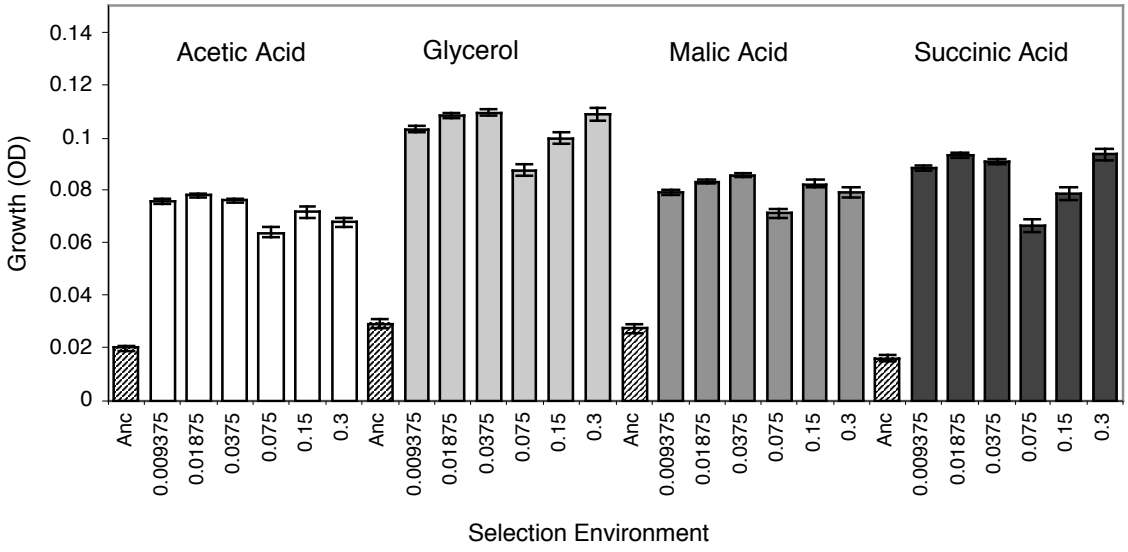


Figure 2.1. Mean growth scores of the ancestral strain (Anc) and evolved lines from each selection environment (shown as resource supply rate in gl^{-1} per 48h) in all four substrates. Bars show means ± s.e.

Total phenotypic diversity increases with resource supply rate

Populations selected at higher resource supply rates show greater overall phenotypic diversity, measured as mean Euclidean distance over all pairwise combinations of genotypes ($F_{5,41}$ = 12.41, p < 0.0001; figure 2.2b). However, this is an overall measure

of metabolic differences between genotypes and can indicate either the existence of different types adapted to different niches, or simply variation in performance between individuals of the same type. Thus, we took variation in growth due to genotype-by-environment interaction as a measure of niche differentiation.

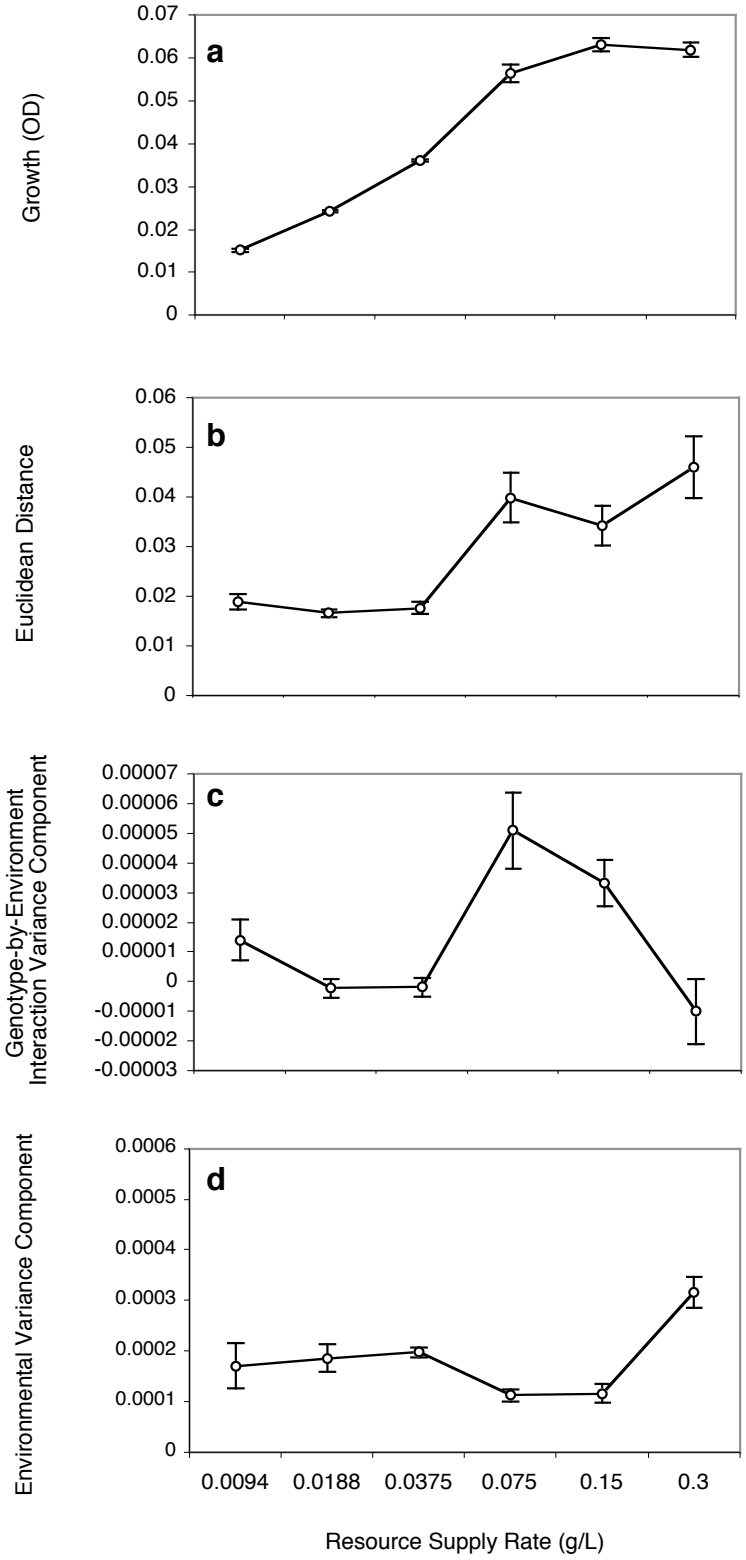
Niche diversification peaks at intermediate resource supply rates

Genotype-by-environment interaction is affected by resource supply rate ($F_{5,41} = 7.84$, $p < 0.001$; figure 2.2c), and peaks at intermediate levels. This shows that in environments with intermediate resource supply rates different genotypes had adapted to different combinations or ratios of substrates, constituting distinct niches. We further partitioned the interaction variance into inconsistency and responsiveness in order to clarify whether the different types within each population had adapted to different combinations of substrates, or had adapted to the same substrates in varying degrees. Inconsistency varies between selection environments ($F_{5,41} = 15.36$, $p < 0.0001$) and is greatest at intermediate resource supply rates, suggesting that high genotype-by-environment interaction in these lines was due to the existence of genotypes that had adapted to different combinations of substrates, indicating niche diversification.

We examined the environmental variance component for each population to test for differences in average growth among the different substrates. This was also affected by resource supply rate ($F_{5,41} = 6.50$, $p < 0.0001$; figure 2.2d), and is greatest at the highest level, indicating a population specialised to a single substrate. Low environmental variance can suggest either a breadth of adaptation or a mixture of genotypes equally adapted to different substrates. Given the trend for niche diversification and overall diversity it is clear that low environmental variance at low resource supply rates indicates relatively low diversification and the existence of generalists, whilst low environmental variance at intermediate resource supply rates is due to equivalent growth of a range of genotypes adapted to different niches.

In summary, at low resource supply rates niche diversification and overall phenotypic variation are relatively low; at intermediate levels different genotypes had adapted to distinct niches and diversity is increased; at very high resource supply rates overall diversity is high but is due to variation within the same phenotypic class that had specialised to a particular substrate.

Figure 2.2. (overleaf). (a) Variation in growth of the ancestral clone, measured over a single two-day transfer, between selection environments with different resource supply rates. Also shown are resulting patterns of (b) total phenotypic variation (measured as mean Euclidean distance), (c) genotype-by-environment interaction (variance component calculated using the interaction mean square relative to the residual mean square for each population), and (d) mean environmental variance, of populations selected at different resource supply rates. The interaction component reveals niche differentiation between genotypes while environmental variance is a measure of variation in growth between substrates. Points show means ± s.e.


a
Growth (OD)
0.07
0.06
0.05
0.04
0.03
0.02
0.01
0
b
Euclidean Distance
0.06
0.05
0.04
0.03
0.02
0.01
0
c
Genotype-by-Environment Interaction Variance Component
0.00007
0.00006
0.00005
0.00004
0.00003
0.00002
0.00001
0
-0.00001
-0.00002
-0.00003
d
Environmental Variance Component
0.0006
0.0005
0.0004
0.0003
0.0002
0.0001
0
0.0094
0.0188
0.0375
0.075
0.15
0.3
Resource Supply Rate (g/L)

2.5. DISCUSSION

In common with previous work (Barrett *et al*. 2005), we find that initially uniform populations of *P. fluorescens* can diversify in a complex environment. Different genotypes emerge that have different performance profiles across the available resources. However, results show that the degree of diversification depends critically on the level of resource supply. Whilst overall phenotypic diversity increased with resource supply, our measure of niche differentiation, which can be thought of as analogous to species richness, peaked at intermediate levels. We can relate these findings to the effects of resource supply on competition and ecological opportunity.

One possible explanation for the fact that overall phenotypic diversity increases with resource supply rate is the effect of variation in population size between environments. Since the serial transfer procedure used during selection involved transferring a fixed volume of media each time, larger populations in environments with higher resource supply rates were maintained throughout the period of selection. In larger populations the number of random mutations per generation is higher (Gerrish & Lenski 1998), potentially leading to the maintenance of higher genetic variance for fitness. The loss of genetic variability by random drift is also generally slower in large populations (Amos & Harwood 1998), but the effective population sizes in all of our selection environments were large enough that drift is unlikely to be an important factor in the observed pattern. Because variation in growth among individuals of the same metabolic phenotype can account for high Euclidean distances, high overall phenotypic diversity does not necessarily indicate stable diversifying selection. This applies at very high resource supply rates where populations comprise a single phenotype that is specialised to a particular substrate.

In contrast to the patterns for overall phenotypic diversity, niche diversification was greatest in environments with intermediate resource supply. How can such a unimodal distribution be explained? When resources are scarce diversification may be limited due

to a lack of ecological opportunity. Here selection favours only a generalist phenotype that can use a wide range of the available resources. As resource supply increases there is a greater number of viable niches (Bell 1997), here meaning different subsets of the available carbon sources. The benefits of specialisation to a particular niche will therefore increase, provided that the population does not adapt equally well to all available niches simultaneously (Levene 1953). In this case, competition for resources may generate divergent selection, favouring adaptation to underexploited niches and the evolution of genotypes adapted to different niches. At very high levels of resource supply, population growth within a given niche is no longer limited by the availability of resources. At this point there will be little selection for diversification and instead the population adapts to exploit the most abundant or productive resource. For example, if a particular resource is more easily exploited then the most successful genotypes are those that are best adapted to it and ecological diversity will be low.

Our results are consistent with known principles of bacterial resource use. When presented with a range of substrates at high concentrations, bacteria generally use them sequentially, metabolising the most easily exploited first and then switching to other substrates as this becomes depleted (Harder & Dijkhuizen 1982). Therefore if the preferred substrate is maintained at very high levels, as in our high resource input treatments, the population may never switch to exploit alternatives. Here only the ability to utilise the preferred substrate will be exposed to direct selection, leading to specialisation to this substrate. In contrast, at low concentrations of resources, exploiting a single substrate does not permit significant growth and so bacteria typically use multiple substrates simultaneously (Lendenmann *et al*. 1996). In this case performance on all substrates is exposed to selection and a generalist strategy may evolve.

Having invoked adaptation to particular substrates or combinations of substrates, we might now ask how adaptation to one substrate affects the ability to exploit alternatives. The stable maintenance of diversity is often predicted to rely upon negative correlations of fitness between alternative habitats or resources (Levene 1953; Futuyma & Moreno

1988; Kawecki 2000). That is, if adaptation to a specific niche does not incur a reduction in fitness elsewhere, then a generalist phenotype would dominate across the environment (Via & Lande 1985). Thus, in the context of bacteria in complex environments, if there is no fitness trade-off between alternative substrates we would ask why different phenotypes emerge instead of a generalist that adapts by increasing its performance on all substrates. At first our results appear to show no such cost of adaptation, since growth increased on all substrates. This agrees with previous findings that when bacteria adapt to particular resources a cost of adaptation may or may not be sustained (Velicer 1999; Velicer & Lenski 1999; MacLean & Bell 2002). However, diversification can still proceed provided that the correlated response to selection is weaker than the direct response (Bell 1997). So even though adaptation to a particular substrate may also increase the affinity for alternatives (Clarke 1984; Lin & Wu 1984; Mortlock 1984b), a fitness trade-off exists if the increase in growth is greater on one substrate than the other.

The mechanism by which adaptive diversification proceeded in this experiment is fundamentally different to that described by Kassen *et al*. (2000, 2004). In static liquid microcosms, diversification results from adaptation to spatially discrete niches, and a unimodal relationship between diversity and resource supply is explained only in the context of spatial heterogeneity. In contrast, we find that selection based solely on exploitation of available resources can explain patterns of diversification and that the outcomes of this process are strongly affected by the availability of resources in the environment. Given broad theoretical evidence that competition for resources and ecological opportunity are key components of divergent evolution (Simpson 1953; Schluter 2000), the mechanism described here may underlie several well-known patterns of diversity.

Increases in resource supply to very high levels frequently lead to a drop in diversity (Rosenzweig 1971; Tilman 1987; Kassen *et al*. 2000). This 'paradox of enrichment' has only been partially explained but is of particular relevance given the environmental impact of human activities such as the widespread use of fertilisers (Vitousek *et al*.

1997; Tilman *et al*. 2001). We find that diversification does indeed decrease at very high levels of resource supply, despite high population density, and that this is due to unchecked growth at a single niche resulting in the dominance of a specialist phenotype. The same principle explains the dominance of individual plant species in eutrophicated ecosystems. However, in most natural communities resources are not constantly available in abundance and the decline in diversity at high resource supply rates is only observed in cases of significant nutrient enrichment.

The relationship between diversity and resource supply has been observed at a range of spatial scales. For example, one of the best-known patterns of diversity is a latitudinal gradient in species richness (Rohde 1992; Rosenzweig 1995; Gaston 1996; Brown & Lomolino 1998). Rising diversity from the poles to the equator appears to reflect changes in energy availability (Wright 1983; Kerr & Packer 1997). Our results suggest that this may be because the limits of diversification are extended when there is greater resource supply, and therefore greater ecological opportunity. The effect of resource supply may be a general, although not exclusive, explanation for variation in the outcomes of adaptive diversification.

3. Decay of Unused Characters by Selection and Drift[§]

3.1. SUMMARY

The reduction and loss of redundant phenotypic characters is a common feature of evolution. However, the mechanisms that drive deterioration of unused characters remain unclear. Here we outline a simple framework where the relative importance of selective and neutral processes varies with environmental factors, because of variation in the fitness costs associated with unused traits. We tested our hypotheses using experimental evolution of the bacterium *Pseudomonas fluorescens* in spatially uniform environments. Results show that an unused character, swimming motility, decayed over evolutionary time and the rate of this decay varied among selection environments with different levels of resource availability. This is explained in the context of an environment-specific genetic correlation between motility and fitness, which is negative when resources are limited but neutral at higher resource levels. Thus, selection against an unused character was most effective in environments where the fitness cost was greatest. This suggests that the same character can decay by different mechanisms depending upon environmental factors and supports previous evidence to show that resource availability can critically affect the outcomes of evolution.

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3.2. INTRODUCTION

The loss of redundant or disadvantageous characters over evolutionary time is widespread and well documented (Darwin 1859; Fong *et al*. 1995; Porter & Crandall 2003). Familiar examples include the loss of functional eyes in cave dwelling species (Jeffery 2005; Romero & Green 2005) and the evolution of flightlessness in birds and insects (McNab 1994). The reduction of such unused characters may result in the evolution of ecological specialisation (Futuyma & Moreno 1988). However, the evolutionary mechanisms that drive character reduction remain unclear and the relative contributions of selective and neutral forces are generally ambiguous (Romero & Green 2005; Maughan *et al*. 2006).

Two principal mechanisms are proposed for the decay of unused characters that need not act exclusively. The first is selection, or antagonistic pleiotropy, where any previous benefit of a character is outweighed by a fitness cost in the present environment and its decay is adaptive (Cooper & Lenski 2000). Here reduction of unused traits is associated with increasing fitness (MacLean & Bell 2002). The second is neutral mutation accumulation, where the character has no significant effect on fitness and decays due to the spread of mutations that are selectively neutral but have a deleterious effect on the unused character (Haldane 1933; Fry 1996; Kawecki *et al*. 1997). Neutral mutation accumulation is expected to proceed stochastically at a rate determined by the genomic rate of mutation (Kimura 1983) and independent of the rate of fitness increase. This is true even in an asexual population that is substituting beneficial mutations at other sites, since although this affects the effective population size and the dynamics of a particular neutral mutation, it will not affect the overall rate of neutral substitution, which is independent of effective population size (Kimura 1983; Cooper *et al*. 2001). Previous studies have successfully distinguished antagonistic pleiotropy and mutation accumulaton in particular sets of conditions (Cooper & Lenski 2000; Collins & Bell 2004; Dorken *et al*. 2004; Maughan *et al*. 2006). However, the relative importance of selection and drift may often be determined by environmental factors.

Theoretical and experimental evidence show that trade-offs between different traits can vary across environments (Stearns 1992; Perrin & Sibly 1993; Sgro & Hoffmann 2004), depending on variation among individuals for resource acquisition and allocation (van Noordwijk & de Jong 1986). This is partly because the energetic cost of a given trait only translates to a fitness cost in certain environments. When resources are limited, expenditure on one trait reduces the amount that can be allocated to other traits. When resources are abundant, expenditure need not compromise allocation to other traits, so it is possible to maintain fitness while allocating resources to both adaptive and unused characters. For example, Gebhardt and Stearns (1988) showed that genetic correlations among life-history traits in *Drosophila mercatorum* vary with the level of dietary yeast. In fact, studies from a range of taxa show that trade-offs are more likely to be detected when resources are limited than when they are abundant (Spitze 1991; Brown 2003; Ernande *et al*. 2004; Blanckenhorn & Heyland 2005; Brockhurst *et al*. 2008; Jessup & Bohannon 2008). It follows that the fitness cost of an unused character, and therefore the mechanism driving its decay, is likely to depend upon resource supply.

The fitness cost imposed by an unused trait should be greatest in environments where resources are scarce, resulting in selection against it and rapid decay that is linked to increasing fitness. In contrast, the cost of an unused character may be independent from fitness when resources are abundant, so that decay occurs stochastically by mutation accumulation. It has not yet been demonstrated experimentally that the same character can decay by different mechanisms because of variation in environmental factors. Here we use experimental evolution of a bacterium *Pseudomonas fluorescens* to test the hypothesis that the fitness cost of an unused function depends upon resource availability and in turn that the same trait can decay by different mechanisms in different environments. Microbes are ideal for this work since they are small and reproduce rapidly; hence evolution can be observed in real time and in controlled laboratory environments (Dykhuizen 1992; Elena & Lenski 2003; Jessup *et al.* 2004). *P. fluorescens* in particular adapts rapidly to novel experimental environments and has been

used in a wide range of evolutionary studies (Rainey & Travisano 1998; MacLean & Bell 2002; Buckling *et al*. 2003; Brockhurst *et al*. 2005).

We treat swimming motility in spatially homogeneous environments as an unused character, since we expect the advantage that flagella-driven motility confers in natural environments to be reduced or lost in spatially uniform conditions. Motility is important for *P. fluorescens* in competitive colonisation and growth in the rhizosphere (Capdevilla *et al*. 2004; Martínez-Granero *et al*. 2006), and may be used in evasion of protozoan predators (Matz & Jurgens 2005). However, flagella are highly complex organelles composed of several protein species with several more for assembly and regulation (Bardy *et al*. 2003), and so the advantage of motility is weighed against an energetic cost. We expected the effects of this cost on fitness to vary with the availability of resources, and for this to affect the deterioration of motility during evolution. We tested this with a two-part experiment. First, we conducted a selection experiment where replicate lines evolved in spatially uniform environments at different resource supply rates. Second, to determine the fitness cost of motility at different levels of resource availability, we estimated the genetic correlation between motility and fitness, measured as growth yield in the experimental environment, at each level of resource supply for a sample of individual genotypes isolated from a single evolved population.

We predicted firstly that motility relative to the ancestral clone would decrease following selection in spatially uniform environments since the fitness advantage conferred by motility would be reduced or lost. Secondly, we predicted that the genetic correlation between motility and fitness would vary with resource availability, since we expect motile individuals to incur a fitness cost when resources are scarce but not when resources are abundant. Thirdly, we predicted that loss of motility would occur more rapidly and to a greater extent in environments with low resource availability since selection against motility will be strongest here, whilst in environments where motility has no significant effect on fitness it should decay stochastically by neutral mutation

accumulation. Fourthly, we expected that changes in motility would be coupled with changes in growth score in environments where motility decayed due to selection.

3.3. METHODS

Selection experiment

Selection was carried out as described in chapter 2: starting with a clonal isolate of *P. fluorescens* SBW25, lines were evolved in 28ml glass universals containing liquid media under shaken conditions at 28°C. Liquid media in each tube comprised 6ml of M9 salt solution plus an equal concentration (gl^{-1}) of each of four carbon substrates. Resource supply rate was manipulated by changing the total concentration of carbon substrates in liquid media at the start of each transfer, so that at different concentrations carbon substrates were supplied at different rates over the course of the experiment. Concentrations were chosen so that growth varied among selection environments and displayed a saturating relationship with resource supply. Although this also generates considerable variation in population size among treatments, effective population sizes were large enough in all lines (N_e between $\sim 7.2\times10^6$ and $\sim 7.2\times10^7$, estimated as the harmonic mean of N following Lenski *et al.* 1991; Hartl & Clark 1997) to discount significant variation in population genetic processes such as the supply of beneficial mutations or the sampling effects of random drift. Population density was measured following growth over 48h by optical density at 600nm using a Jenway 6300 Spectrophotometer. Eight replicate selection lines were evolved at each of six resource supply rates for 50 transfers. Populations were frozen every ten transfers.

Motility and growth assays

Following selection, motility and growth scores were taken for all selection lines at all time points. Growth scores for each population were recorded in the same liquid media that had been used during selection. Prior to assay, all populations were reconditioned

from frozen by growth in KB Medium for 2d at 28°C in shaken conditions. Nine replicate cultures of the ancestral clone were also reconditioned. For each population, the same reconditioned culture was used to estimate growth and motility scores. All assays were done in triplicate.

In preparation for growth assays, each reconditioned population was starved for 2h by dilution in M9 salt solution (MacLean *et al*. 2004). 20µl of starved cells (approx 10^5 viable cells) were transferred to individual wells of 96-well microplates containing 180µl of the appropriate assay media (M9 salt solution plus 0.009375, 0.01875, 0.0375, 0.075, 0.15 or 0.3 $gl^{-1}$of each carbon substrate depending on the selection line). Microplates were then kept at 28°C for 48h. Growth score was then estimated by measuring optical density at 600nm using a SpectraMax M2 microplate reader (Molecular Devices Corporation) and subtracting control well scores (sterile media). Optical density (OD) can be converted to cellular densities (CFU ml^{-1}) as follows: CFU $ml^{-1} = (1.997\times10^9 \text{ OD}) - 4.705\times10^6$. Thus, cell density ranged from approximately 4.0×10^7 to 4.4×10^8 CFU ml^{-1} among our experimental treatments.

Motility was measured by swimming ability on soft agar plates containing 25ml of M9 salts plus 0.15gl^{-1} of each carbon substrate and 3gl^{-1} agar. The same concentration of carbon sources was used for all motility assays to avoid motility measures being confounded by nutrient concentration. 5µl of reconditioned culture was spotted onto the plate and the diameter of swimming haloes was measured with callipers after incubation at 28°C for 24h. Pilot work showed that the duration of assays did not qualitatively affect results since scores after 24h and 40h were strongly correlated ($r^2_{53} = 0.74$, $p < 0.0001$) and that replicate measures for the same culture were highly repeatable, with an intraclass correlation coefficient (Lessells & Boag 1987) between 0.9 and 0.98.

Estimating genetic correlations in different environments

To determine the effect of resource supply on the genetic correlation between growth and motility, we compared the regression of growth on motility at different resource concentrations for a sample of genotypes from a single evolved population. A single population was used so that variation among individuals for growth and motility was not confounded by differences between populations. We chose a population that had been selected at $0.3gl^{-1}$ since this was where we expected variation in motility within populations to be highest. Genotypes were isolated by first reconditioning the population overnight in KB at 28°C in shaken conditions, then the culture was diluted and spread onto a KB agar plate. 78 colonies were then picked off at random, constituting distinct genotypes, and grown for 2d as above in preparation for growth and motility assays. A single motility score was estimated for each genotype as well as growth at each of the six resource supply rates.

Statistical analysis

To test for changes in motility and growth after selection at different resource supply rates, mean motility and growth scores for evolved lines were compared to those for the ancestral clone by student's *t*-tests, with significance levels adjusted by sequential Bonferroni correction, performed in JMP 5.1 (SAS Institute). Then variation of the change in motility among selection environments was tested by one-way ANOVA.

To test for variation in the fitness cost of motility among environments, we tested for an interaction between motility and resource concentration in determining the growth scores of a sample of genotypes isolated from a single population. Because growth scores at different resource supply rates were repeated measures on the same genotypes, we included genotype as a random effect in an analysis of covariance in R v2.2.1. Motility scores were transformed to natural logarithms to account for a non-normal distribution and degrees of freedom were adjusted for within-subject factors (resource supply and the interaction with motility) using a Greenhouse-Geisser estimate of epsilon (ε) as an index of sphericity. To estimate the genetic correlation at each level of resource

supply, linear regressions of growth by motility were fitted using the same parameters as in the overall model, with significance levels adjusted by sequential Bonferroni correction to account for family-wise type I error. We found some extreme values in the distribution of motility scores, which we accounted for by testing the leverage and influence of all data points. Although some points have high leverage scores (above $2(p/N)$ where p is the number of parameters and N is the sample size), they do not have high influence (measured using Cook's D) and plots of residuals confirmed that they did not bias or deviate from fitted models.

Finally, to test whether changes in motility occurred stochastically over time or were coupled with changes in growth score, we compared the predictive power of models including either transfer number or growth score to determine which was more closely linked to changes in motility over the course of selection. We fitted linear mixed-effects models including motility and growth scores at 10-transfer intervals for lines selected at different resource supply rates. Motility score was taken as the response variable, selection environment and either transfer number or growth score as fixed effects, and selection line nested within selection environment as a random effect (accounting for repeated measures on the same selection lines). Each model also included the interaction of either growth or transfer number with selection environment to allow for variation in the rate of decay with resource supply rate. We used the corAR1 function to model the autocorrelation structure of the data. Lastly, because each selection line was started under very similar conditions, we constrained the model of motility over time to a single intercept (Perron *et al*. 2007).

3.4. RESULTS

Changes in motility and growth over the course of selection

Following selection, growth increased in all treatments whilst motility generally declined (figure 3.1; table 3.1). The reduction in motility at the end of selection also varied considerably among selection environments ($F_{5,38} = 17.92$, $p < 0.0001$; figure 3.1a at transfer = 50), with the greatest response to selection at low resource supply rates and little or none at higher levels. The largest increases in growth score occurred within the first 10 transfers in all selection environments but one (0.3 gl^{-1}), and the response to selection for growth at the end of the experiment varied with resource concentration ($F_{5,42} = 29.85$, $p < 0.0001$), peaking at intermediate levels.

The genetic correlation between motility and fitness varies with resource supply

The relationship between motility and fitness depends upon resource availability, which was detected by a significant interaction between resource supply rate and motility in explaining the growth of a large sample of independently isolated genotypes from a single population ($F_{2.86,217.53} = 25.42$, $p < 0.0001$). Specifically, high motility incurs reduced fitness at low resource supply but is independent of fitness when resources are more abundant. This was shown by variation in the slope (b) of growth against motility among environments with different resource supply rates, which is negative at low resource supply but neutral at higher levels (table 3.2; figure 3.2). Thus, high motility was associated with reduced growth score in environments where we detected the greatest deterioration over the selection experiment.

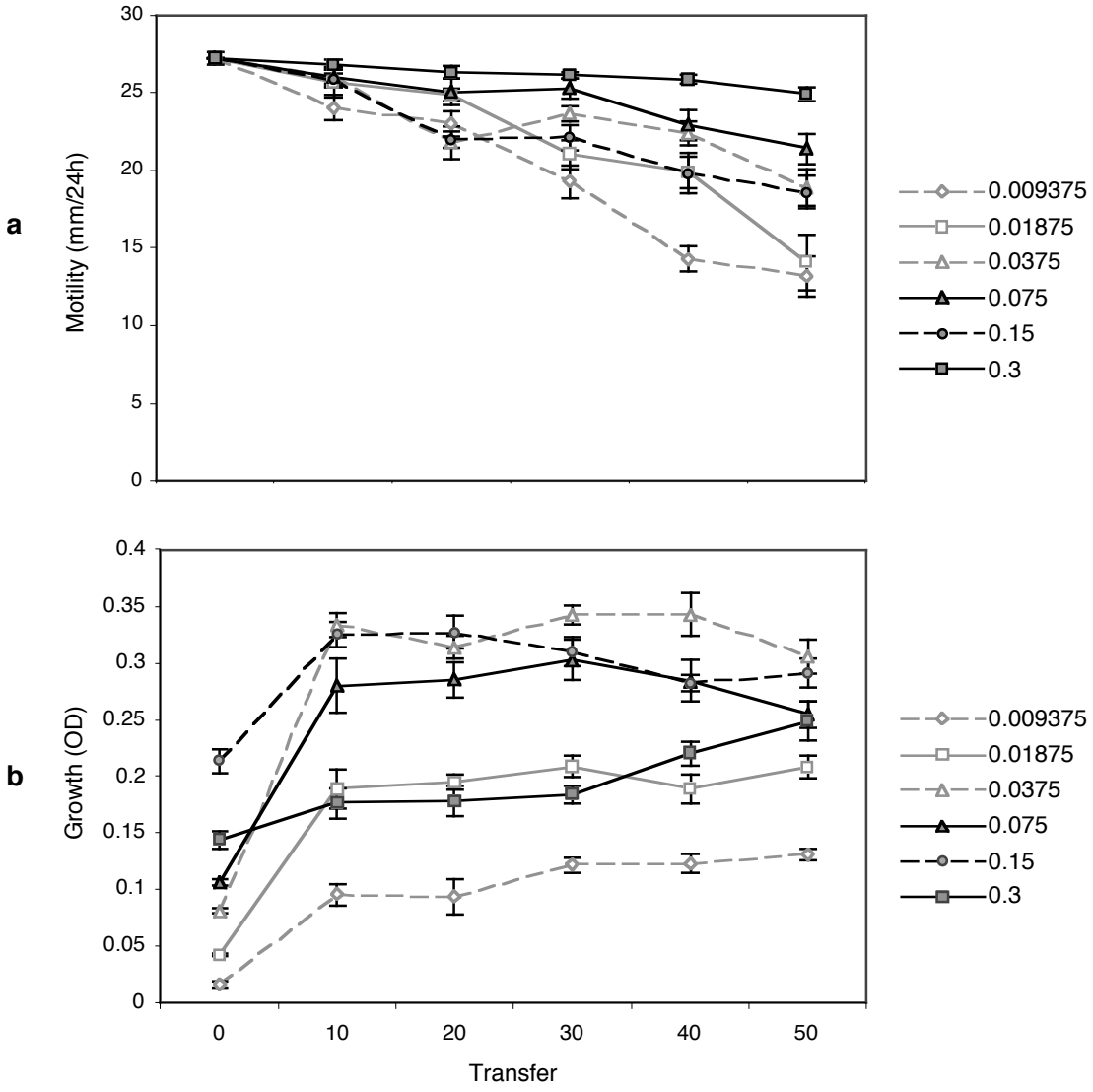


Figure 3.1. (a) Motility and (b) growth scores over the course of selection in different environments (shown at right as resource supply rate in gl^{-1}). Scores at transfer 0 show growth of the ancestral clone, measured over a single two-day transfer, in different selection environments. Points show means ± s.e.

Loss of motility over time and with changes in fitness

If changes in motility were associated with increasing fitness we would expect growth score to have a significant predictive effect on motility scores in a linear model including data from each time point. In contrast, if changes in motility were due to the stochastic effects of genetic drift, they would occur at an approximately constant rate over the course of selection and the number of elapsed generations would be more informative than changes in growth.

Changes in motility during our experiment were better explained by the number of elapsed generations than by changes in growth score, since a model including transfer number was considerably better at explaining the loss of motility ($r^2 = 0.67$, $p < 0.0001$) than one including growth scores at each time point and excluding transfer number ($r^2 = 0.31$, $p < 0.0001$). This was also true in all selection environments when they were tested separately. The inclusion of a quadratic term (growth^2) allowing for a polynomial regression of motility by growth did not significantly improve the predictive power of the model ($r^2 = 0.31$, $p < 0.0001$). Thus, changes in motility and growth occurred over different timescales. Furthermore, the rate at which motility decreased varied among selection environments, shown by a significant interaction between selection environment and transfer number ($F_{5,179} = 29.82$, $p < 0.0001$) and illustrated in figure 3.1a by the non-parallel slopes of motility over time at different resource supply rates. Therefore changes in motility were approximately linear over time, although changes in fitness were not, and the rate of decay varied among treatments.

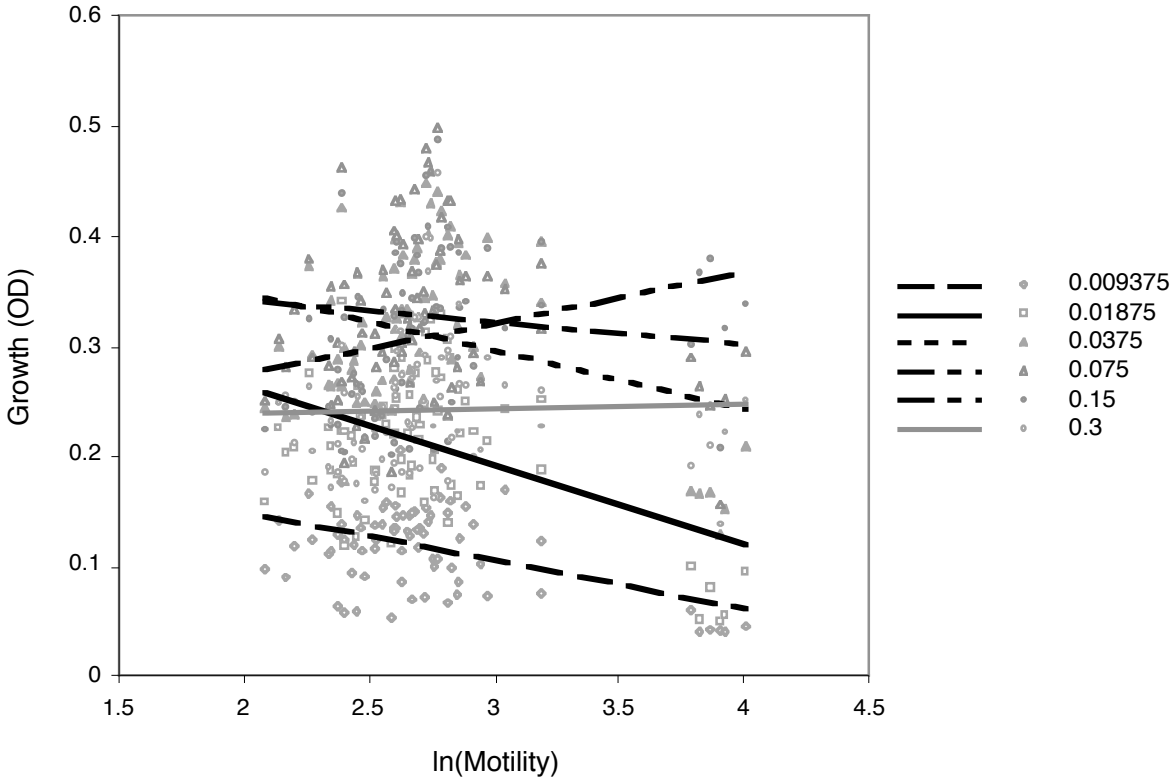


Figure 3.2. Genetic correlation between growth and motility at different resource supply rates. Data show growth scores at each resource supply rate plotted against a single motility score for each of 78 independent genotypes isolated from a single evolved population. Further details are given in table 3.2.

Table 3.1. Student's *t*-tests of growth and motility scores of evolved populations compared to the ancestral clone. Asterisks indicate significance after sequential Bonferroni correction.

Resource supply	Motility			Growth		
(gl^{-1})	*t*	*N*	*p*	*t*	*N*	*p*
0.009375	-10.97	4	0.002*	22.64	8	<0.0001*
0.01875	-7.95	8	<0.0001*	15.76	8	<0.0001*
0.0375	-7.58	8	0.0001*	14.87	8	<0.0001*
0.075	-7.15	8	0.0002*	12.49	8	<0.0001*
0.15	-6.49	8	0.0003*	6.17	8	0.0005*
0.3	-1.81	8	0.11	6.17	8	0.0005*

Table 3.2. Regression analysis of growth against motility at different resource supply rates. Asterisks indicate significance after sequential Bonferroni correction.

Resource supply (gl^{-1})	r^2 ($N = 78$)	*b*	$F_{1,76}$	*p*
0.009375	0.215	-0.043	20.79	< 0.0001*
0.01875	0.202	-0.072	19.21	< 0.0001*
0.0375	0.082	-0.052	6.79	0.011*
0.075	0.013	-0.020	0.98	0.326
0.15	0.071	0.046	5.77	0.019
0.3	0.0008	0.004	0.06	0.801

3.5. DISCUSSION

Swimming motility deteriorated over evolutionary time in spatially uniform conditions, as expected of a trait with little or no function in the prevailing environment. Furthermore, we found that the rate and extent of this deterioration varied among selection environments, with the greatest deterioration in environments with low resource availability. We suggest that this is because the relative effects of selective and neutral forces on an unused character depend upon the availability of resources. Specifically, at low resource supply motility has a negative relationship with fitness, resulting in selection against it and rapid decay over evolutionary time, whilst at high resource supply motility is selectively neutral and decays due to the stochastic spread of mutations that have deleterious effects on motility. Overall, our results support the notion that the importance of trade-offs varies with environmental factors, and that this can determine whether and how quickly phenotypic traits are lost or maintained.

The most compelling evidence that motility is under selection in some environments and not others is that it has a fitness cost at low resource supply but is independent of fitness at higher levels. This interpretation is supported by the relatively rapid deterioration in environments where we expected selection against motility to be strongest. In contrast with some previous studies (Cooper & Lenski 2000; Maughan *et al*. 2006), we found that the effects of selection against an unused character were not coupled to changes in fitness over time. These differences in timescale may be explained by variation in the rate at which different mutations are fixed depending upon their relative contributions to fitness. During adaptation to a novel environment, mutations of large effect tend to be fixed early on (Orr 1998, 2005), so that fitness increases rapidly during the early stages of selection. In our experiment the largest increases in fitness did indeed occur early on, which is concurrent with previous findings for bacteria in general (Elena & Lenski 2003) and *P. fluorescens* in particular (Barrett & Bell 2006; Barrett *et al*. 2006). However, mutations that affect motility presumably have a smaller effect on fitness than those that increase growth yield directly, for instance by enhancing metabolism of different carbon

substrates. Thus, deterioration of motility was approximately linear over the course of our experiment while fitness clearly increased at an inconsistent rate.

The different timescales of increasing fitness and decreasing motility suggest that the deterioration of unused characters occurs more slowly than adaptation to the experimental environment. It follows that changes in the rate of deterioration might occur over thousands as opposed to hundreds of generations. For example, Cooper and Lenski (2000) identified non-linear decay of total catabolic function in *Escherichia coli* over 20,000 generations of evolution. This demonstrates that phenotypic evolution continues long after the initial stages of rapid adaptation in microbial selection experiments. The relative rates of adaptation and decay of unused functions might also be influenced by factors that arise over the course of evolution, such as changes in mutation rate, covariance with other phenotypic characters, or compensatory mutations at other loci.

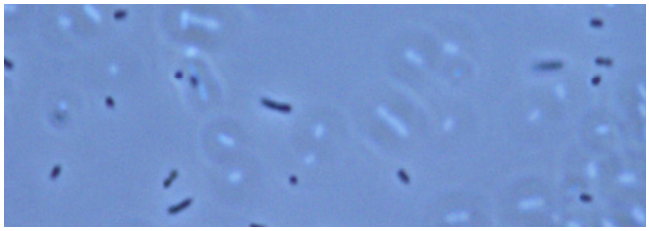
One alternative explanation for the observed pattern is the evolution of mutator genotypes in some treatments. The evolution of higher mutation rates could accelerate neutral mutation accumulation, speeding up the decay of an unused trait. The evolution of mutator strains has been observed in other experimental evolution studies with similar population sizes to those used here (Sniegowski *et al*. 1997). However, to explain the patterns observed here would require a negative relationship between the frequency of mutators and resource supply rate. Although mutators are expected to spread relatively rapidly in poorly adapted populations (Taddei *et al*. 1997; Giraud *et al*. 2001), the low growth yield in our low-resource treatments does not necessarily reflect a lower level of adaptation to the available carbon substrates. Indeed, the response to selection for fitness at the end of the experiment was greatest at intermediate resource supply rates. This suggests that low-resource treatments may not impose significantly novel selection pressures relative to intermediate and high levels when the available carbon substrates are the same in all treatments. Additionally, the evolution of mutators cannot explain variation in the genetic correlation between motility and growth across environments,

since these were estimated for the same sample of genotypes isolated from a single population and any variation in slope is therefore due to environmental and not genetic effects. Unfortunately, we cannot rule out the appearance of mutators in some lines, but we do not expect this to explain the parallel phenotypic deterioration in replicate selection lines at low resource supply rate.

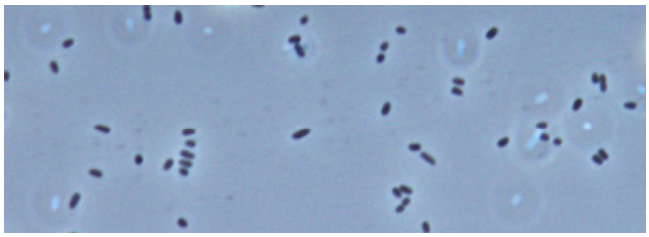
The fitness effects of an unused trait could also be modified by covariance with other phenotypic characters during short-term responses to environmental variation. Environmental factors can affect the growth rate, cell size and swimming motility of microbes concurrently (Matz & Jurgens 2005). In fact, there is evidence from *E. coli* that motility can increase over the short term in low quality environments as part of a broad transcriptional programme that is interpreted as an increase in foraging effort in stressful conditions (Liu *et al*. 2005). Therefore selection against motility in our experiment might have been amplified by such a response at low resource concentrations. We accounted for this possibility by making observations of the ancestral clone at different resource concentrations under the microscope. We found no discernible differences among cultures other than variation in cell density (figure 3.3), providing further evidence that low resource environments did not present unusually stressful conditions here. The discrepancy between this result and that of Liu *et al*. (2005) probably stems from the fact that we used the same carbon substrates in all environments, whereas they detected variation among environments containing carbon substrates of varying quality.

Figure 3.3 (overleaf). Examples of phase contrast microscopy showing no discernible difference in cell size among cultures of wild type *P. fluorescens* grown at different substrate concentrations. Taken at ×1000 magnification with an Olympus BX51 microscope and DB20 camera.

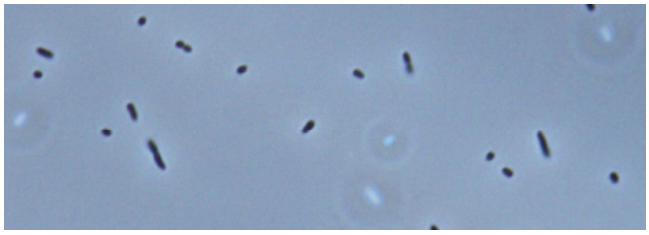
0.3 gl^{-1}



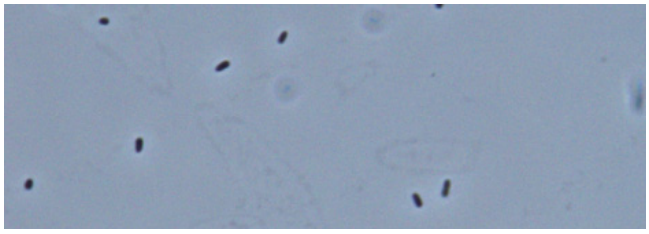
0.15 gl^{-1}



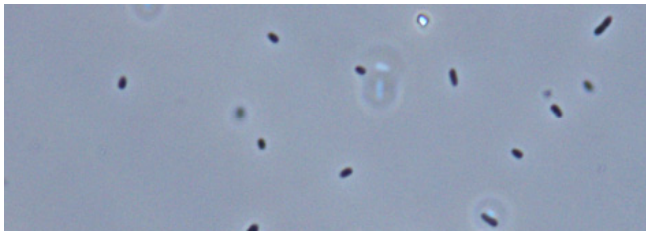
0.075 gl^{-1}



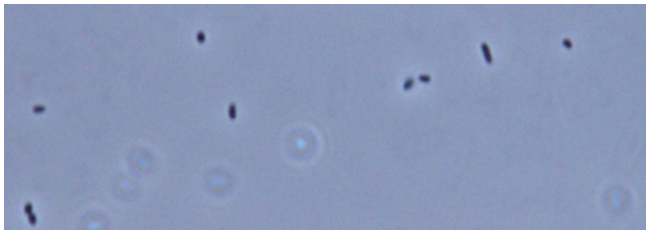
0.0375 gl^{-1}



0.01875 gl^{-1}



0.009375 gl^{-1}



Phenotypic deterioration may be particularly relevant for complex traits like motility since there are several different mutations that can result in loss of function. In contrast, the fitness costs associated with simple traits that can only be knocked out by one particular mutation may be ameliorated by compensatory mutations at other loci that decrease the cost without affecting the redundant character's function (Cohan *et al*. 1994; Levin *et al*. 2000; Maisnier-Patin & Andersson 2004). For example, microbial resistance to antibiotics can be maintained in the short term even in the absence of antibiotics if the fitness cost associated with resistance is reduced (Schrag *et al*. 1997). In other words, compensatory adaptation should be more important when the frequency of loss-of-function mutations is low relative to that of compensatory mutations. Thus, the cost of motility in our experiment was reduced by mutations affecting motility directly and not by compensatory mutations at other loci.

In summary, our results support existing evidence that the importance of trade-offs varies with resource availability (van Noordwijk & de Jong 1986; Sgro & Hoffmann 2004), and agree with recent work showing that the fitness cost of different traits, including biofilm formation and siderophore production in *Pseudomonas* spp., is greatest at low resource supply (Brockhurst *et al*. 2008). Furthermore, we show how this can determine the strength of selection against redundant phenotypic characters. Although the deterioration of unused traits also depends upon a number of genetic factors, the debate over when and why different mechanisms lead to decay might only be resolved by considering how the fitness costs of unused characters vary with environmental factors.

4. Selection for Predator Resistance Varies with Resource Supply in a Model Adaptive Radiation

4.1. SUMMARY

Selection pressures due to predation and resource competition can vary with environmental resource availability, and the mechanisms underlying their interaction remain unclear. We used experimental evolution of the bacterium *Pseudomonas fluorescens* in the presence of a protozoan predator *Tetrahymena thermophila* to show how selection for predator resistance varies with resource supply during adaptive radiation in a spatially heterogeneous environment. Results show that in resource-poor conditions the ancestral 'smooth' morphotype dominates, but as resource supply increases, the frequency of the predator-resistant biofilm-forming 'wrinkly spreader' morphotype rises. Predation increased the frequency of wrinkly spreaders, with the largest effect at intermediate resource concentrations. Thus, coexistence between different prey phenotypes is more likely at intermediate concentrations than at extremes where one type or the other is in the majority, and predation extends the range of concentrations that support high phenotypic diversity.

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4.2. INTRODUCTION

Selection imposed by natural enemies, including predators and parasites, and resource competition are important determinants of community structure and potential drivers of diversification (Levene 1953; Rosenzweig 1995; Doebeli & Dieckmann 2000; Schluter 2000; Abrams 2003). However, the way that these factors interact in different environments remains unclear (Abrams 2000; Chase *et al*. 2002). Here we explore the roles of resource competition and predation during adaptive radiation in environments with different levels of resource availability.

Adaptive radiation, the rapid diversification of a lineage into a range of niche specialists, may be driven by diversifying selection generated by resource competition in the presence of ecological opportunity (Schluter 2000). Predators may affect this process in a number of ways (Chase *et al*. 2002). For example, predation can create additional ecological opportunities in the form of predator-resistant phenotypes (Vamosi 2005; Nosil & Crespi 2006), which may lead to higher levels of diversity than observed under resource competition alone. Alternatively, diversity may be reduced under predation if there is strong selection for a single resistance strategy, or if reduced prey population density reduces the strength of diversifying selection resulting from resource competition (Buckling & Rainey 2002; Meyer & Kassen 2007).

In the absence of predators, diversity is known to vary with environmental resource availability (Tilman 1982; Rosenzweig 1995), and is often seen to peak at intermediate levels (Abramsky & Rosenzweig 1984; Abrams 1995; Rosenzweig 1995). In support of this general pattern, experimental studies with microbes have shown that evolutionary diversification can be constrained in low-resource environments where, in spite of resource competition, ecological opportunity is limited and the only phenotypes that persist are those that can grow at low resource concentrations (Kassen *et al*. 2000; Chapter 2). As resource supply increases, resource competition may generate diversifying selection, leading to the evolution of niche specialists that exploit different

resources or spatially defined habitats. At very high resource supply a single phenotype often dominates, exploiting only the most productive resource or niche.

In the presence of predators, any change in diversity resulting from the evolution of predator-resistant phenotypes may be constrained by resource supply in a similar way. Specifically, if predator resistance incurs reduced growth rate or competitive ability, the selective benefits conferred by resistance may be lowest when resources are in short supply, with selection instead favouring phenotypes that are more successful at competing for the available resources (Leibold 1996). In contrast, when resources are more abundant resource exploitation is less important for fitness and selection is expected to favour predator resistance (Holt *et al.* 1994; Leibold 1996). Coexistence between resistant and susceptible populations may therefore be most likely at intermediate levels of resource supply. Experimental evidence from *Escherichia coli* in the presence of bacteriophage suggests that this is the case in an ecological setting (Bohannan & Lenski 2000).

The theoretical and experimental results outlined in the preceding paragraph lead us to predict that selection for predator resistant phenotypes during an adaptive radiation will vary with resource availability, with energetically costly resistance strategies being less advantageous at low resource supply. In turn, we expect that any increase in diversity resulting from predation will be relatively small in these environments. We tested this hypothesis using a predator-prey system comprising the bacterium *Pseudomonas fluorescens* and a filter-feeding protozoan *Tetrahymena thermophila*. Protozoan predation of bacteria is extremely common and bacteria employ a wide range of resistance strategies (Matz & Jurgens 2003). *P. fluorescens* has been used previously to show that both predation and resource competition can promote adaptive diversification (Rainey & Travisano 1998; Meyer & Kassen 2007), albeit in different ways. Resource competition promotes rapid diversification to spatially defined niches in static microcosms, with the principal morphological classes occupying either the liquid broth phase (ancestral smooth: SM) or the air-liquid interface (wrinkly spreader: WS) due to

the formation of a self-supporting biofilm. This diversity is stably maintained by frequency-dependent selection (Rainey & Travisano 1998). Predation by *T. thermophila* also promotes diversification of SM to WS due to the ability of WS to escape predation in the biofilm (Meyer & Kassen 2007).

Biofilm formation is energetically costly, requiring the over-production of a cellulosic polymer (Spiers *et al*. 2003; MacLean *et al*. 2004), and is therefore severely compromised when resources are limited. Consequently, the success of WS varies considerably with resource availability in the absence of predators (Kassen *et al*. 2000), being very low in resource-poor conditions and higher when resources are abundant and the energetic cost of biofilm formation is outweighed by the benefits of growth at the oxygen-rich air-liquid interface. We expected that the effect of predation would be to increase the relative fitness of WS at all levels of resource supply, reflected by greater WS frequency at the end of diversification. Given the energetic cost of growth at this niche, and aforementioned evidence that selection for costly resistance strategies is relatively weak in resource-poor conditions (Leibold 1996; Bohannan & Lenski 2000), we predicted that the increase in WS frequency resulting from predation would be smallest in low-resource environments. Furthermore, because there are several distinct subclasses within both SM and WS, we expected changes in the frequencies of the principal morphs to have significant effects for total phenotypic diversity.

We first show how predation affects the frequencies of different morphotypes across a gradient of nutrient supply, and how this determines the level of phenotypic diversity at the end of adaptive diversification. We did this by allowing *P. fluorescens* to diversify in static liquid microcosms at different nutrient concentrations both with and without predation by *T. thermophila*. Secondly, we test whether changes in the frequencies of the principal morphotypes are due to the effect of predation on the productivity of the biofilm niche compared to the broth phase, or on competition between SM and WS populations. To do this we carried out growth assays in pure and mixed culture (different morphotypes grown in different vials or the same vial respectively) at three levels of

resource supply both with and without predation. Comparing frequencies in pure and mixed culture reveals the effect of competition, so that reduced WS frequency in mixed culture suggests a negative effect of competition between populations on the relative fitness of WS.

4.3. METHODS

Diversification experiment

A single clone of *P. fluorescens* SBW25 was used to found experimental lines at each combination of resource supply and predation. A gradient of resource supply was constructed by two-fold dilution of KB nutrients (glycerol and proteose peptone) in M9 salt solution eight times, ranging from 1× standard concentration (proteose peptone 20gl^{-1}; glycerol 12 gl^{-1}) down to 0.008× (proteose peptone 0.16gl^{-1}; glycerol 0.09 gl^{-1}). This follows the procedure described by Kassen *et al.* (2000) except that we excluded concentrations of 2× standard and higher because we found that *T. thermophila* does not survive at these levels, presumably because of some physiological stress imposed by the extremely high concentrations of proteose peptone and glycerol. Even so, this range of concentrations generates considerable variation in population density ($F_{7,16} = 15.54$, $p < 0.001$), ranging from ~1×10^8 CFUml $^{-1}$ at the lowest to ~3×10^9 CFUml $^{-1}$ at the highest concentration. The experiment was carried out in static 28ml glass vials containing 6ml of liquid media. Each culture was inoculated with ~10^3 cells of the ancestral clone and then incubated at 28°C for 6d. Three replicate cultures were maintained at each level of resource supply both with and without *T. thermophila*, of which approximately 10^3 cells were introduced at the start of the experiment.

At the end of the experiment all cultures were vortexed for 45s before dilution and spreading on KB agar plates. The frequencies of different morphotypes were then

estimated by counting the number of viable colonies. Visibly distinct morphotypes were propagated in KB and plated again to ensure that differences were heritable.

Frequency data was analysed using a generalised linear model in R version 2.2.1 with a quasi-binomial error structure to account for overdispersion, with predation, resource supply and their interaction as factors. Diversity was measured as the complement of Simpson's index, $1 - \lambda$, where $\lambda = \Sigma p_i^2$ and p_i is the frequency of each morphotype. This measures the probability that two randomly selected colonies are different. Diversity scores were normalised by Box-Cox transformation and population densities were log transformed. We also tested for variation in richness, the number of different morphotypes in a given culture, by analysis of variance with resource supply and predation as factors. Furthermore, we tested for an underlying association between richness and WS frequency using analysis of covariance, with richness as the response variable, predation as a factor and square-root transformed WS frequency as a covariate, with the interaction term testing for a difference in the slope of this relationship between predated and non-predated cultures.

Growth assays in pure and mixed cultures

We tested the effect of competition among morphotypes by comparing the frequencies of the two principal morphs (SM and WS) in pure and mixed culture at three different resource concentrations: 1×, 0.125× and 0.008× standard. In mixed cultures WS and SM were grown in the same vial, and in pure cultures they were grown in separate vials, so that a pair of pure culture vials may be considered a single pure culture growth assay. This experiment was carried out both with and without predation by *T. thermophila*; independent cultures were sampled every day for 4d. Microcosms were inoculated at low density with approximately 10^3 cells of either or both of the ancestral SM and a derived WS of the most common subclass from the end of the experiment. Three cultures at each combination of nutrient concentration, predation and competition were then incubated and destructively sampled as above each day for 4d. The frequencies of WS and SM at each time point were then estimated from colony counts as above.

To analyse the proportion of WS in each assay we fitted generalised linear models as above, with competition (pure or mixed culture), resource concentration, predation and day as explanatory variables in a fully factorial model. The maximal model was then reduced by sequentially removing non-significant interactions using *F*-tests (Crawley 2002). Given that destructively sampled microcosms represent independent data points, these data are not repeated measures in the strict sense. However, because samples were taken over successive days, we accounted for multisample sphericity in testing for day effects by adjusting degrees of freedom using a Greenhouse-Geisser estimate of epsilon (ε).

Low WS frequency in a given assay may be due either to reduced numbers of WS, or to increased numbers of SM. We discriminated between these alternatives by testing the effect of each factor on the log transformed population densities of SM and WS separately and at each level of resource supply. The proportion of explained variance for each factor was estimated as ω^2 using the following equation: $\omega^2 = (SS_{factor} - (p - 1)MS_{residual}) / (MS_{residual} + SS_{total})$, where p is the number of levels of a given factor. In some pure cultures (36/144) diversification was detected over the course of the assay. However, there was no significant reduction in the predictive power or qualitative conclusions of models when these cases were excluded and we found no points with high influence (maximum Cook's $D_i = 0.1$).

4.4. RESULTS

Frequency of resistant individuals varies with resource supply

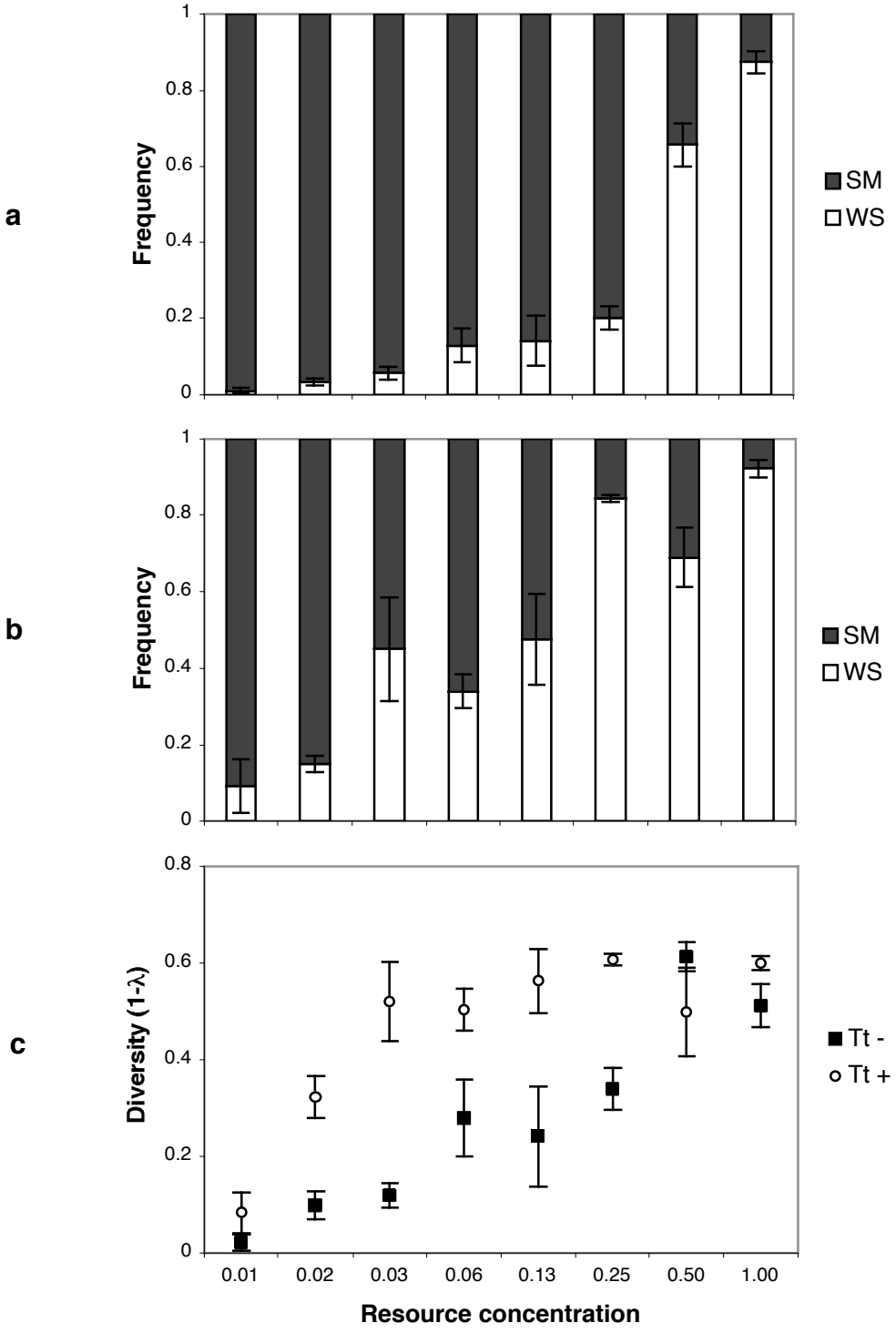
The proportion of cells represented by WS relative to SM was increased under predation ($F_{1,32}$ = 64.40, p < 0.001; figure 4.1a and 4.1b), but the effect varied with resource supply (predation × resource supply interaction: $F_{7,32}$ = 3.45, p < 0.01), being greatest at intermediate levels. Predation by *T. thermophila* also reduced the population density in each culture ($F_{1,32}$ = 111.79, p < 0.0001), decreasing the total number of cells by a factor of ~9.7 on average. While the proportion of the SM population removed by predation appears to be independent of resource supply (effect of predation × resource supply interaction on log(SM population density): $F_{7,32}$ = 1.04, p = 0.42), the proportional reduction in WS population density due to predation was greatest at higher concentrations (predation × resource supply interaction: $F_{7,32}$ = 3.16, p = 0.01), where WS is numerically dominant. In total we observed 4 different types of WS and 2 types of SM, as well as a third morphological class that occupies the bottom of the vial (fuzzy spreader: FS), although this was only found in 2/48 cultures and at very low frequency (< 0.01).

Diversity reflects changes in morphotype frequencies

Increased WS frequency under predation led to an increase in diversity ($F_{1,32}$ = 39.21, p < 0.0001; figure 4.1c). The strength of this effect varied with resource supply (predation × resource supply interaction: $F_{7,32}$ = 3.32, p < 0.01), peaking at intermediate levels where the increase in WS frequency was greatest. This is due to the fact that diversity tended to be greater within WS populations than SM (WS: mean = 0.436, s.d. = 0.151; SM: mean = 0.001, s.d. = 0.004). In turn, because 1 - λ is maximised when the frequencies of morphotypes both within and between the principal classes are approximately equal, diversity was greatest when WS attained a frequency of 0.65-0.75, not when WS and SM were equally abundant.

Diversity can also be interpreted in terms of the number of different morphotypes in each culture, which is analogous to species richness. Richness was not affected by predation overall ($F_{1,32} = 0.25$, $p = 0.62$), but did vary with resource supply ($F_{7,32} = 6.61$, $p < 0.0001$), tending to be higher in high-resource treatments. This probably reflects an underlying relationship between richness and the frequency of wrinkly spreaders, with the number of morphotypes detected being lower for cultures with a smaller proportion of WS (regression against square-root transformed WS frequency: $r^2_{47} = 0.30$, $p < 0.0001$). The slope of this relationship was unaffected by the presence of *T. thermophila* (predation × WS frequency interaction: $F_{1,44} = 1.18$, $p = 0.28$). Thus, the effect of predation on diversity largely reflects changes in the frequencies of different morphotypes, rather than changes in the number of morphotypes at the end of diversification.

Figure 4.1. (overleaf). Frequency of the principle morphotypes following diversification in static microcosms at different levels of resource supply both (a) without and (b) with predation by *T. thermophila*. Bars show means ± s.e. for three populations. Also shown are resulting patterns of diversity (c) in the absence (Tt-) and presence (Tt+) of *T. thermophila*. Points show means ±s.e.


a
b
c
Frequency
1
0.8
0.6
0.4
0.2
0
SM
WS
Diversity (1-λ)
Tt -
Tt +
0.01
0.02
0.03
0.06
0.13
0.25
0.50
1.00
Resource concentration

The interaction between competition and predation varies with resource supply

In pure culture assays, the frequency of WS relative to SM increases with resource supply, both in the presence and absence of predators (figure 4.2). In some treatments WS frequency is reduced by competition with SM in mixed cultures, but this effect depends upon both resource supply ($F_{2,105} = 13.93$, $p < 0.0001$) and predation ($F_{1,105} = 8.76$, $p < 0.005$). Specifically, in low-resource environments WS frequency is reduced by competition with SM both with and without predation by *T. thermophila* ($F_{1,33} = 32.32$, $p < 0.001$; figure 4.2) and there is no interaction between competition and predation ($F_{1,32} = 0.44$, $p = 0.51$). At intermediate resource supply, the effect of competition depends upon the presence of the predator (competition × predation interaction: $F_{1,31} = 5.44$, $p = 0.02$), and WS frequency is decreased by competition only in the absence of *T. thermophila* (no predation: $F_{1,20} = 5.72$, $p = 0.02$, figure 4.2a; predation: $F_{1,18} = 0.06$, $p = 0.81$, figure 4.2b). In high-resource environments, where WS is numerically dominant, there is no significant effect of competition ($F_{1,32} = 1.72$, $p = 0.19$) or predation ($F_{1,32} = 0.93$, $p = 0.34$) on WS frequency.

Is the effect of competition in mixed cultures due to changes in the abundance of WS or SM or both? Overall, competition leads to reduced WS population density ($F_{1,121} = 30.06$, $p < 0.0001$), whilst SM population density is unaffected ($F_{1,128} = 0.28$, $p = 0.60$). The reduction in WS density is greatest in low-resource environments (29% variance explained, compared to 10% and 5% at intermediate and high levels respectively). Having found that the effect of competition on WS frequency is nullified by predation at intermediate resource supply (figure 4.2), we also wanted to know if this was due to increased numbers of WS under predation, or to reduced numbers of SM. We find that the reduction in WS population density due to competition is independent of predation in these treatments (competition × predation interaction: $F_{1,28} = 0.82$, $p = 0.37$), but that SM population density is reduced under predation in mixed cultures ($F_{1,35} = 36.26$, $p < 0.0001$; figure 4.3e and 4.3k). Therefore competition generally reduces WS frequency because of reduced growth and survival in mixed cultures, but this effect is countered by

predation in some treatments, which leads to decreased growth of the competing SM population.

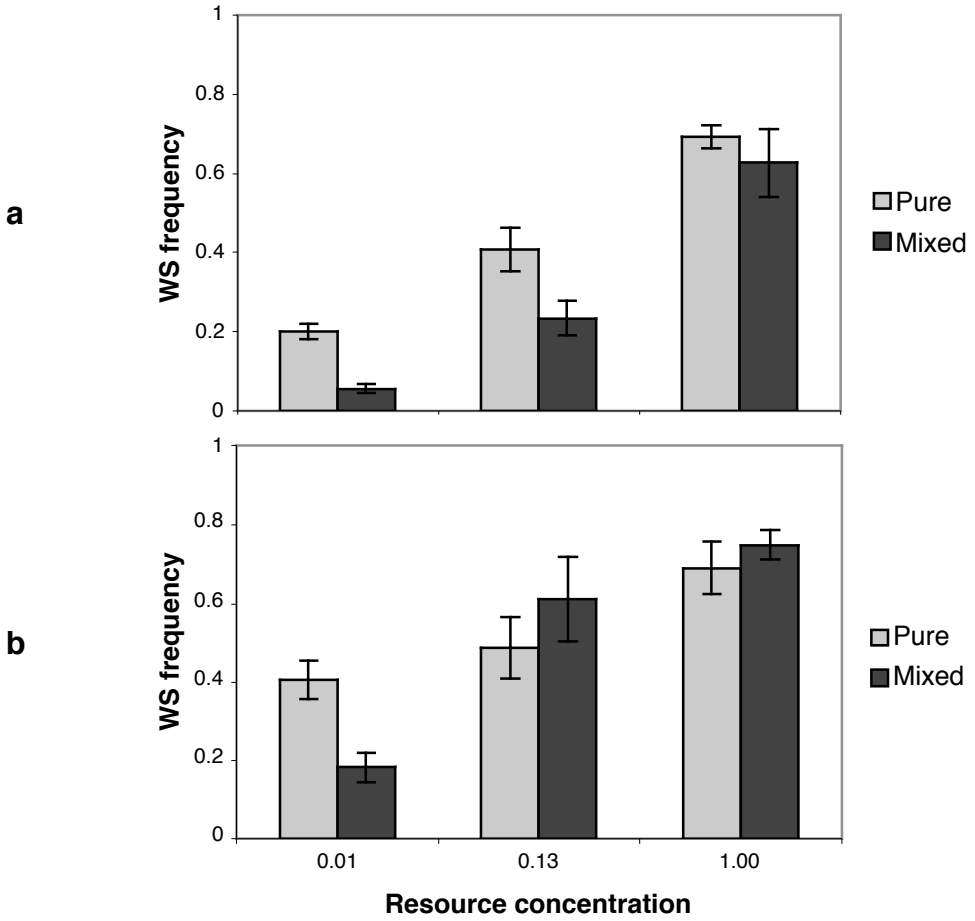


Figure 4.2. Proportion of cells represented by WS relative to SM in pure and mixed culture assays at three levels of resource supply both in the (a) absence and (b) presence of *T. thermophila*. Bars show means ± s.e. over 4 time points.

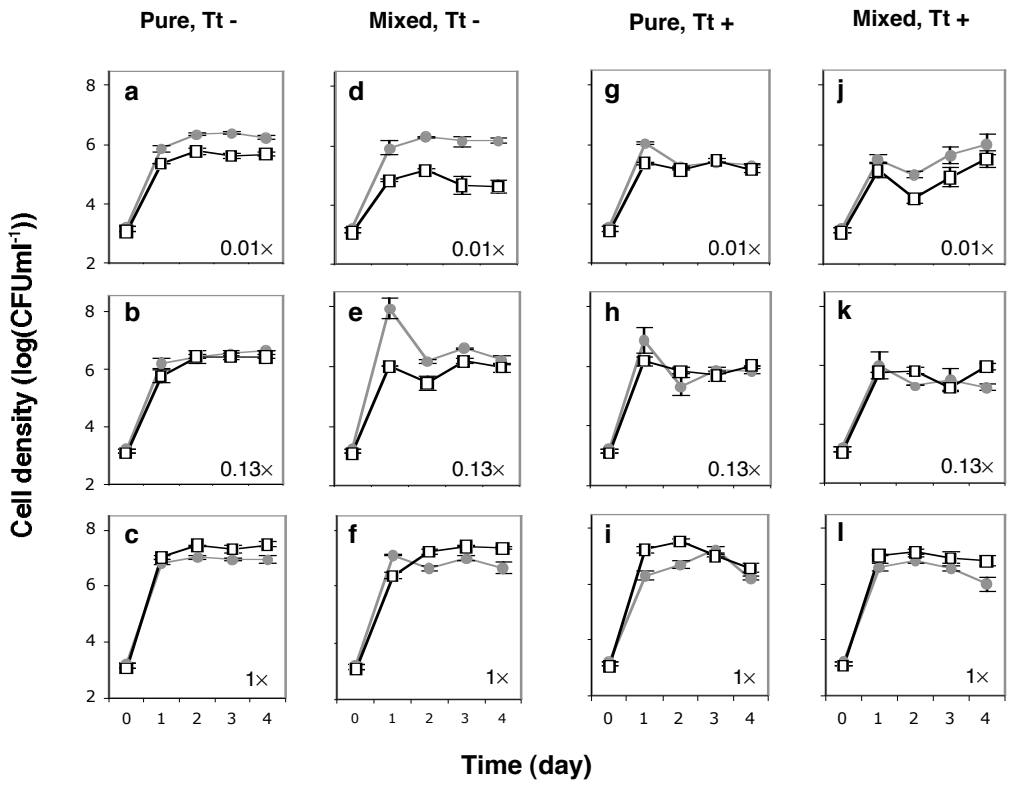


Figure 4.3. Population density of SM smooth (filled circles) and WS wrinkly spreader (open squares) morphotypes over 4d in pure and mixed culture at three different levels of resource supply. Points show means ±s.e.

4.5. DISCUSSION

The overall effect of predation was an increase in diversity due to increased frequency of the biofilm-forming WS phenotype emerging during adaptive diversification. Interestingly, the magnitude of this effect varies across a gradient of nutrient supply, being smallest at the two extremes and largest at intermediate levels. In these treatments a reduction of the competing SM population under predation is combined with sufficient resource supply to maintain a substantial biofilm, leading to increased WS frequency and therefore higher diversity. Thus, predation increased the range of concentrations over which high diversity was supported.

These observations are consistent with our knowledge of the evolutionary ecology of *P. fluorescens* in laboratory microcosms, and also with previous work showing that selection for resource exploitation is strongest when resources are limited (Leibold 1996; Bohannan & Lenski, 2000). In resource-poor conditions, nutrient limitation prevents the formation of the energetically costly WS biofilm, and the pelagic SM population is competitively dominant both with and without predation. As nutrient supply increases, the benefits of growth at the air-liquid interface outweigh the energetic costs of biofilm formation and WS emerges through resource competition in the absence of predation. In the presence of predators, WS has an extra advantage because growth in the biofilm also confers predator-resistance. The competing SM population is more susceptible to predation, as evidenced by the reduction in SM density under predation at intermediate nutrient supply (compare figure 4.3b and e with h and k).

At the highest levels of resource supply, WS are numerically dominant even in the absence of predators, and removal of a proportion of the broth-living SM population in the predator treatments leads to a relatively small change in WS frequency. This effect is compounded by the fact that predators also remove a greater proportion of WS cells when they are more abundant. This is consistent with the previous finding that the advantage of predator resistance in the biofilm is reduced when WS are in the majority

(Meyer & Kassen 2007). Thus, contrary to our expectation that selection for predator-resistance would be more important at high resource concentrations, we find that it has a relatively small effect because the same phenotypes that are resistant to predators are also preponderant in their absence.

Diversity is often seen to display a unimodal relationship with nutrient supply (Tilman 1982; Rosenzweig 1995). This is also true for *P. fluorescens*, as diversity has been shown to drop at concentrations above the highest level used here (Kassen *et al*. 2000). Thus, our results do not contradict the notion of a 'paradox of enrichment' (Rosenzweig 1971) for this system. However, we do find that the range of concentrations that support high diversity is extended under predation, and therefore suggest that the characteristic 'hump-shaped' relationship between diversity and resource supply may be broadened by predation.

A recent study by Benmayor *et al.* (2008) shows that the presence of parasitic bacteriophage in *P. fluorescens* cultures can lead to an analogous increase in diversity, because of selection for the phage-resistant 'fuzzy spreader' FS phenotype that occupies the bottom of the vial. However, Benmayor *et al*. (2008) found no significant variation in FS frequency with either resource concentration or the frequency of disturbance. These apparently contrasting outcomes may reflect ecological differences between growth in the biofilm and at the bottom of the vial. The productivity of the biofilm niche relative to the broth phase varies with resource supply as described above. However, the relative productivity of growth at the bottom of the vial is probably limited by the lack of oxygen at this niche, and so is effectively independent of changes in nutrient concentration. This would mean that the selective benefit of phage resistance for FS is fixed across a gradient of nutrient supply. It is interesting that both parasites (Benmayor *et al*. 2008) and predators (our study) can generate similar patterns of diversity, although the underlying mechanisms responsible may vary.

In our experiment predation modulated the frequencies of phenotypes that also occur during diversification under resource competition alone. However, predators can generate diversification of *P. fluorescens* to distinct morphotypes independently of resource competition. This has been demonstrated by diversification under predation in minimal growth medium that does not support diversity in the absence of *T. thermophila* (Meyer & Kassen 2007). Furthermore, although we looked at the effect of predation in spatially heterogeneous microcosms, spatial structure is not necessary for predation to promote diversification (Levin *et al*. 1977), as demonstrated by recent experimental work with *P. fluorescens* (Gallet *et al*. 2007). All that is required is that there is a trade-off between resistance and competitive fitness or growth rate (Levin *et al*. 1977; Chase *et al*. 2002). Our results support the notion that the effect of such a trade-off varies with resource supply (Holt *et al.* 1994; Bohannan & Lenski 2000). Since the diversifying selection resulting from predation depends upon the relative fitness of resistant and susceptible phenotypes, a single phenotype may dominate at the extremes. In general, diversification may be restricted if there is strong selection for a single resistance strategy (Buckling & Rainey 2002) or for resource exploitation, such as when environmental nutrient supply is extremely low.

In this experiment and several others with *P. fluorescens* (e.g. Rainey & Travisano 1998; Buckling & Rainey 2002; Kassen *et al*. 2004) diversity is measured using the frequencies of different morphotypes. If there were no variation within morphotypic classes then overall diversity would be maximised when SM and WS are equally abundant. However, we found greater diversity within WS populations than SM, and therefore diversity was actually greatest when WS was numerically dominant. High morphological diversity within WS populations is probably due to the differences between growth in a biofilm and in the liquid phase. Biofilms are structurally complex (Spiers & Rainey 2005) and are generally more diverse and dynamic than pelagic communities (Sutherland 2001; Boles *et al*. 2004). Thus, although diversity was greatest in high-resource environments, the ratio of WS to SM was most even at intermediate resource concentrations under predation. This agrees with the notion that coexistence of

resistant and sensitive phenotypes is most likely at intermediate levels of nutrient supply, because one type is limited by predation and the other by competition for resources (Abrams 1993; Holt *et al*. 1994; Leibold 1996).

Recent experimental work has highlighted the importance of predators in evolution (Rundle *et al*. 2003; Meyer *et al*. 2006; Nosil & Crespi 2006). Our results support the idea that predation can promote diversification due to the coexistence of sensitive and resistant prey, both in general (Holt *et al*. 1994; Chase *et al*. 2002) and for this species in particular (Meyer & Kassen 2007; Gallet *et al*. 2007). More importantly, the role of predators during adaptive diversification appears to vary with resource availability.

5. Bottlenecking, Biochemistry, and the Evolution of Antibiotic-Resistant Bacteria

5.1. SUMMARY

When antibiotics are withdrawn, bacteria can evolve to reduce the cost of resistance by reversion mutations that restore susceptibility, or by compensatory mutations at other sites. However, if the population undergoes bottlenecking we expect rare beneficial mutations to be 'washed out' before they can spread, potentially including both compensatory and reversion mutations. We evolved four rifampicin-resistant *Pseudomonas aeruginosa* genotypes under different bottlenecking regimes. We found that the response to selection varied among lines carrying different resistance mutations, and that compensatory adaptation can be constrained by severe bottlenecking. We detected revertant mutants in some populations, but they were at low frequency and were no fitter than other mutants from the same populations. These results show that the evolutionary consequences of drug withdrawal can be altered by bottlenecking, but also vary among genotypes with different resistance mutations on the same gene.

5.2. INTRODUCTION

Most mutations that affect an organism's phenotype are deleterious (Charlesworth & Charlesworth 1998). Genotypes that carry deleterious mutations may go extinct because of their reduced fitness, or persist at some frequency in the population. Alternatively they may evolve to recover the fitness cost associated with the deleterious mutation. This can occur by reversion to the wild type by back mutation at the same site, or by compensatory mutations at other sites that recover the cost of the first mutation (Cohan *et al*. 1994; Maisnier-Patin & Andersson 2004). Adaptation to fitness costs is particularly well studied for antibiotic-resistant bacteria. This is because mutations that confer resistance in the presence of an antibiotic are often costly in its absence, but also because drug-resistant bacteria are a major public health problem (Levy & Marshall 2004; Bergstrom & Feldgarden 2008). Understanding how bacteria adapt to the cost of resistance therefore provides insight on the persistence of resistant pathogens when antibiotic treatment is stopped.

The evolutionary trajectory of resistant bacteria in the absence of antibiotics will depend on the frequency and fitness effects of reversion mutations and compensatory mutations. For example, drug sensitivity should be restored if reversion mutations are common and the wild type is the fittest genotype. Alternatively, if there are several potential compensatory mutations for a given deleterious mutation, then the cost is more likely to be recovered by compensatory adaptation without reverting to susceptibility (Schrag *et al*. 1997; Reynolds 2000; Maisnier-Patin & Andersson 2004). This scenario is analogous to a rugged fitness landscape, with different peaks representing different genotypes with high fitness (Wright 1932). Here the outcome of evolution for a given genotype depends on its initial position on the landscape as well as which mutations arise and in what order (Wright 1932; Colegrave & Buckling 2005). Unfortunately this also means that compensatory adaptation can create stable genotypes that have high fitness and are resistant to antibiotics (Maisnier-Patin & Andersson 2004).

The relative contributions of reversion and compensatory mutations during adaptation to the cost of a deleterious mutation will depend on population dynamics. In the case of pathogenic bacteria, periodic bottlenecks in population size are inherent to a lifestyle involving transmission between hosts, and this can alter the genetic variation available for selection (Bergstrom *et al.* 1999; Levin *et al.* 2000; Elena *et al.* 2001; Wahl *et al.* 2002; Li & Roossinck 2004). Specifically, rare beneficial mutations will be lost under tight bottlenecking. If reversion mutations are less common than compensatory mutations, then they are unlikely to spread in these conditions (Levin *et al.* 2000) and the population will stay resistant. Furthermore, if there is a range of potential compensatory mutations that vary in their fitness effects, as shown for an RNA virus by Burch and Chao (1999), and most mutations have a relatively small effect, then any selection response due to compensatory adaptation may be relatively small under tight bottlenecking.

The role of population biology processes including bottlenecking may also depend on the molecular basis of resistance. There are often several potential resistance mutations for the same drug, each placing specific biochemical constraints on evolution in the absence of antibiotics. Firstly, the selective benefit of potential compensatory and reversion mutations will depend on the magnitude of any cost of resistance, which has been shown to vary among different resistance mutations (Billington *et al.* 1999; Reynolds 2000; Mariam *et al.* 2004; Gagneaux *et al.* 2006; Kassen & Bataillon 2006). Secondly, the frequency of compensatory and reversion mutations may vary among resistant genotypes. Access to beneficial mutations has been shown to vary among genotypes with similar fitness and to constrain the potential for adaptation in RNA viruses (Burch & Chao 2000) and in bacteria carrying deleterious mutations (Moore *et al.* 2000). It follows that bottlenecking should have the greatest effect in genetic backgrounds where beneficial mutations that recover the cost of resistance are rare.

In this chapter we examine whether bottlenecking influences the relative importance of reversion and compensatory mutations, and whether the effect is the same for genotypes

carrying different resistance mutations. To do this we used experimental evolution of rifampicin-resistant (Rif^R) *Pseudomonas aeruginosa* in the absence of antibiotics under different bottlenecking regimes. Rifampicin works by binding to a conserved domain of the β-subunit of RNA polymerase (RNAP), thereby blocking elongation of the RNA transcript (Severinov *et al*. 1993; Campbell *et al*. 2001). Resistance can result from any of several possible mutations on the *rpoB* gene that change the structure of RNAP and prevent binding (Telenti *et al*. 1993; Pozzi *et al*. 1999; Campbell *et al*. 2001; Trinh *et al*. 2006). Rif^R mutations often incur a fitness cost in terms of reduced growth rate, and the magnitude of the cost varies among different mutations (MacLean & Buckling 2009).

We maintained four different Rif^R genotypes for approximately 200 generations by serial transfer. Each genotype was evolved at each of four bottleneck sizes, which we manipulated by changing the dilution factor between transfers. We expected that the spread of revertant genotypes would be less likely under tight bottlenecking, and that any change in fitness due to other beneficial mutations would be relatively small in these treatments. At the end of the experiment we tested whether evolved populations had recovered the cost of resistance, and whether they remained Rif^R or reverted to susceptibility (Rif^S).

5.3. METHODS

Bacterial strains

We used four defined Rif^R genotypes that were isolated at random from a fluctuation test with the Rif^S wild type (*P. aeruginosa* PA01) on agar plates containing 62.11μg/mL rifampicin (MacLean & Buckling 2009). Each Rif^R mutant carries a single mutation on the *rpoB* gene, and each mutation is associated with a different amino acid change in RNAP that confers resistance (MacLean & Buckling 2009). Two mutations were at residues that come into direct contact with rifampicin (H531R and H531Y), one was at a residue that interacts indirectly with rifampicin (S517L), and one was an insertion

mutation (+P518) that may affect residues involved in both direct and indirect Rif-RNAP interactions.

Selection experiment

64 selection lines were grown at 37°C in 6ml of KB Medium in 28ml glass universals with continuous shaking at 180rpm. Upon transfer an aliquot was transferred from each stationary phase culture to fresh media. We manipulated bottleneck size by changing the dilution factor (*d*), which meant transferring a different volume in each treatment. We used the following dilution factors: 0.000001, 0.0001, 0.01 and 0.1, so that the number of cells transferred ranged from ~15 000 at the smallest value of *d* to ~1500 million at the largest. Four replicate selection lines of each of the four Rif^R genotypes were maintained at each of the four bottleneck sizes for approximately 200 generations. We also wanted to see if changes in fitness during the experiment represented compensatory adaptation specific to the defined resistance genotypes, or general adaptation of *P. aeruginosa* to our experimental conditions. To this end we ran four selection lines with the wild type at $d = 0.01$. All selection lines were frozen in 50% v:v glycerol at -80°C every ~50 generations.

Changing the dilution factor (*d*) also changes the number of doublings between transfers (*n*). This potentially confounds our manipulation of bottleneck size with the number of generations each line was selected for. To account for this we ran each selection line for $200/n$ transfers, calculating *n* as follows. Assuming growth by binary fission, the number of cells increases by a factor of 2^n for every *n* doublings. During serial transfer, the proportional increase in the number of cells (2^n) between transfers equals $1/d$ (e.g. $d = 0.01$ amounts to a 100-fold increase per transfer). If $2^n = 1/d$, then $n = \ln(1/d) / \ln(2)$.

Resistance assays

We used selective plating to estimate the proportion of Rif^R and Rif^S genotypes in each population at the end of the experiment. Populations were reconditioned by overnight growth in KB, then diluted in M9 salt solution and plated onto permissive KB agar. At

least 40 colonies per population (mean ±s.d. = 103.6 ±59.7) were then transferred to KB agar with 62.11µg/mL rifampicin and the proportion of resistant colonies was recorded.

Fitness assays

Fitness was calculated as growth rate relative to the wild type (Björkman *et al*. 2000). We estimated growth rate as the slope of $\log(OD_{600})$ over time during exponential growth in KB. For each assay the population was reconditioned from frozen by overnight growth in KB, then diluted in M9 and 20µl inoculated into 180µl of KB in a randomly assigned well of a 96-well plate. We then followed changes in OD_{600} using a SpectraMax M2 microplate reader (Molecular Devices Corporation). Following Novak *et al*. (2006), we corrected measurements by subtracting the scores for blank wells that were measured individually for each well and discarding scores below the detection limit of the spectrophotometer (OD = 0.002). Growth rates were estimated from Baranyi models (Baranyi & Roberts 1994) fitted to each growth curve using the nlstools package (Baty & Delignette-Muller 2007) in R v2.2.1. Four cultures of each unevolved Rif^R mutant and wild type PA01 were tested alongside evolved populations from the end of the experiment. Each population was tested in triplicate in each of two blocks.

To test the relative fitness effects of reversion mutations and other beneficial mutations that occurred during the selection experiment, we isolated five Rif^R and five Rif^S genotypes at random from each of three polymorphic H531R populations and assayed their growth rates as above.

Statistical analysis

We analysed changes in fitness by calculating the selection response for each evolved population at the end of the experiment (fitness minus the fitness of its unevolved Rif^R ancestor). We analysed selection responses using a linear mixed model (lme function in R v2.2.1) with Rif^R genotype and dilution factor as fixed effects and block as a random effect, using the varIdent function (nlme library) to model heterogeneous variances among groups. We were especially interested in any effect of bottlenecking on the

selection response, and whether that effect varied among different Rif^R genotypes (dilution factor × Rif^R genotype interaction). We tested for a difference in fitness between resistant and susceptible genotypes in each of three polymorphic populations by ANOVA with population and rifampicin resistance as factors.

5.4. RESULTS

Loss of resistance

Most selection lines were still resistant to rifampicin after ~200 generations of experimental evolution, although we found susceptible genotypes at low frequency in four H531R populations and one H531Y population (table 5.1).

Change in fitness: effects of Rif^R genotype and bottlenecking

The change in fitness during our experiment varied among lines with different Rif^R mutations ($F_{3,48} = 487.81$, $p < 0.0001$; figure 5.1). Specifically, all selection lines with mutations at H531R and H531Y increased in fitness, reaching growth rates equal or close to that of the wild type (table 5.1; figure 5.1). Most of the +P518 lines showed no improvement, although 3/16 reached fitness equal to or greater than 1.0 (table 5.1). Lines with S517L mutations showed little evidence of adaptation in any treatment. We also evolved PA01 control lines at $d = 0.01$, but found no significant difference in growth rate compared to the wild type at the end of the experiment ($F_{1,7} = 2.27$, $p = 0.18$), suggesting that adaptation was due to compensatory mutations rather than general adaptation to the experimental conditions.

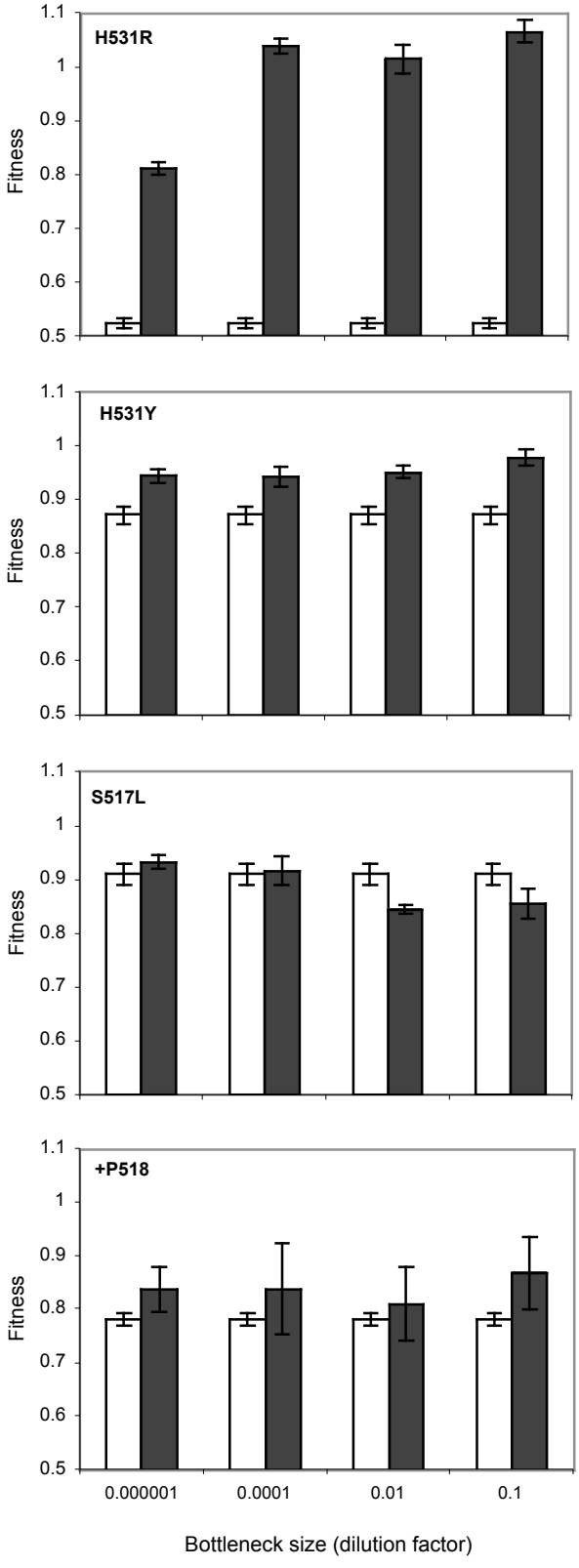
The effect of bottlenecking on the response to selection also varied among different Rif^R genotypes (dilution factor × genotype interaction: $F_{9, 48} = 11.66$, $p < 0.0001$). For H531R lines, where we found the clearest evidence of adaptation, the response to selection was lower under severe bottlenecking ($F_{3,12} = 28.46$, $p < 0.0001$; figure 5.1). In contrast,

bottlenecking had no significant effect on adaptation of H531Y lines ($F_{3,12} = 1.36$, $p = 0.3$). Thus, the capacity to recover the cost of resistance varied among Rif^R genotypes, and can at least sometimes be modified by bottlenecking.

Fitness effects of reversion mutations and other beneficial mutations

We found no difference in fitness between resistant and susceptible clones within each of three polymorphic H531R populations from the end of the experiment ($F_{1,24} = 0.051$, $p = 0.82$; figure 5.2). The average score for the derived clones in each selection line was close to that of the wild type (figure 5.2), suggesting that they carried either reversion mutations or other mutations that had a beneficial effect on fitness.

Figure 5.1. (overleaf). Changes in fitness for selection lines carrying different Rif^R mutations under different bottlenecking regimes. Open and filled bars show fitness before and after experimental evolution for approximately 200 generations. Each panel shows a single Rif^R genotype (from top: H531R, H531Y, +P518, S517L), and each bar shows the mean ±s.e. for four selection lines at a given bottleneck size.


H531R
H531Y
S517L
+P518
Fitness
1.1
1
0.9
0.8
0.7
0.6
0.5
0.000001
0.0001
0.01
0.1
Bottleneck size (dilution factor)

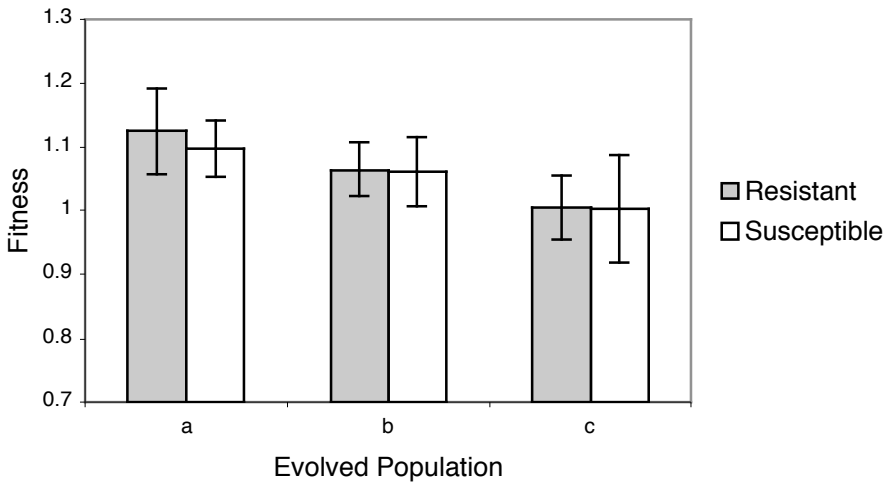


Figure 5.2. Fitness of resistant and susceptible genotypes isolated from polymorphic H531R populations at the end of the experiment. Bars show means ±s.e. for five genotypes from each of three populations (a, b, c as denoted in table 5.1).

Table 5.1. (overleaf). Rifampicin resistance and fitness of all selection lines at the end of the experiment. There are four replicate lines at each combination of Rif^R genotype and bottleneck size (dilution factor, *d*). Fitness scores for the unevolved Rif^R genotypes (baselines) are also shown. Resistance is shown as the proportion of Rif^R genotypes. The three populations from which we isolated Rif^R and Rif^S genotypes are denoted by superscripts $^{a, b, c}$.

Rif^R mutation	Dilution factor (*d*)	Selection line	Relative fitness	Resistance
H531R	-	baseline	0.52	1
"	0.000001	1	0.83	1
"	"	2	0.79	1
"	"	3	0.83	1
"	"	4	0.79	1
"	0.0001	1	1.02	1
"	"	2	1.02	1
"	"	3 [a]	1.05	0.93
"	"	4 [b]	1.07	0.80
"	0.01	1	1.08	1
"	"	2	0.97	1
"	"	3	1.03	0.85
"	"	4 [c]	0.98	0.62
"	0.1	1	1.04	1
"	"	2	1.13	1
"	"	3	1.03	1
"	"	4	1.07	1
H531Y	-	baseline	0.87	1
"	0.000001	1	0.93	1
"	"	2	0.91	1
"	"	3	0.96	1
"	"	4	0.97	1
"	0.0001	1	0.93	1
"	"	2	0.97	1
"	"	3	0.89	1
"	"	4	0.97	1
"	0.01	1	0.94	1
"	"	2	0.98	0.94
"	"	3	0.95	1
"	"	4	0.93	1
"	0.1	1	0.94	1
"	"	2	1.00	1
"	"	3	1.01	1
"	"	4	0.96	1
+P518	-	baseline	0.78	1
"	0.000001	1	0.77	1
"	"	2	0.96	1
"	"	3	0.82	1
"	"	4	0.81	1
"	0.0001	1	0.77	1
"	"	2	0.65	1
"	"	3	0.86	1
"	"	4	1.06	1
"	0.01	1	0.80	1
"	"	2	0.70	1
"	"	3	0.73	1
"	"	4	1.01	1
"	0.1	1	0.75	1
"	"	2	0.78	1
"	"	3	1.05	1
"	"	4	0.89	1
S517L	-	baseline	0.91	1
"	0.000001	1	0.93	1
"	"	2	0.96	1
"	"	3	0.90	1
"	"	4	0.93	1
"	0.0001	1	0.88	1
"	"	2	0.98	1
"	"	3	0.93	1
"	"	4	0.87	1
"	0.01	1	0.86	1
"	"	2	0.86	1
"	"	3	0.83	1
"	"	4	0.83	1
"	0.1	1	0.82	1
"	"	2	0.84	1
"	"	3	0.82	1
"	"	4	0.94	1
PA01	-	wild type	1.00	0

5.5. DISCUSSION

Most populations were still resistant to rifampicin at the end of the experiment. In some cases we found sensitive genotypes at low frequency, but they were not fitter than genotypes with beneficial mutations at other sites. Several populations showed pronounced adaptation, in some cases completely recovering the initial cost of rifampicin resistance. The response to selection and the effect of bottlenecking varied among genotypes carrying different resistance mutations. These results suggest that bottlenecking can be an important factor in the evolution of resistant bacteria when selecting drugs are withdrawn, but that evolution is also constrained by the biochemical constraints particular to each resistance mutation.

Were the observed changes in fitness due to general adaptation of *P. aeruginosa* to our experimental conditions, or because of compensatory adaptation specific to the defined resistance genotypes? Previous authors have identified compensatory mutations whose beneficial effects are specific to the presence of particular deleterious mutations (Schrag & Perrot 1996; Burch & Chao 1999). In agreement with this, we found no increase in fitness for the wild type selection lines, suggesting that the change in fitness in other lines was due to compensatory mutations with epistatic effects specific to costly Rif^R mutations. Furthermore, variation among Rif^R genotypes suggests that compensatory mutations are specific to particular resistance mutations on the same gene. We note that the ideal test for the specificity of compensatory mutations would be to check for a drop in fitness when drug susceptibility is restored by insertion of the wild type allele.

The fact that most bacteria remained resistant agrees with several studies showing that compensatory adaptation is often more likely than reversion to susceptibility (Cohan *et al*. 1994; Schrag *et al*. 1997; Björkman *et al*. 1998; Moore *et al*. 2000; Reynolds 2000; Nagaev *et al*. 2001; Maisnier-Patin & Andersson 2004; Kugelberg *et al*. 2005; Nilsson *et al*. 2006; Schoustra *et al*. 2006; Paulander *et al*. 2007). Presumably compensatory mutations are more frequent than reversion mutations, and we also found that they could

confer an equal fitness benefit. Furthermore, genotypes with compensatory mutations may be even less likely to revert than their uncompensated ancestors, because compensatory mutations can create a genetic background where the reversion mutation is no longer advantageous, or even has a negative effect on fitness (Schrag *et al*. 1997).

If reversion mutations were not lost during bottlenecking, or prevented from spreading by the presence of other beneficial mutations, we would expect them to have reached a much higher frequency than they did. To demonstrate this we calculated the expected change in frequency per generation for a genotype with a reversion mutation, Δp_1, as $sp_1p_2 / \bar{w}$ (Crow & Kimura 1970), where s is the selection coefficient (difference in fitness between the revertant genotype and resistant mutant), p_1 and p_2 are the proportions of the two genotypes, and $\bar{w}$ is mean fitness. We assume that reversion to the wild type restores fitness to 1.0, and take population size as the harmonic mean of N over the transfer cycle. We estimate that a single reversion mutation in an H531R population would reach fixation within ~80-100 generations (depending on bottleneck size) unless it was lost to drift or faced competition from other beneficial mutations. Even for S517L populations, where the selection coefficient is smallest, a revertant mutant should reach a frequency of between 0.06 and 0.99 within 200 generations. Thus, the lack of reversion is not simply due to the slow spread of beneficial mutations.

There are at least two possible explanations for variation in the potential for adaptation among Rif^R genotypes. First, the frequency of beneficial mutations varies among genotypes, and in some cases they are rare enough to be lost before they can spread in any bottlenecking treatment. Second, selection is weaker when the cost of resistance is small, so that beneficial mutations spread more slowly in some genetic backgrounds. Our results lend some support to both of these explanations. The complete fitness recovery for costly H531R mutations is concurrent with Moore *et al*.'s (2000) finding that compensatory adaptation proceeds more rapidly when deleterious mutations have a larger effect. However, this does not explain all of our results because we found adaptation more frequently in H531Y lines than +P518, which had lower fitness

initially. Indeed, Moore *et al*. (2000) also found variation in the potential for compensatory adaptation among selection lines carrying different deleterious mutations with similar fitness effects. This suggests that the supply of compensatory mutations varies among genotypes independently of the fitness cost, even though their selective benefit is greatest when the cost is larger.

The effect of bottlenecking on the response to selection also varied among Rif^R genotypes. Bottlenecking had the effect we predicted in H531R lines, where the cost was largest, presumably because it takes rare compensatory mutations of big effect to recover the fitness cost completely, and under tight bottlenecking the population only has access to the most common mutations rather than the best ones. In contrast, there was no effect of bottlenecking in H531Y lines. The most parsimonious explanation for this is that compensatory mutations are more frequent for this genotype, so that the best ones are accessible even under tight bottlenecking. This also agrees with our expectation that access to beneficial mutations would vary among genotypes (Burch & Chao 2000; Moore *et al*. 2000). An alternative explanation is that a greater proportion of the potential compensatory mutations in H531Y lead to full fitness recovery. However, this seems unlikely given that the shape of the distribution of effect sizes for beneficial mutations is expected to be robust across fitness levels and genetic backgrounds (Orr 2003; Silander *et al*. 2007). Instead, the frequency and fitness effect of a given beneficial mutation vary among resistant genotypes, leading to different capacities for compensatory adaptation and variation in the effects of bottlenecking.

These results support the view that fitness landscapes can be rugged for microbial populations that have been displaced from a fitness peak by a deleterious mutation (Burch & Chao 1999). Instead of recovering fitness by ascending the original peak, they can reach high fitness at another peak by mutations that do not restore the original phenotype. In a rugged fitness landscape the evolutionary path of a given genotype will depend strongly on its initial position on the landscape and which beneficial mutations

are accessible (Colegrave & Buckling 2005). This is evident in our experiment as variation among genotypes with different deleterious mutations.

The finding that bacteria do not simply revert to the wild type when antibiotics are withdrawn offers little encouragement regarding the eradication of resistant bacteria. Furthermore, our resistant mutants were isolated at random in the laboratory, whereas in nature selection will probably act to minimise the cost of resistance (Spratt 1996), potentially making selection for reversion even less likely in pathogenic populations than in our experiment. These and other results suggest that prudent use of antibiotics is important to avoid creating widespread populations of resistant bacteria that are unlikely to revert to susceptibility (Billington *et al*. 1999; Levin 2001; De Gelder *et al*. 2004) without infection controls and the immigration of susceptible bacteria.

6. Evolution of Bacterial Growth Parameters in Complex Environments – Does Diversity Matter?

6.1. SUMMARY

When presented with a range of novel substrates in the laboratory, bacteria can evolve to exploit some or all of them more effectively. Here we show that adaptation of *Pseudomonas fluorescens* to novel substrates in batch culture environments involves increasing both the rate and efficiency of growth relative to the wild type, as well as reducing the duration of the lag phase. The same qualitative response was seen at a range of different resource supply rates, despite variation in the level of phenotypic diversity supported by each environment. These results show that adaptation to novel substrates affects different components of the growth cycle simultaneously, and that the effect is the same in populations adapted to different combinations of resources.

6.2. INTRODUCTION

Bacteria in nature have largely evolved in complex environments containing a range of different resources that are in limited supply (Harder & Dijkhuizen 1982; Münster 1993; Lendenmann *et al*. 1996). To simulate these conditions in the laboratory we used batch culture environments with a defined range of carbon substrates simultaneously available for growth. We showed in Chapter 2 that *Pseudomonas fluorescens* can evolve to exploit the available substrates more effectively in these conditions, and that the metabolic phenotypes that evolve depend on the total substrate concentration. Here we test the effect of adaptation to different combinations of substrates at different resource supply rates on three key growth parameters (lag time, growth rate and total cell yield) that reflect changes in population size at different stages of the growth cycle.

When bacteria adapt to a novel environment, do they evolve to grow faster or to grow more efficiently and produce more cells per unit of substrate? Because of a fundamental trade-off between the rate and yield of ATP-producing pathways (Pfeiffer *et al.* 2001; Helling 2002), we might expect adaptation to be constrained to maximising either growth rate or total cell yield, but not both. However, when bacteria adapt to a new substrate in the laboratory, they tend to improve both rate and yield simultaneously (Luckinbill *et al*. 1978; Novak *et al*. 2006), rather than evolving to a particular point along a rate-yield trade-off. This is not so surprising given that some of the adaptations associated with resource exploitation, such as improved substrate uptake through the cell wall, are not under the same constraints as ATP-producing pathways and may therefore have a positive effect on both rate and yield. Adaptation to the novel substrates in our experiment may therefore have occurred by mutations that had a positive effect on different growth parameters simultaneously. If this were true for all four of the substrates that we used, then adaptation to any combination of substrates would lead to increased growth rate and yield relative to the wild type.

A different outcome could be observed in a complex environment if adaptation to different substrates, or different strategies for resource exploitation, have independent effects on the different growth parameters. For example, selection for rapid growth on the most productive substrate during the early exponential phase might lead to an increase in growth rate but not yield, whereas efficient exploitation of less favourable substrates at the onset of stationary phase may lead to increased yield but not growth rate. In this case, a diverse population with a range of genotypes using different strategies would show increases in both rate and yield relative to the wild type. In contrast, if diversity is low and a single strategy dominates, such as rapid growth during exponential phase using only the most productive substrate, the population may show a relatively strong selection response for a single growth parameter. We therefore hypothesized that variation in phenotypic diversity at the population level could be associated with variation of the response to selection for different growth parameters.

Selection for rapid or efficient growth on a given substrate might also vary with resource supply because of the constraints imposed by nutrient stress at low concentrations. Trade-offs between pairs of traits can be more important under resource scarcity (Brockhurst *et al*. 2008; Chapter 3). Thus, it may be impossible to maximise both the rate and yield of growth on a given substrate when it is in short supply, while an abundance of resources at higher concentrations permits fast growth without compromising total cell yield. This mechanism is not supported by evidence from *Escherichia coli* showing concurrent increases in rate and yield during adaptation to both resource scarcity and abundance in environments with a single substrate (Luckinbill 1984; Velicer & Lenski 1999). However, the same will not necessarily be true in complex environments where resource use strategies vary with total substrate concentration (Harder & Dijkhuizen 1982; Lendenmann *et al*. 1996) and different sets of mutations may be required for adaptation to high and low resource supply.

We predicted that the three mechanisms described above would lead to different outcomes for the evolution of growth parameters. First, if nutrient stress constrains

adaptation at low resource supply, we would see a positive selection response for yield but not growth rate in these conditions, and for both rate and yield at high resource supply. Second, if the selection response for different growth parameters depends on the level of phenotypic diversity, this would lead to a relatively strong response across growth parameters at intermediate resource concentrations that support high diversity. Third, if neither of the first two mechanisms is important, and adaptation to any concentration of a given substrate has the same effect on the different growth parameters, we would see the same qualitative response to selection across resource supply rates.

We used populations of *P. fluorescens* that had evolved in complex environments containing different total concentrations of four novel carbon substrates. We first measured variation in the growth kinetics of the wild type (SBW25) with resource concentration, and then tested for changes in growth rate, total cell yield and lag time in each environment after long-term evolution. We included lag time in our analysis because it may covary with the other growth parameters, and has been shown to decrease during adaptation to batch culture environments (Vasi *et al*. 1994).

6.3. METHODS

Bacterial strains and selection experiment

We used a subset of the frozen samples from the selection experiment described in Chapter 2. Briefly, populations of *P. fluorescens* were maintained for 50 transfers, with 100-fold dilution at each transfer, in liquid growth media. We ran six different treatments, each with a different total substrate concentration. The wild type and evolved populations were stored at –80°C in 50% v:v glycerol.

Assay procedure

At the end of the experiment, we measured lag time, growth rate and total cell yield for three populations from each of the six selection environments. All evolved populations were assayed in the same environment (i.e. resource concentration) that they were selected in, and three replicate cultures of the ancestral clone were also assayed at each concentration. Prior to assay, populations were reconditioned from frozen by overnight growth in microplates in liquid KB, then transferred to dilute M9KB (glycerol 1 gl^{-1}, NH_4Cl 0.1 gl^{-1}, Na_2HPO_4 0.6 gl^{-1}, KH_2PO_4 0.3 gl^{-1}, NaCl 0.05 gl^{-1}) for a further 24h to obtain stationary phase cultures. To ensure that growth during the assay was not due to metabolism of nutrients in the rich media used during reconditioning, the cells were then transferred to M9 salt solution and starved for 2h (MacLean *et al*. 2004). Then 20µl (~4×10^4 cells) were added to the appropriate assay media (M9 salts plus 0.009375, 0.01875, 0.0375, 0.075, 0.15 or 0.3gl^{-1} of each substrate) in flat-bottomed microplates to begin the assay. We then estimated the number of cells every hour over a 24h growth cycle, measured using OD at 600nm with a SpectraMax M2 microplate reader (Molecular Devices Corporation). Three independent populations for each combination of selection history (ancestral or evolved) and assay concentration were tested in duplicate in each of two blocks.

Estimating growth parameters

Following Novak *et al*. (2006), we corrected OD scores by subtracting scores for sterile media for each well individually, and then discarding measurements below the detection limit of the spectrophotometer (OD = 0.002). We estimated growth parameters by fitting a three-phase linear model to the log-transformed OD scores for each microplate well (Buchanan *et al*. 1997). This model fits three straight lines to the data: a lag phase where there is no change in OD, an exponential growth phase where log(OD) increases linearly over time, and a stationary phase where there is no growth. We used R v2.2.1 with the nlstools package (Baty & Delignette-Muller 2007) to fit models, using the criterion of minimal residual mean squares to find the best fit.

In these assays the inoculum size was close to or below the detection limit of the spectrophotometer. In principle lag times could be estimated indirectly by calculating the intercept between the slope of the exponential phase and the hypothetical log(OD) of the inoculum, calculated from standard curves relating cell numbers to log(OD) and estimating the number of cells in the inoculum from colony counts after spreading onto agar plates. However, we found estimates from this method to have low repeatability, which we calculated as the intraclass correlation coefficient (Lessells & Boag 1987), across blocks of assays ($r = 0.31$). This inconsistency is probably due to variation in the inoculum size among individual assays. We had more success estimating lag times directly, using an inoculum size well above the detection limit of the spectrophotometer ($r = 0.94$), and we present data from these assays below. Note that we did not use these assays to estimate growth rates, because increasing the inoculum size reduces the number of data points during the growth phase.

Statistical analysis

Growth parameter estimates were used in secondary models to test for variation in rate, yield and lag time with resource concentration for the ancestral clone, and then for an effect of evolution by comparing the ancestral clone with populations from the end of the experiment. This was done by analysis of variance with growth rate, yield or lag time as the response variable and selection history (ancestral or evolved) and resource concentration as factors. Block was included as a random effect and all models were run using the lme function in R. During analysis of lag time data the varIdent function (nlme library) was used to account for heterogeneous variance among groups. We used the evolution × resource concentration interaction term to test for variation in the response to selection among populations evolved in different treatments.

6.4. RESULTS

Growth kinetics of the wild type at different concentrations

The rate of increase in population size during exponential growth did not vary significantly with resource concentration for the ancestral clone ($F_{5,12} = 2.73$, $p = 0.07$; figure 6.1). The borderline p-value for this analysis reflects a slight dip in growth rate at the highest resource concentration; when that level is excluded the result is clearly non-significant ($F_{4,10} = 1.51$, $p = 0.27$). In contrast, changing the total substrate concentration had a noticeable effect on total cell yield ($F_{5,12} = 13.41$, $p < 0.0001$; figure 6.1), showing the same saturating relationship reported previously (Chapter 2). The duration of the lag phase did not vary much among treatments, except for a particularly high value at a resource concentration of $0.01875 gl^{-1}$ ($F_{5,12} = 3.90$, $p = 0.025$; figure 6.1).

Evolution of growth parameters

Populations at the end of the experiment had significantly higher growth rates than the wild type ($F_{1,24} = 19.52$, $p < 0.001$; figure 6.1). Evolved populations also produced a greater yield over the growth cycle ($F_{1,24} = 25.66$, $p < 0.0001$; figure 6.1), and had a shorter lag phase than the ancestral clone ($F_{1,24} = 25.32$, $p < 0.0001$; figure 6.1). Taking data across both blocks of assays, the response to selection did not vary significantly with resource supply for any of the three parameters (evolution × resource supply interaction for rate, $F_{5,24} = 1.29$, $p = 0.30$; yield, $F_{5,24} = 1.44$, $p = 0.25$; lag time, $F_{5,24} = 1.18$, $p = 0.35$). Yield scores were positively correlated with those obtained for the same populations by a slightly different assay procedure in Chapter 3 ($r^2_{22} = 0.46$, $p < 0.001$). Thus, our selection lines evolved to respond more quickly than their ancestors to resource renewal in batch culture, to reproduce more quickly during exponential growth, and to produce a greater biovolume over the course of the growth cycle.

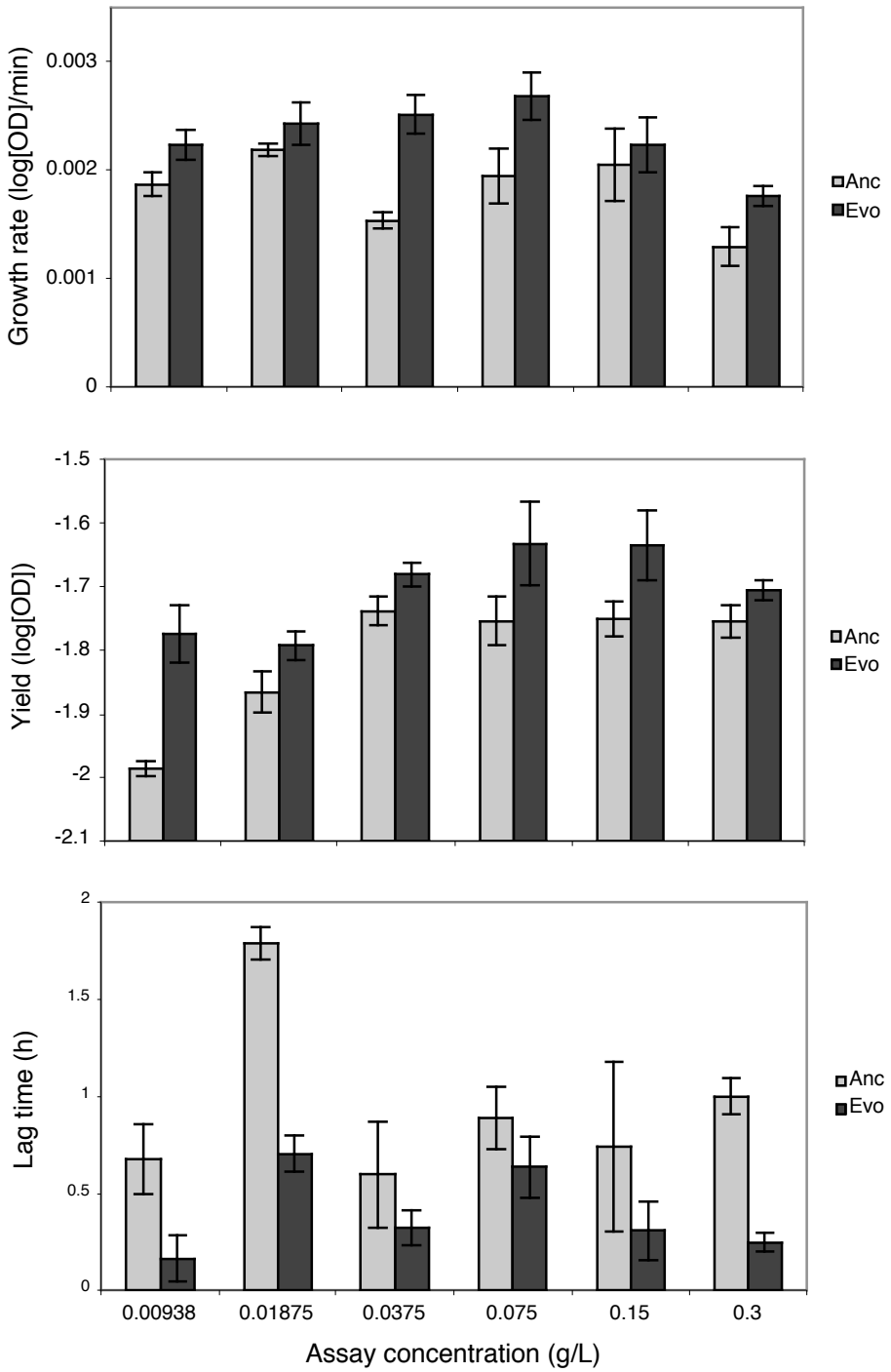


Figure 6.1. Growth parameters of *P. fluorescens* in complex batch culture environments at different resource concentrations. Each panel shows a single parameter (from top: rate, yield, lag time), both before (Anc) and after (Evo)

long-term evolution at each resource concentration. Bars show means ± s.e. for three populations.

6.5. DISCUSSION

After long-term evolution, selection lines had adapted by increasing growth rate and total cell yield, and reducing the duration of the lag phase relative to the wild type. This was true across a range of resource supply rates, even though populations that evolved at intermediate resource supply contained a diversity of genotypes exploiting different combinations of the available substrates (Chapter 2). This suggests that adaptation to any of the available substrates had the same qualitative effect on bacterial growth kinetics.

The positive selection response of the three key growth parameters agrees with results from batch culture experiments with *E. coli* (Luckinbill *et al*. 1978; Vasi *et al*. 1994; Novak *et al*. 2006) and *Pseudomonas pseudoalcaligenes* (Shi & Xia 2003). Growing quickly and producing more cells is obviously advantageous in a selection environment where the probability that a given genotype is transferred to the next tube of growth media depends on its frequency at the end of the growth cycle. The advantage of a shorter lag phase is less straightforward, although Vasi *et al*. (1994) found both experimental and theoretical evidence of direct selection for a rapid response to resource renewal. Interestingly, in the same study Vasi *et al*. (1994) found that evolved cells were also much larger than the wild type, producing a greater biovolume in the form of larger cells that were actually fewer in number. Microscopic observations of our samples showed no discernible effect of evolution on cell size for *P. fluorescens* (figure 6.2), suggesting that they adapted by increasing the number of cells produced over the growth cycle instead. We note that our experiment was relatively short compared to the ~2000 generations of Vasi *et al*.'s (1994) selection lines, so we do not discount the possibility of changes in cell size for *P. fluorescens* during later stages of adaptation.

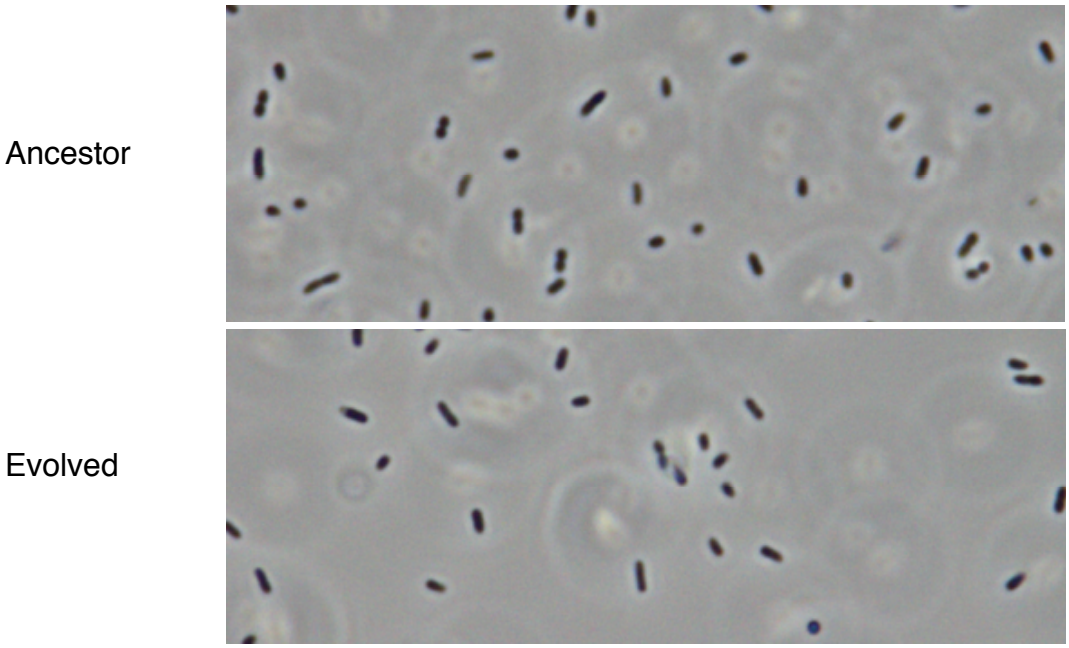


Figure 6.2. Phase contrast microscopy showed no discernible difference in cell size between ancestral and evolved bacteria. Images show cells after 24h of growth in media with 0.15gl^{-1} of each carbon substrate. Taken at $\times$1000 magnification with an Olympus BX51 microscope and DB20 camera.

These results do not support the notion that adaptation to a particular substrate will require a different balance between rapid and efficient growth under resource scarcity and abundance. This is in apparent contrast with results showing that trade-offs between traits can be more important in microbial populations in low resource environments (Bohannan & Lenski 2000; Jessup & Bohannan 2008; Brockhurst *et al* 2008; Chapter 3). One explanation for this is that the same mutation may affect both rate and yield for growth on a given substrate. For example, a mutation that increases affinity for the

available substrate (Clarke 1984) could increase both the rate of metabolism and the ability to grow at low substrate concentrations at the onset of stationary phase. Alternatively, the positive selection responses for growth rate and yield could be due to independent beneficial mutations that have correlated effects on fitness.

How can we determine whether simultaneous changes in rate and yield are due to the same or different mutations? Oxman *et al*. (2008) suggested that the selective benefit of mutations that affect different parameters would vary over evolutionary time, with mutations affecting lag phase being most beneficial early on during adaptation and those affecting growth rate being more advantageous later. In this scenario, the spread of mutations that affect a single growth parameter can be detected by following the trajectories of different parameters over evolutionary time. However, Oxman *et al*. (2008) concede that selection can also act on different parameters simultaneously, making it difficult to rule out epistasis with this approach. An alternative strategy would be to sequence the entire genome of a sample of evolved genotypes and identify the mutations responsible for adaptation. Then by inserting the wild type sequence at different loci we could check for the effect of each mutation on different parameters. If the beneficial mutations affecting each parameter were independent, it would suggest that selection acts independently on rate and yield. In our experiment this would indicate covariance of the selective benefits of rapid and efficient growth across environmental conditions.

Despite the positive correlation of yield scores obtained here and in Chapter 3, we did not observe the relatively large selection responses for populations evolved at intermediate resource supply that we found previously. This discrepancy may reflect the fact that the present assays only captured the first 24h of the 48h growth cycle, and therefore do not reflect any adaptation that may be expressed during the late stationary phase. The emergence of mutants that have a growth advantage at stationary phase has been reported for *E. coli* (Zambrano *et al*. 1993), and adaptation to the different stages of growth may even be associated with the evolution of microbial diversity (Finkel &

Kolter 1999). Diverse populations at intermediate resource supply may therefore contain genotypes that express relatively high fitness during the later stage of the growth cycle, which is ignored by the relatively short assay procedure described above. The possibility of adaptation to growth at stationary phase emphasizes the potential importance of measuring adaptation in the same conditions as those used during selection.

Our results also highlight an important difference between batch culture and chemostats. In chemostats the biproducts of growth are washed out of the system as fresh nutrients are added. As such they are never at stationary phase and substrate concentration directly determines population growth rate. Growth rate therefore generally shows a saturating relationship with substrate concentration (Monod 1949; Shehata & Marr 1971). In batch culture, population growth rate is only transiently limited by nutrient supply, undergoing a feast-famine cycle (Ferenci 1999) so that resources may be exploited at similar rates even when they are supplied in different concentrations at the start of the growth cycle. In agreement with this view, we found little evidence for a positive relationship between growth rate and substrate concentration. At very high concentrations, osmotic stress can inhibit the growth of bacteria (Reis & Döbereiner 1998), and the marginal dip in growth rate at the highest concentration in our experiment may reflect the beginning of this effect.

Adaptation to particular carbon substrates has been repeatedly shown to involve simultaneous changes to several components of fitness. This can be advantageous from the perspective of experimental design, because we can be confident of detecting adaptation by measuring a single parameter, such as total cell yield at the end of the growth cycle, in experiments where bacteria evolve to exploit novel resources.

7. General Discussion

7.1. SUMMARY

The preceding chapters each contain detailed discussion of the experiments described therein. In this chapter I outline some of the broad themes that emerge from this research, and point to potential areas for future work. The main issue I address is the role of environmental variation in the evolution of phenotypic diversity, which I studied using experimental evolution of microbes. My results show that environmental resource availability can determine the potential for adaptation to different niches within a complex environment (Chapters 2 & 4). Resource availability also influences selection for allocation of resources to different phenotypic traits (Chapters 3 & 4). Finally, I showed how population dynamics and genetic constraints can influence adaptation (Chapters 5 & 6). These findings suggest that environmental heterogeneity plays a central role in evolutionary diversification, but only within boundaries imposed by the genetic mechanisms of adaptation.

7.2. ENVIRONMENTAL HETEROGENEITY & DIVERSITY

At the beginning of this thesis I outlined the importance of environmental heterogeneity for the stable maintenance of phenotypic diversity. The presence of a range of alternative niches to which organisms may adapt is expected to be important for the evolution of diversity (Levins 1979; Schluter 2000). I enacted environmental heterogeneity experimentally in two ways. First, I used mixtures of alternative carbon substrates that can be exploited for growth by bacteria. Second, I used static microcosms with different spatially defined niches. Both of these types of environmental heterogeneity have been shown previously to support phenotypic diversity (Rainey & Travisano 1998; Barrett *et al.* 2005). My results show how the evolution of diversity in these conditions depends on the relative productivities of the different niches, which are determined by environmental factors including resource availability and predation (Chapters 2 & 4). For example, when resources are scarce in a complex environment there is effectively a single viable niche, which is to be a carbon generalist exploiting all of the available substrates.

In spatially structured microcosms, resource supply influences the viability of the predator-resistant niche in a similar way. The benefits of bacterial resistance to protozoan predators are traded against an energetic cost, and therefore resistant phenotypes are less likely to evolve when resources are in short supply (Chapter 4). Just like in spatially uniform microcosms, total resource availability constrains the viability of different ecological niches, and I found that this restricts the potential for evolutionary diversification. At the other extreme, when resources are in excess, resistant types dominate across the environment because competition for resources has little effect, rendering the energetic cost of growth in the biofilm irrelevant. Likewise, in spatially uniform conditions an excess of the most favourable substrate allows a single specialist phenotype to dominate.

Taken together, these results suggest that the coexistence of different phenotypes requires sufficient productivity to support growth at different niches. However, excessive nutrient enrichment leads to unchecked growth at the most productive niche and a drop in diversity. The latter scenario is directly analogous to eutrophication of aquatic ecosystems, or strong selection for a single phenotype that is resistant to a disease or parasite. Thus, environmental heterogeneity itself does not guarantee evolutionary diversification, because adaptation to different niches depends on their relative productivities, which are determined by abiotic variables including resource supply, as well as biotic factors like predation and competition.

7.3. RESOURCE SUPPLY CONSTRAINS ADAPTATION & ALLOCATION

Results from Chapters 2 and 4 show how environmental resource supply determines the ecological opportunity represented by different niches. However, even within a given niche, resource supply can influence the optimal strategy of energy allocation to different phenotypic traits. My results suggest that the fitness cost associated with swimming motility was greatest under resource scarcity (Chapter 3). Similarly, the energetic cost of growth in the biofilm had a relatively large effect on fitness in static microcosms with low resource supply (Chapter 4). These results are in step with theoretical and empirical evidence from a range of taxa that the allocation of resources to different traits, or adaptation to different niches, is more likely to be constrained by trade-offs under conditions of resource scarcity or environmental stress (van Noordwijk & de Jong 1986; Ernande *et al*. 2004; Blanckenhorn & Heyland 2004; Brockhurst *et al*. 2008; Jessup & Bohannon 2008). Thus, even during adaptation to a single niche, resource supply can influence the phenotypes that evolve.

7.4. MOLECULAR DETAILS MATTER

As stated in Chapter 1, the frequency and fitness effects of beneficial mutations determine the rate and extent of adaptation. This was observed indirectly in Chapter 5, where some genotypes showed relatively little evidence of adaptation, presumably because of infrequent or ineffective beneficial mutations. Another important property of beneficial mutations is their identity, or the physiological mechanism by which they influence fitness. An increase in fitness can be attained by different mutational pathways, resulting in alternative phenotypes with similar levels of fitness (Travisano *et al*. 1995; Woods *et al*. 2006). In the case of antibiotic-resistant genotypes, increased fitness can be attained either by reversion mutations that restore susceptibility, or by compensatory mutations at other sites. In common with several other studies (e.g. Schrag *et al*. 1997; Reynolds 2000; Paulander *et al*. 2007), I found that resistant bacteria tended to increase their fitness without losing resistance (Chapter 5). This is in contrast with results for swimming motility, where the fitness cost was recovered by loss-of-function mutations that reduced the allocation of resources to the costly trait (Chapter 3). The difference probably reflects variation in the number of genes involved in regulating each trait, with loss-of-function mutations being relatively common for complex traits like motility that are regulated by many different genes. To summarise, while I have emphasized the importance of environmental effects throughout this thesis, my results also highlight the importance of genetic constraints on the rate, extent and phenotypic outcomes of adaptation.

7.5. ARE MICROBES SPECIAL?

I performed experiments with two related species of bacteria in tightly controlled conditions. In nature, microorganisms are more or less ubiquitous; they occupy every conceivable niche and have vast phenotypic diversity that is barely represented by the small proportion of species that can be cultured in the laboratory (Dykhuizen 1998;

Giovannoni & Stingl 2005). Is there a link between the rapid adaptation to new ecological opportunities that we see in the laboratory and the ecological diversity of microbes in nature? The potential to acquire new metabolic functions by regulatory mutations certainly suggests a capacity for adaptation to ephemeral nutrient supply in nature, as does the rapid adaptation to growth at different physical niches. Furthermore, there is little reason to suspect that these are special properties of *Pseudomonas*. Even though pseudomonads have a relatively large number of regulatory genes (Spiers *et al*. 2000; Stover *et al*. 2000), other microbes show a similar capacity for growth and adaptation in a range of different conditions (Madigan & Martinko 2006).

In some ways the evolutionary biology of prokaryotic microbes is distinct from that of eukaryotes. They are small, reproduce rapidly, and have comparatively huge effective population sizes. Furthermore, their capacity to withstand prolonged periods of physical stress and starvation (Schimel *et al*. 2007) makes them less prone to extinction than eukaryotic species (Dykhuizen 1998). These are quantitative differences, but there are two properties of bacteria in particular that make them qualitatively distinct from many non-microbes. First, they reproduce asexually, and the fate of beneficial and deleterious mutations will be different in organisms that do or don't recombine their genes when they reproduce (Maynard Smith & Szathmary 1995). Nevertheless, evolutionary theories developed for sexual and asexual organisms often overlap. For example, clonal interference in microbes (Gerrish & Lenski 1998) describes a similar process to the Hill-Robertson effect in sexual species (Hill & Robertson 1966).

The second distinctive property of bacteria is their ability to exchange genetic material horizontally (Lederberg & Tatum 1946; Ochman *et al*. 2000). Transferring DNA independently of cell division potentially allows novel functions to be obtained from distantly related bacteria and integrated into new ecological contexts without having to acquire entire genetic sequences by mutation (Cohan & Koeppel 2008). The frequency and evolutionary significance of lateral gene transfer is not yet entirely clear (Souza *et al*. 2002), but in the extreme it may mean that microbial phylogenies resemble networks

or webs more closely than the branching trees in which eukaryotic phylogenies are usually cast (Doolittle 1999; McInerney & Pisani 2007).

7.6. FUTURE RESEARCH

Co-evolution

Ecological interactions with other species, including predators and parasites, are expected to play a major role in the evolution of phenotypic diversity. Recent results show that the evolution of bacteria is altered by the presence of natural enemies (Buckling & Rainey 2002; Meyer & Kassen 2007; Chapter 4). Furthermore, interactions between species can change over evolutionary time, with reciprocal adaptation leading to co-evolution of host and parasite traits (Brockhurst *et al*. 2003, 2007). However, the role of natural enemies in the laboratory varies with genetic and environmental factors. Therefore a major challenge for understanding the evolution of diversity is to identify the ecological mechanisms that determine the consequences of co-evolution for properties such as host diversity and parasite virulence. Recent work suggests that this can be achieved by examining longstanding theories of co-evolution in the context of new experimental systems such as bacteria-phage microcosms that allow genetic and environmental effects to be readily discriminated (Morgan *et al*. 2005; Forde *et al*. 2008; Lopez-Pascua & Buckling 2008).

The influence of environmental quality on the specificity of interactions between co-evolving hosts and parasites is of particular interest. Laboratory studies have shown that the fitness cost associated with resistance to natural enemies can be altered by environmental factors, tending to be greater under environmental stress or resource scarcity (Kraaijeveld & Godfray 1997; Bohannon & Lenski 2000). Interestingly, the magnitude of any cost of resistance is expected to influence long term co-evolutionary dynamics, because fitness costs may determine the specificity of host-parasite interactions that evolve (Agrawal & Lively 2002). Specifically, when resistance to a

given parasite genotype incurs a large fitness cost, generalist host genotypes with resistance to several parasite genotypes will be selected against. In contrast, when the cost of resistance is lower, host genotypes that maintain resistance to a range of parasite genotypes may be at an advantage, leading to a less specific interaction between hosts and parasite genotypes. Thus, we can predict that host-parasite interactions will evolve to be more specific in stressful environments where generalist resistance strategies are most costly. This hypothesis can be tested using co-evolution of bacteria and phage in different abiotic environments, and measuring the resistance and infectivity ranges of hosts (bacteria) and parasites (phage) after long-term co-evolution. If interactions evolve to be more specific under stress, then host genotypes that evolve in stressful environments will be resistant to a relatively small range of parasite genotypes.

Antibiotic resistance

The probability that resistant bacteria persist in the absence of selecting drugs is partly determined by the fitness cost associated with resistance. A key area for future work is therefore studying the genetic and ecological factors that influence costs of resistance and the potential for them to be recovered by compensatory adaptation. In general selection will favour resistant genotypes that have little or no fitness cost (Spratt 1996). However, recent work suggests that some resistant genotypes occur at a much greater frequency than others, because they carry resistance mutations at hypermutable sites (MacLean & Buckling 2009). We may therefore expect that in some scenarios, such as severe population bottlenecking or small effective population sizes, the most frequent resistant genotypes, rather than rare genotypes with high fitness, will spread. These predictions may be tested using methods similar to those employed in Chapter 5, by evolving bacteria in the laboratory in the presence of antibiotics, with experimental manipulation of population dynamics. Comparing the fitness of resistant genotypes that emerge in different treatments will allow us to identify the ecological conditions that maintain fitness costs, and potentially to predict the clinical scenarios that will lead to the evolution of resistant clones with high fitness.

A related issue is the interaction between different antibiotics when they are applied in combination. Bacteria can maintain resistance to different antibiotics simultaneously, and increasing levels of multiple drug resistance (MDR) are of particular importance given the difficulty of treating MDR infections (Levy 1998, McCormick *et al*. 2006). Recent work has shown that mutations on the same genome that confer resistance to different antibiotics can have epistatic effects on fitness (Ward *et al*. 2009), although the molecular mechanisms driving these interactions are often unclear. One way to identify combinations of antibiotics that are most likely to engender a large fitness cost would be to use experimental evolution of MDR in the laboratory, screening different treatment combinations to find ones where multiple resistance incurs a significant cost. By extension, the potential to recover the costs of MDR by compensatory adaptation can be compared for different MDR genotypes. Ultimately, employing antibiotic combinations that require costly resistance mechanisms may help to minimise the risk that MDR bacteria persist after treatment is stopped.

The relevance of evolutionary theory and laboratory experiments for understanding the global problem of antibiotic resistance is clear to many biologists (Bergstrom & Feldgarden 2008; Read & Huijben 2009). However, some of the most pressing questions lie at the interface of epidemiology, evolution and microbiology. For example, how long will it take to restore drug-susceptibility by prudent use of antibiotics (Levin 2001)? Will it be easier to eradicate resistance in the hospital or in the community (Lipsitch 2001)? Which pathogens are most likely to evolve resistance (Wijngaarden *et al*. 2005)? Is it better to use antibiotics one at a time or in combination (Bonhoeffer *et al*. 1997)? Evolutionary studies are required to understand how resistance emerges, and why it does not disappear when drugs are withdrawn. Molecular tools for microbiology can identify the genetic basis of resistance, and therefore inform evolutionary studies such as that described in Chapter 5. Finally, epidemiological data on the population dynamics of infection may be combined with evolutionary and microbiological information to predict the spread of resistant and susceptible bacteria in their hosts, us.

7.7. CONCLUDING REMARKS

The motivation for the experiments described above was to better understand general patterns of adaptation and diversity, such as latitudinal gradients in species richness, the evolution of flightlessness in birds and insects, the variable role of predators in nature, and the persistence of drug-resistant bacteria. Using microbial microcosms I have studied the mechanisms that generate analogous patterns in controlled environments. One theme that runs throughout is the role of environmental heterogeneity in the evolution of phenotypic diversity. However, the level of diversity that is sustained by heterogeneous environments, and the properties of the constituent phenotypes, depends on resource supply, genetic constraints and ecological interactions with other species. Therefore patterns of phenotypic diversity are probably not explained by a single mechanism, and instead require us to consider the environmental and genetic factors that alter the importance of different ecological and evolutionary processes.

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