The effects of competition and ecological opportunity on adaptation and diversification

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Abstract

Ecological processes have the potential to influence evolution through their effects on selection. This thesis explores the effects of two ecological factors - competition and ecological opportunity.

Intraspecific (within-species) competition is often expected to drive adaptation and diversification by increasing selection for the use of novel resources, thereby alleviating the detrimental effects of competition. However, this is not always the expected outcome; theory suggests that intraspecific competition can also drive convergent evolution. On the other hand, interspecific (between-species) competition is usually expected to impede adaptation and diversification because competitor species occupy potential available niches, preventing the focal species from diversifying to do so. In this thesis, I review previous experimental studies exploring the effects of competition on adaptive diversification, and then directly test these effects using experimental evolution of the bacterium *Pseudomonas fluorescens*. I confirm that intraspecific competition drives adaptive diversification, while the effects of interspecific competition are varied. Strong interspecific competition impedes adaptation and diversification, while the presence of weak, non-diversifying interspecific competitors drives diversification through increased resource competition.

The presence of ecological opportunity is essential for adaptation and diversification, and so variation in attributes of those opportunities is expected to have important effects on the dynamics of adaptive evolution. In another evolution experiment with *P. fluorescens*, I tested the effects of variation in ecological opportunity on adaptive evolution and found that the type and arrangement of ecological opportunities drives adaptation but, in this system, not diversification. I also show that ecological opportunity drives differences in the degree of parallel evolution at the phenotypic and genotypic level. Finally, I explore some unexpected genetic changes identified in one of these evolved

populations - two synonymous mutations that conferred fitness benefits, and show that the observed fitness improvements are the result of increased gene expression.

I have shown that ecological processes can play an important role in shaping the evolutionary trajectories taken by populations. Understanding the interactions between ecological and evolutionary processes is vital for our understanding of evolutionary dynamics as a whole, and the studies laid out in this thesis represent valuable contributions to this field of study.

Résumé

Les processus écologiques peuvent agir sur l'évolution du fait de leurs effets sur la sélection. Cette thèse explore les effets de deux facteurs écologiques particuliers, la compétition et les opportunités écologiques.

En théorie, la compétition intra-spécifique (entre individus d'une même espèce) peut souvent conduire à l'adaptation et à la diversification écologique en augmentant la sélection par rapport à l'usage de nouvelles ressources, ce qui conduit à une réduction de l'effet négatif de la compétition. Cependant, ceci n'est pas toujours le cas. Certains modèles théoriques suggèrent que la compétition intra-spécifique peut aussi conduire à une convergence évolutive. Par ailleurs, la compétition inter-spécifique (entre différentes espèces) empêche théoriquement l'adaptation et la diversification - la présence d'espéres compétitrices occupant les niches potentiellement disponibles empêche l'espèce focale d'occuper ces niches en se diversifiant. Dans cette thèse, je répertorie les études expérimentales ayant exploré les effets de la compétition sur la diversification adaptative, et je teste directement ces effets en utilisant une approche d'évolution expérimentale chez la bactérie *Pseudomonas fluorescens*. Mes résultats valident l'hypothèse d'une augmentation de la diversification du fait de la compétition intra-spécifique; en revanche les résultats concernant les effets de la compétition inter-spécifique sont plus compliqués. En effet, une forte compétition inter-spécifique empêche l'adaptation et la diversification, alors qu'une compétition inter-spécifique faible conduit à une diversification via une augmentation de la compétition pour l'acquisition de ressources.

La présence d'opportunités écologiques est essentielle pour l'adaptation et la diversification; en conséquence, les différents facteurs affectant ces opportunités peuvent avoir un effet sur la dynamique de l'adaptation. Dans une autre expérience d'évolution expérimentale avec *P. fluorescens*, j'ai testé les effets de la variation des facteurs écologiques sur l'adaptation.

Les résultats montrent que, dans ce système, le type d'opportunité écologique et leur arrangement déterminent l'adaptation, mais pas la diversification. Je montre aussi que les opportunités écologiques déterminent les différences dans le degré d'évolution parallèle aux niveaux phénotypique et génotypique. Enfin, j'explore des changements génétiques inattendus observés dans une de ces populations expérimentales. Je montre que deux mutations synonymes confèrent une plus grande aptitude et que ce changement est dû à une augmentation de l'expression du gène concerné.

J'ai montré que les processus écologiques peuvent jouer un rôle important dans l'adaptation en déterminant la trajectoire évolutive des populations. L'analyse des interactions entre processus écologiques et évolutifs est primordiale pour une compréhension globale des dynamiques évolutives. Les études présentées dans cette thèse apportent une contribution précieuse à ce domaine d'étude.

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Chapter 1

General introduction

Adaptive evolution is the outcome of two processes: mutation and selection. Random mutations arise and selection acts on them, purging mutations with deleterious fitness effects while allowing beneficial mutations to persist, the result of which is increased fitness, or adaptation, of a population (Bell, 1997). The specifics of how these processes proceed and interact in a given population determine the details of the particular evolutionary trajectory taken, including the rate and extent of adaptation, and whether or not diversity evolves. Ecological processes have the potential to have important effects on adaptive evolution through their effects on selection (Schluter, 2000b), and my thesis focuses on the evolutionary consequences of two ecological factors: competition and ecological opportunity. Competition can both drive and impede adaptation and diversification, depending on the strength of competition, the relatedness of the competitor, and potential for the competitor itself to evolve and diversify. Variation in the number, type, and arrangement of ecological opportunities, or more generally, environmental heterogeneity, can influence adaptation and diversification by generating spatial and/ or temporal variation in the patterns of selection. There is great variety in strength and type of competition and environmental heterogeneity across habitats and species, and so tests

of how these ecological factors influence evolutionary processes have important general implications for understanding how the diversity of life came to be.

Evolutionary change occurs slowly, over many generations, and so can be difficult or even impossible to study directly. One widely used strategy for dealing with this problem is to quantify the phenotypic and/or genetic patterns of current species and communities, and use that information to infer what past events led to those observed patterns (e.g. Zhang et al., 2013). An alternate, complementary strategy is to directly observe evolutionary processes by experimentally evolving organisms with short generation times in controlled environments - experimental evolution of microbes. Originating with an experiment by William Henry Dallinger in the 1880s (Hass, 2000), but truly gaining popularity since the initiation of Richard Lenski's long-term *Escherichia coli* evolution experiment on February 15, 1988 (Lenski et al., 1991), experimental evolution of microbes has been instrumental in pushing forward our understanding of evolution (Kawecki et al., 2012). Paired with our ever improving ability to quickly and accurately sequence genes and genomes, experimental evolution has proven to be an extremely powerful approach to probing evolutionary questions, providing answers to fundamental questions about the dynamics of adaptation and genome evolution (Barrick et al., 2009), diversification (Rainey and Travisano, 1998) and speciation (Dettman et al., 2007), to name a few.

The general aim of my thesis is to examine how ecological processes influence evolutionary dynamics using experimental evolution of *Pseudomonas fluorescens*. This particular microbe is a plant-colonzing, ubiquitous, Gram-negative bacteria, part of a group of species (Pseudomonads) known for their incredible physiological diversity in nature (Spiers et al., 2000). The ancestral strain (SBW25) used in my experiments was orginally isolated in 1989 from a sugar beet leaf in Oxfordshire (Bailey et al., 1995) and has undergone only a few generations of growth in the lab. *P. fluorescens* SBW25

has previously been shown to adapt readily to a variety of novel sugar resources (e.g. Jasmin and Kassen, 2007a) and to rapidly diversify in static, rich-media microcosms (e.g. Rainey and Travisano, 1998), making it an ideal organism for exploring adaptation and diversification in the lab.

I begin the thesis with a review of the literature on the effects of competition on the evolution of adaptive diversity (chapter 2), followed by an experimental test of those effects in the *P. fluorescens* model adaptive radiation (Rainey and Travisano, 1998) (chapter 3). The next three chapters describe various aspects of an evolution experiment aimed at testing the effects of ecological opportunity on evolutionary adaptation and diversification using *P. fluorescens* populations cultured in a range of simple sugar environments. Chapter 4 provides a detailed description of the evolution experiment design and examines the effects of variation in the number, type, and arrangement of ecological opportunities on the dynamics of adaptation and diversification measured at the level of fitness and phenotype. Chapter 5 examines the changes in those same populations at the genomic level, focusing on comparisons of parallel changes across populations evolving in the same and differing selection environments. Chapter 6 reports on some particularly interesting genetic changes seen in one population that was part of the larger evolution experiment, specifically two synonymous mutations that confer positive fitness effects. I finish with a concluding chapter (chapter 7), briefly summarizing and bringing together the main findings of the thesis.

Much of the work described in this thesis was a collaborative effort, however I took a lead role on each of the projects. The details of my collaborators' contributions are described at the beginning of each chapter where applicable.

Chapter 2

A review of the role of competition in the evolution of adaptive diversity

2.1 Introduction

The notion that competition influences evolutionary adaptation and diversification has been around since Darwin first presented his theory of evolution by natural selection. Competition can influence the dynamics of adaptation quite simply because in a limiting environment, as is often the case in nature, competition between individuals has the potential to drive differences in relative fitness. Diversity evolves when different lineages take advantage of different niches, or ecological opportunities, as they adapt to their environment (Simpson, 1949, 1953; Losos, 2010), and it has been suggested that particularly abundant ecological opportunity is often present when other competing species are low in numbers or even absent (e.g. on islands compared to mainland, Lister, 1976; Schluter, 1988). However, the presence of competitors of a different species (interspecific competitors) is also implicated in driving the evolution of diversity in the form of character displacement (Brown and Wilson, 1956; formally modelled by Slatkin, 1980;

most recently reviewed by Stuart and Losos, 2013). Within-species competition, or intraspecific competition, on the other hand, is thought to drive diversification and even speciation (Maynard Smith, 1966; Rosenzweig, 1978; Dieckmann and Doebeli, 1999), however some have suggested that this may only occur under a relatively restricted set of conditions (e.g. Slatkin, 1980; Abrams, 1987).

Over the years, numerous observational/ comparative and some experimental studies have been aimed at examining competition's role in adaptive diversification (for overviews of some of this literature see Schluter, 2000b; Pfennig and Pfennig, 2012). Much emphasis in the adaptive diversification literature has been placed on the diversifying effects of competition, however it is clear from both theoretical and empirical studies that competition can, and does, both drive and impede the evolution of adaptive diversity. Thus, the goal of this review is to use the experimental evidence to outline some general expectations for when competition drives versus impedes adaptive diversification and suggest future experiments aimed at identifying key mechanisms that may mediate the multifarious effects of competition. It is important to note that this review is aimed at addressing ideas regarding the origins, not simply the maintenance, of adaptive diversity. While it may be that a number of the processes influencing the maintenance also influences the origin of diversity, not all of these mechanisms are shared and so I focus here on studies that explicitly examine the origins of diversity via adaptive evolution.

Most of the traditional ideas and hypotheses regarding the influence of competitive processes on adaptive diversification stem from theory specific to exploitative competition for resources. However, it is important to keep in mind that the definition of "resource" can be quite broad, ranging from food items, to territories, to pollinators, to anything else that somehow aids in the perpetuation of a lineage. While other types of competition such as interference competition (e.g. Robinson and Pfennig, 2013) and apparent competition

(e.g. Van Nouhuys and Hanski, 2000) may also influence adaptive diversification, the mechanisms are different and may require different theoretical frameworks that are less well developed, and so I restrict my discussion here to studies of exploitative resource competition.

I begin this review by first outlining the ways in which adaptive diversity can evolve. I then identify and distinguish the circumstances under which one might expect competition to drive versus impede adaptive diversification, focusing on the experimental evidence for these contrasting effects. In particular I focus on experiments that manipulate the strength of competition (see table 2.1), with an aim to observe its effects on patterns of selection and/ or the evolution of diversity. Because much emphasis has been placed in previous studies on distinguishing the effects of intra- and inter-specific competition, I start from this perspective but follow with a discussion of the utility of this dichotomy for understanding the potential effects of competition on adaptive diversification. I finish by identifying outstanding questions in the field and providing suggestions for future work.

2.2 Adaptive diversification

Diversity can be broadly defined as a measure of variation, quantified at the level of individuals, eco-types/ subspecies, species, or any other level of biological organization of interest. When talking about diversity, it is very important to be clear about the level of focus, as processes that drive diversification at one level of organization may simultaneously reduce diversity at another level. For diversity to be adaptive, it must be tied to fitness. There are many ways to measure diversity, however two broad categories are: 1) measures of continuous diversity - estimates of variation in a phenotypic trait or set of traits (i.e. niche breadth); and 2) measures of discrete diversity - simply the number of different types (e.g. species, morphotypes) or a calculation that also incorporates the

proportions of each of those types (e.g. Simpson index; Simpson, 1949).

While adaptive changes in both continuous and discrete measures of diversity can be initiated by the same general mechanisms, an increase in the number of discrete types or species often requires additional processes. Indeed, in sexual species, divergence leading to speciation requires that assortative mating evolves within those emerging specialist lineages (Maynard Smith, 1966; Dieckmann and Doebeli, 1999; Kondrashov and Kondrashov, 1999). However, there is evidence that ecological and reproductive divergence are often linked (Rundle and Nosil, 2005; Schluter, 2009) and so I leave further discussion of the details of speciation to others. For the purposes of this review I draw on studies that consider both types of diversity.

For adaptive diversity to evolve, a few conditions must hold. First and foremost, ecological opportunities or novel niches must be available for the diversifying population to utilize. Second, heritable variation must be available (either as standing variation or arising through de novo mutation) in those traits required to utilize the novel ecological opportunities. However it has been suggested that plastic variation in phenotype may also facilitate population divergence because variation that emerges as a plastic response can eventually become canalized (Pfennig and Murphy, 2002; Price et al., 2003). Once these two requirements are met, diversity will arise if selection drives different lineages in different evolutionary directions. This can occur quite easily if diversifying lineages become spatially or temporally separated (i.e. evolution in allopatry). On the other hand, lineages that occur in overlapping habitat (i.e. in sympatry) can only evolve diversity if there are genetic constraints generating trade-offs in fitness improvements on the multiple available ecological opportunities. Without these constraints, the expected outcome is the evolution of a population of generalists, adapted to utilize all available opportunities with equal success (Via and Lande, 1985). When lineages and populations evolve in

Table 2.1: Important definitions

Divergent selection: Selection for extreme values of a trait. Often used interchangeably in the literature with the term *disruptive selection*, however the first term emphasizes selection for extreme types, while the second term emphasizes selection against intermediate types.
Stabilizing selection: Selection for intermediate values of a trait. In theoretical models, disruptive and stabilizing selection are often considered as a continuum of effects with sign indicating the type of selection (by convention positive values indicate disruptive selection, while negative values indicate stabilizing selection), and the magnitude of those values indicates the strength of selection (e.g. Abrams et al., 2008).
Strength of competition: The per capita proportional decrease in a population's growth rate resulting from the presence of individuals of either the same (*intraspecific competition*) or a different species (*interspecific competition*). Actually quantifying this metric beyond relative measures such as 'high'/ 'low' is difficult, and debate continues over what approach is most appropriate (e.g. Wootton and Emmerson, 2005).

sympatry, individuals and lineages can compete with each other, potentially influencing the evolutionary outcome. Thus, when asking questions about how and when competition might influence diversification, evolution in sympatry is the scenario of interest.

2.3 Intraspecific competition

Intraspecific competition for limited resources can result in strong selection for the utilization of alternate resources. If genetic constraints generate adaptively relevant trade-offs (as outlined earlier), selection is divergent or disruptive (see table 2.1) and may lead to the evolution of adaptive diversity. In addition, as strong selection continues to drive adaptation, initially continuous diversity can eventually evolve into discrete diversity.

Thus the traditional expectation is that increased intraspecific competition drives increased adaptive diversification, however contradictory observational and experimental studies suggest that this is not the whole story. For example, Svanbäck and Persson (2004, 2009) showed a positive relationship between strength of intraspecific competition, and diversity in populations of Eurasian perch (*Perca fluviatilis* L.), as traditional expectation would suggest. However, with different populations of the same species, Olsson et al. (2006) detected the opposite - a negative relationship. Theoretical models show that intraspecific competition can, in fact, generate both divergent and stabilizing selection Abrams (1987, 1990) depending on the strength of competition (Abrams et al., 2008).

Figure 2.1 depicts the expected patterns of selection and evolved diversity as the strength of intraspecific competition ranges from very weak to very strong. This figure summarizes the general expectations of both verbal and mathematical theory on the relationship between intraspecific competition and adaptive diversification. Region B of figure 2.1 shows the more traditionally held view of how intraspecific competition influences divergent selection and so adaptive diversification, however the extremes of this relationship look quite different.

When intraspecific competition is weak, disruptive selection is also weak or can even be stabilizing (region A of figure 2.1). At this strength, intraspecific competition has such an insignificant impact on resource uptake that the best strategy is for individuals to use the most abundant, easiest-to-use resource. The details of the competition-selection relationship in this region - i.e. the switch from disruptive to stabilizing - depends on the specifics of the resource utilization function (Abrams et al., 2008).

When intraspecific competition increases to very high strengths (region C of figure 2.1), the strength of disruptive selection eventually begins to decrease. This is because intense competition can act to lower population density, decreasing the adaptive benefit of utilizing

alternate resource types. In addition, in a situation where competition is so intense that population size is significantly reduced, the resulting reduction in standing variation and/or mutation supply (the variation that evolution by natural selection operates on) can slow or even halt diversification even if selection is still divergent. Furthermore, analysis of a range of theoretical models and under a variety of conditions suggests that the relationship seen in region C in figure 2.1 is likely to be a general pattern (Abrams et al., 2008).

2.3.1 Empirical background

A number of observational studies exploring adaptive diversity associate the evolution of that diversity with strong intraspecific competition. These observational studies tend to make the link between intraspecific competition and diversification based on one of two types of observations: 1) the detection of disruptive selection (for continuous diversity) or negative frequency-dependent selection (for discrete diversity) (e.g. Smith, 1993; Martin, 2012; Hori, 1993; Hendry et al., 2009), or 2) an association between intraspecific population density and diversity (e.g. Svanbäck et al., 2008; Svanbäck and Persson, 2004, 2009).

While in many of these studies, intraspecific competition is indeed likely to have driven diversification, this is not a given for a number of reasons. To start, while an observed pattern of selection can certainly influence the ongoing maintenance of diversity, it does not reveal how that diversity arose to begin with. Patterns of selection could look very different now, compared to what they looked like when diversity was initially evolving (Schluter, 2000a; Connell, 1980). Secondly, while both a positive correlation between population density and diversity, are consistent with intraspecific competition driving diversification, these observations are necessary but not sufficient - it is possible that other processes could generate these patterns.

Finally, even if intraspecific competition does generate disruptive selection in a given population, this does not necessarily lead to adaptive diversification. Plasticity could generate enough phenotype diversity to alleviate the detrimental effects of competition and so evolutionary processes need not come into play at all (e.g. Tinker et al., 2008). Another possibility is that correlations between traits and other genetic constraints might not allow the population to diverge at all (e.g. Smith and Rausher, 2008b). These alternative possibilities mean that experiments are necessary in order to definitively establish the effects of intraspecific competition on the evolution of adaptive diversity. The crucial test is to manipulate the strength of intraspecific competition and then observe changes in the pattern and strength of selection, or even more definitively, actual changes in diversity, and critically, confirm that those changes are heritable.

2.3.2 Experimental evidence

A few studies do actually manipulate the strength of intraspecific competition with an aim to observe its consequences on divergent selection and the evolution of diversity. Table 2.2 summarizes these studies. Strength of competition is a function of per capita resource availability and so there are two main strategies for manipulating strength of competition: 1) changing population size, or 2) changing resource availability. Studies that vary strength of competition via population size do so either directly by initiating populations with different numbers of individuals (Maret and Collins, 1997; Agashe and Bolnick, 2010; Martin and Pfennig, 2010; Bolnick, 2004; Svanbäck and Bolnick, 2007), or indirectly - Buckling and Rainey (2002) and Meyer and Kassen (2007) introduce an antagonist that reduces population size, while Bailey et al. (2013a) initiate populations with strains that are identical save for single mutations that affect their growth rates. Studies that vary strength of competition via resource availability also do so directly (Bolnick, 2001; Olsson

et al., 2006; Martin and Pfennig, 2009), or indirectly - Maret and Collins (1997) adjust the abundance of a zooplankton food resource by manipulating nutrient availability. It is not clear whether one strategy is more appropriate than the other, but it is likely that direct manipulations of these factors will lead to fewer unidentified effects on selection than the indirect manipulations. That being said, it is also important to explore the outcomes of indirect manipulations of intraspecific competition, as these manipulations may be closer to the way that strength of competition varies in nature.

Despite the range of systems and types of manipulations seen in experimental studies of intraspecific competition and the evolution of diversity, all but one of these studies report the same general pattern - increased intraspecific competition results in increased within-population diversity. The one exception, Olsson et al. (2006), tested the effects of intraspecific competition on diversification in populations of Eurasian perch (*Perca fluviatilis* L.) grown in aquaria by varying food supply and found that increased intraspecific competition led to reduced morphological variation (traditional theory suggests variation should increase). They also found the same relationship in a comparison of field populations from 10 different lakes that varied in their population density. These observations are even more notable because other studies on different populations of the same species have found the opposite, more traditional relationship - increased diversity correlated with increased population density (Svanbäck and Persson, 2004, 2009).

So, while theory suggests a wide range of possible responses to increased intraspecific competition, the current experimental evidence weighs heavily on the side of a positive relationship between intraspecific competition and diversification. This could simply be because most experiments have not varied the strength of intraspecific competition widely enough to reveal patterns outside of region B in figure 2.1. It is important to

point out, however, that varying strength of competition to its extremes may be quite difficult experimentally, in particular at the high end, where populations are no longer sustainable in the long term. In other words, selection becomes so strong that extinction occurs before evolution does. In addition, because of difficulties in actually quantifying strength of competition, it is difficult to tell where any given study lies on this scale.

2.4 Interspecific competition

Interspecific competition for limited resources can drive further phenotypic divergence of those distinct groups (i.e. character displacement, MacArthur and Levins, 1964). Selection pushes those lineages to diverge in order to alleviate the detrimental effects of competition, thus increasing phenotypic diversity at the community level. On the other hand, the absence of interspecific competitors is thought to allow for niche expansion or competitive release (Grant, 1972). If a population's niche expansion occurs via specialization of individuals, as opposed to general niche expansion of all individuals, then within-species/ within-lineage diversity will increase. The flip side to the idea of competitive release is that the presence of interspecific competitors will impede diversification of a focal species or lineage by occupying potential niches and so preventing adaptation and diversification into those niches (Schluter, 2000b).

These apparently opposing positive and negative effects of interspecific competition depend on the level at which diversity is observed. The amount of available niche space in an environment may place bounds on the total phenotypic variation that can arise and be maintained. However, this total variation can be partitioned within or between species depending on the balance between intra- and inter-specific competition (e.g. Wehenkel et al., 2006). When interspecific competition is strong, selection may drive the two competing species to diverge, increasing between-species variation via character

displacement, but often at the cost of within-species variation (see the top panels of figure 2.2 for cartoon examples). When interspecific competition is weak, selection for the competing species to diverge is lessened and the focal species experiences "character release" - increased within-species phenotypic variation (bottom right panel compared to top right panel in figure 2.2).

Much of the empirical basis for the theories of character displacement and character release are observational studies comparing the phenotypes of populations living in sympatry and allopatry - i.e. in the presence or absence of a competitor species (e.g. Schoener, 1970; Huey and Pianka, 1974; Malmquist, 1985; Diamond et al., 1990; Losos, 1990). However, subsequent to the formation of these theories, there has been a great deal of debate about the appropriateness of many of these empirical comparisons, leading to more rigorous explorations of these mechanisms (see Losos, 2000 for a review) and the development of six criteria for the establishment of character displacement as the mechanism for phenotypic divergence between sympatric species (Schluter and McPhail, 1992). More recently, experimental studies manipulating interspecific competition provide direct tests of these theories. Table 2.3 summarizes these studies.

2.4.1 Presence/ absence of interspecific competitors

The evidence for increased *between*-species diversity

A number of studies provide experimental evidence for interspecific competition driving between-species diversity and/or impeding within-species diversity by comparing the effects of the presence/ absence of a competitor species. The majority of these experiments show that the presence of a competitor species results in changes in the patterns of selection, driving selection in the focal species for phenotypes that differ from the competitor phenotype (Guénard et al., 2012; Forseth et al., 2003; Schluter, 1994), or even results

in the evolution of increased between-species divergence (Tyerman et al., 2008; Melville, 2002; Pfennig and Pfennig, 2010).

However, there are a few interesting exceptions. A study examining protists (*Colpoda spp.*) cultured in the presence of a competitor species (*Pseudocyrtolophosis alpestris*) showed both increases and decreases in between-species phenotypic divergence depending on the trait observed (TerHorst, 2011).

Smith and Rausher (2008b,a) detected divergent selection between Ivyleaf morning glory (*Ipomoea hederacea*) and its competitor (*I. purpurea*) when grown in the presence of that competitor, but saw that the actual evolution of divergent Ivyleaf morning glory phenotypes was impeded by genetic constraints in the form of trait correlations (Smith and Rausher, 2008a). These results underline the important point that while divergent selection is necessary for divergent adaptive evolution, it is not sufficient.

Finally, Rundle et al. (2003) saw that decreases in the strength of interspecific competition via decreases in population density due to predation resulted, unexpectedly, in an increase in between-species divergent selection. The authors suggest that in this experiment predation-induced selection on divergent foraging behaviour may be more important than the effects of competition-driven disruptive selection. This experiment highlights the difficulty of interpreting indirect manipulations of competition, and also underscores the advantage of experimental tests over observational comparisons, as the mechanisms driving these observed patterns of selection are difficult to resolve even in this relatively simple system.

The evidence for decreased *within*-species diversity

A few experimental studies confirm that the presence of interspecific competition increases between-species diversity, while at the same time reducing within-species diversity

(Melville, 2002; Pfennig and Pfennig, 2010). This apparent trade-off between within-species and between-species diversity lends support to the idea that the amount of opportunity or niche space defines a limit for the total ecological variation that can arise and be maintained in any given environment, and the balance between intra- and inter- specific competition determines how that variation is partitioned. Hendry et al. (2009) report on what may be an interesting example of the balance between intra- and inter- specific diversity driven by different sources of competition. The authors detected a pattern of disruptive selection for different beak sizes in a population of Galápagos finches (*Geospiza fortis*), likely driven by intraspecific resource competition for seeds that varied in size. However, at the phenotypic extremes they detected balancing selection, suggesting that this pattern may occur, at least in part, because interspecific competition restricts any further niche expansion. Thus, when examining the effects of interspecific competition on diversity, it is important to define the level of focus appropriately or, if at all possible, examine diversity across multiple levels of organization.

2.4.2 Interspecific competition over a range of strengths

Even with the increased rigour in the examination of the potential effects of competition on diversity since the criteria for character displacement were developed (Schluter, 1994), most studies (as discussed above and in table 2.3) still tend to focus on identifying changes in patterns of selection or diversity in the presence/ absence of competitor species, ignoring the details of how this relationship might vary over a range of strengths of interspecific competition.

However, a few observational studies do compare diversity across populations that vary in the number of different competitor species present (Parent and Crespi, 2009; Harmon et al., 2003; Robinson et al., 2000). Making the assumption that strength

of interspecific competition increases with the number of competitor species present, these studies all suggest that diversity of the focal species decreases with strength of interspecific competition. More specifically, results from these studies indicate that community-level diversity decreases approximately linearly with increasing strength of competition. Another observational study correlates evolutionary changes in diversity with strength of interspecific competition in a 'natural experiment' with the Galapágos finches *Geospiza fortis* and *G. magnirostris* (Grant and Grant, 2006). These populations ranged in density due to fluctuations in precipitation over 33 years of study, and population density was positively correlated with interspecific divergence in beak size.

A single experimental study directly manipulates the strength of interspecific competition over a range of values and quantifies evolved diversity within a focal species, *Pseudomonas fluorescens* SBW25 (Bailey et al., 2013a). Here, *P. fluorescens* evolved less diversity with increased strength of interspecific competition, suggesting that stronger competitors constrain diversification by taking up potential niches more successfully than weaker competitors.

2.4.3 When interspecific competitors act like intraspecific competitors

If there is a great deal of niche overlap between a focal species and its competitor, interspecific competition may instead function in a way similar to that of intraspecific competition, potentially driving disruptive selection within the focal species population and so adaptive diversification. Under this scenario, adaptive diversification of the focal lineage can occur if 1) interspecific competition is not so strong that it leads to competitive exclusion, and 2) the focal lineage is able to diversify and take over available niches before the competitor species does. The bottom right panel of figure 2.2 depicts this possible outcome. However, if the competitor species does diversify rapidly, taking over potential

niches, interspecific competition reverts to impeding within-species diversity as discussed earlier. This pattern is seen in an experiment following the adaptive diversification of *Pseudomonas fluorescens* in the presence of interspecific competitors that, at least initially, overlap completely in their resource-use (Bailey et al., 2013a). Here, the presence of weak interspecific competitors actually drives *P. fluorescens* diversity to levels higher than in the absence of competitors, while stronger interspecific competitors impede diversification because they diversify into initially unoccupied niches before the focal species can.

2.5 Can we predict the evolutionary consequences of competition?

The majority of the experimental evidence to date supports the idea that intraspecific competition drives the evolution of within-species diversity, and interspecific competition impedes within-species diversity while driving between-species diversity. However, there are a number of notable exceptions as discussed above, and so one must be cautious when making predictions about the effects of competition on the evolution of adaptive diversity. Adding to this caution, it is important to keep in mind that observational studies, as well as many of the experimental studies, that examine the effects of competition on adaptive diversity tend to do so in systems selected for study because of their particularly impressive or unique patterns of diversity. As a result, a lack of an effect and patterns of decreased diversity are likely to be under-represented in the literature.

It is also important to remember that competition can only further drive diversity if the conditions for sympatric adaptive diversification are already met, and it is not clear how common an occurrence this is. Two of these conditions - the presence of ecological opportunity and the generation of heritable variation in relevant traits - are often difficult

to quantify until after the diversification has already occurred. On the other hand, a heterogeneous novel environment is likely to provide plenty of ecological opportunity and in theory, given enough time, the appropriate heritable variation should arise. The third condition, the existence of genetic constraints generating fitness trade-offs, has been tested to some extent and while this is still up for debate, some evidence suggests that the necessary types of constraints are not as common as originally thought (Hereford, 2009).

In predicting the effects of intraspecific competition at moderate strengths of competition, the pattern of increasing diversity with increasing competition is experimentally reasonably well supported, however data are lacking at the extremes. Experiments testing the effects of intraspecific competition across a wider range of competitive strengths are needed to help us to better understand this relationship.

In trying to predict interspecific competitive effects on diversity, it is important to make a distinction between the multiple ways in which the strength of interspecific competition can be altered (see figure 2.2). Interspecific competition can change with increases or decreases in competitor density resulting in a change in overall consumption of the shared resource(s), or with shifts in the particular combination of resources competitors consume (i.e. their niche), resulting in a change in overlap with the focal population's niche. These different types of changes may have different effects on diversity. Changes in the strength of interspecific competition via changes in interspecific density have been examined experimentally, however changes to the strength of interspecific competition via changes in the degree of niche overlap have not explicitly been tested. The closest thing to a test of the effects of competitor niche overlap on the evolution of diversity are the few observational studies that look at the effects of different numbers of interspecific competitor species (Parent and Crespi, 2009; Harmon et al., 2003; Robinson et al., 2000). One might assume the total combined interspecific competitor niche width might expand

with the number of interspecific species present. However it is not clear how consistent this relationship is, and as the addition of more species likely also affects the total interspecific density, it is difficult to know what effects are actually due to niche changes. Experimental tests teasing apart these two types of changes to interspecific competition are needed and may help explain some of the observed inconsistencies in the effects of interspecific competition.

2.6 The necessity of distinguishing intra- and inter-specific processes

Throughout this review I have considered intra- and interspecific competition as two distinct mechanisms. Is this dichotomy appropriate? As discussed earlier, it does appear that interspecific competition can act in a similar way to intraspecific competition when interspecific competitors overlap greatly in niche use. However, I would argue that the distinction between these two types of effects is still useful because an interspecific competitor, regardless of its phenotypic overlap with the focal species, is still an evolutionarily independent unit and evolutionary adaptation may play out differently in the competitor versus focal species. Evolutionary changes in an interspecific competitor population may result in its own diversification and so prevention of focal species diversification, or even complete competitive exclusion.

Another important reason for keeping the processes of intra- and inter- specific competition separated in our exploration and interpretation of adaptive evolution, is that strength of intra- and inter- specific competition can change independently of one another. So even if their effects align at a given point in time, this can change as evolution progresses. For example, in a system containing a focal species and an interspecific

competitor, changes to resource availability (as in Grant and Grant, 2006) are likely to influence the strength of both intra- and inter- specific competition. It can be difficult to predict the ecological consequences of changes to resource supply because different species can respond differently and so result in changes to the balance of intra- to inter-specific competition. It quite likely that the balance of these competitive strengths, not just the total strength of competition, is important in determining evolutionary outcomes. Future experiments explicitly testing the effects of interactions between intra- and inter- specific competition on the evolution of diversity will be key to understanding these two linked processes.

2.7 Conclusions

Enthusiasm about the importance of competition in adaptive diversification has waxed and waned through the years (e.g. Stuart and Losos, 2013), however more recent empirical and experimental work have built a solid argument for its influence on the evolution of diversity (e.g. see Schluter, 2000b, this review). Our understanding of the effects of competition at moderate strengths is quite good, but notable exceptions to the traditional expectations means that there is still much more that we need to understand. Based on our current empirical and experimental understanding of these processes, it is still difficult (or even impossible) to predict when competition will drive versus impede adaptive diversification particularly in complex systems, and in this light, future work should be focused on looking closely at the exceptions to the more traditionally predicted competition-diversity relationships. Perhaps a greater awareness of the potential multi-faceted roles of intra- and inter-specific competition will help us to uncover more examples of exceptions. Identifying and exploring more of these alternate relationships will help us to build a fuller understanding of the role of this important ecological process in helping or hindering

adaptive evolution of the diversity of life we see today.

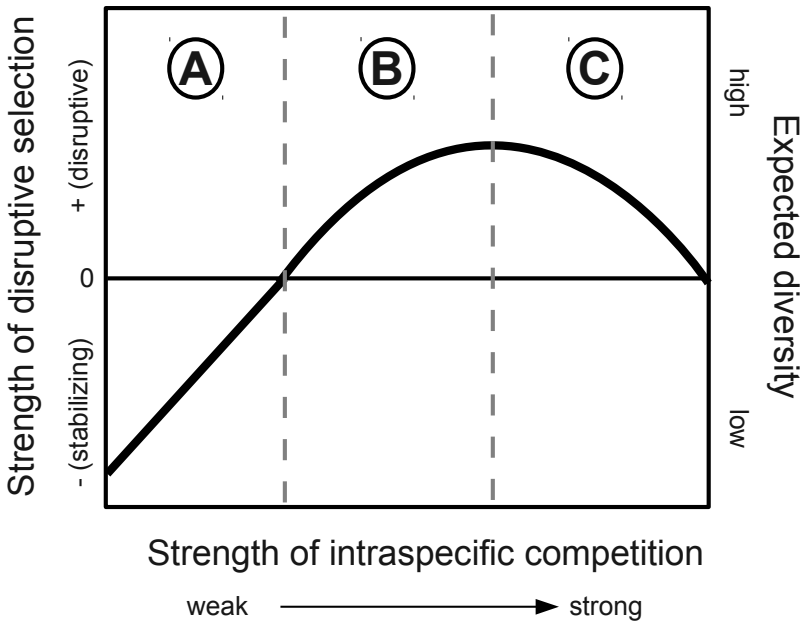

Figure 2.1: Expected relationship between strength of intraspecific competition, and disruptive selection and diversity. Three distinct regions are identified. Region A: When intraspecific competition is weak, selection is weakly disruptive or even stabilizing, leading to low expected diversity. Region B: As the strength of intraspecific competition increases, strength of disruptive selection increases, and so the potential for adaptive diversification increases. Region C: As the strength of intraspecific competition continues to increase, strength of disruptive selection begins to decrease as intense competition lowers population density and so decreases the benefit of diversifying onto alternate resource types. Region B follows traditional ideas regarding the relationship between intraspecific competition and diversity, however theoretical models suggest that the relationship in region C may also be a general pattern. The switch to stabilizing selection in region A may or may not hold for any given system, and depends on the details of resource utilization (Abrams et al., 2008).

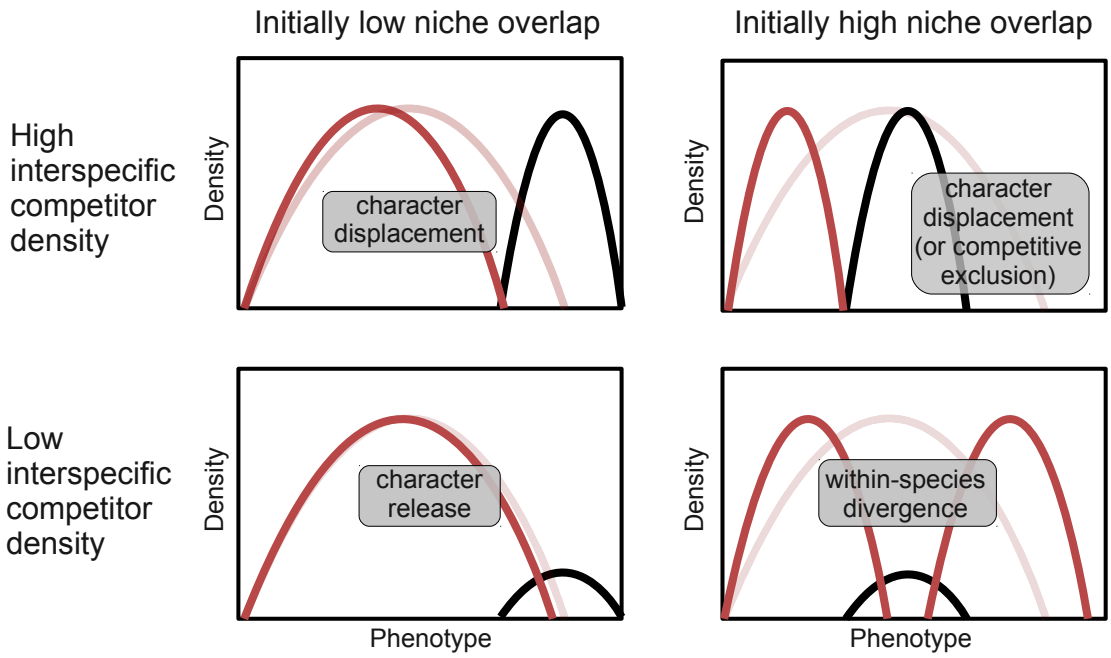

Figure 2.2: Expected consequences of changes in the strength of interspecific competition via 1) changes to competitor density (top panels: high density, bottom panels: low density) and 2) changes in the degree of niche overlap (left panels: low overlap, right panels: high overlap). The black curves indicate the phenotype distribution of the competitor populations, while the red curves represent the phenotype distribution of the focal population. Transparent red indicates the distribution in the absence of interspecific competition and solid red shows the realized distribution when interspecific competitors are present.

Table 2.2: Experimental tests of the effect of intraspecific competition on adaptive diversification.

	Study system	Effect of increased intraspecific competition on diversity	How was competition manipulated?	Evidence	Heritable variation?	References
1)	Arizona tiger salamander (*Ambystoma tigrinum nebulosum*)	increased	Initiated populations at different densities and added nutrients to increase abundance of food resource.	Decreases in per capita resource availability increased diversity.	no	Maret and Collins (1997)
2)	*Drosophila melanogaster*	increased	Varied amount of available food resource.	Diversity increased more rapidly with decreased resource availability.	yes	Bolnick (2001)
3)	Eurasian perch (*Perca fluviatilis* L.)	decreased	Varied amount of available food resource.	Morphological divergence decreased with decreasing food supply.	no	Olsson et al. (2006)
4)	Flour beetles (*Tribolium castaneum*)	increased	Initiated populations at different densities.	Diversity in resource consumption increased with increased density.	likely yes	Agashe and Bolnick (2010)
5a)	*Pseudomonas fluorescens*	increased	Initiated populations with different genotypes that varied in their growth rates and so population densities.	Diversity increased with increased population density.	yes	Bailey et al. (2013a)
5b)	*Pseudomonas fluorescens* with a phage	increased	Presence of phage decreased population density.	Less diversity evolves when population density is reduced by the presence of phage.	yes	Buckling and Rainey (2002)
5c)	*Pseudomonas fluorescens* with a predator	increased	Presence of predator decreased population density.	Diversity evolves more slowly and frequency dependent selection is reduced when population density is reduced by the presence of predators.	yes	Meyer and Kassen (2007)
6)	Spadefoot toads (*Spea multiplicata* and *S. bombifrons*)	increased	Initiated populations at different densities and varied amount of available food resource.	Frequency dependent selection between morphotypes, disruptive selection, and diversity all increase with increased population density.	no, but*	Martin and Pfennig (2010, 2009)
7)	Three-spine stickleback (*Gasterosteus aculeatus*)	increased	Initiated populations at different densities.	Population niche breadth (but not individual niche breadth) and disruptive selection increased with increasing population density.	yes	Bolnick (2004); Svanbäck and Bolnick (2007)

*Plastic response in this study, but there are heritable differences in the degree of plasticity between natural populations (Martin and Pfennig, 2011).

Table 2.3: Experimental tests of the effect of interspecific competition on adaptive diversification.

	Study system	Effect of increased interspecific competition on: within-pop'n diversity	Effect of increased interspecific competition on: between-pop'n diversity	How was competition manipulated?	Evidence	Heritable variation?	Key references
1)	Arctic charr (*Salvelinus alpinus*) and brown trout (*Salmo trutta*)	not addressed	increased	Initiated charr populations with and without trout competitors.	Presence of trout results in directional selection in charr, away from the trout phenotype; co-adapted charr are less affected by the presence of trout.	yes	Guénard et al. (2012); Forseth et al. (2003)
2)	Caribbean damselfish (*Stegastes planifrons*, *S. partitus* and *S. variabilis*)	decreased	not addressed	Competitor species was removed/ not removed from reef patches.	Detected differences in population size and habitat use resulting from competitor species removal.	not tested	Robertson (1996)
3)	*Escherichia coli*	not addressed	increased	Sympatrically evolved divergent morphotypes were grown separately, then grown together.	Detected phenotypic convergence of morphotypes when grown separately and then divergence when grown together again.	yes	Tyerman et al. (2008)
4)	Ivyleaf morning glory (*Ipomoea hederacea*)	not addressed	same or increased	Initiated populations with and without competitor species (*Ipomoea purpurea*).	The presence of the competitor species drives directional selection away from the competitor phenotype, but genetic correlations between traits impede this process.	yes	Smith and Rausher (2008b,a)
5)	Protists (*Colpoda* spp.)	not addressed	decreased and increased	Initiated populations with and without competitor species (*Pseudocyrtolophosis alpestris*).	Observed both divergence and convergence, depending on the trait, in the presence of a competitor species.	not tested	TerHorst (2011)
6)	*Pseudomonas fluorescens*	decreased[1,2] and increased[2]	not addressed	Initiated populations with and without competitor strains. Competitor strains varied in ecotype[1], fitness[2], and propensity to diversify[2].	The presence of many competitor strains impeded diversification[1,2], however some weak competitors drove diversification[2].	yes	[1]Brockhurst et al. (2007) [2]Bailey et al. (2013a)
7)	Skinks (*Niveoscincus greeni* and *N. microlepidotus*)	decreased	increased	Initiated populations with and without competitor species.	*N. microlepidotus* restricts its habitat range in sympatry, and species differences in body size were greater in sympatry than in allopatry.	not tested	Melville (2002)

8)	Spadefoot toads (*Spea bombifrons* and *S. Multiplicata*)	decreased	increased	Initiated populations with and without competitor species.	Increased interspecific competition results in increased character displacement and increases disruptive selection.	no, but*	Pfennig and Pfennig (2010)
9a)	Threespine stickleback benthic and limnetic (*Gasterosteus aculeatus* species complex)	not addressed	increased	Initiated populations of benthics with and without limnetics.	Presence of limnetic species selects for divergent phenotype in the benthic species.	yes	Schluter (1994)
9b)	Threespine sticklebacks (*Gasterosteus aculeatus*) with trout (*Oncorhynchus clarki*) and sculpin (*Cottus asper*) competitors	decreased	not addressed	Initiated populations with and without sculpin and/or trout competitor species.	Absence of trout resulted in population-level niche expansion, while absence of sculpins resulted in individual-level niche expansion.	yes	Bolnick et al. (2010)
9c)	Three-spine stickleback benthic and limnetic (*Gasterosteus aculeatus* species complex) with predators	not addressed	decreased	Initiated populations with and without cutthroat trout predators.	Presence of predators decreased benthic and limnetic population densities (and so competition), which resulted in increased divergent selection.	yes	Rundle et al. (2003)

*Plastic response in this study, but there are heritable differences in the degree of plasticity between natural populations (Martin and Pfennig, 2011).

Chapter 3

Competition both drives and impedes diversification in a model adaptive radiation

This chapter is reproduced from:

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Collaborator contributions:

J. Dettman conducted the experiments for the intraspecific competition part of this study. P. Rainey provided strains, lab facilities, and much guidance during my 4 month visit to his lab at Massey University, New Zealand.

Abstract

Competitors are known to be important in governing the outcome of evolutionary diversification during an adaptive radiation but the precise mechanisms by which they exert their effects remain elusive. Using the model adaptive radiation of *Pseudomonas fluorescens*, we show experimentally that the effect of competition on diversification of a focal lineage depends on both the strength of competition and the ability of the competitors to diversify. We provide evidence that the extent of diversification in the absence of interspecific competitors depends on the strength of resource competition. We also show that the presence of competitors can actually increase diversity by increasing interspecific resource competition. Competitors that themselves are able to diversify prevent diversification of the focal lineage by removing otherwise available ecological opportunities. These results suggest that the progress of an adaptive radiation depends ultimately on the strength of resource competition, an effect that can be exaggerated or impeded by the presence of competitors.

3.1 Introduction

The diversity of life is thought to have arisen in part through repeated adaptive radiations, the rapid diversification of a single lineage into an array of ecologically and phenotypically distinct species (Benton et al., 1995; Simpson, 1953). Competition is commonly cited as an important driver of diversification, especially when individuals share very similar resource requirements or niches and a wide range of under-utilized resources (ecological opportunities) are available in the environment (Yoder et al., 2010; Schluter, 2000b; Dieckmann and Doebeli, 1999). However competition can also prevent diversification if competitors use substantially different resources or sets of resources, thereby eliminating

ecological opportunity. The effects of competitors on diversification during an adaptive radiation should thus depend on the extent to which their niches overlap: closely overlapping niches promote diversification while very different niches prevent it.

The idea that competitors can have manifold, and even opposing, effects on diversification has not received focused attention. The traditional dichotomy is that competition between close relatives (often intraspecific competition) drives divergence via character displacement, whereas competition from other taxa (interspecific competition) inhibits divergence via niche pre-emption (Lack, 1947; Simpson, 1953; Schluter, 2000b). However this distinction is somewhat artificial as it fails to capture the idea that the essential differences are in the degree of niche overlap. In addition, the bulk of existing theory focuses on the conditions required to maintain diversity but not its origin. We thus know much about, for example, limiting similarity - the minimum niche differences required for two types to coexist (Kassen and Bell, 2000; Abrams, 1983), but we know much less about the details of the mechanisms that drove the evolution of those niche differences in the first place. Consequently, understanding of the multiple ways in which competition influences evolutionary adaptation and diversification in novel environments remains understudied.

Experimental work has shown that intraspecific competition can promote phenotypic divergence (Tyerman et al., 2008; Bolnick, 2004; Schluter, 1996) and expanded resource use (Bolnick et al., 2010; Persson and Hansson, 1999; Pacala and Roughgarden, 1985) by a population. Likewise, resource competition is often cited as an important driver of lineage diversification in microbial experiments (e.g. Rainey and Travisano, 1998) although this is more often because other potential mechanisms promoting diversification, like predation or parasites, are excluded by design. However, direct manipulative tests linking resource competition to lineage diversification are lacking. There is stronger

experimental evidence that competitors prevent diversification through niche pre-emption. Two microbial experiments, both involving the adaptive radiation of *P. fluorescens* in static microcosms, have shown that niche pre-emption can prevent diversification of the ancestral strain (Brockhurst et al., 2007; Fukami et al., 2007). Abundant comparative evidence has implicated interspecific competition in preventing diversification (e.g. Losos and Ricklefs, 2009; Svanbäck et al., 2008; Jonsson and Jonsson, 2001; Losos and Queiroz, 1997; Robinson and Wilson, 1994), and niche expansion (e.g. Dayan and Simberloff, 1994; Feinsinger and Swarm, 1982) during a radiation as well.

These results thus suggest that there is a continuum of effects that competitors can have on the diversification of a focal strain. At one end, individuals of the same or very similar genotype will tend to be ecologically similar (Burns and Strauss, 2011) and so will likely compete directly and intensely for resources (Cahill Jr et al., 2008; Violle et al., 2011), generating disruptive selection leading to diversification when ecological opportunities are available. The strength of competition under these conditions is determined by the degree of niche overlap, realized as the per capita effect of a competitor on an individual (i.e. competition coefficient; e.g. Brauer and Castillo-Chavez, 2001), and the number of individuals exerting that effect (i.e. population density). Thus, for a given competition coefficient, species or genotypes with lower population densities experience weaker resource competition, and so are expected to diversify to a lesser extent compared to those with high-density populations. At the other end are genetically and ecologically divergent genotypes, or even different species, whose resource use profiles are so distinct that they effectively do not compete with the focal strain for resources. Rather, they occupy what would (in their absence) be available niche space and so prevent diversification of the focal lineage. In between these two extremes, it is at least conceivable that a competitor can impact diversification through both mechanisms. Indeed, results from a

recent experiment show that evolutionary diversification of *P. fluorescens* occurs more rapidly in the presence an interspecific competitor - *P. putida*, and the authors suggest that this likely occurs because of increased competition for resources (Zhang et al., 2012).

Here we examine the manifold effects of competitors on diversification using the model adaptive radiation of *P. fluorescens* SBW25 (hereafter SBW25) cultured in spatially structured static microcosms. Previous work has shown that frequency-dependent selection is operating in this system (Rainey and Travisano, 1998) and this observation is highly suggestive that intra-specific competition for resources drives the resulting diversification. Our study is aimed at explicitly testing this mechanism and exploring in detail the potentially opposing effects of competition on adaptive diversification. Our strategy is to follow the rate and extent (measured as the number of morphologically distinct types) of diversification of SBW25 when it is co-cultured alongside competitors that vary in the strength of competition and in their ability to diversify. This approach allows us to disentangle experimentally the diversification-promoting effects of resource competition from the diversification-preventing effects of niche pre-emption, the two most common modes by which intra- and interspecific competitors, respectively, are thought to exert their effects on a radiating lineage.

We first ask how variation in the strength of intraspecific resource competition impacts diversification. In the absence of interspecific competitors, diversification is expected to occur only when resource competition is sufficiently strong to generate disruptive selection in the presence of abundant ecological opportunity. We test this prediction, manipulating the strength of resource competition experienced by a radiating lineage, by manipulating population density. While the effect of changes in population density driven by changes in the environment, on the evolution of diversity in this system has been previously documented (e.g. nutrient concentrations: Kassen et al., 2000; predation:

Meyer and Kassen, 2007), here we take a complementary approach holding environment constant and manipulating population density via genetic differences.

We then ask how competing genotypes (analogous to interspecific competitors), that differ both in their competitive fitness and in their ability to diversify, impact diversification in SBW25. While strong interspecific competitors are expected to out-compete a radiating lineage before the latter has diversified, weaker competitors may either have no effect or promote diversification by increasing the strength of resource competition experienced by the radiating lineage. However, if those competitors also exclude SBW25 from potential niches (i.e. niche pre-emption), their presence is expected to suppress the evolution of diversity in SBW25. Our use of diversifying and non-diversifying competitors allows us to test this idea directly.

3.2 Materials and methods

3.2.1 The *P. fluorescens* radiation

On its own, SBW25 diversifies rapidly and repeatably into characteristic niche specialist morphotypes smooth (SM), wrinkly spreader (WS), and fuzzy spreader (FS) (Rainey and Travisano, 1998) resulting from competition for nutrients and oxygen (Koza et al., 2010; Rainey and Rainey, 2003). These morphotypes are genetically distinct (Rainey and Travisano, 1998; Bantinaki et al., 2007) and are easily distinguishable on agar plates and employ distinct ecological strategies for acquiring oxygen in static microcosms: motile SM morphotypes are aerotactic and swim towards the air-broth interface, WS morphotypes construct a cellulose-like biofilm at the air-broth interface, and FS morphotypes form thin rafts at the air-broth interface that quickly collapse under their own weight and collect in the anoxic zone at the bottom of the microcosm (Ferguson et al., shed). Diversity

among the major morphs is stably maintained by negative frequency dependent selection (Rainey and Travisano, 1998) and within each morphotype class there are often additional phenotypically distinct colony morphs. Note that *P. fluorescens* is strictly asexual under our experimental conditions, meaning that all genotypes whether arising de novo through mutation or introduced by design as part of the experiment are evolutionarily independent lineages that are formally equivalent to species in a sexual system (Rainey and Travisano, 1998).

3.2.2 Fitness measures

We measured the relative fitness of any given strain compared to SBW25 with head-to-head competition experiments. Strains were inoculated as pure cultures from frozen for 24 hours in 6 ml shaken KB medium (28° C, 150 rpm). Then, 6 ml KB static microcosms were inoculated with 30 μl each of the focal strain and SBW25, and their frequencies estimated at 0 and 24 hours by plating on KB agar. We calculated the relative fitness of each strain using the following equation:

$$\omega = \left(\frac{F_{initial}/f_{initial}}{F_{final}/f_{final}} \right)^{(1/d)} \tag{3.1}$$

where $F_{initial}$ and F_{final} are the initial and final frequencies of the strain of interest, $f_{initial}$ and f_{final} are the initial and final frequencies of SBW25, and d is the number of generations or doublings.

3.2.3 Diversification dynamics

We tracked diversification over 7 days after inoculating replicate, static microcosms with a total 60 μl of culture containing 6 ml of KB at 28° C (as in Rainey and Travisano

(1998)). We destructively sampled three replicate microcosms every twelve hours for five days, then once a day for another two days. Evolved diversity was estimated by plating on KB agar and noting the morphology of at least 50 colonies. Diversity was measured as morphotype richness, the number of morphologically distinct types. When diversity is estimated using Simpson's index, which takes into account relative abundances in addition to richness, similar patterns over time and across treatments were observed; we report only richness estimates here. To prevent potential biases arising from differences in the number of colonies counted per sample, we exclude any morphotypes making up less than 2% (or 1 in 50 colonies) of the population from our diversity estimates.

The diversification dynamics of the strain of interest in each experiment were summarized by fitting the following modified logistic equation to diversity over time:

$$N(t) = \begin{cases} 1, & \text{if } t < t_{lag} \\ k/(1 + (k-1)e^{-r(t-t_{lag})}), & \text{if } t \geq t_{lag} \end{cases} \tag{3.2}$$

where $N(t)$ is diversity at time t, t_{lag} is the time until diversification begins to occur, r is the rate of diversification, k is the extent of evolved diversity. Using these parameter estimates, we took the value of t when $N(t) = 0.99k$ as an estimate of time until maximum diversity is reached, and k as an estimate of the extent of diversification. All model fitting and statistical analyses were performed using R version 2.13.1 (R Development Core Team, 2011).

3.2.4 Intraspecific competition

To examine the effect of varying the strength of intraspecific resource competition on diversification, we allowed genetically distinct strains of SBW25 carrying costly antibiotic resistance mutations (see Table A.1 in supplementary information) to diversify in static KB

microcosms. The rationale behind this manipulation of intraspecific resource competition comes from the simple logistic model describing density-dependent population growth (e.g. Brauer and Castillo-Chavez, 2001),

$$\frac{dN}{dt} = rN\left(1 - \frac{N}{K}\right) \tag{3.3}$$

where N is the population density, t is time, r is the intrinsic growth rate, and K is the carrying capacity. The genetically distinct strains used in this experiment do not differ in their carrying capacity (K in equation 3.3) when grown in static KB microcosms (see final densities in Fig. A.1 in supplementary information) and so do not differ in their per capita interaction strength ($1/K$). However, the strains do differ in their intrinsic growth rate (r) and so differ in their population densities until carrying capacity is reached after 2 to 6 days (see Fig. A.1 in supplemental materials). Those differences in density generate differences in the overall strength of competition (or the degree to which growth rate is slowed due to density dependence) experienced by individuals of each strain during the first 2 to 6 days - the same time period over which adaptive diversification tends to occur in this system (Rainey and Travisano, 1998). Thus, otherwise isogenic strains that have lower intrinsic growth rates and so lower initial population densities experience weaker resource competition. Strains resistant to the quinolone antibiotic nalidixic acid were originally generated using a conventional fluctuation assay (Kassen and Bataillon, 2006) to minimize the number of genetic differences from SBW25. Resistance results from mutations in genes not known to affect SBW25 diversification (gyrases: gyrA, gyrB; topoisomerases: parC, parE; efflux pump regulation: nfxB, mexR). Population density after 24 hours and fitness measured in head-to-head competitions (as described above), are well correlated in our strains (see Fig. A.2 in the supplemental material); we report competitive fitness measures here to facilitate comparison with our other experiments.

An examination of our fitness estimates for these eight strains showed that four strains fell into a "low fitness" group (mean fitness ± SE: 0.694 ± 0.012), and the other four strains fell into a "high fitness" group (mean fitness ± SE: 0.971 ± 0.010). Within these groups we were unable to detect significant differences in fitness, despite each strain having a unique genotype. For this reason we test for an effect of fitness on the extent of evolved diversity and time until maximum diversity is reached in a categorical manner, grouping the strains appropriately and using two-sample t-tests.

3.2.5 Interspecific competition

To examine the combined effects of resource competition and niche pre-emption on diversification, we tracked the dynamics of SBW25 diversification in the presence of competitor strains that varied in both their propensity to diversify and their fitness relative to SBW25. We use relative fitness of the competitor strain as a measure of relative strength of interspecific competition against SBW25 as competitor strains with a high relative fitness will grow more quickly, resulting in a higher density and so greater total competitive strength compared to competitor strains with low relative fitness. Six strains were evolved derivatives of SBW25 capable of diversifying into the same range of niche specialists as SBW25 (DIV+: strains A-F), while four were evolved from PBR716 (McDonald et al., 2009), an SBW25 deletion strain that lacks the key operons involved in adaptation to the air-broth interface making it severely compromised in its ability to diversify in static microcosms (DIV-: strains 1-4). DIV- strains thus exert their effects on the focal strain through resource competition while DIV+ strains compete via resource competition and niche pre-emption. All competitor strains are morphologically SM. Fitness relative to SBW25 ranged from 0.71 (weak competitors) to 1.22 (strong competitors). The four low fitness DIV+ competitors were isolates from *P. fluorescens*

SBW25:lacZ+ populations evolved for 1000 generations in 2 ml shaken M9 salts media plus either glucose, mannose, xlyose, or all three sugars (from Bailey and Kassen, 2012). The two high fitness DIV+ competitors were isolates from *P. fluorescens* SBW25:lacZ+ populations evolved in 6 ml static KB for 8 days (from Meyer et al., 2011). The four DIV- competitors were isolates evolved from *P. fluorescens* PBR716 in static microcosms containing 6 ml KB, transferred to fresh microcosms every day for 2-6 days. See Table A.1 in the supplemental materials for further details about the strains used. All competitor strains contain a neutral genetic marker enabling us to distinguish them from SBW25 (Zhang and Rainey, 2007).

The experiment was initiated with 30 μl of the focal SBW25 strain and 30 μl of a competitor strain. For logistical reasons, this experiment was run in four blocks (block 1: SBW25 + strain A, SBW25 + strain B; block 2: SBW25 + strain C, SBW25 + strain D; block 3: SBW25 + strain E, SBW25 + strain F; block 4: SBW25:lacZ + strain 1, SBW25:lacZ + strain 2, SBW25:lacZ + strain 3, SBW25:lacZ + strain 4). Each block included a replicated control treatment - SBW25 evolving in the absence of any competitor strain. As in the intraspecific experiment, we destructively sampled three replicate microcosms every twelve hours for five days, then once a day for another two days. To quantify diversity over time, we again aimed to classify and count at least 50 plated colonies per strain (i.e. both SBW25 and the competitor) per sample; however this was not always possible for some replicates of the two weakest competitor strains treatments after day 5, as they were essentially out-competed.

Diversification dynamics were summarized for both the focal strain and the competitor strain in each treatment by fitting equation 4.2. Parameter estimates for the focal strain were then adjusted by estimates from the appropriate competitor-absent control in order to remove any potential block effects. The adjusted extent of diversity and time

until maximum diversity is reached were then compared across competitor fitness and competitor diversification potential (DIV-/ DIV+) using ANOVAs.

3.2.6 Density-diversity relationships

To investigate the role played by resource competition stemming from interspecific competition on diversification, we regressed SBW25 diversity on density in the presence and absence of DIV+ and DIV- competitors. To control for block effects and to account for temporal autocorrelation in the data, we first subtracted the density and diversity, respectively, of SBW25 diversifying on its own from the comparable value when it diversified in the presence of a competitor. The resulting data thus represent the marginal increase (or decrease) in density and diversity caused by the presence of a competitor at a given time point. The SBW25 density-diversity relationship was tested using an ANOVA with adjusted diversity as the dependent variable and adjusted density, competitor treatment (no competitors/ DIV- / DIV+), and their interaction as independent variables, nesting strain within competitor treatment. Adjusted density data was log-transformed before analysis to meet model assumptions.

3.2.7 Carbon niche similarity

Although the strains used in this study differ in their competitive fitness, they are all morphologically and ecologically identical to the ancestral SBW25, that is, they have smooth colony morphologies and grow in the broth phase of the static microcosms. We attempted to further characterize the niche of each strain by measuring its carbon metabolism profile using BIOLOG GN2 plates. BIOLOG GN2 plates are 96-well microwell plates containing 95 different carbon sources plus a carbon-absent control well. Each strain was grown-up overnight from frozen in vials containing 6 ml KB media (28° C, shaken at

150 rpm), starved for 2 hours (20 μl of each culture in 20 ml M9 minimal salts at 28° C, 150 rpm), and then 150 μl was transferred into each well of the BIOLOG plates. Optical density (at 660 nm) was measured at the time of inoculation and after 24 hours of growth, and growth on each carbon substrate was calculated as r = ln($OD_{initial}$)-ln(OD_{final}). These growth rates then were adjusted by subtracting the maximum growth rate estimate obtained from all the control wells. Growth rate data were then converted into binary growth/ no-growth data by setting all positive growth estimates to 1 and all zero or negative growth estimates to 0. Niche similarity with SBW25 was calculated as the percentage of a focal strain's growth/ no-growth niche profile that is identical to that of SBW25. Niche similarity was then compared across strain fitness and between DIV+ and DIV- groups to look for any potentially confounding effects. Data from this study were deposited in the Dryad repository (Bailey et al., 2013b).

3.3 Results

3.3.1 Intraspecific competition

We found a clear effect of population density, and so strength of resource competition, on diversification (Fig. 3.1 A and B) with low fitness strains (light grey) diversifying less than high fitness strains (dark grey), as expected. Specifically, the extent of diversity (estimated using a modified logistic model) increases significantly with fitness (two-sample *t*-test, $T_6 = 5.31$, P = 0.002; Fig. 3.1 B) as expected. Time to maximum diversity showed no relationship with fitness (two-sample *t*-test, $T_6 = 0.0613$, P = 0.953; see Table A.2 in the supplemental materials for parameter estimates).

3.3.2 Interspecific competition

We found a striking difference in the effect of DIV+ and DIV- competitors on the rate and extent of SBW25 diversification. Inspection of Figs 3.2 A and B reveals that strong DIV+ competitors (dark grey lines) almost completely prevent SBW25 diversification, as expected, but strong DIV- competitors do not. More quantitatively, DIV+ strains slow the rate of SBW25 diversification (*t*-test, $T_5 = 3.7214$, $P = 0.01369$) but DIV- strains do not (*t*-test, $T_3 = 1.6102$, $P = 0.2057$) independently of competitor fitness (linear regression, $P = 0.2914$; Fig. 3.3 A). There is a negative relationship between competitor fitness and the extent of SBW25 diversity for both DIV+ and DIV- strains (Fig. 3.3 B; ANOVA: main effect of competitor fitness, $F_{1,6} = 31.08$, $P = 0.0014$), although this relationship is stronger for the DIV+ competitors than for the DIV- competitors (ANOVA: competitor fitness x competitor type, $F_{1,6} = 7.63$, $P = 0.0327$; DIV+ slope: -6.15 , DIV- slope: -1.41). Note that the sign of the effect depends on the fitness of the competitors: weak competitors cause diversity to be higher, while strong competitors cause it to be lower, than would be the case in the absence of competitors. Proximately, this extra diversity is not due to the occurrence of unusual or rare morphotypes but rather is associated with more types within either the WS or SM niche classes. Note also that a regression of the realized extent of competitor diversity on SBW25 diversity shows a significant negative relationship for DIV+ competitors, but no relationship for DIV- competitors (Fig. 3.4 A; DIV+: $P = 0.0032$, DIV-: $P = 0.819$), however the two slopes are not significantly different from each other (ANCOVA, interaction of competitor type and competitor diversity: $F_{1,6} = 1.2488$, $P = 0.3065$). These results are consistent with the idea that niche pre-emption is the mechanism by which DIV+ competitors prevent diversification.

3.3.3 Density-diversity relationship

Figure 3.4 B shows a significant positive relationship between SBW25 density and SBW25 diversity across all treatments (nested-ANOVA; adjusted density: $F_{1,12} = 262.452$, $P < 0.0001$). The marginal effect of an increase in SBW25 density on diversity is larger in the presence of non-diversifying (DIV-) competitors and smaller in the presence of diversifying (DIV+) competitors than when competitors are absent altogether, a result confirmed both by a marginally significant effect of competitor type ($F_{2,12} = 3.691$, $P = 0.05631$) and a significant interaction between adjusted density and competitor type ($F_{2,12} = 7.631$, $P = 0.00727$). Over the range of densities where SBW25 experiences antagonistic effects (adjusted log(density) < 0), the effect of adding competitors is to increase SBW25 diversity when the competitors are unable to diversify (DIV- competitors) and to lower it when they can (DIV+ competitors). This result provides direct support for the idea that weak, non-diversifying competitors increase the effective amount of resource competition experienced by the radiating lineage. Weak diversifying competitors, on the other hand, act to impede diversification of SBW25 by radiating to occupy what would otherwise be available niche space.

3.3.4 Carbon niche similarity

There is no evidence of any relationship between competitor strain fitness and carbon-niche similarity with SBW25 (Fig. A.3; linear regression: $P = 0.702$). In addition, there is no relationship between competitor carbon-niche overlap with SBW25 and evolved diversity in SBW25 (Fig. A.4; linear regression: $P = 0.9779$).

3.4 Discussion

We have investigated the effects of different kinds of competition on the rate and extent of diversification in a model adaptive radiation. Briefly, our main results are: (1) the extent of diversification is directly, positively related to the strength of intraspecific resource competition; (2) interspecific competitors can prevent diversification through niche pre-emption or, if they are particularly strong competitors, through resource competition; (3) weak interspecific competitors can, counter-intuitively, promote diversification by increasing resource competition. We discuss each of these results in turn below.

3.4.1 Intraspecific competition

We found that the extent, but not the rate, of diversification increases significantly with the strength of intraspecific resource competition. More specifically, a cost of adaptation caused by the presence of antibiotic resistance mutations kept population densities low enough to prevent substantial diversification. Evidently there is some threshold level of population density that must be reached for the pronounced and rapid diversification characteristic of other experiments with the *P. fluorescens* system to proceed. To the extent that this model system represents a good guide to adaptive radiations in the 'real' world, it suggests that simply gaining access to novel ecological opportunities is not by itself sufficient to drive diversification. Population densities must also be high enough to generate strong resource competition capable of generating disruptive selection.

This result is somewhat different from that observed in previous experiments with this system where the presence of predators that reduce population densities, and so the strength of resource competition, slowed the emergence of diversity but did not change the extent of diversification (Meyer and Kassen, 2007). Predation has been shown to promote the emergence of wrinkly spreader morphs that gain protection from predation by virtue

of being part of the biofilm (Meyer and Kassen, 2007; Hall et al., 2008), suggesting that the high levels of diversity observed under predation are due to a fortuitous alignment of selective forces where predation-resistance and resource competition both favour biofilm formation. Without the additional selective advantage of predation-resistance driving diversification, in our experiment diversity differences between the strong and weak competition treatments emerge early on and are then maintained throughout the course of the experiment.

An alternative explanation for our results is that the resistance mutations themselves have unknown pleiotropic effects that in some way compromise diversification independently of their effects on resource competition. This explanation seems unlikely because, as we noted earlier, none of the mutations conferring resistance occur in genes known to be involved in diversification into the broad niche classes in this system (SM, WS, FS). Two of the naladixic acid resistant strains we used do have large multi-gene deletions that might be expected to have wide reaching effects, however removal of those two strains from the analysis did not qualitatively change our results. Furthermore, all resistant strains diversified into the three available broad niche specialist classes; differences in diversity arise from differences in the number of types that evolve within the broad niche classes. We were also unable to detect any significant differences between these strains in their carbon-niche profiles (see Fig. A.5 in the supplemental materials). Thus, there appears to be no reason to expect potential pleiotropic effects to vary systematically across fitness and/or diversification potential in a way that would confound our results.

3.4.2 Interspecific competition

Our co-culture experiments show clearly that the dynamics of diversification can be modulated by the strength of interspecific competition. Strong competitors can prevent

diversification through a combination of niche pre-emption and competitive exclusion. Weak competitors, surprisingly, can actually promote diversification, presumably because competitor density is sufficiently high in the initial phases of the radiation to increase the strength of resource competition experienced by the radiating lineage but not so high that they themselves diversify.

Direct evidence for both niche pre-emption and resource competition as significant mechanisms modulating the outcome of competition during the radiation comes from a regression of the extent of competitor diversity on SBW25 diversity. The slope of this regression is negative for DIV+ competitors but not for DIV- competitors, consistent with the idea that competitor diversification leads to niche pre-emption and prevents SBW25 diversification (Fig. 3.4 A). This result is also consistent with that seen in other experiments with this system where niche pre-emption has been directly manipulated (Brockhurst et al., 2007; Fukami et al., 2007) and cannot be explained by pre-existing differences in metabolic niche space, as the competitors and SBW25 do not differ in carbon-use profiles (t-test, $P = 0.1604$). Our results thus lend further support to the idea that interspecific competitors can prevent diversification of a focal lineage by removing or reducing the extent of ecological opportunity through niche pre-emption. Note also that strong, non-diversifying competitors also prevented diversification but, because they were unable to radiate themselves, they necessarily did so through direct resource competition that resulted in the competitive exclusion of SBW25. Taken together, these results provide direct evidence that interspecific competitors can prevent diversification through niche pre-emption, and at its extreme this leads to competitive exclusion.

In general, it is competition for resources that drives the adaptive radiation of SBW25 in this system, and the positive relationship seen between density and diversity in fig. 3.4 B supports this. While the presence of interspecific competitors modifies the details of

this relationship, it is always the case that a higher density SBW25 population results in increased SBW25 diversity. However, the marginal effect of an increase in SBW25 density on diversity is larger in the presence of non-diversifying (DIV-) competitors, and smaller in the presence of diversifying (DIV+) competitors, than when competitors are absent altogether. This result suggests that non-diversifying competitors tend to increase the effective amount of resource competition experienced by the radiating lineage, while diversifying competitors tend to impede the radiation through niche pre-emption. Interestingly, the additional diversity that arises in the presence of weak interspecific competitors is not due to the occurrence of unusual or rare morphotypes but rather is associated with more types within the SM and WS broad niche classes. As before, these results cannot be explained by pre-existing differences in carbon-use as there is no relationship between competitors strain carbon-use and competitor strain fitness, and no relationship between competitor strain carbon-use and evolved SBW25 diversity.

3.4.3 Summary

We have provided direct evidence that the extent of diversification under intraspecific competition is determined by the strength of resource competition. Interspecific competitors, by contrast, can either increase the strength of resource competition experienced by a radiating lineage, promoting diversification, or remove ecological opportunities available to it, and so prevent diversification. Which of these two outcomes will be realized in any 'real' radiation will thus depend on both the strength of the competitor itself as well as its ability to diversify. In our study, "ability to diversify" was a constructed genetic property particular to our experimental design; however in nature, we expect differences in species' underlying genetic architecture to cause populations to vary in their propensity to diversify in any given environment (McDonald et al., 2009).

An incipiently radiating lineage that gains access to novel ecological opportunities in the presence of a weak competitor, or a competitor that is incapable of diversifying rapidly, will find little obstacle to diversification and may even diversify more extensively than it would in the absence of the competitor. It is possible that additional diversity driven by the presence of weak competitors contributes to the 'overshooting' pattern observed in some extant radiations (e.g. Seehausen, 2006), with diversity peaking and then dropping again as those competitors are eventually lost. The extra diversity that comes through the presence of a weak competitor also constitutes an example of how the presence of one type can cause diversification in another, an example of 'diversity begetting diversity' (e.g. Forbes et al., 2009; Emerson and Kolm, 2005). By contrast, the presence of a strong competitor that is itself capable of diversifying, or one that has already diversified (Brockhurst et al., 2007; Fukami et al., 2007), will prevent diversification in the focal lineage. These results also bear on the issue of the evolutionary fate of invasive species outside their native range: an exotic species that colonizes a novel environment that lacks the usual suite of competitors, or is itself a stronger competitor against existing native species, should be expected to diversify. As a whole, our study underlines the complexities of competition's important role in the evolution of diversity and helps to focus our understanding of the ways in which competition may be at work in other systems.

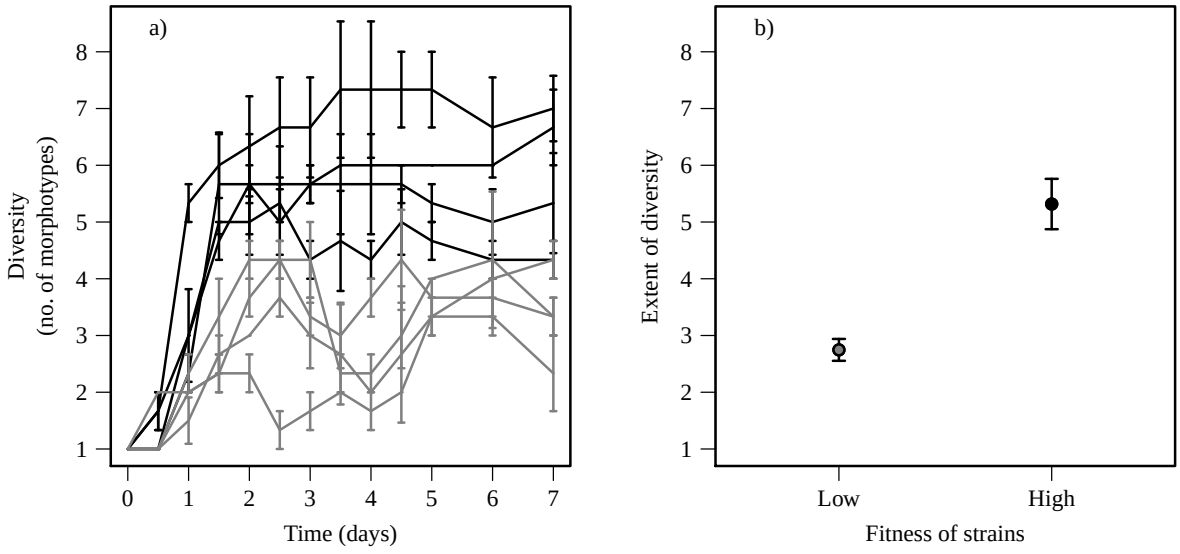


Figure 3.1: a) Diversity of low-fitness (grey) and high-fitness (black) naladixic acid resistant strains over time. Diversity is the number of unique colony morphotypes making up more than 2% of the population (mean ± 1 s.e.m., N=3). b) Extent of diversity of low-fitness and high-fitness naladixic acid resistant strains. Points represent the mean extent of diversity of four strains ± 1 s.e.m. Extent of diversity was estimated for each strain using a 3-parameter logistic model.

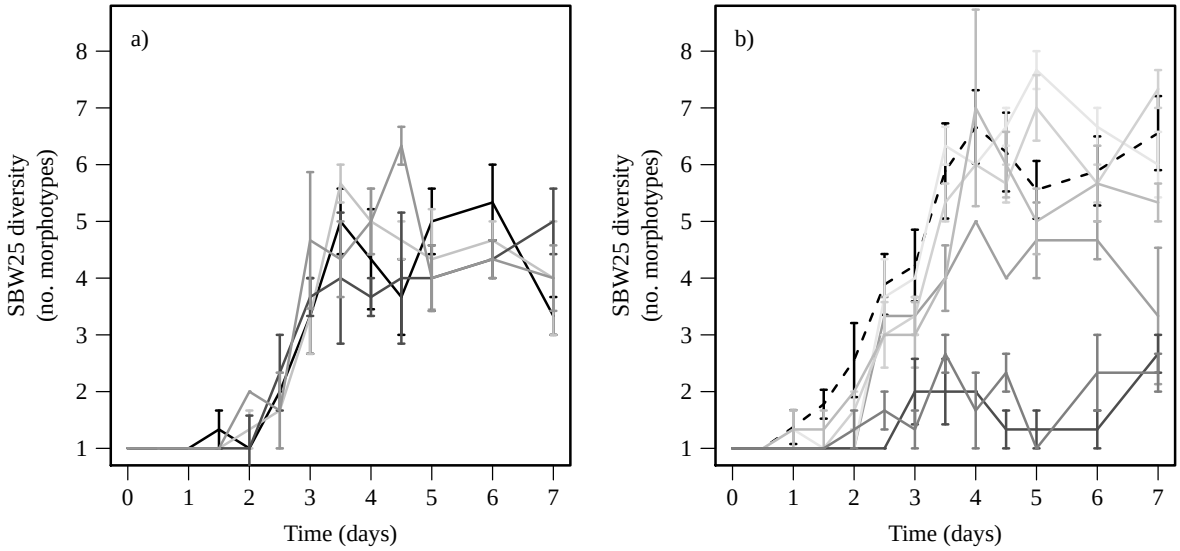


Figure 3.2: Diversity of SBW25 in the presence of a) DIV- competitors and b) DIV+ competitors over time, where light to dark grey corresponds to low to high competitor fitness. Each panel includes SBW25 in the absence of competitors (dashed black line), for that experiment. SBW25 diversity is the number of unique colony morphotypes making up more than 2% of the population (mean ± 1 s.e.m., N=3).

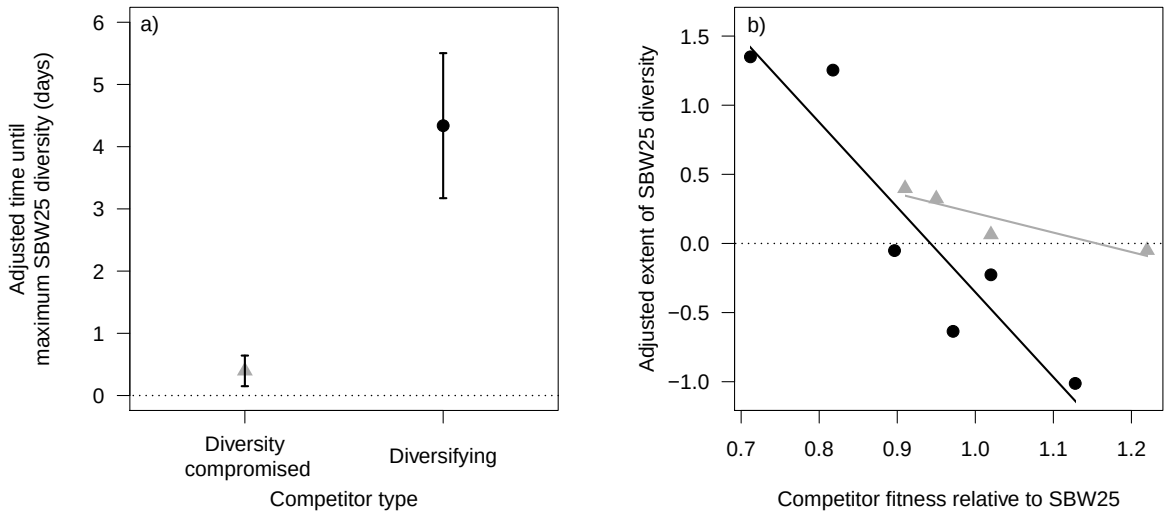


Figure 3.3: a) Adjusted time until maximum SBW25 diversity is reached the presence of DIV- and DIV+ competitors. b) Adjusted extent of SBW25 diversity in DIV- (grey triangles) and DIV+ (black circles) competitors, plotted against fitness of the competitor relative to SBW25. Statistically significant regression lines are shown. Time until maximum diversity and extent of diversity were estimated using a 3-parameter logistic model (see 3.2.1) and adjusted by parameter estimates for SBW25 in the absence of competitor.

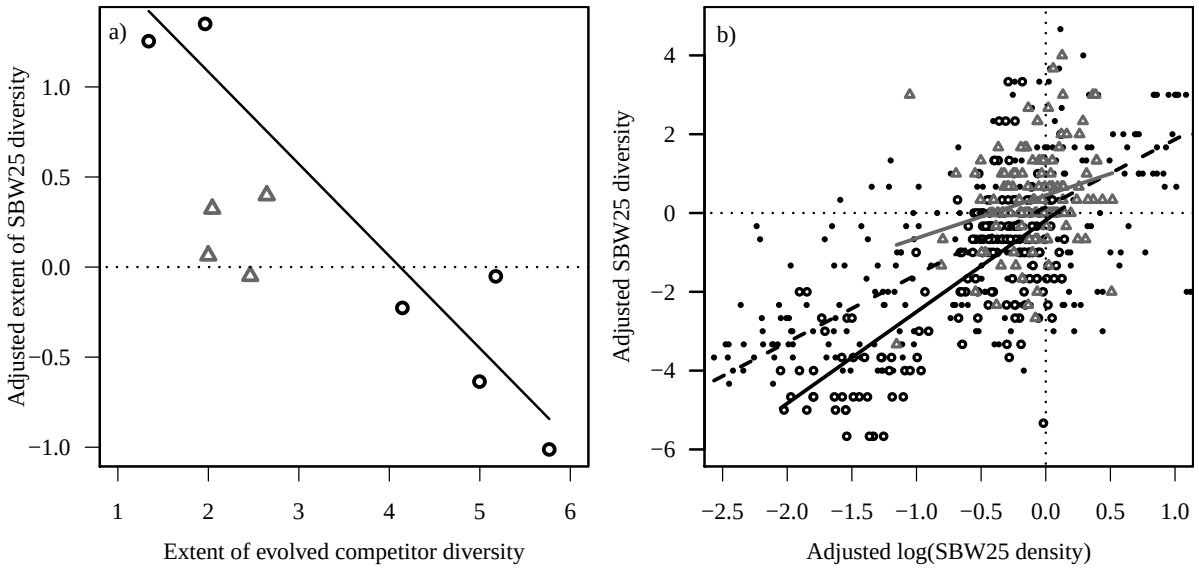


Figure 3.4: a) Adjusted extent of SBW25 diversity in the presence of DIV+ (black circles) and DIV- (grey triangles) competitors, plotted against extent of evolved competitor diversity. Statistically significant regression lines are shown. b) SBW25 diversity in the presence of DIV+ (black open circles, black solid line), the DIV- (grey open triangles, grey solid line) competitors, and the absence of competitor strains (black points, black dashed line), plotted against log-transformed SBW25 density. Diversity and density measures were adjusted by SBW25 density and diversity measures in the absence of competitor strains at the same time point. Lines show regression fits for each group.

Chapter 4

Spatial structure of ecological opportunity drives adaptation

This chapter is reproduced from:

Bailey, S.F. and Kassen, R. 2012. Spatial structure of ecological opportunity drives adaptation in a bacterium. *American Naturalist* 180(2): 270-283.

Abstract

Abundant ecological opportunity is thought to drive adaptation and diversification. The presence of multiple opportunities leads to divergent selection, which can slow adaptation when niche-specific beneficial mutations have antagonistically pleiotropic effects. Alternately, competition for multiple opportunities can generate divergent selection leading to high rates of adaptive differentiation. Which outcome occurs may depend on the spatial structure of those ecological opportunities. In a mixture of resources, competition for multiple opportunities can drive divergent selection; however if each resource is available in a spatially distinct patch, competition for multiple opportunities

simultaneously cannot occur. We report the effects of extent and spatial structure of ecological opportunity on the evolutionary dynamics of populations of *Pseudomonas fluorescens* over 1000 generations. We varied extent of ecological opportunity by varying the number of sugar resources (mannose, glucose, and xylose), and varied spatial structure by providing resources in either mixtures, or spatially distinct patches. We saw that a particularly novel resource (xylose) drove the rate of adaptation when in a mixture but had no effect on diversity. Instead we saw the evolution of a single adaptive strategy that differed with respect to phenotype and degree of specialization, depending on both the extent and spatial structure of ecological opportunity.

4.1 Introduction

It is widely appreciated that rates of both adaptation and diversification can vary markedly across taxa and environments (Gaston, 2000). At one extreme are groups of living fossils that exhibit extremely low rates of diversification and have persisted nearly unchanged for long periods of evolutionary time (e.g. Ginkgo trees: Royer et al., 2003; Lungfish: Lee et al., 2006). At the other are adaptive radiations, where rates of both adaptation and diversification are typically very high (e.g. Columbines: Hodges, 1997; Galapagos finches: Grant, 1999). A range of hypotheses have been ventured to explain what might control this remarkable variation including serendipity, genetic constraints (Blows and Hoffmann, 2005), and sexual selection (Panhuis et al., 2001), although perhaps the most compelling explanations - in part because they are the most general - are those that involve ecological factors such as the pattern of environmental variation and the extent of ecological opportunity. That both factors are commonly cited drivers of adaptive radiation lends support to this idea (Schluter, 2000b; Kassen, 2002; Benton and Emerson, 2007; Kassen, 2009). Here we explore their contribution to governing the dynamics of

ecological diversification and adaptation in evolving populations of microbes.

Ecological diversification occurs because divergent selection, generated ultimately by environmental heterogeneity, leads to the evolution of niche specialist types that have high fitness in one environment and lower fitness in others. The conditions favouring the evolution of specialization are well understood. Briefly, specialization evolves most readily in spatially, rather than temporally, variable environments (Felsenstein, 1976; Futuyma and Moreno, 1988; Kassen, 2002) when selection is strong and dispersal among patches is low. The extent of diversification, measured as the number of distinct niche specialists, depends on the number of ecological opportunities afforded by the environment (ecological opportunity here refers to the number of niches or underutilized resources available): the greater the ecological opportunities, the more diversity can be supported (MacArthur and Levins, 1964; Tilman, 1982).

Understanding the rate and extent of adaptive divergence, by which we mean the rate of phenotypic change and final difference in fitness between an evolved population and the ancestor from which it was derived, requires that we consider more precisely the genetics of adaptation to a spatially heterogeneous environment. As a starting point, it is useful to consider the simplest case, selection in an unchanging, uniform environment in the absence of dispersal. We know from Fisher's fundamental theorem that under these conditions the rate of adaptation is governed by the strength of selection and the quantity of genetic variation available (Fisher, 1930). Adaptation will proceed by first sorting among extant variation, if it exists, and then through the sequential substitution of beneficial alleles until all new variation introduced by mutation is deleterious. The rate at which niche specialization evolves depends on how the genes that are substituted in this environment affect fitness in alternate environments. If these genes have antagonistically pleiotropic fitness effects in different environments, then specialization can evolve rapidly.

But, if pleiotropic effects in a novel environment are neutral or positive, specialization will take much longer to evolve because it requires the accumulation of mutations neutral in the environment of selection but deleterious elsewhere.

In spatially varying environments, the situation is more complex for two reasons. The first is that selection can act in multiple directions at the same time. This effectively pulls the population in different directions at once, which has the effect of slowing the rate at which genes with environment-specific effects are substituted (Whitlock, 1996) and reducing the extent of adaptation due to genes with antagonistically pleiotropic effects because progress in one direction means regress in another (Dickerson, 1955; MacLean et al., 2004). The second is that dispersal will reduce the effectiveness of selection at generating adaptation because mal-adapted types are constantly re-introduced into the population from elsewhere (Lenormand, 2002; but see Perron et al., 2007). The result is that both the rate and the extent of adaptation, and diversification, should be reduced in a heterogeneous environment compared to a uniform environment.

The opposite result - faster rates of diversification and more extensive adaptation in heterogeneous environments - can occur if resource competition is strong and genes affecting resource use have antagonistically pleiotropic effects (i.e. there are trade-offs in fitness across resources). Resource competition generates selection against phenotypically intermediate or generalist types, and promotes the evolution of specialization by causing divergence of phenotypic characters associated with resource use. The result can be character displacement, exaggerated phenotypic differences compared to what would have been observed in the absence of resource competition (MacArthur and Levins, 1964; Dayan and Simberloff, 2005; Tyerman et al., 2008), and faster rates of diversification (e.g. Barrett and Bell, 2006).

Whether environmental heterogeneity promotes or slows diversification thus depends on

the effectiveness of resource competition at promoting phenotypic divergence and ecological specialization. We suggest that the opportunity for competition-driven divergence will depend on the nature of environmental heterogeneity experienced by an evolving lineage. If the environment is composed of a series of patches each containing a different resource but linked through dispersal, then a lineage may experience just one patch over its lifetime and competition for alternative resources cannot occur. Under these conditions, rates of diversification are expected to be slow. On the other hand, rapid diversification is most likely to occur when the environment is composed of a mixture of resources that remain available through time, because now it is possible for genotypes to switch to alternative resources as a result of intense competition for a preferred resource. Such an environment affords the opportunity for more effective selection against intermediate phenotypes and for types that specialize on alternative, and perhaps less preferred, resources. We are unaware of any studies that have tested this prediction directly. The relationship between ecological opportunity and diversification, on the other hand, is well established. A positive relationship between diversity and the level of environmental heterogeneity (used as a proxy for ecological opportunity) has been observed in numerous natural systems (e.g. Tews et al., 2004) and a few experimental tests also support this relationship (e.g. Rainey and Travisano, 1998; Barrett et al., 2005). Moreover, increased diversity due to competition for novel ecological opportunities has been inferred in a number of field (MacArthur and Levins, 1964; Schluter, 2000b; Grant and Grant, 2006) and laboratory (Barrett and Bell, 2006; Jasmin and Kassen, 2007a; Tyerman et al., 2008) systems. Here we present an experiment designed to disentangle the effect of ecological opportunity per se from the manner in which those opportunities are experienced by an evolving lineage. To the extent that competition is important in driving diversification, we expect that when resources are experienced as a fine-grained mixture (all resources are available in a

single patch), resource competition will be more effective at driving diversification than when they are experienced as coarse-grained mixture (each resources is available in its own patch).

To test this idea we allowed clonally reproducing *Pseudomonas fluorescens* populations (Spiers et al., 2000) to evolve over approximately 1000 generations in environments that varied in the extent and spatial structure of ecological opportunity. Measures of adaptation and diversity in each population were tracked as they evolved throughout the course of the experiment. To maximize the potential for the evolution of adaptive diversity, we provided ecological opportunities in the form of different carbon sources that previously had resulted in the presence of fitness trade-offs in *P. fluorescens* after 600 generations of evolution (Jasmin and Kassen, 2007b,a) and the carbon concentrations were adjusted such that the initial productivity of each available resource was equal. Environments containing multiple resources (heterogeneous environments) were expected to generate more adaptive diversity than those with a single resource (homogeneous environments). We also manipulated the spatial structure of heterogeneous environments, and so the potential for resource competition to drive divergent selection, by combining all resources into a mixture or keeping them separate and allowing the population to disperse among resource patches approximately every 6.6 generations. The opportunity for resource competition to drive adaptive diversification should be higher in the mixed multi-resource environment compared to the spatially-structured environments.

Our results show that the presence of multiple ecological opportunities and the potential to compete for those different opportunities simultaneously can drive adaptation, but we saw no significant effect on diversity in our system. Instead, we saw evidence for the evolution of a single adaptive strategy in each population, with different strategies evolving under different kinds of spatial structure. Our results thus suggest that the

spatial structure of ecological opportunities can have markedly different effects on the dynamics of adaptation and phenotypic differentiation.

4.2 Material and methods

4.2.1 Selection experiment

We used a clonal isolate of the bacterium *P. fluorescens* SBW25:lacZ to found five selection lines, each replicated three times. This strain is isogenic to *P. fluorescens* SBW25 save for the insertion of the lacZ gene, a selectively neutral marker (Zhang and Rainey, 2007). Neutrality of the lacZ marker was confirmed in our experimental environments by measuring the fitness of the marked strain relative to the unmarked strain (see fig. B.1). Colonies with lacZ are blue on agar plates supplemented with 40 mg/L of 5-bromo-4-chloro-3-indolyl-beta-D-galactopyranoside (X-Gal), and can easily be distinguished from the pale yellow colonies of the unmarked strain. All strains and evolving populations were frozen at -80° C in 16 % (v/v) glycerol.

Populations were cultured in 24-well plates (Costar, Corning Incorporated), with 2 ml of media in each well, in an orbital shaker (150 rpm) at 28° C. The culture media consisted of M9 minimal salts (1 g/L NH_4Cl, 3 g/L KH_2PO_4, 0.5 g/L NaCl, and 6.8 g/L Na_2HPO_4) supplemented with 15 mg/L $CaCl_2$, 0.5 g/L $MgSO_4$, and a source of carbon. Three monosaccharides were used: mannose, glucose (both $C_6H_{12}O_6$), and xylose ($C_5H_{10}O_5$). Extent of ecological opportunity was manipulated by constructing single-resource and multi-resource environments containing one sugar type and three sugar types respectively (fig. 4.1). In the multi-resource environments, we created a fine-grained mixture of carbon sources by mixing multiple sugars in a single well (MIX) and a coarse-grained, spatially-structured environment (SPAT) by filling each well with a different sugar and

dispersing a sample of the population among all three wells (and so all three sugars) at each transfer. MIX conditions thus allowed individuals to compete for multiple resources in the same well, while this could not happen under SPAT conditions because only one resource was available to an individual at any given point in time.

P. fluorescens SBW25 grows well on mannose and glucose but poorly on xylose (Jasmin and Kassen, 2007b,a). We were thus unable to simultaneously control for initial population density, population size, and the molar concentration of sugar in each environment - all factors that have the potential to affect the dynamics of adaptation. We chose to control for density (initially at approximately 3×10^7 CFU/ml after 24 hours) instead of population size because competition for resources is often density dependent. This manipulation also ensures that the contribution of individuals to the total population arising from growth on each sugar making up the multi-resource treatments is roughly equal. Thus, the concentrations of each sugar used are different (table B.1). While initial effective population size in the single-well environments (single- resource and MIX) were about 6×10^5 colony forming units (CFUs), as a result of controlling for density, the three-well environment (SPAT) supported an effective population size approximately 2.4 times larger. Note also that it is well known that diauxic or biphasic growth (where one resource is preferentially used before another when both are available) can occur in high resource concentration mixtures but when resource concentrations are low, bacteria tend to scavenge resources as they become available (Harder and Dijhuizen, 1982). We therefore used a much lower concentration of sugar in our experiments than has been used in comparable work with the same strain (e.g. Hall and Colegrave, 2007). Observations of the growth curve of the founding strain over 24 hours showed no evidence for the primary growth phase followed by a lag phase, followed by a secondary growth phase - the characteristic pattern of diauxic growth, as expected.

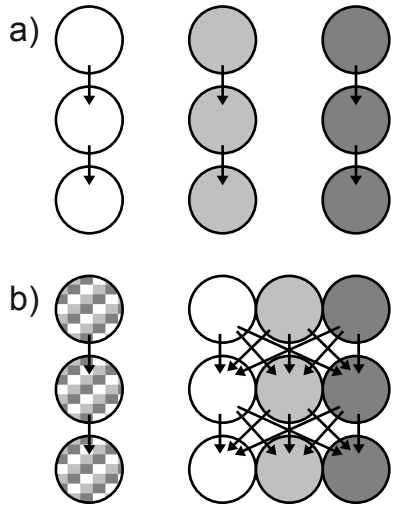


Figure 4.1: a) Single-resource and b) multi-resource selection environments. Circles represent wells containing 2 ml of media made up of some combination of glucose, mannose, and xylose. The multi-resource combination included both a MIX (resources mixed within a single well) and SPAT (resources spatially separated in multiple wells) treatment. Arrows represent daily transfers, with each well receiving a total of 20 μl from a transfer event.

The experiment consisted of three replicates each of five different selection environments. A sample of each culture was transferred to fresh media every 24 hours ($\sim$ 6.6 generations). In treatments consisting of a single micro-well (the single-resources and MIX), 20 μl of culture was transferred. In treatments consisting of multiple micro-wells (SPAT), each micro-well also received 20 μl but this transferred volume was made up of equal parts by volume of samples from each of the micro-wells making up that selection environment (see fig. 4.1). Consequently, in the SPAT environments approximately three times as many cells initiated a population after each transfer compared to the other environments. Note that although bottleneck size differed across treatments as a result of this manipulation, bottleneck size is still quite large under all conditions, ranging from approximately 1×10^5 to 3×10^5 CFUs. We thus do not expect genetic drift to play a substantive role in any treatment. This transfer regime was repeated for 150 transfers resulting in approximately 1000 generations of selection. Samples from each evolving population were routinely checked for contamination and frozen for further analysis every five days.

4.2.2 Estimating population fitness

The fitness of the evolved populations in their selection environment was estimated every 100 generations by competing the evolved population against the ancestral genotype lacking the lacZ neutral marker. Each population and the ancestral strain were first acclimated from frozen separately on the substrate of interest for two growth cycles (48 hours). Evolved and ancestral cultures were then mixed at a 1:3 ratio for a transfer volume of 20 μl. A skewed ratio was chosen to reduce the possibility of the evolved populations completely out-competing the ancestor by the end of the fitness assay. Strains were grown together for three growth cycles, with estimates of the relative frequency of both types quantified after the first (24 hours) and third (72 hours) growth cycles by

plating on agar plates containing M9 minimal salts and glucose, and supplemented with X-Gal. We estimated relative fitness, ω, by calculating the rate at which the frequency of the evolved population changes relative to its ancestor, with the following equation:

$$\omega = \left(\frac{f_{final}}{f_{initial}} \right)^{(1/doublings)} \tag{4.1}$$

where $f_{initial}$ and f_{final} are the ratios of the frequency of the evolved population to the frequency of ancestral populations before and after competition respectively, and *doublings* refers to the number of doublings or generations that occur between the initial and final measurements.

We used model fits of the fitness time series data to estimate the rate of adaptation for each evolved population. Although there is no widely-accepted, simple equation to capture the dynamics of fitness over time, both theory and empirical studies suggest a general heuristic pattern of rapid initial increase in fitness followed by little to no increase thereafter as the population approaches evolutionary equilibrium (e.g. de Visser and Lenski, 2002). There are a number of ways that this pattern could be described mathematically; however we chose to use a logistic model of the form

$$\omega(t) = \frac{k}{1 + (k-1)e^{-rt}}, \tag{4.2}$$

where ω is the measure of relative fitness, k is the maximum fitness achieved (the fitness plateau), r is the maximum rate of adaptation, and t is time measured in number of generations (0 to 1000). The logistic function is appropriate for fitting the dynamics of adaptation as it describes a rapid initial increase followed by a slowing, asymptotic approach to some maximum value (k). Comparing the fit of the logistic against two alternative models, linear and exponential, using AIC values confirms that the logistic

provides a more accurate description of our data (table B.2). We use logistic model parameter estimates to compare rate of adaptation across treatments. Some populations do not reach a fitness plateau within the time frame of our experiment and so estimates of maximum fitness, k, from the logistic model are not always biologically reasonable. For this reason we also estimate maximum fitness specific to the time frame of our experiment by taking the mean of fitness measures in the final two time steps (generations 900 and 1000) for each population. Estimates of the rate of adaptation were compared across treatments using ANOVAs following log-transformation to meet the assumption of homogeneity of variances.

4.2.3 Extent of adaptation

We measured the extent of adaptation, the amount by which fitness increased relative to the ancestor on each component resource, by isolating six genotypes from each evolving population every 200 generations throughout the experiment and then estimating their fitness on each of mannose, glucose, and xylose separately. In these assays the concentration of each sugar was the same as that used in the single-resource selection environments. Competitive fitness assays were conducted using the same protocol as the population fitness assays described above and relative fitness for each isolate in each assay environment was calculated using equation 4.1.

Using this data we conducted two analyses. The first characterizes a phenotype for each isolate as its combined fitness across all three resources and asks how this changes through time. We used repeated measures analysis of variance (RM-ANOVA; see supplementary information for R code) to examine the effects of both time and treatment on this combined phenotype. The second asks specifically after the extent of adaptation to each component resource for isolates from the multi-resource environments.

For this analysis we use only data collected for isolates from the end of the experiment at generation 1000. Estimates of extent of adaptation to each component resource were then compared to the extent of adaptation of isolates selected in the single-resource environment for that component resource using ANOVAs and, when the model was significant, multiple-comparison corrected pairwise *t*-tests were used to distinguish specific differences between selection environments.

4.2.4 Degree of specialization

The degree of specialization was estimated for each population over time by calculating the mean coefficient of variation in fitness across the three assay environments mannose, glucose, and xylose. These measures of specialization were then compared across treatments and over time using repeated measures analysis of variance. Estimates of the degree of specialization were log-transformed to meet ANOVA assumptions of homogeneity of variance.

4.2.5 Resource-use diversity

Within-population resource-use diversity was estimated as the genotype-by-environment interaction variance in fitness (G $\times$ E; following Kassen and Bell (2000) and Barrett et al. (2005)). We calculate the G $\times$ E of six genotypes isolated from a given selection line at a given point in time, repeating this calculation for isolates from each line every 200 generations up to generation 1000. In this context, G $\times$ E is a measure of within-population diversity because it quantifies the differences in the fitness among the six genotypes from each population on each of the three carbon sources used in our selection environments (glucose, mannose, and xylose). G $\times$ E is the sum of two variation components: 1) a measure of the differences in the environmental variance among the six genotypes, and

2) a measure of the lack of correlation between the fitness of the six genotypes across environments:

$$\sigma^2_{G\times E} = \sum \frac{(\sigma_{Ei} - \sigma_{Ej})^2}{2G(G-1)} + \sum \frac{\sigma_{Ei}\sigma_{Ej}(1-\rho_{EiEj})}{G(G-1)}, \tag{4.3}$$

where σ_{Ei} and σ_{Ej} are the standard deviations in fitness across environments for genotypes i and j respectively, ρ_{EiEj} is the correlation in fitness in each environment comparing genotypes i and j, G is the number of genotypes tested, and these estimates are summed over all pairwise combinations of genotypes giving an estimate of within-population diversity for each replicate population. Resource-use diversity estimates were compared across treatments and over time using repeated measures analysis of variance.

4.2.6 Metabolic diversity

Genotypes from generation 1000 of each population were further characterized by their growth profiles on GN2 Biolog plates (Biolog Inc.). Biolog plates are 96-well microwell plates containing 95 different carbon sources plus a negative control well. Genotypes were grown from frozen overnight in 20 ml vials containing 6 ml King's B media (28° C, shaken at 150 rpm), starved for 2 hours (20 μl of each culture in 20 ml M9 minimal salts at 28° C, 150 rpm), and then 150 l was transferred into each well of the Biolog plates. Optical density (OD) was measured at the time of inoculation and after 24 hours of growth (28° C, 150 rpm), and growth rate on each carbon substrate was calculated as $r = \ln(\mathrm{OD}_{\mathrm{initial}})\text{-}\ln(\mathrm{OD}_{\mathrm{final}})$. These growth rates were then adjusted relative to the control well by subtracting the maximum growth rate estimate obtained from the control wells (0.09), and setting all negative growth estimates to zero. With these adjustments, our estimates of a strain's metabolic profile are quite conservative. With these data we again quantified within-population diversity by calculating the genotype-by-environment interaction variance in fitness (G $\times$ E) for six genotypes from each population, this time

measured across 95 environments (the 95 different carbon sources on the Biolog plates), thus estimating within-population metabolic diversity for each replicate population at generation 1000. The rationale for this second diversity measure is that the Biolog plates provide a more comprehensive view of the evolved metabolic phenotype than simple assays of performance on the three sugars used in the experiment, thus potentially increasing our power to detect subtle differences in diversity. Metabolic diversity measures were compared across selection environments using an ANOVA. For all statistical analyses in this study, model assumptions were tested and data were transformed when necessary. Analyses were performed using R version 2.13.2 (R Development Core Team, 2011).

4.3 Results

4.3.1 Rate of adaptation

Fitness of all populations increased a mean (± SE) of 24.6% ± 2.36% by the end of the experiment (t-test, $T_{14} = 10.44$, $P < 0.0001$). Mean fitness over time in the single-resource and multi-resource selection environments is shown in fig. 2a and fig. 2b, respectively. In all environments fitness increased, often levelling off at what may be a fitness plateau. The fluctuations in fitness seen here are not unusual in microbial evolution experiments (e.g. Lenski et al., 1991) and likely reflect small changes in environ- mental conditions (despite our efforts to control them), and experimental or sampling error. Moreover, the observed fitness increases are comparable to that of microbial evolution experiments over similar time frames (e.g. Lenski et al., 1991; Melnyk and Kassen, 2011). As an additional check we repeated the fitness assays for five of the fifteen selection lines (G1, X1, X3, MIX MGX2, and SPAT MGX2), this time obtaining replicate measures of fitness (n=3) at generations 100, 200, 400, 600, 800, and 1000. Now, most of the temporal fitness

fluctuations observed in the original, un-replicated, time series disappear suggesting that they are indeed simply a result of measurement error (see fig. B.2).

Estimates of the rate of adaptation and maximum fitness for each replicate population are provided in table B.3 and logistic model fits are shown in fig. B.2. Selection environment significantly affected the rate of adaptation (fig. 4.3a; ANOVA on log-transformed rate data, $F_{4,10} = 6.99$, $P = 0.00593$; Kruskal-Wallis rank sum test on untransformed rate data, $\chi^2 = 10.8333$, df = 4, $P = 0.0285$). Rates of adaptation in the single carbon source environments tended to be low and of the same magnitude as rates in the multi-resource SPAT treatment; they are highest in the multi-resource MIX treatment. Inclusion of the additional replicate fitness measures did not qualitatively change these results and, in fact, marginally increased the significance of those patterns (ANOVA on new log-transformed rate data, $F_{4,10} = 8.72$, $P = 0.00269$).

The maximum fitness achieved by evolved populations did not vary across selection environment over the course of the experiment (fig. 4.3b; ANOVA, $F_{4,10} = 0.767$, $P = 0.571$), although at least four lineages show little evidence that they have reached a fitness plateau. These four lineages are not consistently associated with any particular selection environment. Notably, where the logistic model provided reasonable estimates of k, these were not significantly different from estimates of the extent of adaptation directly from the data (paired sample t-test: $T_{10} = 1.248$, $P = 0.241$) suggesting that using either method does not introduce a consistent bias to our results.

4.3.2 Extent of adaptation

Fig. 4.4 shows the responses to selection in each carbon source for each treatment. To calculate these values, we first took the mean fitness of the six isolates within a selection line and then averaged these values for all three replicate lines from a given treatment. The

dynamics of mean fitness were highly variable across treatment (RM-ANOVAs, assayed in mannose: selection environment (S) × time (T): P = 0.0011; assayed in glucose: S × T: P = 0.262; assayed in xylose: S × T: P = 0.0501; see table B.4), but some broad patterns emerge. First, the best-adapted lineages in each assay environment were those that had been selected in that environment. Second, correlated responses to selection (that is, the relative fitness of lines in environments in which they were not evolved), were often positive relative to the ancestor except in the case of the xylose and MIX lines assayed in mannose, where there was little evidence of a consistent response in either direction (fig. 4.4a), and in glucose, where a cost of adaptation was evident in the xylose line and no response was seen in the MIX line (fig. 4.4b). Third, selection in the variable environments resulted in the greatest advance in xylose and little or no progress in glucose or mannose (compare response of MIX and SPAT in fig. 4.4c with responses in fig. 4.4a and fig. 4.4b). Fourth, environmental heterogeneity slowed the rate of adaptation to xylose relative to what the rate could have been in xylose alone, although the MIX lines adapted faster than the SPAT lines (fig. 4.4c).

Fig. 4.5 contrasts the extent of adaptation in each resource for isolates from the multi-resource environments against those from each single-resource environment at generation 1000. Lines selected in both types of multi-resource environments (MIX and SPAT) adapted to xylose to the same extent as the xylose-only lines (fig. 4.5c; ANOVA, selection environment: P = 0.803; see table B.5), while the extent of their adaptive response to glucose was significantly less compared to the glucose-only lines (fig. 4.5b; ANOVA, selection environment: P = 0.0003194 ; Holm adjusted pairwise *t*-tests, MIX: P = 0.0012, SPAT: P = 0.0012). However, in mannose, MIX and SPAT lines differed in the extent of their adaptive response (fig. 4.5a; ANOVA, P = 0.002978) - SPAT lines adapted to the same extent as the mannose-only adapted lines (Holm adjusted *t*-test, P

= 0.2780), while MIX lines adapted significantly less (Holm adjusted t-test, P = 0.0025).

In fig. 4.6 we show how the degree of specialization (measured as the within-population coefficient of variation in fitness across the three assay environments) changes over the course of the experiment. There is a significant effect of selection environment (RM-ANOVA, selection environment: P = 0.0066; see table B.6), with populations evolved in xylose tending to be most specialized, followed by the populations evolved in MIX. Populations evolved in xylose and MIX were significantly more specialized than all the other populations (multiple pairwise t-tests, Holm adjusted, all P < 0.05), but not significantly different from each other (pairwise t-test, Holm adjusted, P = 0.714). In contrast to MIX, the SPAT treatment supported a more generalist population, although they become nearly as specialized as MIX and xylose by the end of the experiment.

4.3.3 Diversity

The genotype-by-environment variance (G × E) was used to characterize within-population diversity. G × E appears to increase over time, although this was not statistically significant (fig. 4.7a; RM-ANOVA, time: P = 0.3728; see table B.7). Similarly, selection environment did not have a significant effect on G × E (RM-ANOVA, selection environment: P = 0.0897). The two components of G × E variance, the difference in genetic standard deviations across environments and the genetic correlation in fitness across environments, similarly revealed no significant treatment effects for either measure (data not shown). To ensure that this result was not an artefact of limited sampling, we isolated an additional 8 strains from a xylose population, a MIX population, and a SPAT population at generation 1000 and re-estimated diversity using the same method. The additional isolates did not significantly change our estimates of GxE (paired sample t-test, $T_2 = 1.989$, P = 0.185).

We also asked whether we could detect broader patterns of metabolic variation among selection lines by examining the variation in metabolic profile within a lineage using Biolog microwell plates. For the evolved populations at generation 1000, within-population metabolic diversity appears to be highest for populations selected in the mixed multi-resource environment (MIX in fig. 4.7b), however this trend is not significant (ANOVA, $P = 0.559$).

4.4 Discussion

We have attempted to evaluate more rigorously the link between ecological opportunity and diversification by examining the dynamics of adaptation and diversity in experimental populations of *P. fluorescens*. The main comparison of interest is between those populations that evolved in heterogeneous environments that share the same extent of ecological opportunity but differ in how those opportunities are structured in space. Our leading results are that the spatial structure of ecological opportunity can affect the rate of adaptation, with fine-grained environments composed of a mixture of resources (MIX) supporting faster rates of adaptation than coarse-grained environments where resources are separated in space (SPAT). Spatial structure can also affect the ecological characteristics that evolve, with specialists evolving more readily in fine-grained than in coarse-grained environments. Notably, we did not observe any effect on the rate or extent of diversification in our experiment, regardless of the number of resources available or how they were arranged in space. Taken together, our results lend support to the idea that the spatial structure of ecological opportunities can have a profound effect on the emergence and fate of diversity in heterogeneous environments, although not necessarily in the way that we would have expected. Below, we consider the implication of each of these results for our understanding of selection in heterogeneous environments.

4.4.1 The dynamics of adaptation

In the broadest terms, the pattern of adaptation we see in our experiments is consistent with the standard model of selection on rare beneficial mutations. All populations increased in fitness as beneficial mutations arose and escaped stochastic loss, sometimes after an initial period of little or no fitness increase. In some populations this waiting time was quite substantial: over 200 generations in the slowest-evolving populations in mannose, glucose, and the spatially coarse-grained environments. By contrast, the waiting time was shorter, and the rate of adaptation much faster, in the spatially fine-grained environment and in xylose. This result suggests that either selection was much stronger and/or genetic variation much more abundant in these environments than in the others.

A number of lines of evidence suggest that both factors play a role in governing the faster rates of adaptation in these treatments. Xylose is a sugar that *P. fluorescens* is initially very poorly adapted to and so it is not surprising that beneficial mutations would become available and spread through these populations rapidly due to strong selection. Moreover, the increased variation seen among isolates of the xylose and MIX lines at generation 200 disappears by generation 400 (G $\times$ E, fig. 4.7 a), consistent with the idea we have caught these populations in the middle of a selective sweep. While it may be possible that such rapid changes in fitness are physiological or epigenetic, this seems unlikely because the differences in fitness we observe are maintained upon sub-culturing and whole genome sequence analysis has identified a number of non-synonymous mutations in the evolved populations. Details of this genome sequence study will be provided in a future manuscript. Previous work showed that the availability of beneficial mutations was higher in xylose than in mannose (Jasmin and Kassen, 2007b) and we have shown, using a similar mutant collection from the same library, that the same is true when we compare xylose with glucose (see fig. B.3). Thus not only is selection likely to be stronger

in environments that contain xylose, it is genetically based and the effective amount of genetic variation available for adaptation to xylose is also likely higher than it is for mannose or glucose.

Adaptation was also substantially faster in the fine-grained environment than it was in the coarse-grained environment. It is tempting to explain this difference as a result of resource competition in the former but not in the latter, especially considering that coarse-grained environments supported populations that were initially 2-3 times larger than fine-grained environments and so should have adapted faster. Resource competition is clearly not the appropriate interpretation for three reasons. First, competition for alternative resources was entirely absent in the xylose-selected lines, yet these showed a rate of adaptation comparable to the lines in the fine-grained environment. Second, there was no evidence for the evolution of diversity or character displacement in the fine-grained environment, as would be expected if resource competition was driving adaptive diversification. Third, xylose specialists evolved in the fine-grained environments, while more broadly adapted generalists evolved in the coarse-grained environment. This result is consistent with those of a previous experiment with the same founding strain: Jasmin and Kassen (2007b) found that xylose specialists evolved in spatially fine-grained two-resource environments containing mannose and xylose, while more of a generalist strategy evolved in spatially coarse-grained environments with the same two resources. Taken together, these results suggest that the rapid adaptation observed in the fine-grained environment was driven primarily by adaptation to xylose.

Given that xylose was also present in the coarse-grained environment, why did we not observe a similar result there? We attribute this outcome to two processes. The first is dispersal. The imposition of dispersal in the spatially structured environment causes a given lineage to experience the environment as at least partially temporally variable, which

will tend to favour the evolution of generalists (Kassen, 2002) and slow the rate of fixation of genes with environment-specific effects (Whitlock, 1996). Individuals in the fine-grained mixture environment, on the other hand, do not experience the same degree of temporal variation since all resources are available simultaneously and so the direction of selection does not fluctuate through time. This allows mutants with environment-specific effects to be fixed more rapidly in mixtures compared to spatially structured environments.

The second is the evolution of unequal productivity among the available resources. Both theory (Holt, 1985) and experiment (Jasmin and Kassen, 2007a) suggest that adaptation tends to occur more readily to the most productive resource. We adjusted the concentrations of each sugar to balance productivity at the beginning of the experiment such that each sugar in a mixture contributed equally to the growth of the population (see Methods). This necessitated having a much higher concentration of xylose in the medium because the founding strain, which lacks a gene (xylB) that is a central component of the xylose utilization operon in other Pseudomonads and Gram-negative bacteria (Rainey, 1999), grew so poorly on this substrate. This initial genetic constraint was readily overcome by selection, as we see the rapid evolution of xylose specialists when the ancestor is selected both in xylose and in the fine-grained environment, and this is accompanied by an increase in stationary phase population density (see fig. B.4). Thus the relative productivity of the different resources is now heavily weighted in favour of xylose, which in turn biases the subsequent evolution of the populations by vastly reducing the benefit of any adaptation to the other two less productive substrates. We stress that these evolved differences in productivity - sometimes an almost ten-fold increase in population size - are the result of adaptation to xylose and so could not have contributed to the initial rapid specialization, but only reinforce the benefit of continuing to specialize on xylose.

4.4.2 The absence of diversification

These same two processes, dispersal and relative productivity, also explain why we failed to see diversity, measured both as genotype-by-environment interaction and metabolic profiles, arising and being maintained in the heterogeneous environments. The presence of dispersal in the spatially coarse-grained environment generates a form of temporal variation that selects for the evolution of generalists and makes it difficult for diversity to be stably maintained (Kassen, 2002). In the fine-grained environment, by contrast, we suspect that a difference in relative productivity among substrates caused by adaptation to xylose is responsible. Theory suggests that when there are strong imbalances in productivity, adaptation will occur predominantly to the most productive substrate and diversity cannot be maintained (Levene, 1953; Kassen and Bell, 2000). This interpretation is consistent with what we have observed here, where the evolved differences in productivity in favour of xylose reinforce the evolution of a xylose-specialist population. This result is also consistent with our previous work where we have manipulated, by design, the relative productivity in favour of xylose and observed the evolution of xylose specialists in coarse-grained heterogeneous environments (Jasmin and Kassen, 2007a).

It is always possible that we were unable to detect differences simply because we did not sample enough isolates from each population. To ensure that we had sufficiently sampled our populations, we isolated an additional nine strains from populations selected in xylose, the fine-grained environment, and the coarse-grained environment at generation 1000 and re-estimated diversity. The additional isolates did not change our results. It is still possible that there is more genetic variation that arose in these heterogeneous environments that we are missing with our assays, but clearly if it exists, it is either quite rare or is neutral with respect to fitness in these environments. Preliminary results from full genome sequence analysis also suggest that while genomic diversity can be detected

in some of these populations, it did not vary in a consistent way across environments. Further analysis of both within and between population genomic diversity in this system will be reported in a future manuscript.

4.4.3 The extent of adaptation in heterogeneous environments

Our analysis of the extent of adaptation on each component resource can provide insight into the nature of the evolved genotypes in each complex environment. If resource competition is important in driving diversification in the fine-grained environment, we should see the extent of adaptation of isolates from the fine-grained environment exceeding that of those from the single-resource environments on some or all of the component resources. In the absence of resource competition, the extent of adaptation of isolates from both kinds of heterogeneous environment will be equal to or less than that observed for isolates selected and assayed on the same resource. The amount by which the extent is less depends on the degree to which the mutations selected have antagonistically pleiotropic effects. As noted earlier, we failed to see any evidence of resource competition promoting adaptation in our experiment. The fact that isolates from both fine-grained and coarse-grained environments failed to adapt to glucose and only the coarse-grained isolates showed evidence of adapting to mannose indicates that the main effect of selection in heterogeneous environments was to prevent adaptation to all component substrates due to the substitution of genes with antagonistically pleiotropic effects. Notably, the difference in the patterns of adaptation across resources suggests that the specific mutations substituted under fine- and coarse-grained situations are different. Results from genome sequence analysis of these populations also support this (see Chapter 5); there is only a single example of the same mutation arising in both the fine-grained and coarse-grained environments (out of a total of 19 unique mutations arising in 6 populations - 3 replicate

lines in each of the two environments) and, more broadly, only two examples of the same gene being targeted in the two types of environments (out of 12 unique genes targeted in the 6 populations - 3 replicate lines in each of the two environments).

4.4.4 The evolutionary dynamics of niche specialization

Our approach to studying the dynamics of adaptation in multiple environments provides some novel insight into the evolution of specialization, in particular when specialization is underlain by costs of adaptation. Recall that we chose the resources for this experiment based on previous work that demonstrated the evolution of negative correlations in fitness across these resources (Jasmin and Kassen, 2007b,a). In our experiment, negative correlations in fitness do evolve but not immediately. In the mixture environment, for example, adaptation to xylose occurs within the first 200 generations but results in a loss of fitness on glucose only at generation 600. This fitness loss is then largely recovered by generation 1000 (fig. 4.4b). This dynamic is not observed in the xylose-selected line, where adaptation to xylose results in a consistent cost of adaptation on glucose that is maintained throughout the experiment. This result suggests, first, that adaptation to xylose occurs through a different route in the mixture environment than it does in the xylose-only environment and, second, that the evolution of trade-offs can be more complex than we usually think. We suspect that the alternate substrates in the mixture environment played an important sieving role in determining what kinds of genes are substituted.

4.4.5 Genetic and physiological targets of adaptation

The underlying genetic and physiological mechanisms associated with adaptation in our experiment are not known because sugar metabolism is not well understood in

P. fluorescens. In general pseudomonads metabolize glucose and mannose via the Entner-Doudoroff (ED) pathway and/ or the pentose pathway, while xylose can be metabolized only via the pentose pathway (Lessie and Phibbs, 1984). In another *P. fluorescens* strain (Pf 52-1C) where metabolic pathway use has been quantified, the ED pathway is used much more heavily than the pentose pathway for glucose metabolism (Fuhrer et al., 2005). Although there appears to be much variation across the pseudomonad complex in their ability to metabolize different carbon sources, our best guess is that in *P. fluorescens* SBW25 the ED pathway is the dominant route for the metabolism of glucose and other similar sugars like mannose, suggesting that this strain is likely to be well-adapted to using the ED pathway. By contrast, this strain is likely to be less well adapted to using the pentose pathway. If this interpretation is accurate, then there should be more genetic routes to adaptation in xylose than in mannose or glucose. This result is consistent with recent work from our lab showing that the adaptive landscape in xylose was more rugged than it was for glucose (Melnyk and Kassen, 2011) and it could also explain why there are more beneficial mutations available in xylose than glucose or mannose. An analysis of the flux through the potential metabolic pathways as glucose, mannose, and xylose are consumed by the evolved populations (similar to analysis performed by Fuhrer et al., 2005) would help to characterize the details of these possible adaptive changes in metabolism.

4.4.6 Summary

Taken together, our study emphasizes the importance of considering the spatial structure of ecological opportunities for understanding the variation in rates of adaptation and diversification. Our results suggest that rapid adaptation is more likely to occur in fine-grained environments than in coarse-grained ones because the absence of dispersal

eliminates any temporal variation in the direction of selection. The relative productivity of different resources that make up a heterogeneous environment can also change as a direct result of adaptation, and this can have consequences for the rate of adaptation, the ecological characteristics of the genotype selected, and the maintenance of diversity. At this point it is difficult to say with confidence how general an effect these evolved differences in productivity are to the outcome of selection in heterogeneous environments, because few experiments have been able to track the evolutionary dynamics of adaptation and diversity as we have. Moreover, our results may be unique in the sense that they are largely driven by the presence of a particularly novel ecological opportunity, xylose. However, to the extent that the dynamics and fate of diversity is associated with adaptation to novel ecological opportunities, as it often appears to be in many adaptive radiations (e.g. Mitter et al., 1988; Thomas et al., 2009), then our results provide important insight into how this process works, and why an incipient radiation may ultimately fail to diversify. More specifically, while we did see rapid adaptation in a multi-resource environment, that adaptation was largely confined to a single ecological opportunity, and selection for improvement on this ecological opportunity appeared to swamp the benefit of improving on all other available opportunities. Our results thus add an additional explanation for why some clades fail to radiate despite the apparent presence of ecological opportunity (reviewed in Losos, 2010) - imbalances in the "novelty" of the ecological opportunities available. Perhaps the importance of environmental context, as seen here, may be one of the reasons that rapid adaptive radiations appear to be such unique events in the history of life.

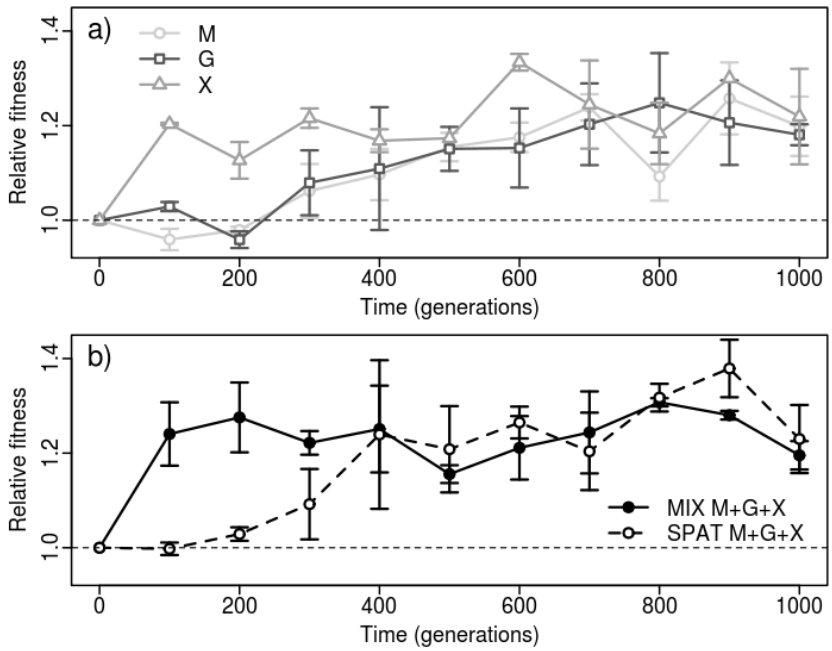


Figure 4.2: Mean relative fitness (± SE) for three replicate populations every 100 generations in each: a) single-resource environments (M: mannose, G: glucose, and X: xylose), and b) multi-resource environments (MIX: mixed multi-resource, and SPAT: spatially-separated multi-resource) over 1000 generations.

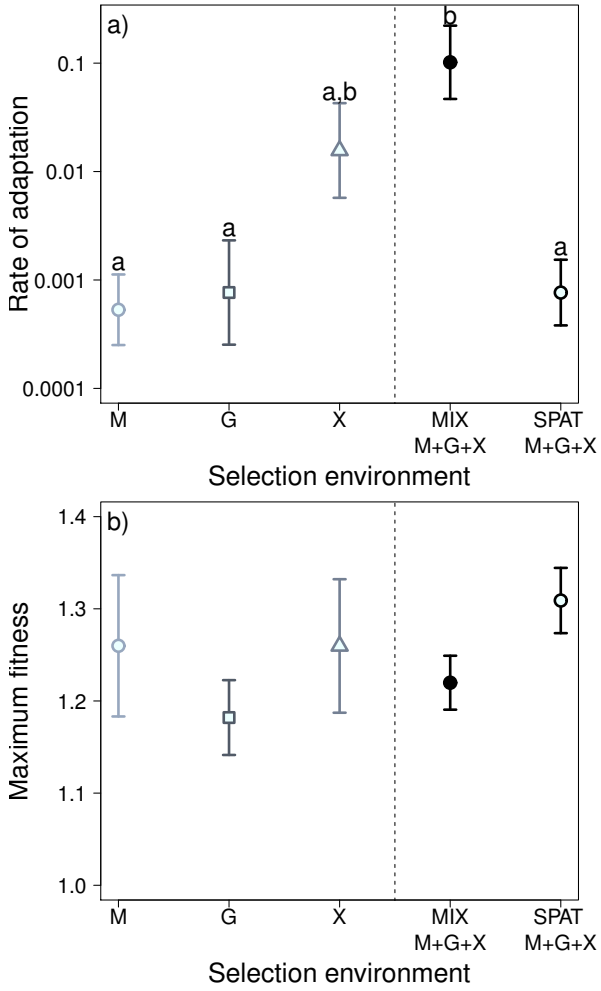


Figure 4.3: a) Mean (± SE) rates (from logistic model fits) and b) maximum fitness reached (mean relative fitness at the final two time points) estimated for three replicate populations in each single-resource environment (M: mannose, G: glucose, and X: xylose), and multi-resource environments (MIX: mixed multi-resource, and SPAT: spatially-separated multi-resource). In panel a), statistically significant differences are indicated by a and b groupings (Bonferroni adjusted pairwise t-tests, $P < 0.005$).

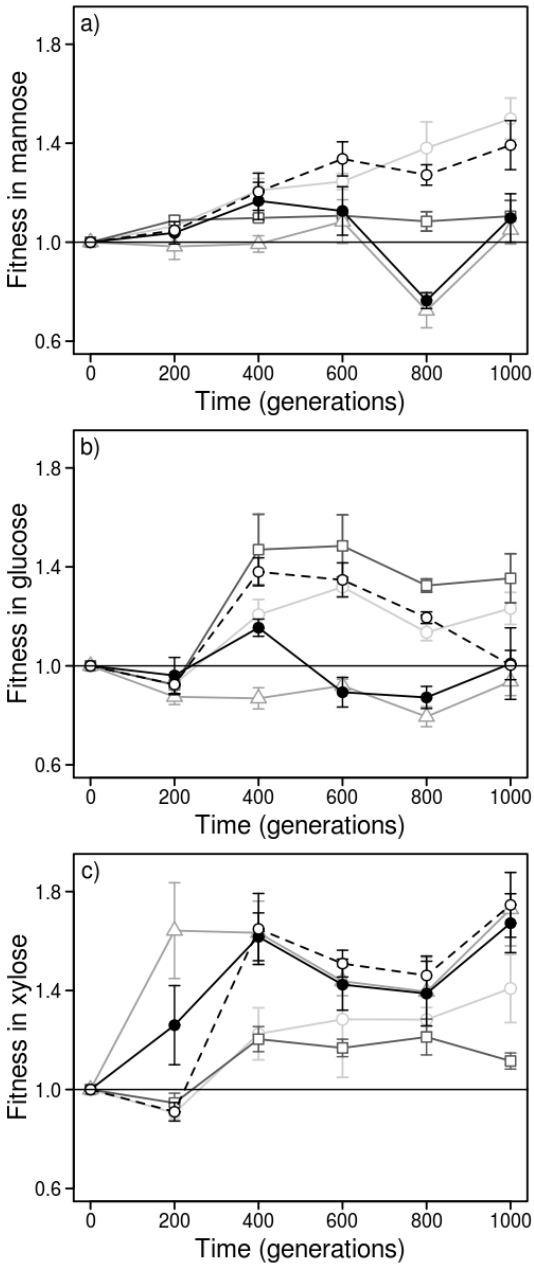


Figure 4.4: Mean three-trait phenotype over time. Measured as relative fitness in a), mannose, b) glucose, and c) xylose, every 200 generations for isolates from populations selected in mannose (M: light grey circles, solid line), glucose (G: dark grey squares, solid line), xylose (X: medium grey triangles, solid line), and competitive (MIX: black circles, solid line) and non-competitive (SPAT: black open circles, dashed line) multi-resource environments. Each point represents the mean of the means of the six isolates from three replicate populations ± SE of the population means.

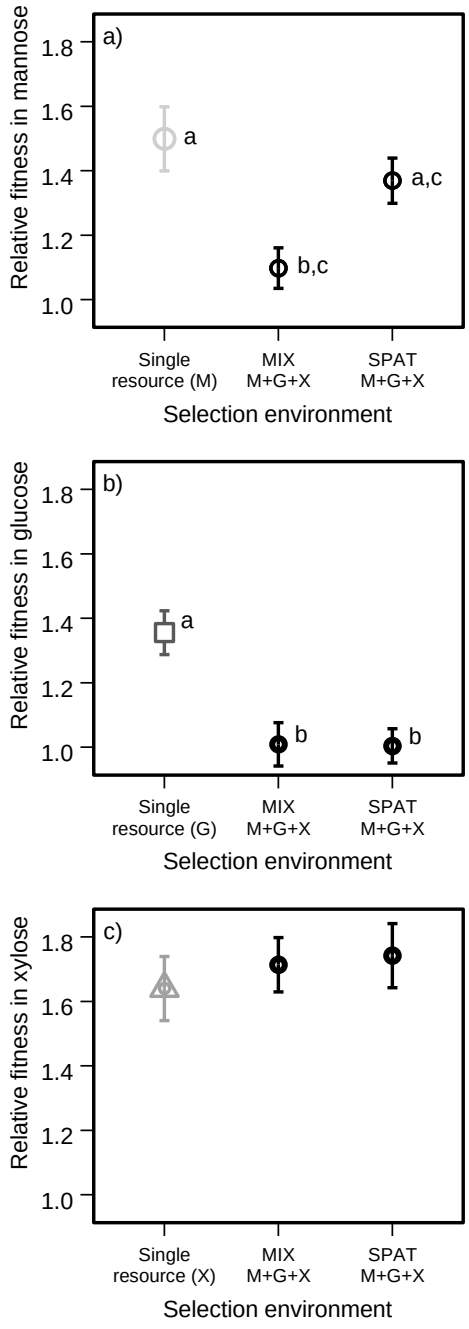


Figure 4.5: Extent of adaptation to a) mannose, b) glucose, and c) xylose of isolates from lines evolved in the two multi-resource environments (MIX: black, filled circles; SPAT: black, open circles) and isolates from lines evolved in the single-resource selection environment containing the resource of interest (Single resource (M / G / X): light grey, circles / dark grey, squares / medium grey, triangles). Points represent the mean relative fitness at generation 1000 ($\pm$ SE) of 6 isolates each from three replicate populations. In panels a) and b), statistically significant differences are indicated by a and b groupings (Holm adjusted pairwise t-tests, $P < 0.05$).

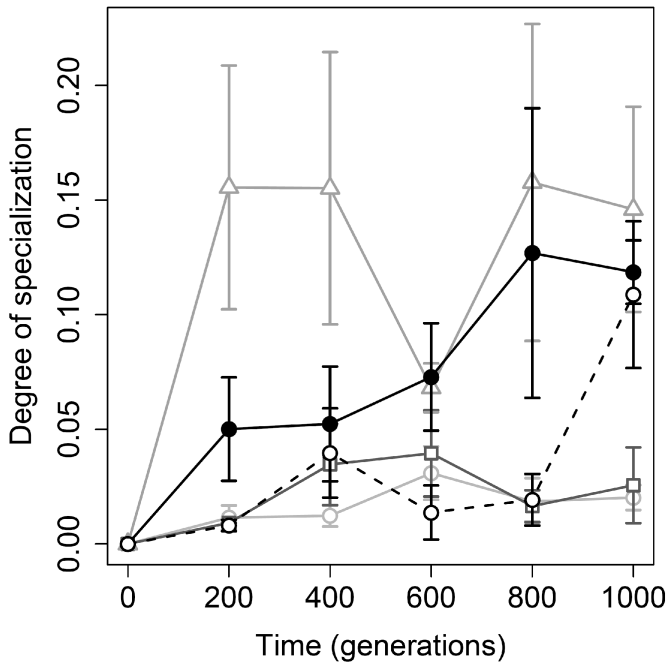


Figure 4.6: Degree of specialization of evolving populations, measured as the coefficient of genetic variation within a population, over time. Points represent the mean (± SE) of three replicate populations selected in mannose (M: light grey, circles), glucose (G: dark grey, squares), xylose (X: medium grey, triangles), mixed multi-resource (MIX: black, filled circles, solid line) and spatially-separated multi-resource (SPAT: black, open circles, dashed line).

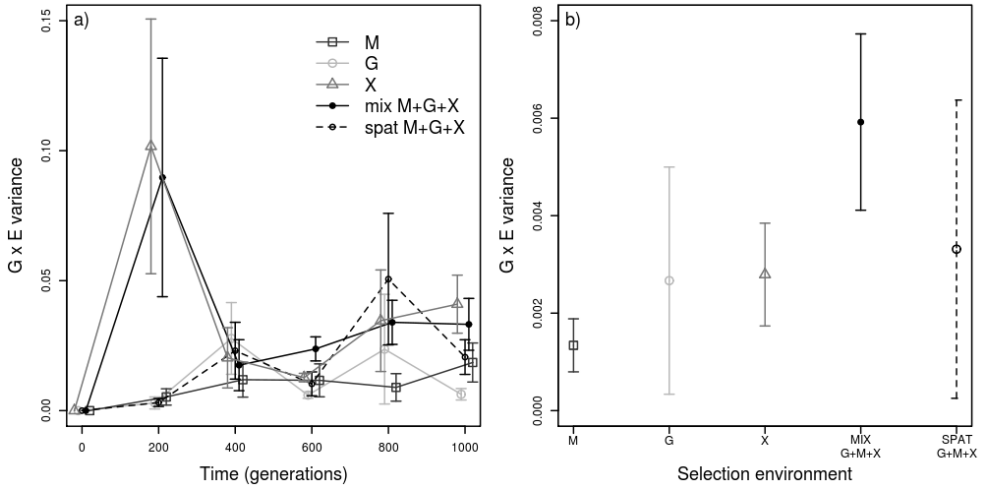


Figure 4.7: Genotype-by-environment (G × E) variance estimated using i) resource-use phenotype (estimated from fitness measures in mannose, glucose, and xylose) measured every 200 generations for 1000 generations, and ii) metabolic phenotype (estimated from Biolog data) at generation 1000, in each selection environments. G: glucose, M: mannose, X: xylose, MIX: mixed multi-resource, and SPAT: spatially-separated multi-resource. Each point represents a mean of the G × E variance in three replicate populations ± SE.

Chapter 5

Selection environment drives the degree of parallel evolution

This chapter is reproduced from:

Bailey, S. F., Rodrigue, N. and Kassen, R. (in prep. for submission to *PLoS Genetics*) Selection environment drives the degree of parallel evolution in experimentally evolved populations of *Pseudomonas fluorescens*.

Collaborator contributions:

N. Rodrigue aligned sequence reads and identified the mutations.

5.1 Introduction

Abundant and varied ecological opportunity drives populations to evolve different adaptive solutions, the result of which is the rich diversity of life we see today (Schluter, 2000b; Yoder et al., 2010). In fact, even populations evolving under the same environmental conditions have the potential to adapt in completely different ways. Despite the potential

for differences, there are numerous examples of populations that have evolved to meet similar challenges in similar ways, both within species and across species, and across levels of biological complexity - from fitness, to phenotype, to genotype (e.g. Zhang and Kumar, 1997; Ödeen and Håstad, 2003; Harrison et al., 2005; Liu et al., 2010). The terms 'parallel evolution' and 'convergent evolution' are usually used to distinguish similarities that were independently derived within- and between-species respectively. However, these terms refer to the same phenomenon at two ends of a continuum of relatedness (Arendt and Reznick, 2008). For this reason, we simply use a single term - parallel, to refer to all instances of independently derived similarities. Understanding the circumstances under which evolution drives populations along divergent versus parallel adaptive paths helps add to our understanding of the interplay between the processes of mutation, drift, and selection that drive evolutionary adaptation.

Parallel evolution has previously been examined, from phenotype to genotype, in a number of systems where striking phenotypic parallels are seen in naturally replicated populations (e.g. Wittkopp et al., 2003; Colosimo et al., 2005; Steiner et al., 2009). The molecular details underlying a number of traits that appear repeatedly across diverse groups of species have also been explored (e.g. Yokoyama and Radlwimmer, 2001; Copley, 2004; Fernald, 2006; Castoe et al., 2009). These studies often use parallel evolution as evidence that selection is driving the evolutionary changes in question, but tend not to explore potential factors affecting the probability of repeated evolution (except for some broad comparisons of within and between species parallelisms, e.g. Arendt and Reznick, 2008). A few studies use experimental evolution of microbes to explore the probability of parallel evolution across replicate populations at both the phenotypic and genetic level, some probing target genes (Woods et al., 2006), others using whole genome sequence comparisons (Barrick et al., 2009; Herron and Doebeli, 2013). However, experiments

of this nature aimed at comparing variation in the degree of parallel evolution across organisms and environments are lacking.

Differences in the degree to which independent populations evolve in parallel must arise from differences in the supply and fixation of beneficial mutations (Orr, 2005; Chevin et al., 2010). However those differences could be generated in a number of ways, both through environmental drivers and differences specific to the organism of interest. Previous studies suggest that replicate populations of some species are intrinsically more likely to exhibit parallelism compared to others, perhaps due to details of their genetic architecture such as mutation rates and genome complexity (Dettman et al., 2012). For example, experiments with replicate populations of viruses, which have very simple genomes made up of few genes, tend to show a great deal of parallel evolution all the way down to the nucleotide level (e.g. Bull et al., 1997; Wichman and Brown, 2010; Miller et al., 2011), compared to replicate populations of bacteria, which have many more genes and gene interactions, where examples of identical nucleotide changes are rare (e.g. Woods et al., 2006; Barrick et al., 2009; Wong et al., 2012). Differences in the selection environment may also impact the degree of parallel evolution that arises through changes to the supply and/or fixation of beneficial mutations. Theoretical work by Chevin et al. (2010) showed that shorter distances to the fitness optimum and increased pleiotropy (both potentially driven by selection environment) result in a higher probability of parallel evolution. However, the effects of environment on the degree of parallel evolution have not been tested empirically.

When independently evolving populations appear to adapt in the same way, those observed similarities often depend on the level at which the observations are made. For example, parallels observed at the phenotypic level often have a very different basis when looked for at the gene level (e.g. Wittkopp et al., 2003; Steiner et al., 2009; Fry et al., 2009). This is not surprising given the hierarchical nature of these types of observations.

However, the particularities of the underlying supply of beneficial mutations might mean that parallels are seen all the way down to gene and nucleotide level observations in some types of selection environments, while under other types of selection conditions, independent populations might reach the same phenotype via different genetic changes. The potential environmental factors driving these kinds of differences have not been explored either theoretically or empirically.

In this paper we report evolutionary changes in fifteen independently evolved populations of the bacteria *Pseudomonas fluorescens*, all initiated from the same starting genotype, and selected in five different selection environments for approximately 1000 generations. The selection environments varied in the particular food resources available, the number of food resources available, and arrangement of those resources. In a previous paper, we tested the effect of selection environment on the rate and extent of evolutionary changes in fitness and phenotype in these populations (Bailey and Kassen, 2012). We observed clear differences between populations in their evolutionary trajectories, prompting us to further explore the generation-1000 populations using whole genome sequencing, in an attempt to link the evolved phenotypic differences to underlying genomic differences. This exploratory genome sequencing work brought to light, among other things, interesting differences in the degree of parallel evolution between selection environments and so we focused our questions to examine this phenomenon. The resulting study provides a unique perspective on the probability of parallel evolution spanning multiple levels of biological organization (from fitness to phenotype to genes) and provides evidence that differences in the type of selection environment can influence the degree of parallel evolution.

5.2 Materials and methods

5.2.1 Selection experiment

We used a clonal isolate of the bacterium *P. fluorescens* SBW25:lacZ to found fifteen independent lines. This strain is isogenic to *P. fluorescens* SBW25 save for the insertion of the lacZ gene, a selectively neutral marker (Zhang and Rainey, 2007). Colonies with lacZ are blue on agar plates supplemented with 40 mg/L of 5-bromo-4-chloro-3-indolyl-beta-D-galactopyranoside (X-Gal), and can easily be distinguished from the pale yellow colonies of the unmarked strain. All strains and evolving populations were frozen at $-80°$ C in 16% (v/v) glycerol.

Populations were cultured in 24-well plates (Costar, Corning Incorporated), with 2 ml of media in each well, in an orbital shaker (150 rpm) at 28° C. The culture media consisted of M9 minimal salts (1 g/L NH_4Cl, 3 g/L KH_2PO_4, 0.5 g/L NaCl, and 6.8 g/L Na_2HPO_4) supplemented with 15 mg/L $CaCl_2$, 0.5 g/L $MgSO_4$, and a carbon source. Three monosaccharides were used as carbon sources: mannose, glucose (both $C_6H_{12}O_6$), and xylose ($C_5H_{10}O_5$). When grown in a 1M sugar concentration for 24 hours (150 rpm at 28° C), SBW25 reaches cell densities over 10-fold higher in glucose (mean ± s.e. = $5.33 \pm 1.09 \times 10^8$ colony forming units (CFUs)) and mannose ($5.76 \pm 0.54 \times 10^8$ CFUs) compared to xylose ($1.33 \pm 0.15 \times 10^7$ CFUs). For this reason, concentrations were adjusted to control for population size resulting in environments containing 9.57 mg/L of mannose and glucose, and 255.22 mg/L of xylose. Single-resource and multi-resource environments were constructed containing one sugar type and all three sugar types respectively. Two types of multi-resource environments were created 1) fine-grained: three sugars in a single well (MIX), and 2) coarse-grained: three sugars available, each in a different well with population mixing and redistribution at each 24 hour transfer

(SPAT). In total, the experiment consisted of three replicates in each of five different selection environments. Every 24 hours ($\sim$ 6.6 generations), a sub-sample of each culture was transferred to fresh media. In treatments consisting of a single micro-well (the single-resource environments and MIX), 20 μl of culture was transferred. In treatments consisting of multiple micro-wells (SPAT), each micro-well also received 20 μl but this transferred volume was made up of equal parts by volume of samples from each of the micro-wells making up that selection environment. This transfer regime was repeated for 150 transfers resulting in approximately 1000 generations of selection. See chapter 4 or Bailey and Kassen (2012) for further methodological details.

5.2.2 Fitness and phenotype assays

Fitness of the evolved populations in their selection environment and in the three single resource environments was estimated by competing evolved strains against the ancestral genotype lacking the lacZ neutral marker. Each strain was first acclimated from frozen on the substrate of interest for two growth cycles (48 hours), then evolved and ancestor strains were mixed at a $\sim$1:1 ratio for a transfer volume of 20 μl. Estimates of the relative frequency of both types were quantified after the first (24 hours) and third (72 hours) growth cycles by plating on M9 minimal salts + glucose + X-Gal agar. We estimated relative fitness, ω, with the following equation:

$$\omega = \left(\frac{f_{final}}{f_{initial}} \right)^{(1/doublings)} \tag{5.1}$$

where $f_{initial}$ and f_{final} are the ratios of the frequency of the evolved population to the frequency of ancestral populations before and after competition respectively, and doublings refers to the number of doublings or generations that occur between the initial and final measurements. Resource-use phenotype was compared across selection environment and

population using a mixed effects nested-ANOVA, where population was modelled as a random effect nested within selection environment, and all other variables were modelled as fixed effects.

The catabolic profiles of the evolved strains were characterized using GN2 Biolog micro-well plates containing 95 different carbon sources plus a negative control well. Strains were grown from frozen overnight in 6 ml King's B media (28° C, shaken at 150 r.p.m.), starved for 2 hours (20 μl of each culture in 20 ml M9 minimal salts at 28° C, 150 r.p.m.), and then 150 μl was transferred into each well of the Biolog plates. Optical density (OD) was measured at the time of inoculation and after 24 hours of growth (28° C, 150 r.p.m.), and growth rate on each carbon substrate was calculated as r = ln(ODinitial)-ln(ODfinal). Growth rates were then adjusted relative to the control well by subtracting the maximum growth rate estimate obtained from the control wells (0.09), and setting all negative growth estimates to zero. The adjusted growth estimates were transformed into their principle components (first adjusting and scaling the data, then transforming using the 'pr.comp' function in R). We then restricted our analysis to the first two principle components (all other principle components described less than 5% of the total variation) and then compared these summary catabolic phenotypes across population and selection environment using a mixed effects nested-ANOVA as in the resource-phenotype comparison.

5.2.3 Genome sequencing and analysis

For each evolved population and the ancestral SBW25 genotype, genomic DNA was extracted from an overnight culture using the Promega Wizard Genomic DNA Purification kit. 75-bp paired-end Illumina sequencing was performed by the Michael Smith Genome Sciences Centre. Mean coverage across all 15 populations was ∼60-fold (mean = 60.91;

range 43.15 - 89.88) at a quality score of 20. We performed pair-end mapping of reads on the Pf SBW25 reference genome number NC_012660.1, called and filtered snps/ indels, and annotated with respect to the reference genome using the pipeline outlined in the supplementary information of Dettman et al. (2012). We called and filtered snps/ indels again using the BRESEQ pipeline (Barrick et al., 2009) to validate the previously identified snps/indels and also to identify larger-scale insertiondeletions that the BRESEQ pipeline is better suited to detect. Mutational changes in the sequence data identified by either pipeline were checked by viewing the mapped reads, and a subset of those mutational changes were then confirmed using targeted sequencing. A number of mutations initially detected in the whole genome sequencing analysis were located in intergenic repeat regions. However, these putative mutations were not well supported due to very low coverage (repeat regions are difficult to accurately sequence), and are unlikely to have fitness effects even if they are real. Thus we removed those putative mutational changes from any further analyses. Because we sequenced population genomic DNA, as opposed to that of single clones, we were able to detect evolved polymorphisms in our populations. We classified variation in reads at a given locus as a true population polymorphism (as opposed to sequencing error) when at least 10% of the reads differed from the others for a given allele.

We classified the mutation-bearing genes into functional groups using predicted COG classifications (Tatusov et al., 2000) provided in the Pseudomonas Genome Database (Winsor et al., 2011). In particular, we were interested in identifying genes with predicted 'cell motility' functions, as mutations in these genes may simply be general adaptations to living in a well-mixed lab environment, while we suggest that mutations in genes with non-cell motility related functions are more likely to be resource specific adaptations.

5.2.4 Parallel evolution comparisons

We quantified similarity in genetic changes between pairs of populations using the Jaccard Index, J. Given two sets G_1 and G_2 of the mutation-bearing genes found in populations 1 and 2, respectively,

$$\mathrm{J}(G_1, G_2) = \frac{G_1 \bigcap G_2}{G_1 \bigcup G_2} \tag{5.2}$$

That is, J is the number of genes mutated in both populations divided by the total number of genes mutated in population 1 or in population 2. J ranges from 0 to 1, with 1 indicating identical genotypes and 0 indicating no shared mutation-bearing genes. This value was calculated for all pairs of evolved populations, both within and between selection environments. Due to the non-independence of J estimates, statistical significance of the mean J value for each "comparison set" was estimated using a resampling procedure. By "comparison set" we mean a set of all pairwise population comparisons of a particular type; for example, all mannose-evolved to glucose-evolved population comparisons would make up a single "comparison set". For each comparison set, the populations' mutation-bearing genes were resampled from the set of all mutation-bearing genes identified in the combined fifteen population dataset. For each population, the number of resampled mutations was set equal to the number of actual mutations in that population. J was recalculated for each comparison in the set, and a new mean J was calculated. This procedure was repeated 50,000 times for each comparison set, generating distributions of mean J values. Using these distributions, we calculated the probability of observing the actual mean J values or greater by chance alone (P-value). Significance was assigned to probabilities less than 0.0033 - an experiment-wide P-value of 0.05, Bonferroni-adjusted for fifteen comparison sets. Calculation of J and significance testing was performed for all mutation-bearing genes and also for just the motility-related mutation-bearing genes.

We also quantified the difference in similarity between within- and between- selection environment comparisons by subtracting the average between-environment J values from the average within-environment J values for each replicate population. For this metric, a positive value indicates a population is more similar on average to populations selected in the same selection environment, while a negative value indicates a population is more similar on average with populations selected in different selection environments. For reasons of non-independence we again used resampling methods, testing whether the mean differences are significantly greater than zero, and if the effect differs with selection environment. For this test, the calculated within/between environment differences were randomly reassigned to a selection environment, and t-statistics were calculated using an ANOVA procedure. This was repeated 50,000 times, generating distributions to which the actual t-statistics were compared and P-values were then calculated.

Finally, we used the Jaccard similarity measures to look for a relationship between distance to the fitness optimum and degree of parallel evolution at the gene level. We used the log difference between ancestral and evolved absolute fitness in each selection environment, where absolute fitness was measured as cell density of a population after 24 hours growth. A re-sampled linear regression model was used to quantify and test for the significance of this relationship. All calculations and analyses were performed using R version 2.15.2 (R Development Core Team, 2011).

5.3 Results

5.3.1 Phenotypic changes

The relative fitness of all populations increased by 24.6%($\pm$2.3% SE) after 1000 generations of evolution and this change in fitness did not differ significantly across selection

environments (ANOVA, $F_{4,10} = 0.767$, $P = 0.571$; see Bailey and Kassen, 2012 for further details). However, the way in which populations adapted to gain those fitness improvements differed across selection environments. Figure 5.1 A shows how the populations differ in their fitness in mannose and xylose (two of the three component sugar resources used in the resource-use fitness assays) relative to each other, and relative to 1 - the fitness of the ancestor. Selection environment drove the evolution of different resource-use phenotypes (nested-ANOVA, interaction of selection environment and assay environment (S $\times$ A): $F_{8,140} = 15.192$, $P < 0.001$), with the exception of the X and MIX environments. However, replicate populations did not differ in their resource-use phenotype - including the interaction of population and assay environment (P $\times$ A) did not significantly improve the ANOVA model fit (χ^2 test comparing model log-likelihoods: $P = 0.9966$).

Biolog growth assay data was transformed into principle components, and the first 2 principle components (explaining 15.8% and 6.53% of the total variation respectively) were used as a summary of catabolic phenotype. With this phenotypic measure, populations do appear to group loosely by selection environment (Fig. 5.1 B), but this effect was not significant (nested-ANOVA, S $\times$ A: $F_{4,158} = 1.5066$, $P = 0.200$). Catabolic phenotype differed significantly by population, independent of selection environment including P $\times$ A significantly improved model fit (χ^2 test comparing model log-likelihoods, $P < 0.001$).

5.3.2 Genotypic changes

Mutational changes were detected in all populations after 1000 generations of selection, with the number of changes per population ranging from 1 to 6 (mean = 3.4667) for a total of 52 mutational changes across 15 independent populations. The detected mutations were made up of 24 SNPs, 8 insertions, and 20 deletions. We detected much within-population

genetic variation - 26 of the 52 detected mutations were polymorphic, however there was no significant difference in the frequency of detected polymorphisms across selection environments (ANOVA, $F_{4,10} = 0.6557$, $P = 0.6362$).

Most insertions and deletions were small and predicted to result in loss of function to a single gene, with three notable exceptions - a 2,864 bp deletion (spanning three genes) in a xylose-selected population, a 3,175 bp deletion (spanning two genes) present in two independent MIX-selected populations, and a 35,110 bp deletion (spanning 32 genes) in a glucose-selected population. We did not detect any gene-scale duplication events. The number of mutations per population varied by selection environment (Table 5.1; resampled ANOVA, $P = 0.0385$), with populations selected in the SPAT environment tending to have the largest number of mutations. These differences appear to be driven by mutations in genes not known to be related to motility (resampled ANOVA on non-motility-related genes: $P = 0.0361$; resampled ANOVA on motility-related genes: $P = 0.8215$). Details of the observed mutations are summarized in Table C.1.

5.3.3 Parallel evolution

The majority of the mutational changes detected by whole genome sequencing were unique nucleotide changes (45 of the total 52), however there were three examples of the same nucleotide change occurring in multiple independently evolved populations. Those changes were: 1) a 11 bp deletion resulting in a frameshift in the fruR gene, seen in two SPAT populations; 2) a SNP resulting in a stop gained in the PFLU1301 gene, seen in a MIX population and two SPAT populations; and 3) a 3,175 bp deletion resulting in the loss of the part of the PFLU2574 and all of the PFLU2575 gene, seen in two MIX populations.

Selection env.	Replicate	Non-mot.	Mot.	All mutations	Proportion of mutations that are polymorphic
G	1	1	2	3	0.67
	2	2	1	3	0.67
	3	1	–	1	0
M	1	1	1	2	0
	2	1	1	2	0.5
	3	3	1	4	1
X	1	1	3	4	0.75
	2	3	1	4	0.25
	3	2	1	3	0
MIX	1	2	2	4	0.25
	2	3	1	4	0.5
	3	2	1	3	0
SPAT	1	5	1	6	0.5
	2	3	1	5	0.6
	3	3	1	4	1
Totals		34	18	52	0.5

Table 5.1: Number of genic mutations detected in each evolved population. Mutations are categorized by whether the predicted function of the mutation-bearing gene is motility-related ('Mot.') or not ('Non-mot.'). Frequencies of mutations that were polymorphic are also listed.

At the gene-level, there was much more overlap between populations in the changes that occurred - 50% of the 22 mutation-bearing genes were seen in at least two independently evolving populations (Fig. 5.2 A), and the gene with the greatest number of repeat changes (PFLU4443, likely coding for a flagellar regulatory protein) was mutated in eight independent populations. The mutation-bearing genes for which function could be predicted were distributed across seven functional categories with the greatest proportion of mutation-bearing genes found in the cell motility category - about 25% (Fig. 5.2 B, white bars). Some mutation-bearing genes were shared across selection environments

that did not share any carbon resources (e.g. between populations in the mannose environment and the glucose environment), and those genes were significantly associated with motility ($\chi^2 = 7.0911$, df = 1, P = 0.0077). Unique mutational changes were more evenly distributed across functional categories, with cell motility, signal transduction, transcription, cell envelope biogenesis, and energy production/ conservation each making up about $10 - 15\%$ of the total (Fig. 5.2 B, grey bars).

Similarity at the gene-level, estimated with the Jaccard Index (J), ranged the full spectrum of possible values from no similarity (J = 0) to complete similarity (J = 1) (Fig. 5.3). The distributions of J values in each selection environment tended to be quite zero-heavy, with the possible exception of the SPAT environment. Fig. 5.4 A summarizes the mean degree of similarity in gene-level changes (J) between populations from different selection environments (black lines and boxes), and between populations evolved within the same selection environment (grey lines and boxes). Mean J values that are significantly higher than expected, given the set of all mutation-bearing genes observed, are indicated by solid lines. Some degree of overlap in genetic change was seen between populations in almost all pairs of selection environments (with the exception of the xylose-mannose comparison), and the mannose-SPAT comparison showed significantly more overlap than expected. Replicate populations evolving within the same selection environment all overlapped genetically to some extent, and populations evolving in the mannose and the SPAT environments were significantly more similar than expected. J values calculated with only the motility related genes showed a slightly different picture (see Fig. 5.4 B). Many selection environment pairs showed no overlap at all - indicated by the absence of a connecting line. However, populations evolving in the mannose and the SPAT environments were again more similar than expected and also more similar to each other than expected.

Examining the difference in similarity (estimated as J) between within- and between-selection environment comparisons across environments (Fig. C.1), we saw that within-environment similarity was significantly greater than between-environment similarity in mannose (resampled t-test, P = 0.0015) and SPAT (resampled t-test, P = 0.0005) and marginally significant in xylose (resampled t-test, P = 0.0500), but not significantly different in glucose and MIX (P = 0.9231 and P = 0.6805 respectively). Additionally, while the degree of parallel evolution does differ by selection environment, these differences do not appear to be driven by distance to the fitness optimum (Fig. 5.5; linear regression, P = 0.9225).

5.4 Discussion

We have shown that evolutionary parallelism occurs across multiple levels of biological organisation, from fitness and phenotype to genotype, in experimentally evolved populations of *P. fluorescens* selected in a range of environments. The degree of parallelism varied from complete overlap at the level of fitness where all fifteen evolved populations were indistinguishable, to only three repeated changes out of a total of 48 unique changes at the nucleotide level. In between, at the level of phenotype and the level of the mutation-bearing genes, the degree of parallelism varied both by selection environment and by individual population, from none to complete overlap.

5.4.1 Phenotypic and genotypic changes

Selection environment drove a number of interesting phenotypic and genomic changes in our experiment. In terms of resource-use phenotype, we saw that populations adapted to different selection environments tended to have different phenotypes, while populations

evolved in the same selection environment did not differ significantly. The exceptions to this pattern were the xylose-selected and MIX-selected populations. Populations evolved in these two types of environments were indistinguishable from each other with resource-use phenotype measures - these populations evolved improvements that were quite specific to xylose. However, the genes (and to some extent the catabolic changes) involved in those adaptations still tended to differ between the two environments. For example, all populations selected in xylose evolved what appear to be loss-of-function mutations in PFLU2366 - a gene coding for a putative GntR family regulatory protein. But this is not a distinguishing feature of populations evolved in the MIX environment. These gene-level differences suggest that the details of the environment are very important in driving selection and there may be evolved phenotypic differences too subtle to detect with our fitness assays.

5.4.2 General versus environment-specific adaptations

Some of the observed genetic changes detected in these populations appear to be general adaptations to well-mixed lab conditions. Mutations in genes known to be related to motility arose in all but one of the evolved populations and those mutation-bearing genes that are shared between the three single-resource selection environments - those environments that don't share any carbon resources - were significantly associated with motility. These motility related mutations likely all result in loss-of-function of flagella assembly (allelic replacements for a subset of these genes confirm this phenotype, data not shown). Repeated evolution of loss of motility is not surprising for populations selected in well-mixed liquid media where the ability to move is unnecessary, while assembly and use of the complex machinery required for locomotion is likely quite costly (Bardy et al., 2003). However, while loss of motility does appear to be a general adaptation to "life in

the lab", the availability and arrangement of resources still tends to constrain, to some extent, which motility genes bear the mutations in a given population. These differences between selection environments must be due to pleiotropic effects of motility genes on resource transport and/or catabolism, and future work will be aimed at characterizing some of these potential differences.

5.4.3 Parallel evolution

Differences between selection environments also drove differences in the degree of gene-level parallel evolution. Not surprisingly, gene-level changes tended to be more similar between populations selected under the same environmental conditions than between populations selected under different environmental conditions. But interestingly, within-environment gene-level similarity differed between selection environments. In particular, the degree of gene-level parallel evolution was significantly higher than expected for populations evolved in the mannose and the SPAT environments. Higher overlap in gene-level changes suggests that in these environments, the supply and/or fixation probability of beneficial mutations may be restricted, constraining the number of the potential routes for adaptation (Orr, 2005; Chevin et al., 2010).

While there are many factors that may act to constrain evolution and so increase the probability of parallel evolution, recent theoretical work by Chevin et al. (2010) highlights the importance of two mechanisms that could play a role in our system: 1) distance from the evolutionary optimum - the closer to the optimum, the higher the probability of parallel evolution, and 2) the degree of pleiotropy - as pleiotropy increases, so does the probability of parallel evolution. In the selection environments used here, ancestral populations started at a range of distances from an evolutionary optima. However, we were unable to detect a significant relationship between our estimates of distance from the optimum and

degree of parallel evolution in this system. This lack of a relationship between distance to the optimum and degree of parallel evolution is perhaps not surprising given the multitude of other factors that could generate variation in the degree of parallelism (as well as the small number of replicates we tested). For example, it is also possible that the variation we see in the degree of parallel evolution is generated by differences in realised pleiotropy across the different selection environments. It would be interesting to try to quantify potential differences in realised pleiotropy between our selection environments and relate these measures to estimates of the degree of parallel evolution, however this is beyond the scope of the current study.

It is also possible that the variation in the degree of parallel evolution seen in this study is influenced by variation in mutation rates across the genome. Mutation rate is known to vary across genomes across many scales - from increasing mutation rates with increasing distance from the origin of replication (Mira and Ochman, 2002), to the general mutational bias towards AT (Hershberg and Petrov, 2010). Some genes under selection may have higher mutation rates than the other genes under selection in that environment, and that potential bias in mutation rate might lead to biases in the particular mutations that fix. Thus, one would expect populations evolving in environments where mutation rate varies widely across the genes under selection to show a high degree of parallel evolution, while populations evolving in environments where mutation rates are relatively consistent across the genes under selection would be much less likely to evolve in parallel. To examine this potential mechanism, future work will be aimed at using comparative genomics to characterize genome-wide variation in mutation rates and identify the genes under selection in *P. fluorescens*.

5.4.4 Effects of environmental structure

Increased parallel evolution in SPAT-evolved populations compared to MIX-evolved populations suggests that environmental complexity may increase the probability of parallel evolution. While both types of environments provide the same multiple food-resources, the spatial-structure of the SPAT selection environment pushes the populations to evolve a generalist strategy as individuals and their offspring are required to utilize all three available sugars over the course of the experiment. In the MIX environment, all three resources are provided simultaneously and so populations in this environment can exist on a single resource, effectively ignoring the complexity in the environment. And indeed, the MIX populations appear to do this, evolving to specialize on a single sugar - xylose. Thus, under these conditions evolution in the SPAT environment appears to be more constrained than evolution in the MIX environment.

Adaptation in the SPAT environment also seems to require more genetic changes than adaptation to the other selection environments (Table 5.1) and the additional genetic changes that arise are in non-motility-related genes - suggesting that these changes may be environment specific improvements as opposed to more general adaptations to well-mixed lab conditions. Gene-level parallelism was quite high in the SPAT-evolved populations, and these populations shared many mutation-bearing genes, many of them shared with populations evolved in different selection environments. However, the large number of mutation-bearing genes shared between the SPAT populations, over and above those they shared with populations in other environments, particularly in light of the larger number of mutational changes seen in those populations, also suggests that fitness improvements in the SPAT environment require a specific set of rare genetic changes.

The other possibility is that differences in degree of parallel evolution were generated instead through differences in fixation probabilities of those beneficial mutations. Increases

in population size can increase the supply of mutations (both beneficial and deleterious), however as a population becomes increasingly large, fixation probabilities can also be affected. In very large population, clonal interference occurs (Gerrish and Lenski, 1998; de Visser et al., 1999), potentially increasing the probability of parallel evolution by constraining the set of beneficial mutations that are likely to fix to those of larger effect. However, there is no evidence to suggest that this mechanism drives differences between environments in our experiment. If clonal interference was driving increased parallel evolution in the SPAT populations, we would expect the mutations arising to be, on average, of larger effect. However the lack of significant differences in final fitness across selection environments combined with the significantly larger number of mutations detected in the SPAT populations suggests that, on average, the mutational changes arising in the SPAT environment actually have a smaller effect size compared to mutations arising in the other selection environments. In addition, if clonal interference is driving differences, we might expect more genetic polymorphisms in the SPAT populations - evidence of multiple clones competing for fixation. While a number of genetic polymorphisms were detected in our populations, the proportion of mutations that are polymorphic did not differ across selection environments.

5.4.5 Conclusions

As genome sequencing continues to become faster and cheaper, evolution experiments with large numbers of replicate populations will help to identify more clearly the factors affecting the probability of parallel evolution - a value inherently difficult to characterise simply because it is the product of so many stochastic events. Our ability in this study to detect differences between selection environments in the number of genetic changes and degree of parallelism with only three replicate populations per environment is indicative

of how important environmental context is for the rates and processes of adaptation. Distance from an evolutionary optimum and realized pleiotropy may play a role in determining the repeatability of evolution that is consistent with current theory, but not surprisingly, these factors are not the whole story. Future theoretical, comparative, and experimental work is necessary to further expound the mechanisms driving parallel evolution, and to continue working towards an understanding of how and when evolution is predictable.

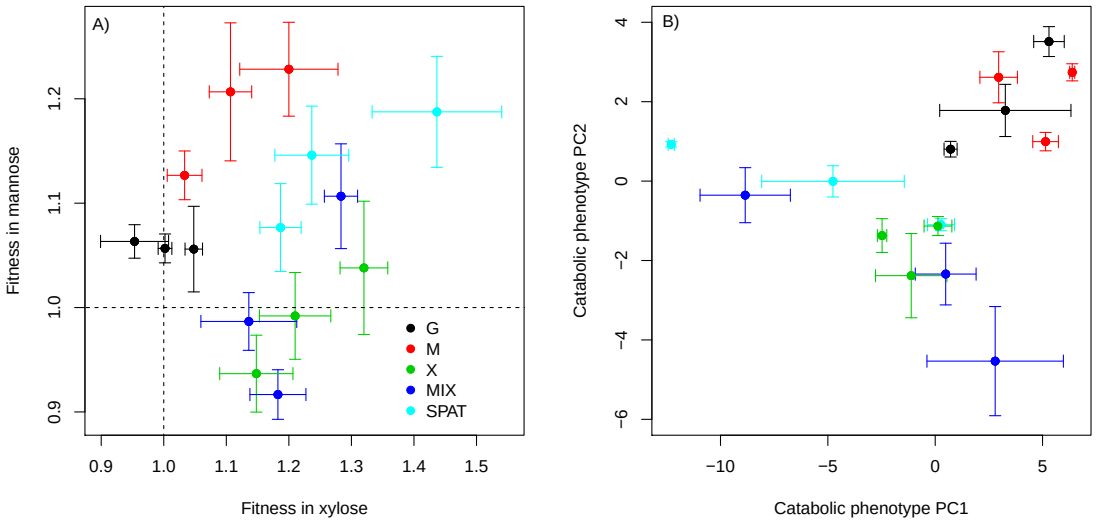


Figure 5.1: A) Fitness relative to the ancestor in mannose and xlyose (two of the three component resources), for each of the 15 populations. B) Catabolic phenotype (measured as growth on 95 carbon substrates) for each of the 15 populations summarized by their location in the first two dimensions of principle component space. Points and error bars represent mean ± standard error (N=6) for each population.

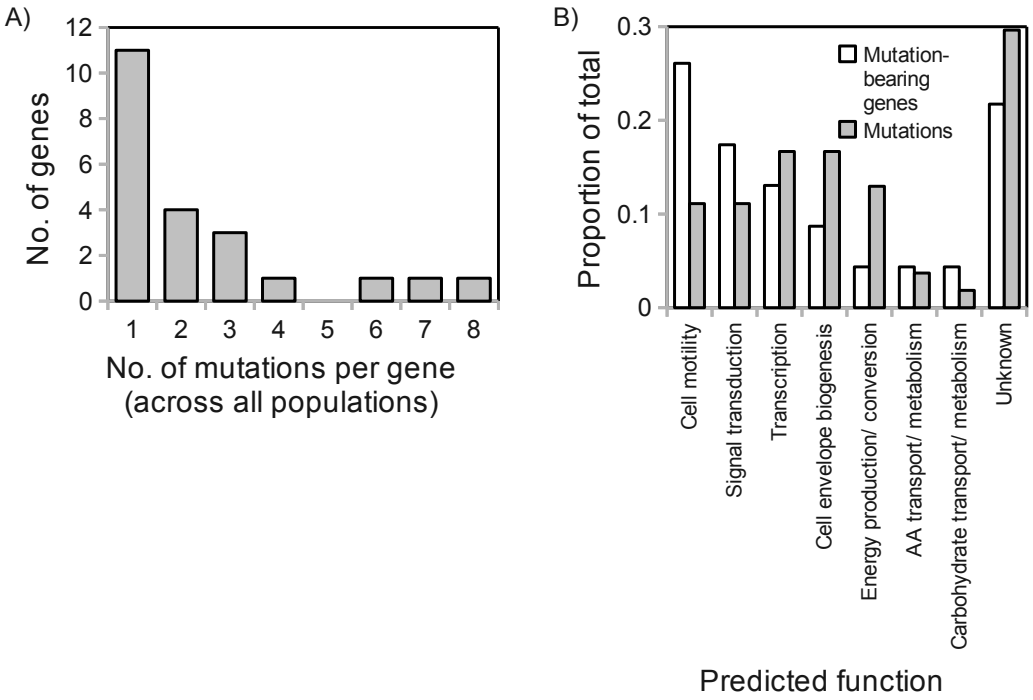


Figure 5.2: A) Distribution of mutations across the mutation-bearing genes of the 15 independent populations. B) Proportion of mutation-bearing genes and mutations from all 15 populations categorized by predicted function.

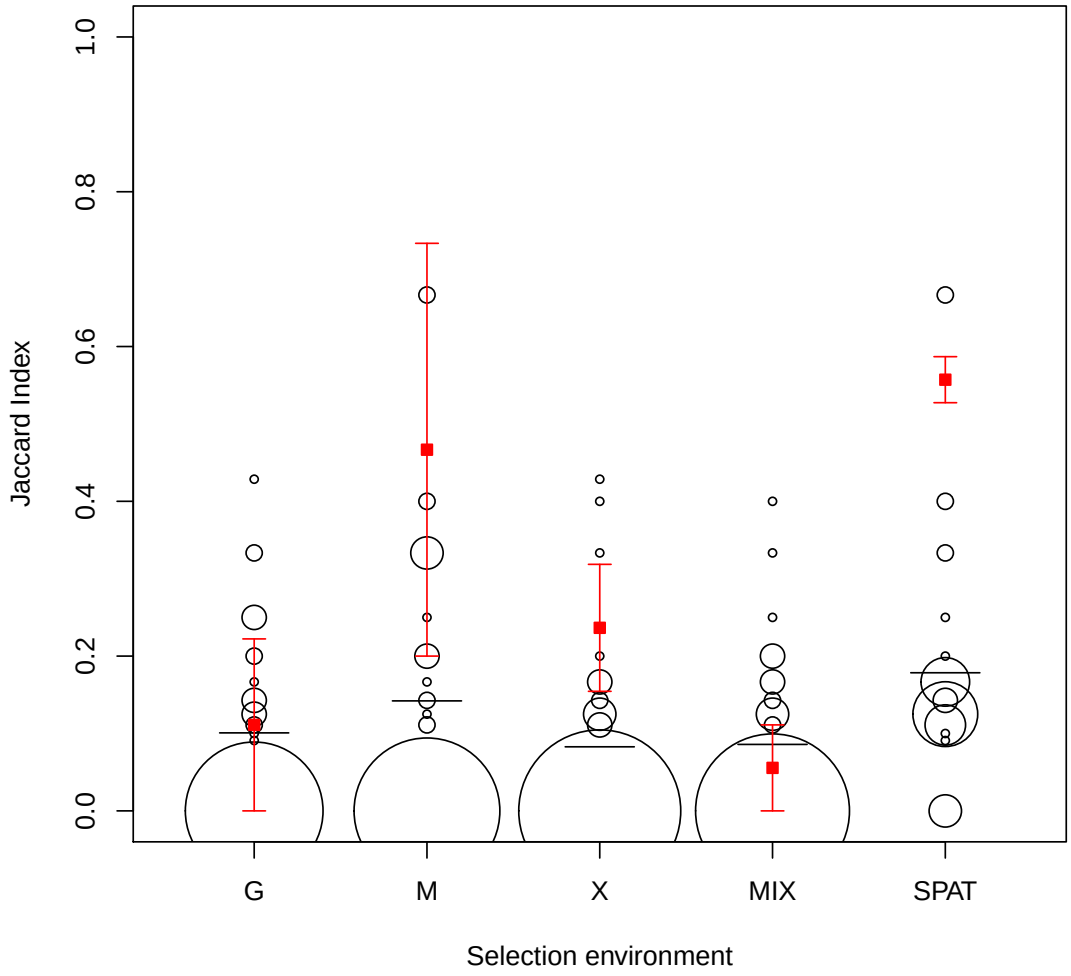


Figure 5.3: Jaccard index (J) by selection environment for all population comparisons of the genes baring mutations. Diameter of each circle is proportional to the number of observations at that value. Open circles indicate between-environment comparisons, black lines indicate the mean between-environment J value for each selection environment. Red squares indicate mean within-environment J-value ± s.e. (n=3).

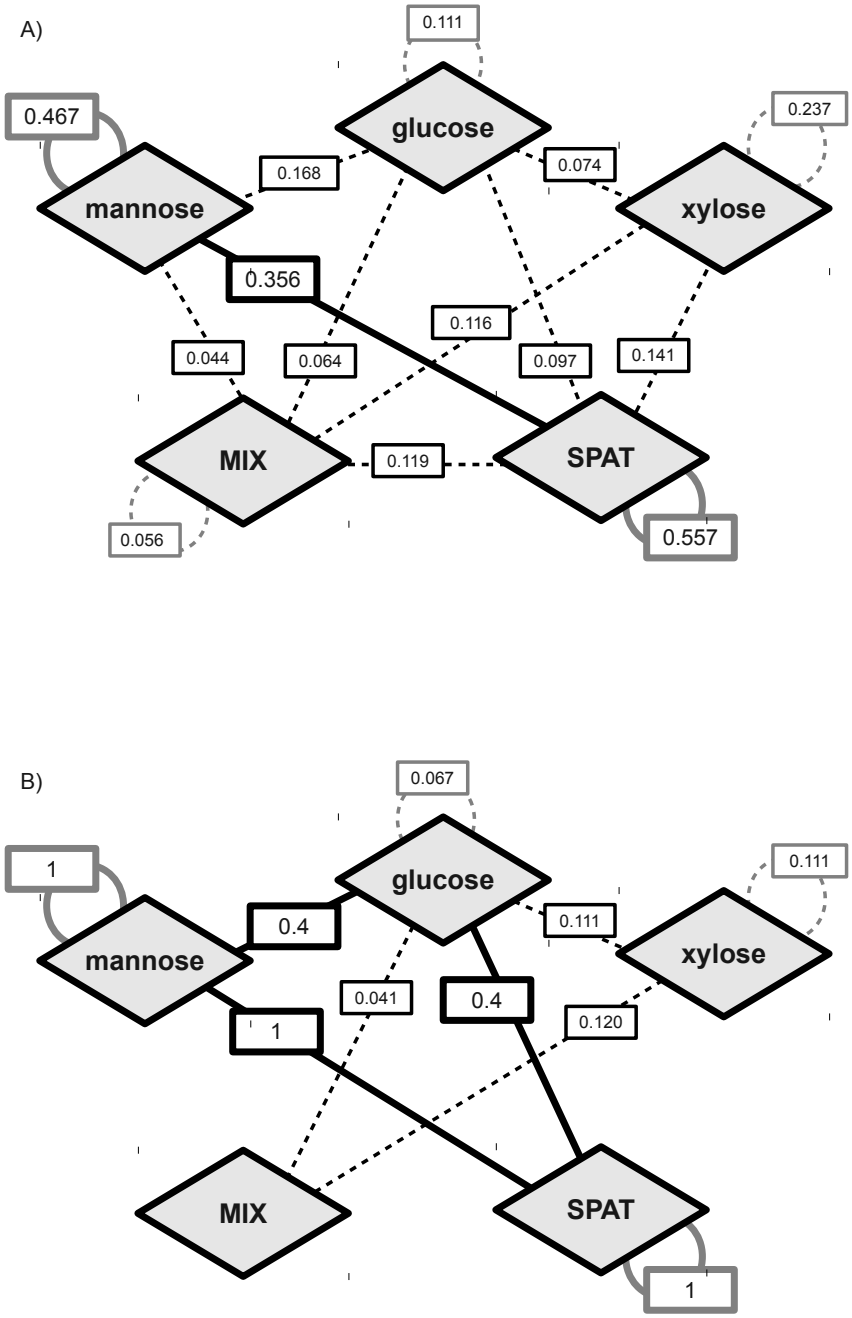


Figure 5.4: Mean Jaccard similarity index for a) all mutation-bearing genes, and b) motility-related mutation-bearing genes, both within (grey) and between (black) selection environments. Solid lines indicate comparisons with significantly higher similarity than expected.

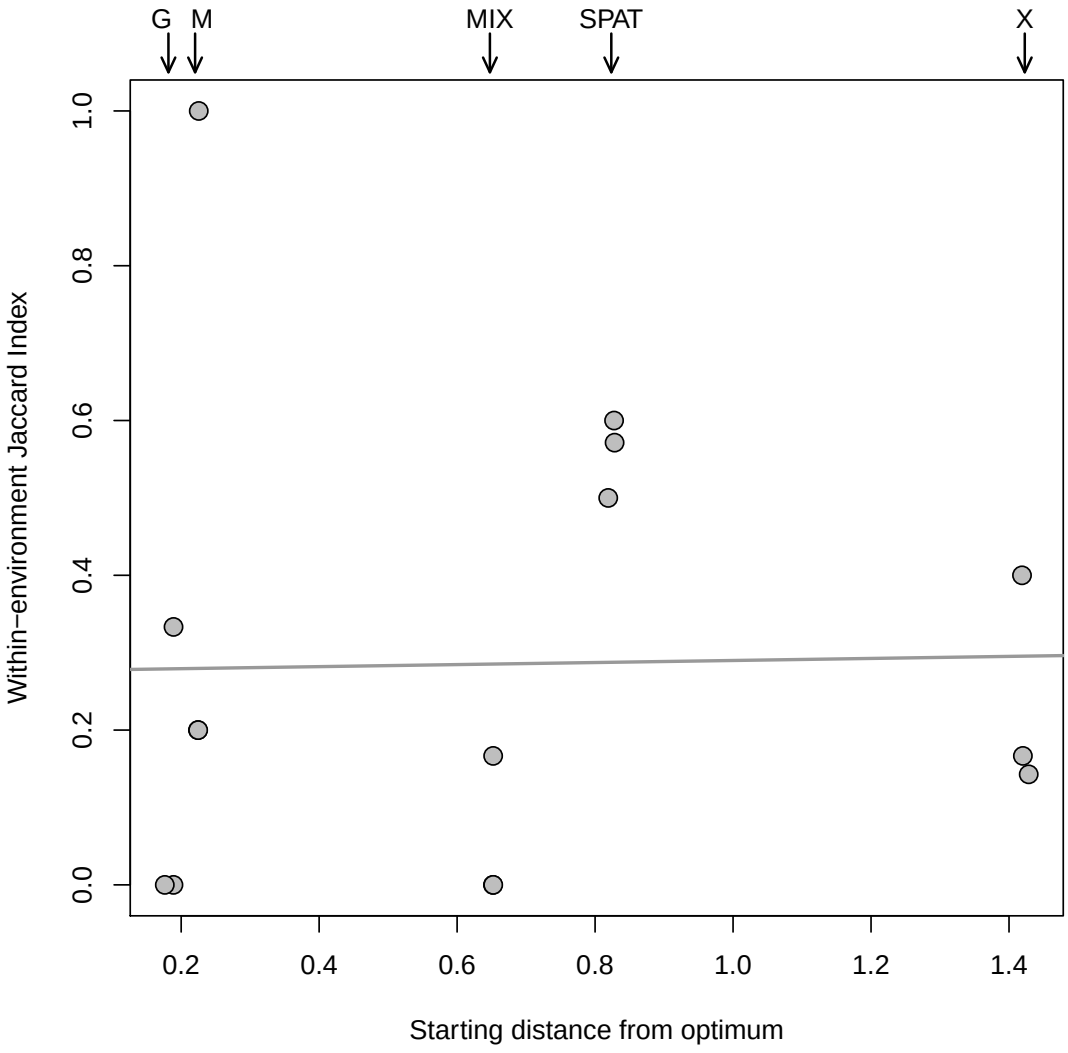


Figure 5.5: Within-environment similarity (estimated for each replicate population as the mean within-environment Jaccard Index), over starting distance from the fitness optimum (estimated in each selection environment as the log difference in 24-hour density between ancestral and evolved populations). Grey line shows the non-significant linear regression fit.

Chapter 6

Adaptive synonymous mutations in an experimentally evolved population

This chapter is reproduced from: Bailey, S. F., Hinz, A. and Kassen, R. (in review at *Science*) Adaptive synonymous mutations in an experimentally evolved population.

Collaborator contributions:

A. Hinz generated the *gtsB*(A10T), Δ*gtsB*, and all Δ*crc* strains, and conducted the luciferase transporter assay and induced-expression fitness experiments.

Abstract

Synonymous mutations, which do not change the amino acid produced, are often thought to have no effect on an organism's fitness. However, evidence from a variety of sources has been accumulating to suggest that synonymous mutations may sometimes affect fitness,

although the underlying mechanisms have remained elusive. Here we report the recovery of two synonymous single base pair changes that arose independently in an experimentally evolved population of *Pseudomonas fluorescens* and are clearly beneficial. We further show that the fitness advantage they confer is associated with increased gene expression. Our data provide direct, experimental evidence that synonymous substitutions can have beneficial fitness effects and drive the adaptive evolution of a population, and suggest that the use of synonymous mutations as a measure of the rate of neutral evolution should be reconsidered.

6.1 Introduction

Conventional wisdom holds that synonymous mutations, nucleotide changes that do not alter the encoded amino acid, should have no detectable effect on fitness. However, three lines of evidence suggest otherwise. First, pervasive variation in the frequencies of synonymous codons across genes and genomes (codon usage bias) (Sharp et al., 2005), and the association of some synonymous mutations with human diseases (Chamary et al., 2006) suggest that selection can favour some synonymous codons over others. Second, nucleotide replacement experiments show that certain synonymous mutations can reduce fitness, although on occasion some are recovered that appear to be beneficial (Lind et al., 2010; Cuevas et al., 2012; Schenk et al., 2012; Agashe et al., 2013). Third, a recent examination of deep population sequencing data from *Drosophila melanogaster* indicated that 22% of synonymous sites experience strong purifying selection (Lawrie et al., 2013). Taken together with the observation that synonymous mutations have been observed to affect gene expression (Agashe et al., 2013; Kudla et al., 2009), presumably through changes to the rate or accuracy of transcription and/or translation (Plotkin and Kudla, 2010), mRNA stability and folding (Shabalina et al., 2013), or even protein secondary

structure (Kimchi-Sarfaty et al., 2007; Zhang et al., 2009), evidence is accumulating that the conventional wisdom is sometimes wrong.

To date, no study has uncovered a mechanistic link between single synonymous mutations and their effects on fitness. Fitness changes are often assumed to be associated with changes in gene expression, but this has only been experimentally demonstrated for extensive changes in codon usage (Agashe et al., 2013). Here we show that synonymous substitutions that have arisen spontaneously during a laboratory evolution experiment increase fitness by an amount comparable to other, independently occurring, non-synonymous mutations. We also provide direct evidence to show that the fitness benefit conferred by these single synonymous mutations is caused by increased gene expression.

6.2 Results and Discussion

As part of a larger experiment (see chapter 4 or Bailey and Kassen, 2012), we propagated an initially isogenic population of *Pseudomonas fluorescens* SBW25 for 1000 generations in a minimal medium containing glucose as the sole carbon source. Fitness, assayed every 200 generations, increased significantly by 14.79% (t-test, P=0.01939) relative to the ancestor by the end of the experiment (Fig 6.1A). Comparing whole genome sequences of the generation 1000 population with the ancestor revealed two synonymous single base pair changes in the *gtsB* gene (A15A: GCA→GCG; G38G: GGC→GGT; see Fig 6.2A) and a 35,107 base pair deletion, Δ(PFLU4435-4466). We reconstructed the dynamics of substitution by isolating 24 colonies every 200-generations and probing each isolate for the relevant mutation (Figs 6.1A, B). The Δ(PFLU4435-4466) genotype appeared first around generation 200 and was nearly fixed by generation 400 before being replaced by a second genotype with the *gtsB*(A15A) mutation in the Δ(PFLU4435-4466) background, which rose to high frequency by generation 800 and was itself replaced by the

Δ(PFLU4435-4466) *gtsB*(G38G) genotype by the end of the experiment. This pattern, with each new genotype arising and nearly fixing within 100 generations, is consistent with natural selection acting on strongly beneficial mutations.

Assays of competitive fitness confirm that both evolved genotypes containing the synonymous substitutions confer a significant fitness advantage over the ancestor and, moreover, that Δ(PFLU4435-4466) *gtsB*(G38G) is fitter than Δ(PFLU4435-4466) *gtsB*(A15A) (Fig 6.1C), consistent with the substitution dynamics observed (Figs 6.1A, B) and strongly suggesting that the synonymous substitutions have beneficial effects. We confirmed that the synonymous mutations confer an independent fitness advantage using an allelic replacement strategy to introduce each synonymous mutation into the ancestral genetic background that lacked the Δ(PFLU4435-4466) mutation (see Materials and Methods). Competitive fitness assays reveal that *gtsB*(A15A) confers a 7.25 % (± 0.55 s.e.) and *gtsB*(G38G) a 8.73 % (± 0.79 s.e.) fitness advantage relative to the ancestor (Fig 6.1C).

Four lines of evidence suggest that the physiological effects of these synonymous mutations are specific to glucose and not general adaptation to laboratory conditions. First, both synonymous mutations occur in a gene predicted to encode a permease subunit of an ABC transporter, and homologues of this transport system in *P. aeruginosa* and *P. putida* are glucose-inducible and responsible for glucose uptake (Fig 6.2) (Hancock and Carey, 1980; Wylie and Worobec, 1993, 1995; del Castillo et al., 2007, 2008). Second, the benefits conferred are specific to glucose: neither mutation significantly affected fitness on alternate sugars (Fig 6.3). Third, from the same experiment we identified a beneficial non-synonymous mutation (A10T) in *gtsB* that arose in an independently evolved population in glucose (Fig 6.2A and Fig 6.3), while populations selected in mannose or xylose never showed mutations in this gene. Finally, a *gtsB* knockout strain (Δ*gtsB*) shows a significant fitness decrease in glucose of 14.23 % ± 1.42 s.e. compared

to the SBW25 ancestor ($P < 0.0001$; Fig 6.3), but no significant difference in fitness from the ancestor on alternate sugars ($P = 0.9563$; Fig 6.3). These results strongly suggest that the synonymous mutations confer an adaptive advantage specifically on glucose, and that the fitness gains do not stem from a loss of gene function.

What is the mechanism by which the synonymous substitutions confer high fitness? As the amino acid sequence is not changed, the mechanism presumable involves changes to gene expression at the level of transcription or translation, or perhaps even changes to protein secondary structure resulting from altered translation rates (Plotkin and Kudla, 2010; Zhang et al., 2009). We obtained measures of *gtsB* gene expression by assaying transcript abundance using a luciferase reporter assay (see Methods; Fig. D.1). We found that synonymous mutations and the independently evolved non-synonymous mutation in the same gene increased gene expression by approximately two-fold in glucose (Fig 6.4A; $P < 0.0001$). Both the wild-type and mutant alleles were specifically expressed on glucose, indicating that there was no shift from inducible to constitutive expression. Although our assay cannot distinguish between an increase in gene expression due to increased rates of transcription or decreased rates of transcript degradation, we can confirm a link between increased gene expression and fitness. While transcriptional induction of the wild-type *gtsB* gene alone did not increase fitness, induction of the entire operon containing the gene was beneficial (Fig 6.4B). Taken together, these results indicate that increased expression of the ABC transporter operon is likely responsible for the increased fitness caused by these synonymous mutations.

The proximate cause of increased expression in this system remains unresolved but we find little support that the most common explanations, codon usage bias and mRNA stability, explain our results. Both the A15A and G38G mutations result in slightly less preferred codons (see Materials and Methods; table D.1), indicating that the fitness

increase is not due to aligning codon bias within the gene to that of highly expressed genes. Synonymous mutations can affect the stability of mRNA secondary structure through altered base pair interactions. Less stable secondary structures near a ribosomal binding site can lead to increases in RNA and protein levels, presumably via more efficient ribosome binding (Kudla et al., 2009). Although the G38G mutation does decrease the predicted mRNA stability relative to the ancestor, the A15A mutation does not (table D.1), suggesting that changes in mRNA folding energy do not fully explain the effects of the substitutions. It is conceivable that the fitness benefit of these mutations comes about via their effect on catabolite repression. Glucose metabolism in Pseudomonads is regulated in part by the binding of the catabolite repression control protein (Crc) to target mRNA, the effect of which is to inhibit translation (Rojo, 2010). It may be that the synonymous mutations inhibit binding of Crc to *gtsB* mRNA, leading to increased GtsB expression. While the operon containing *gtsB* is a predicted target of Crc (Browne et al., 2010), in a Δcrc background the synonymous mutations remain beneficial and gene expression elevated, suggesting that the increase in fitness we observed is independent of this mechanism of catabolite repression (fig D.1). It remains possible that differences in translational efficiencies, if they exist, could affect protein folding (Kimchi-Sarfaty et al., 2007) but this seems unlikely given that we observed an increase in fitness associated with increasing gene expression in the wild type allele (Fig. 6.4C), however further examination of this hypothesis must await a complete characterization of the GtsB protein.

6.3 Conclusions

Our results demonstrate unequivocally that synonymous mutations can increase fitness by causing increases in gene expression. It is notable that the nearby non-synonymous mutation we uncovered in this experiment increases fitness by a comparable amount,

displays the same resource-specificity, and shows a similar increase in gene expression to the synonymous mutations. These results suggest that the increase in fitness conferred by this mutation may arise through the same mechanism as that used by the synonymous mutations (e.g., by increasing gene expression) rather than by changing the protein structure. Although the precise molecular mechanism responsible for these increases in fitness remains elusive, our results show that synonymous mutations can contribute to adaptation and be positively selected. Moreover the selection coefficients associated with these mutations are comparable to those of many other beneficial mutations that arise from non-synonymous changes in microbial selection experiments. These results, together with the observation that as many as 22% of synonymous sites may be under strong selection in *Drosophila melanogaster* (Lawrie et al., 2013), thus call into question the validity of using estimates of the relative rates of non-synonymous to synonymous mutations to infer the strength of selection from comparative gene sequence data. The fact that we have uncovered an example of adaptive evolution driven by the substitution of synonymous mutations in an experimental system evolving over a few hundred generations suggests that this class of mutation is vastly under-appreciated as a cause of adaptation and evolutionary dynamics in nature.

6.4 Materials and Methods

Bacterial strains, plasmids, and media. The strains, plasmids, and primers used in this study are listed in Tables D.2 and D.3. Plasmids were propagated in *E. coli* DH5α or DH5$\alpha\lambda$pir (for pUIC3 derivatives). Transformation of DNA into *E. coli* and *P. fluorescens* was accomplished by chemical transformation, electroporation, or conjugation according to standard protocols (Sambrook and Russell, 2001; Choi et al., 2006).

E. coli was grown on LB media at 37°C and *P. fluorescens* on LB or minimal (M9)

media at 28°C. The minimal media contained 48 mM Na_2HPO_4, 22 mM KH_2PO_4, 9 mM NaCl, 19 mM NH_4Cl, 2 mM $MgSO_4$, and 0.1 mM $CaCl_2$ with either glucose (53 μM), succinate (80 μM), mannitol (53 μM), mannose (53 μM), or xylose (1.4 mM) as sole carbon source.

Antibiotics were used at the following concentrations: 100 g/ml ampicillin (Ap), 25 μg/ml kanamycin (Km), and 25 μg/ml gentamicin (Gm) for *E. coli*; 100 μg/ml nitrofurantoin (Nf), 10 μg/ml tetracycline (Tc), and 10 μg/ml Gm for P. fluorescens. Media was supplemented with 5-Bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-gal) at 20 μg/ml or Isopropyl β-D-1-thiogalactopyranoside (IPTG) at 1 mM when appropriate.

6.4.1 Selection experiment and competitive fitness assays

As part of a larger experiment, we propagated an initially isogenic population of *Pseudomonas fluorescens* SBW25-lacZ for 1000 generations in a minimal medium containing glucose as a sole carbon source (see chapter 4 or Bailey and Kassen, 2012). We estimated relative fitness of the population every 200 generations using 48 hour head-to-head competitions against the SBW25 ancestor. Fitness was calculated as $\omega = (f_{final}/f_{initial})^{1/doublings}$, where $f_{initial}$ and f_{final} are the ratios of the frequency of the evolved population to the frequency of ancestral population before and after competition respectively, and *doublings* refers to the number of doublings or generations that occur between the initial and final measurements. Six isolates from the generation-1000 population were also assayed for their relative fitness in glucose, mannose, and xylose minimal media environments to determine whether the detected fitness improvements were glucose-specific. Fitness of the SBW25 allelic replacement constructs was estimated in competitions against SBW25-lacZ.

6.4.2 Whole-genome sequencing and genotyping

Chromosomal DNA was extracted from the generation-1000 population using a Promega Wizard Genomic DNA Purification kit and Illumina sequencing was performed by the Michael Smith Genome Sciences Centre. The sequence data were aligned to the *P. fluorescens* SBW25 reference genome NC_012660.1, and mutations were called and annotated using a custom computational pipeline (Dettman et al., 2012) and BRESEQ (Barrick et al., 2009). The time-dynamics of the detected mutations were characterized using 24 isolated colonies from every 200 generations over the course of the experiment. Single-nucleotide polymorphisms (SNPs) were detected by amplifying a $\sim$ 700 bp PCR product containing the putative SNP from each isolate, and directly sequencing the PCR products (Genome Quebec, Montreal). We probed for the large 35,107 bp deletion in each isolate by characterizing them as motile (ancestral state) versus non-motile (evolved state) on semi-solid agar plates, a trait associated with this deletion.

6.4.3 Allelic replacement constructs

The *gtsB* synonymous mutations (A15A and G38G) were individually introduced into SBW25 using a previously described allelic exchange method involving the pUIC3 vector (Rainey, 1999; Goymer et al., 2006). PCR fragments containing the mutations and 1-kb of flanking sequence on each side were amplified using primers 4845-BglII-F and 4845-AvrII-R from chromosomal DNA isolated from the evolved bacteria. The DNA was digested with BglII and AvrII and cloned into the compatible BglII and SpeI sites of pUIC3 to generate pUIC3-A15A and pUIC3-G38G. The plasmids were mobilized into SBW25 by triparental mating with the help of pRK2013. Exconjugates carrying the plasmid integrated by a single homologous recombination were selected on LB agar with Nf and Tc. To allow for plasmid excision by a second homologous recombination, bacteria

were grown for two 24 h subcultures in LB broth, and plated on LB agar containing X-gal (Goymer et al., 2006). The rare white colonies do not express pUIC3-encoded *lacZ* and were screened for tetracycline sensitivity to verify loss of the plasmid backbone. The *gtsB* locus of independent white colonies was sequenced to identify clones with the desired SNPs (Genome Quebec, Montreal).

Additional *gtsB* mutations (A10T and $\Delta gtsB$) and the *crc* (PFLU5989) deletion were recombined into SBW25 using a pUIC3 derivative in which the *lacZY* genes were replaced by the sacB counterselectable marker. This vector was generated by excising the *lacZY* genes from pUIC3 by EcoRI digestion, followed by self-ligation, transformation into DH5$\alpha lambda$pir, and screening for white colonies on LB with Ap and X-gal. A 2-kb fragment containing the *sacB* gene was amplified from pEX18Tc (Hoang et al., 1998) using primers SacB-EcoRI and SacB-MfeI and inserted at the EcoRI site of the *lacZY*-excised pUIC3. The resulting plasmid (pAH79) confers sucrose-sensitivity, Ap^r, and Tc^r. Transcription of the *sacB* gene is oriented towards the multiple cloning site, which carries an additional unique EcoRI site suitable for cloning.

The pAH79 derivative containing the *gtsB*(A10T) mutation was generated by amplifying the 2-kb *gtsB* locus using primers 22F-5324097 and 22R-5324097 from DNA isolated from an evolved clone carrying the mutation. The fragment was digested with BglII and XbaI and cloned into the BglII and SpeI sites of pAH79 to generate pAH79-A10T. The *gtsB* deletion plasmid was constructed by amplifying 1-kb fragments upstream and downstream of the *gtsB* gene with primer pairs 4845-del-F1/R1 and 4845-del-F2/R2. The two products were digested and ligated together using engineered SacI sites, and the 2-kb hybrid product was amplified with primers 4845-del-F1/R2. The resulting fragment contains a gtsB allele with an in-frame deletion of codons 7 through 274 (of 302 total codons). The fragment was digested with BamHI and XbaI and inserted in the compatible BglII and SpeI sites of

pAH79 to generate pAH79-Δ*gtsB*. The *crc* deletion plasmid (pAH79-Δ*crc*) was generated similarly with primer sets crc-del-F1/R1 and crc-del-F3/R3. The allele carries a deletion of codons 31 to 216 (of 259 total), with a stop codon introduced after codon 31.

Chromosomal integration of the pAH79 derivatives in *P. fluorescens* was achieved by triparental mating as described for the pUIC3 constructs. *sacB* counterselection enabled selection for the second recombination event by streaking Tc^r exconjugates on LB agar containing 5 % sucrose. Sucrose-resistant colonies were tested for Tc-sensitivity and screened for the presence of the desired mutations by sequencing (for A10T) or a PCR test (for the *gtsB* and *crc* deletions).

6.4.4 Transcriptional luciferase fusions

Gene expression of the *gtsB* alleles was examined using transcriptional luciferase fusions delivered by a site-specific mini-Tn7 transposon (Choi and Schweizer, 2006). A 2.1-kb PCR fragment including the 350-bp *gtsA* promoter region, *gtsA* open reading frame, and 115 codons (out of 302) of the *gtsB* open reading frame was amplified with primers 4845-lacZ-F1 and 4845-lacZ-R1. The fragment was digested with XmaI and XhoI and ligated at the corresponding sites of the vector pUC18-mini-Tn7T-Gm-*lux*. The truncated *gtsB'* open reading frame encounters a vector-encoded stop codon 36 nucleotides downstream of the XhoI site, and the start codon of the initial *lux* gene is located 432 nucleotides further. Constructs containing four *gtsB* alleles (wild-type, A15A, G38G, and A10T) were introduced into *P. fluorescens* by co-electroporation with the pTNS2 helper plasmid by a previously described method (Choi et al., 2006), with selection on LB Gm_{10} (for SBW25) or LB Gm_1 (for SBW25 Δ*crc*). Insertion at the *att*Tn7 locus was verified as recommended (Choi and Schweizer, 2006) by PCR amplification with mini-Tn7 and SBW25-specific primer sets (PTn7R/PglmS-up-SBW25 and PTn7L/PglmS-down-SBW25).

Activity of the transcriptional fusions was determined in exponential phase liquid cultures. Overnight cultures grown in M9-glucose or M9-succinate media were diluted (1:100) in 1.5 ml fresh media and incubated 7 hours with shaking in 24-well plates. Luminescence at 520 nm was measured using a microplate reader (Tecan Infinite 200 Pro). The luminescence values were standardized according to cell densities, which were determined by dilution plating.

6.4.5 Inducible gene expression constructs

Regulated expression of *gtsB* and *gtsABCD-oprB* by the P_{tac} promoter was achieved using mini-Tn7T-LAC constructs (Choi and Schweizer, 2006). A 1-kb fragment containing the *gtsB* open reading frame and 53-bp of upstream sequence was amplified with primers SacI-F2-Tac and XhoI-R1-Tac, digested with SacI and XhoI, and cloned into the corresponding sites of pUC18-mini-Tn7T-LAC to generate pAH308. The 5.8-kb *gtsABCD-oprB* fragment was amplified with primers SacI-F1-Tac and NheI-R3-Tac and included sequences from 31-bp upstream of the *gtsA* start codon to 32-bp downstream of the *oprB* stop codon. The product was digested with SacI and NheI and cloned into the compatible SacI and SpeI sites of pUC18-mini-Tn7T-LAC to generate pAH305. Transposition of the expression constructs to the SBW25 *att*Tn7 site was performed as described for the luciferase fusions. The fitness effects of inducing the *gtsB* and *gtsABCD-opr* genes were measured via 48 h head-to-head competitions against SBW25-*lacZ* mini-Tn7T-LAC under inducing (1 mM IPTG) and non-inducing (no IPTG) conditions.

6.4.6 Codon bias estimates and mRNA structure comparisons

To look for potentially important changes in codon bias as a result of the two synonymous mutations, we compared the codon usage of highly expressed genes in SBW25 to codon

usage within the *gtsB* gene using the codon adaptation index (CAI) (Sharp and Li, 1987). We used the SBW25 ribosomal protein genes as our sample of highly expressed genes. To calculate change in CAI caused by each mutation, we used the 'cai' function in the 'seqinr' package (Charif and Lobry, 2007) in R. We then used 'mfold' (Zuker, 2003) to predict and compare mRNA structure and stability of the ancestral *gtsB* gene and the three mutated versions.

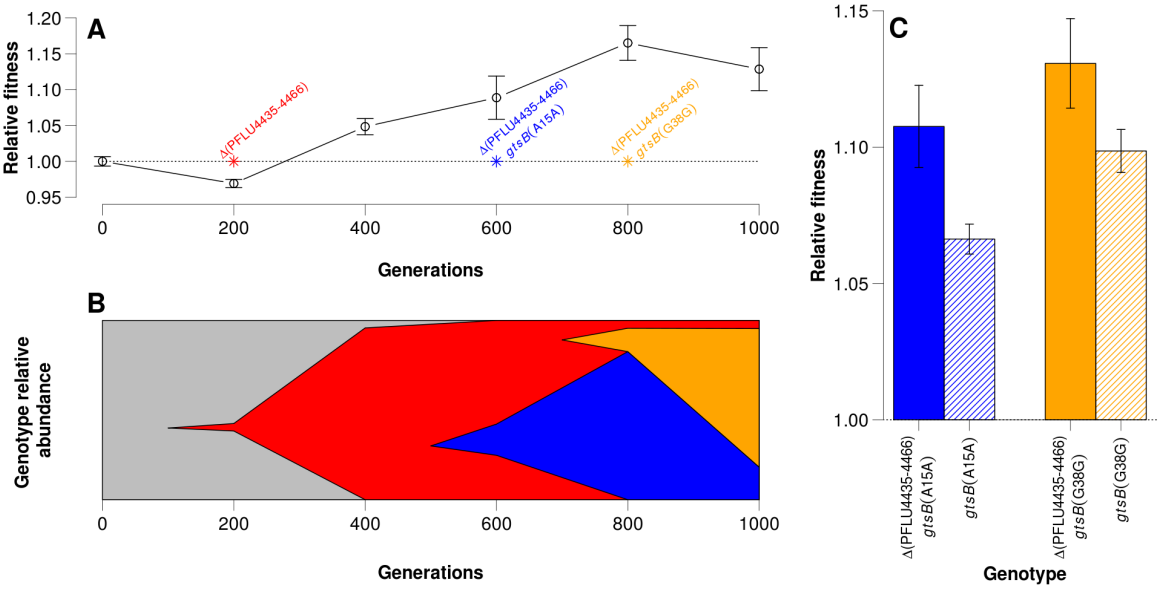


Figure 6.1: Fitness and genotype trajectories of a *P. fluorescens* population adapting to glucose minimal media over 1000 generations. A) Population fitness relative to the ancestor (SBW25), over 1000 generations (mean ± s.e.; N=3). * indicates when genotypes were first detected. B) Relative abundance of the ancestor (grey), Δ(PFLU4435-4466) (red), Δ(PFLU4435-4466) *gtsB*(A15A) (blue), Δ(PFLU4435-4466) *gtsB*(G38G) (yellow) genotypes over 1000 generations. C) Fitness of evolved (solid bars) and constructed (hashed bars) synonymous substitution genotypes relative to the ancestor (mean ± s.e.).

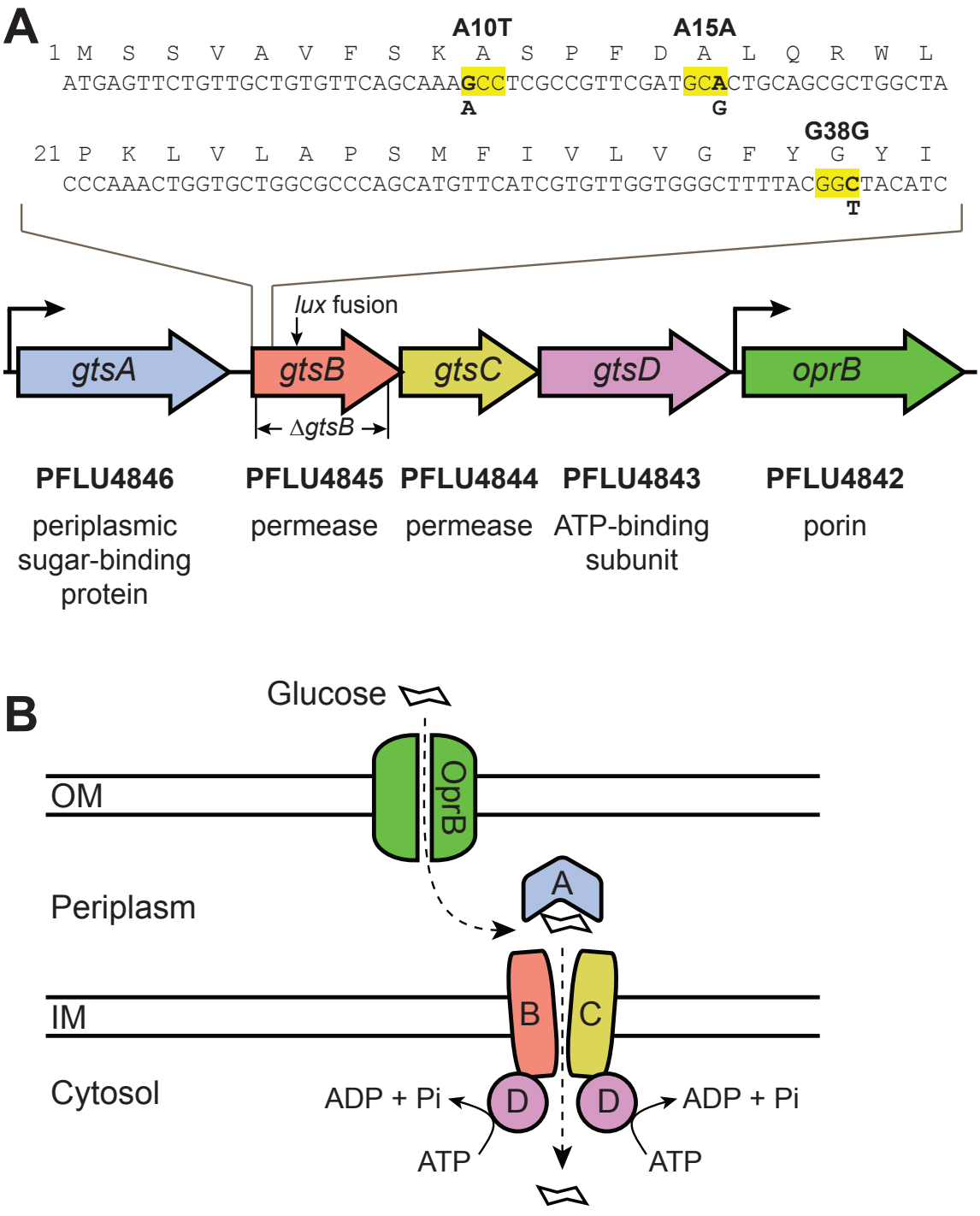


Figure 6.2: Continued on following page.

Figure 6.2: Genomic and functional context of the synonymous (A15A and G38G) and nonsynonymous (A10T) substitutions. (A) The substitutions occurred in in the 5' region of PFLU4845, which encodes a permease subunit of an ATP-binding cassette (ABC) transporter. The DNA and amino acid sequence of the first 40 codons are shown, with the nucleotide substitutions indicated below the highlighted codons. Neighboring genes encode three additional ABC transporter subunits and an outer membrane porin. Homologues of these gene products are glucose-inducible and responsible for glucose uptake in *P. aeruginosa* and *P. putida* (Hancock and Carey, 1980; Wylie and Worobec, 1993, 1995; del Castillo et al., 2007, 2008). The transporter gene names (*gts*; **g**lucose **t**ransporter **s**ubunit) are based on those of the *P. putida* KT2440 homologues (del Castillo et al., 2007). The open reading frame lengths, position of the luciferase transcriptional fusion junction, and $\Delta gtsB$ deleted region are drawn to scale. Bent arrows indicate promoters predicted by the Softberry BPROM program (http://linux1.softberry.com/berry.phtml). (B) The schematic depicts the probable subunit organization of the gene products during glucose uptake (Davidson and Chen, 2004; Schneider, 2001). Glucose crosses the outer membrane by facilitated diffusion through the OprB porin, interacts with the periplasmic glucose-binding protein (GtsA), and is transported across the inner membrane channel ($GtsBCD_2$) in a process energized by ATP hydrolysis. IM, inner membrane; OM, outer membrane.

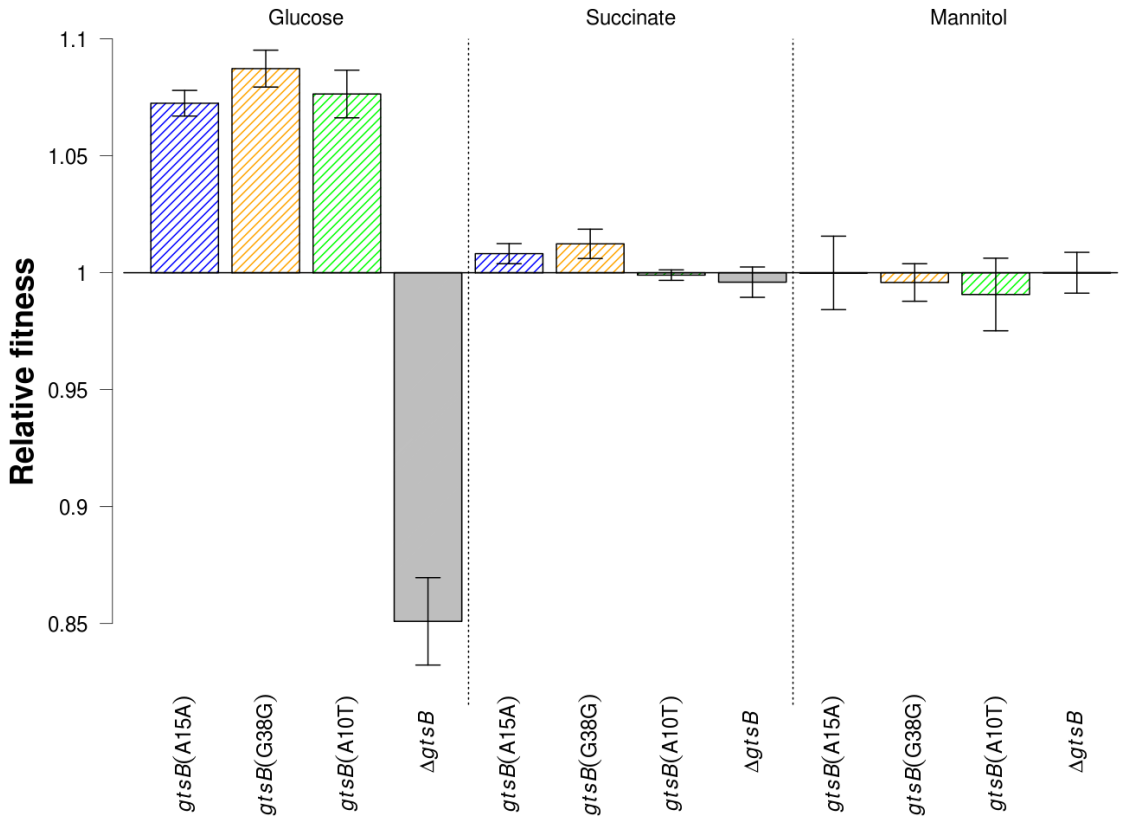


Figure 6.3: Relative fitness of constructs containing the two synonymous mutations, *gtsB*(A15A) and *gtsB*(G38G), the non-synonymous mutation, *gtsB*(A10T), and the *gtsB* knock-out, Δ*gtsB*), in glucose and two alternate carbon sources - succinate and mannitol. The mean ± s.e. for each treatment is shown.

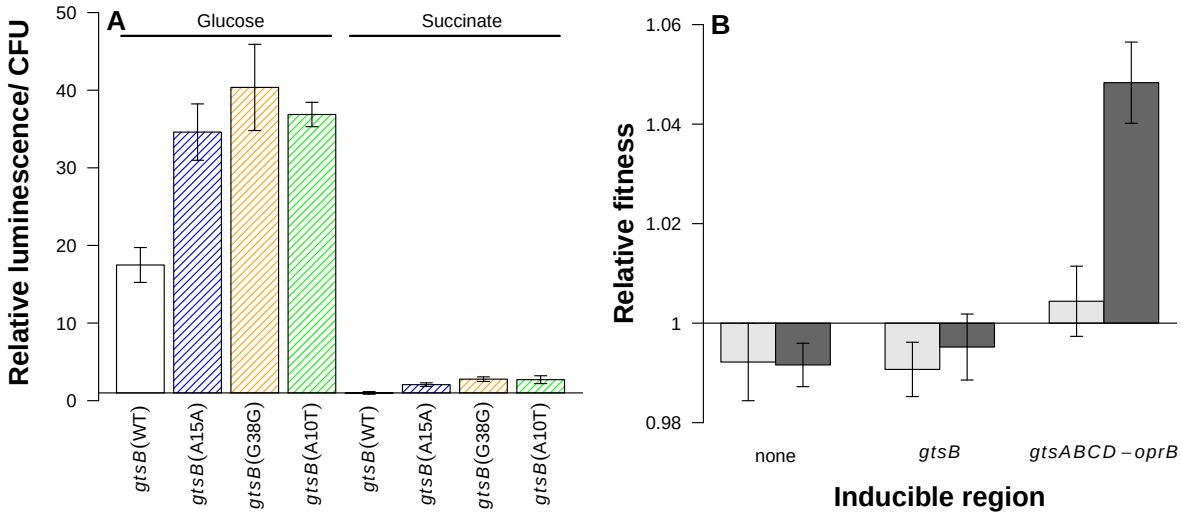


Figure 6.4: The effects of the *gtsB* mutations on gene expression, and the effects of increased expression on fitness. A) Reporter gene expression measured in luminescence/ colony forming units (CFU), relative to the minimum expression (mean ± s.e.). Transcriptional reporter strains with the wild-type (WT), synonymous (A15A and G38G), and non-synonymous (A10T) alleles of *gtsB* driving luciferase expression were tested in the selection environment (glucose) and with an alternate carbon source (succinate). B) Mean fitness (± s.e.) of constructs containing inducible inserts. Light grey shows fitness in the absence of the inducer (IPTG) and dark grey shows fitness in the presence of the inducer. The mean ± s.e. for each treatment is shown.

Chapter 7

General conclusions

Competition and ecological opportunity are two ecological factors with the potential to influence adaptation and diversification, and for this thesis I have tested the effects of both of these factors using experimental evolution of the bacterium *Pseudomonas fluorescens*. I found that intraspecific competition drives adaptive diversification and that this effect increased with increasing strength of competition. The presence of interspecific competitors, on the other hand, either increased or decreased adaptive diversification depending on both the strength of competition and the ability of the competitor itself to diversify. Strong interspecific competitors prevented diversification of the focal population simply by out-competing them before they had a chance to evolve diversity, while diversifying competitors prevented diversification of the focal population by diversifying into new niches, essentially blocking the focal population from diversifying. However, the presence of weak interspecific competitors drove diversity of the focal population, by increasing the strength of resource competition and so driving divergent selection.

I have also shown experimentally that variation in ecological opportunity can have important effects on evolution. I report that both the novelty and spatial structure of the available ecological opportunities can drive rates of adaptation, however I found no

significant effect of this factor on evolved diversity. I also found that spatial structure in ecological opportunity increased the average number of mutations required for adaptation, as well as increasing the degree of parallel changes seen across replicate evolved populations.

The final section of my thesis, while not strictly addessing my general questions about the effects of ecological factors, still has important implications for understanding how ecological processes influence evolutionary processes. I have shown that the traditional assumptions about evolution at synonymous versus nonsynonymous sites in genomes do not always hold true, and so strategies for identifying patterns of selection using comparative genomics need to be reassessed. Allowing for the possibility of adaptive synonymous mutations may expand future interpretations of genomic evolution in evolution experiments, providing a more accurate representation of adaptive evolutionary outcomes.

Although I have tested two ecological factors using separate sets of experiments, these factors also vary and interact with each other in natural systems. In fact, without the availability of multiple ecological opportunities, competition is unable to drive the evolution of diversity. Furthermore, only certain types and arrangements of ecological opportunity are expected to drive adaptation and diversification. It is clear that an understanding of ecological interactions and their effects on evolution is necessary for predicting the progress of adaptation and diversification, and my thesis represents a valuable contribution to this growing body of work.

Appendix A

Appendix to Chapter 3

A.1 Supplemental figures and tables

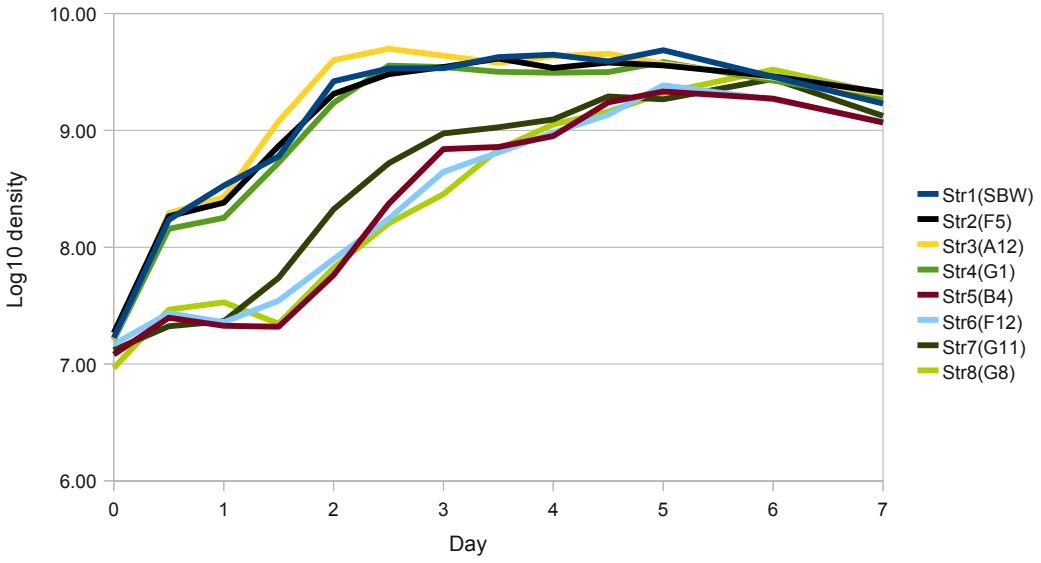


Figure A.1: Log-transformed mean density (CFU/ml) of the naladixic acid-resistant mutant strains grown in static Kings B microcosms for seven days.

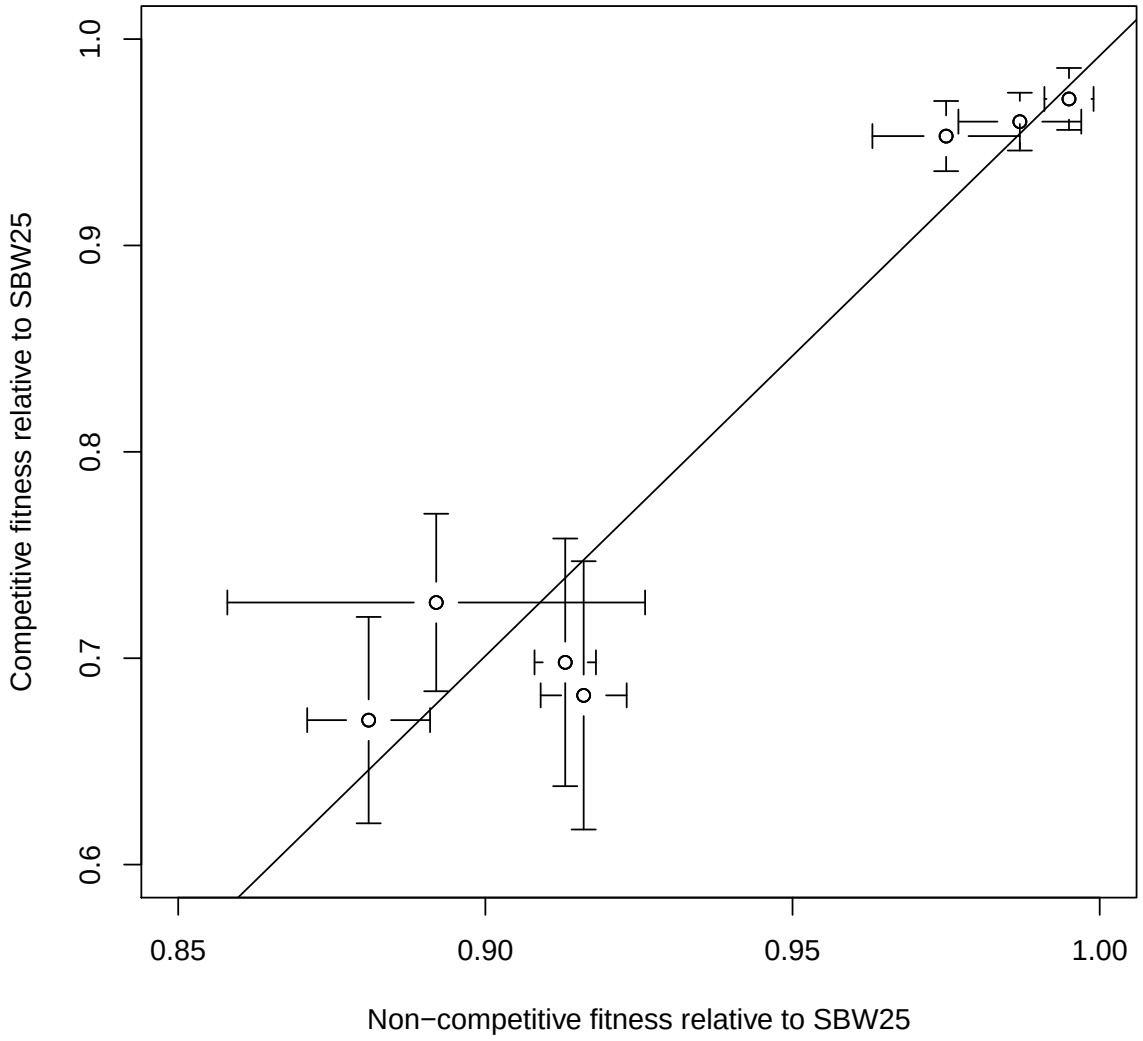


Figure A.2: Relationship between competitive and non-competitive fitness measures for the naladixic acid resistant mutants. Competitive fitness was measured in 24 hour head-to-head competitions with SBW25. Non-competitive fitness was measured as the log change in density over 24 hours, relative to the same measure in SBW25. These two measures are highly correlated (Pearson correlation, r = 0.958, P = 0.0007).

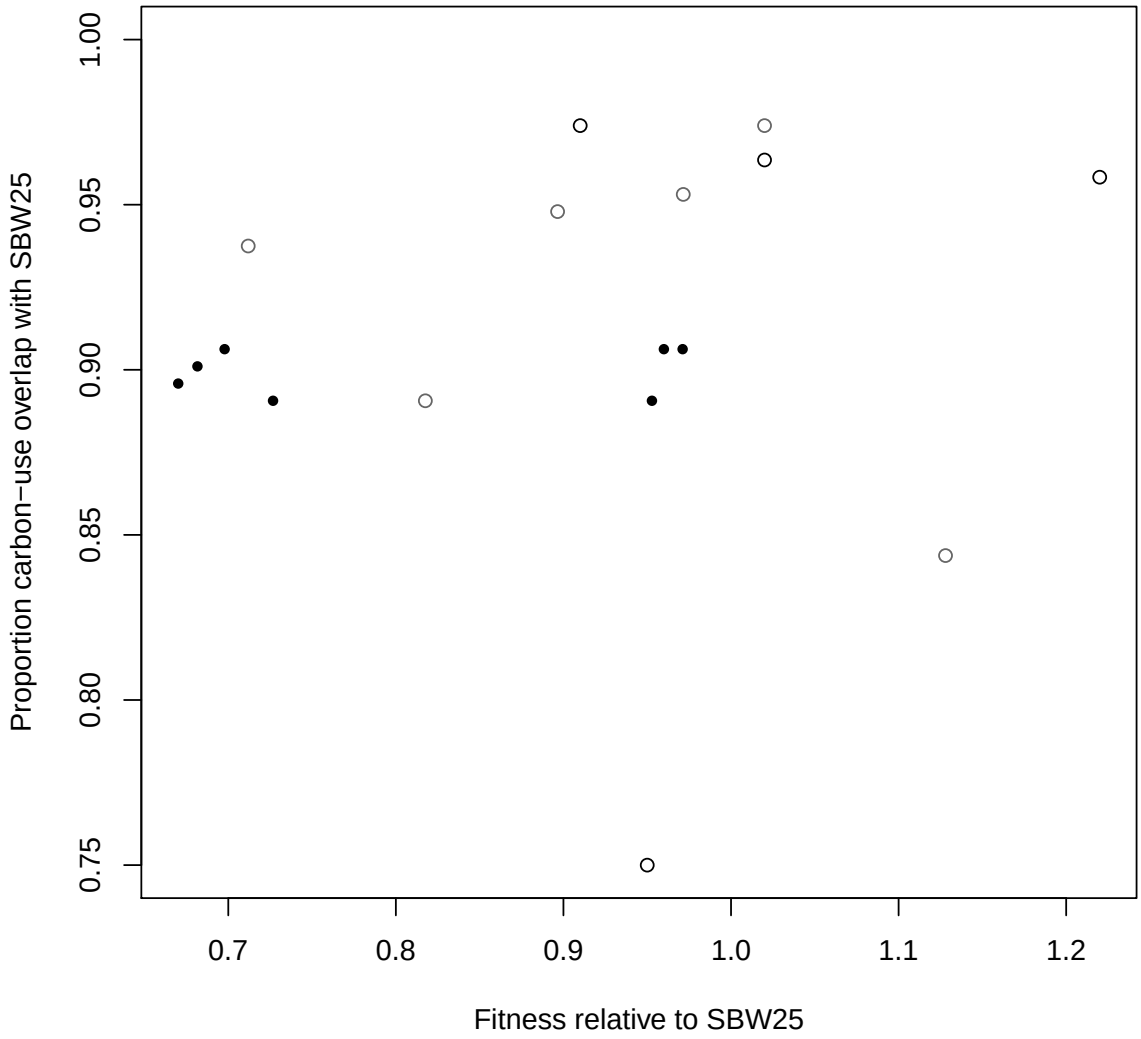


Figure A.3: The absence of a relationship between fitness and carbon-niche overlap of the strains used in this study. Black circles: DIV- strains; grey circles: DIV+ strains; black dots: SBW25 naladixic acid resistant mutants.

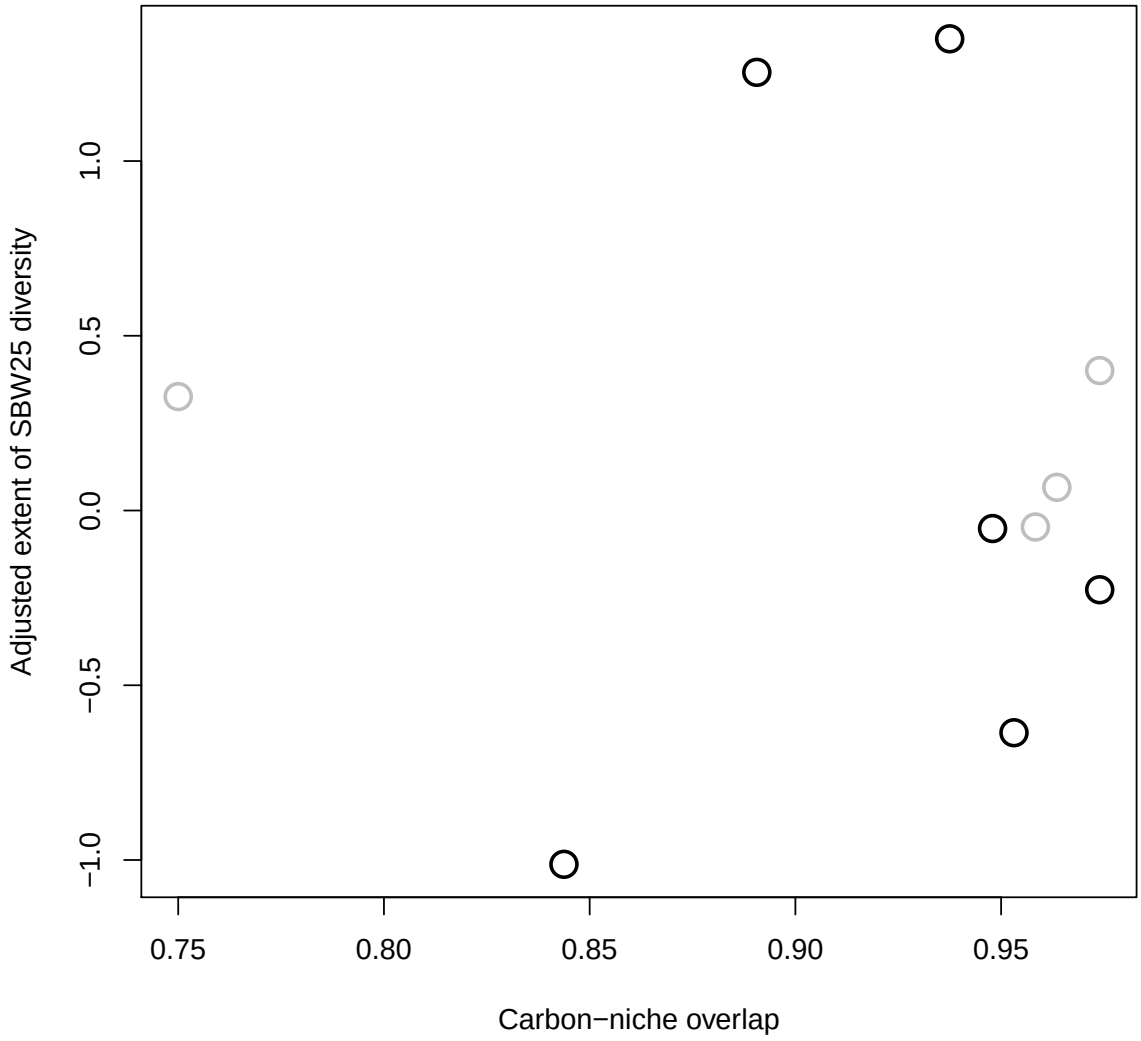


Figure A.4: The absence of a relationship between carbon-niche overlap and the extent of evolved SBW25 diversity. Black circles: DIV- strains; grey circles: DIV+ strains.

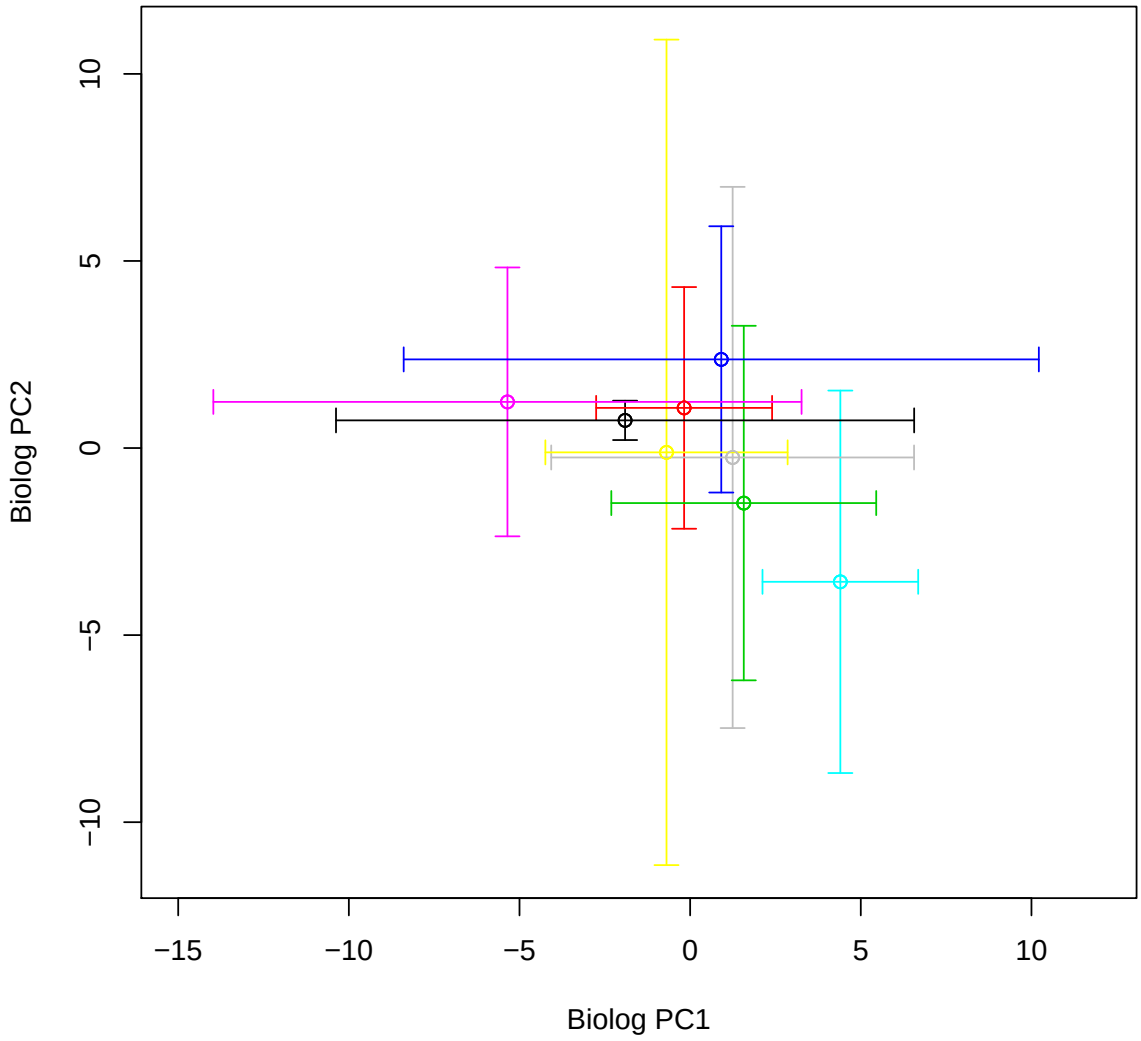


Figure A.5: Naladixic acid resistant mutants in principle components 1 and 2 of carbon-niche space as calculated using Biolog data. Points show mean ± 2 standard errors (n=2).

Name	Ancestor	Strain history	Fitness relative to SBW25 (mean ± s.e.m.)	% niche overlap with SBW25 (mean ± s.e.m.)
F5	SBW25	isolated from Luria Bertrani (LB) + nalidixic acid agar[1]	0.960 ± 0.014	90.63 ± 1.04
A12	SBW25	isolated from LB + nalidixic acid agar[1]	0.971 ± 0.015	90.63 ± 1.04
G1	SBW25	isolated from LB + nalidixic acid agar[1]	0.953 ± 0.017	89.06 ± 2.60
B4	SBW25	isolated from LB + nalidixic acid agar[1]	0.727 ± 0.043	89.06 ± 2.60
F12	SBW25	isolated from LB + nalidixic acid agar[1]	0.670 ± 0.050	89.58 ± 0.00
G11	SBW25	isolated from LB + nalidixic acid agar[1]	0.698 ± 0.060	90.63 ± 1.04
G8	SBW25	isolated from LB + nalidixic acid agar[1]	0.682 ± 0.065	90.10 ± 2.60
A	SBW25:lacZ+[2]	M9 salts + mannose, transferred daily, 150 days[3]	0.9713 ± 0.0232	95.31 ± 0.52
B	SBW25:lacZ+[2]	M9 salts + xylose, transferred daily, 150 days[3]	0.7103 ± 0.0154	93.75 ± 1.04
C	SBW25:lacZ+[2]	M9 salts + mannose, transferred daily, 150 days[3]	0.8165 ± 0.0097	89.06 ± 5.73
D	SBW25:lacZ+[2]	M9 salts + glucose + mannose + xylose, transferred daily, 150 days[3]	0.8959 ± 0.0077	94.79 ± 0.00
E	SBW25:lacZ+[2]	static King's B media (KB), undisturbed, 8 days[4]	1.1284 ± 0.0177	84.38 ± 3.12
F	SBW25:lacZ+[2]	static KB, undisturbed, 8 days[4]	1.0198 ± 0.0140	97.40 ± 0.52
1	PBR716[5]	static KB, transferred daily, 4 days[6]	1.0186 ± 0.0235	96.35 ± 0.52
2	PBR716[5]	static KB, transferred daily, 2 days[6]	0.9452 ± 0.0437	75.00 ± 1.04
3	PBR716[5]	static KB, transferred daily, 6 days[6]	1.2242 ± 0.0204	95.83 ± 0.00
4	PBR716[5]	static KB, transferred daily, 4 days[6]	0.9077 ± 0.0503	97.40 ± 0.52

Table A.1: Fitness measures are from 24 hour head-to-head competitions with the alternately marked SBW25 (lacZ- for SBW25:lacZ+ derived strains, lacZ+ for SBW25 and PBR716 derived strains) in 6 ml static King's B media (n = 3). Niche overlap is a measure of the overlap between SBW25 and the strain of interest in their ability to use 95 different carbon sources (see 3.2.1). [1]from Kassen and Bataillon, 2006; [2]from Zhang and Rainey, 2007; [3]from Bailey and Kassen, 2012; [4]from Meyer et al., 2011; [5]from McDonald et al., 2009, PBR716 is SBW25$\triangle wsp \triangle aws \triangle mws$; [6]isolated for this study.

Treatment	Strain(s)	Time until maximum SBW25 diversity (days)	Extent of SBW25 diversity (no. morphotypes)	Time until maximum competitor diversity (days)	Extent of competitor diversity (no. morphotypes)
Competitors absent	F5	0.5954	4.8922	-	-
	A12	2.4542	6.6300	-	-
	G1	2.4017	5.0559	-	-
	B4	0.5001	2.5000	-	-
	F12	0.5400	3.2369	-	-
	G11	1.2361	2.3846	-	-
	G8	0.5218	2.8649	-	-
	SBW25 control	1.4017	4.6906	-	-
DIV- competitors present	SBW25 + 1	3.8572	4.4365	3.3366	1.9993
	SBW25 + 2	3.7590	4.6961	2.6580	2.0435
	SBW25 + 3	4.8447	4.3230	2.3186	2.4622
	SBW25 + 4	4.2362	4.7705	3.3704	2.6455
	SBW25 control	3.7781	4.3700	-	-
DIV+ competitors present	SBW25 + A	3.3038	3.9060	1.9023	4.9962
	SBW25 + B	5.8929	5.8916	1.4997	1.9643
	SBW25 + C	9.5696	6.4560	0.0000	1.3403
	SBW25 + D	5.8880	5.1507	1.6663	5.1765
	SBW25 + E	12.8489	3.7585	0.0000	5.7671
	SBW25 + F	10.2569	4.5441	1.3732	4.1441
	SBW25 control (for A, B)	2.1114	4.5417	-	-
	SBW25 control (for C, D)	4.5270	5.2021	-	-
	SBW25 control (for E, F)	4.2286	4.7707	-	-

Table A.2: Logistic model parameter estimates from logistic model fits.

Appendix B

Appendix to Chapter 4

B.1 R code for repeated-measures ANOVA

Generalized version of R-code used to perform repeated-measures ANOVAs in this study:

names(our.data) = c('Generation', 'Replicate', 'SelectionEnvironment', 'DepVar')

our.data$id = interaction(our.data$SelectionEnvironment, our.data$Replicate)

model = aov(DepVar ~ Generation*SelectionEnvironment + Error(id), data = our.data)

summary(model)

B.2 Supplemental figures and tables

Resource combination	Resource arrangement	Conc. of mannose	Conc. of glucose	Conc. of xylose	Total vol. of media
M	(1 well)	9.57 mg/L	-	-	2 ml
G	(1 well)	-	9.57 mg/L	-	2 ml
X	(1 well)	-	-	255.22 mg/L	2 ml
M+G	MIX (1 well)	4.79 mg/L	4.79 mg/L	-	2 ml
	SPAT (2 wells)	4.79 mg/L	4.79 mg/L	-	4 ml
M+X	MIX (1 well)	4.79 mg/L	-	127.61 mg/L	2 ml
	SPAT (2 wells)	4.79 mg/L	-	127.61 mg/L	4 ml
G+X	MIX (1 well)	-	4.79 mg/L	127.61 mg/L	2 ml
	SPAT (2 wells)	-	4.79 mg/L	127.61 mg/L	4 ml
M+G+X	MIX (1 well)	3.19 mg/L	3.19 mg/L	85.07 mg/L	2 ml
	SPAT (3 wells)	3.19 mg/L	3.19 mg/L	85.07 mg/L	6 ml

Table B.1: Concentrations of sugars and total volumes of media in each selection environment. MIX: resources were provided in a single mixture; SPAT: resources were provided in spatially separated patches. Sugar concentrations were chosen to control for ancestral population density after 24 hours.

Model	No. Parameters	AIC
linear	15	-230.4287
exponential	15	-223.5656
logistic	30	-276.5293

Table B.2: Comparison of linear, exponential, and two-parameter logistic model fits to all population fitness time series data simultaneously. AIC values indicate that the logistic model is the most appropriate for these data.

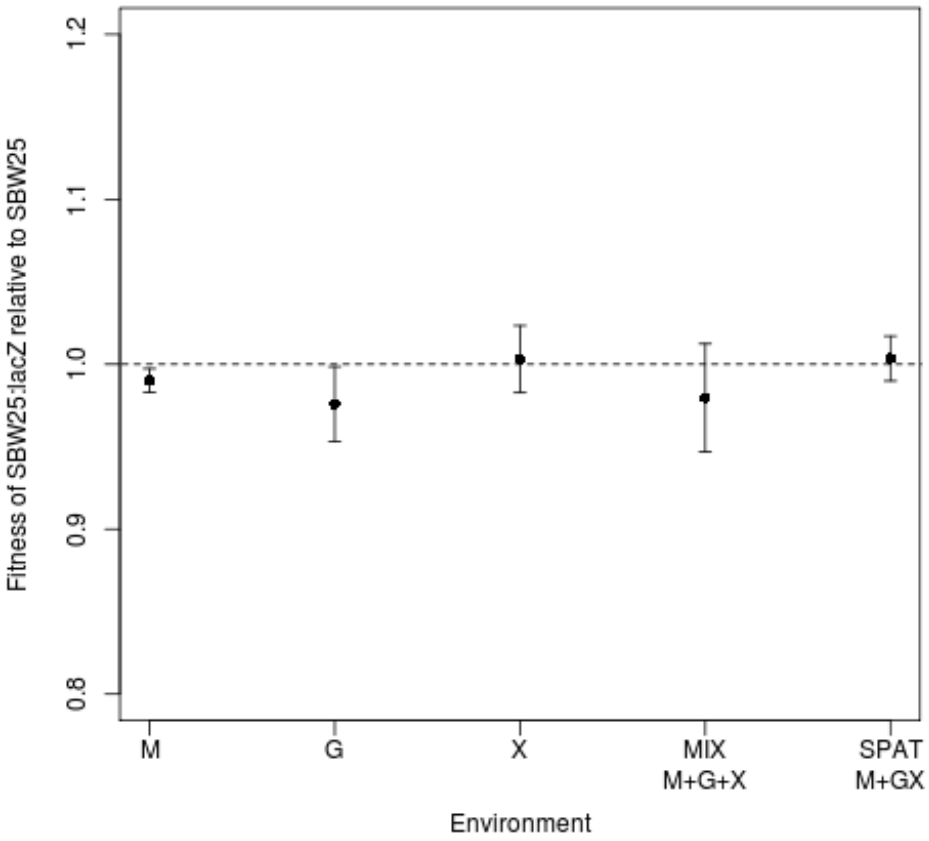


Figure B.1: Fitness of SBW25:lacZ relative to SBW25 in each selection environment used in this study. Fitness estimates were calculated from head to head competition assays, following the protocol outlined in the methods. Points represent the means ± SE of 6 replicate competitions. The lacZ insertion does not have a significant effect on fitness in any of the tested environments (*t*-tests; M: P = 0.2292, G: P = 0.3348, X: P = 0.8851, MIX M+G+X: P = 0.5619, SPAT M+G+X: P = 0.8094).

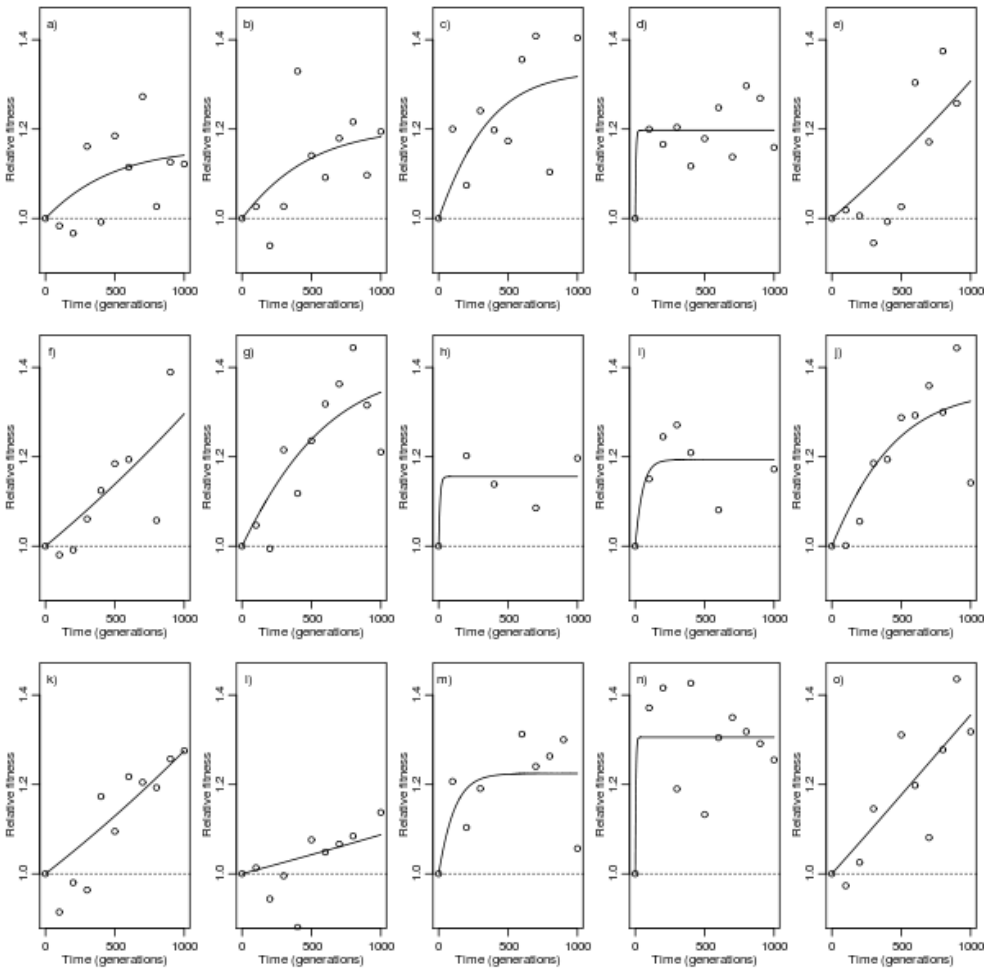


Figure B.2: Relative fitness over 1000 generations and logistic model fits used to estimate the rate of adaptation. Each panel shows one selection line with each column indicating selection environments M, G, X, MIX MGX, and SPAT MGX respectively. Circles show unreplicated estimates of fitness for each population over time; triangles show fitness estimates (mean ± SE) from additional replicate fitness assays (n=3). Solid lines show logistic model fits for unreplicated fitness data; dashed lines show logistic model fits for all fitness data combined.

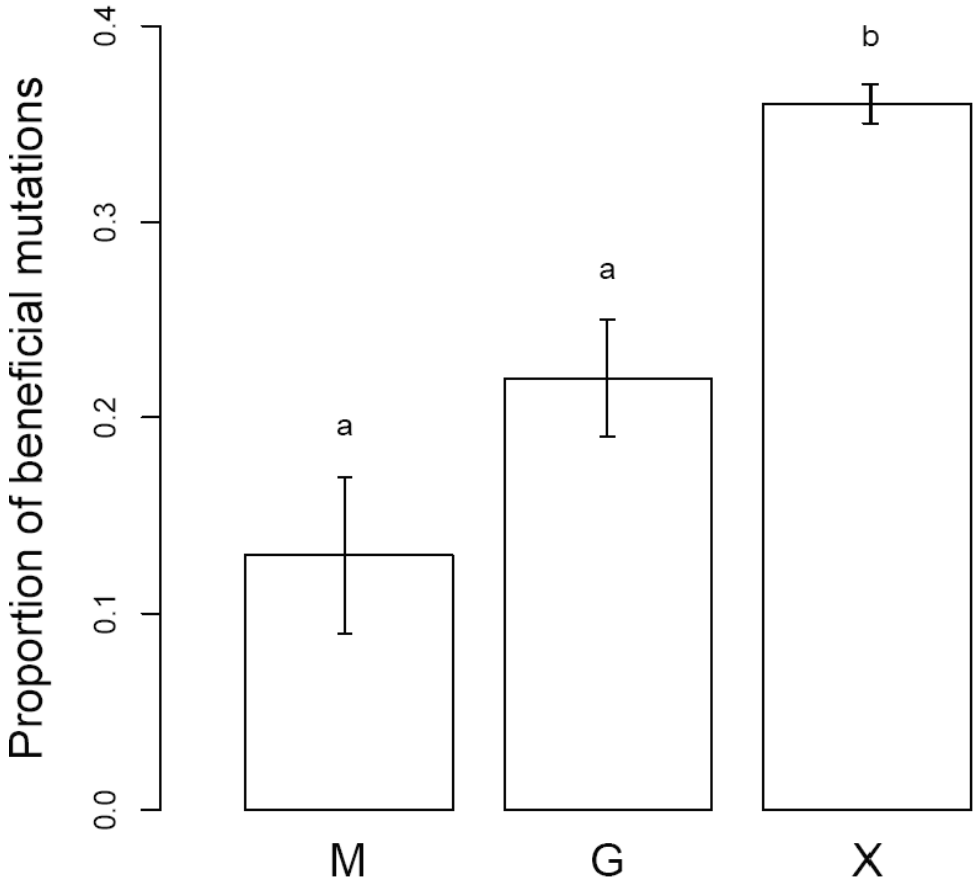


Figure B.3: The mean proportion of beneficial mutations in 46 single-step *P. fluorescens* SBW24 mutants (from the mutant library detailed by Kassen and Bataillon, 2006) assayed in mannose (M), glucose (G), and xylose (X). Data are the mean of three replicate fitness assays ± SE. Significantly different groups are indicated by a and b (Bonferroni corrected pairwise *t*-tests , $p < 0.05$).

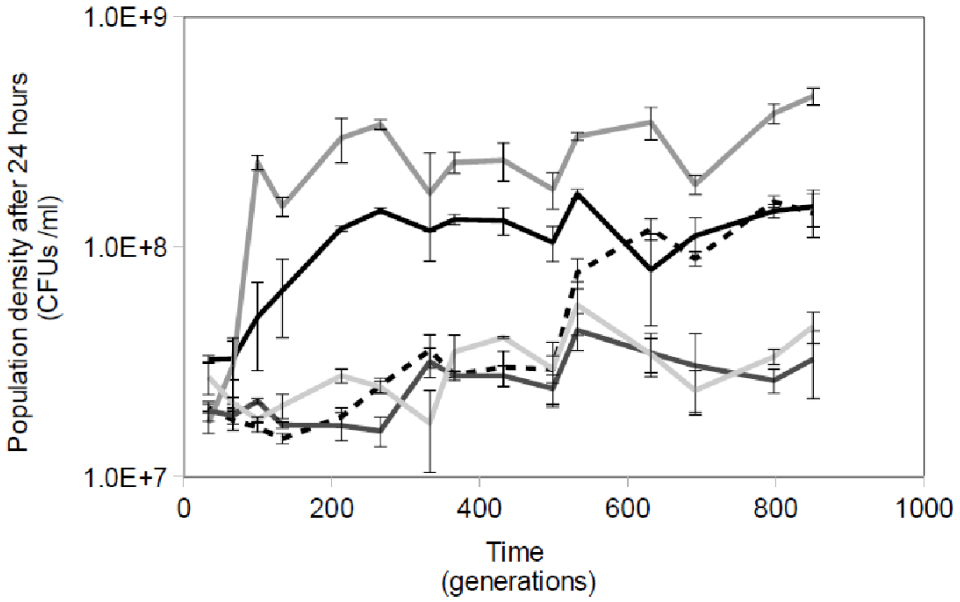


Figure B.4: Mean population density after 24 hours of growth (just prior to transfer) in each environment over time. Time points are from every 5 - 15 days throughout the 1000 generations of evolution when the populations were plated out to check for contamination. Data are the mean ± SE of the three replicate lines from each environment: mannose (light grey), glucose (dark grey), xylose (medium grey), mixed multi-resource (black solid line) and spatially-separated multi-resource (black dashed line).

		Logistic model parameters		
Env	Rep	r (rate of adaptation)	k (extent of adaptation)	Mean of gen 900-1000 fitness
M	1	0.0023772749	1.158166	1.123955
M	2	0.000259369	41721.833898	1.389389
M	3	0.0002432381	41234.350321	1.266263
G	1	0.0024187815	1.204155	1.145617
		(0.0007555)	(1.556002)	(1.147895)
G	2	0.0022236822	1.403144	1.263047
G	3	0.0000836702	25539.433147	1.137312
X	1	0.0035258947	1.330153	1.40418
		(0.008245)	(1.287118)	(1.329467)
X	2	0.1070481017	1.155664	1.19659
X	3	0.0102427209	1.224485	1.178279
		(0.007902)	(1.296596)	(1.289238)
MIX MGX	1	0.2098851315	1.19757	1.213972
MIX MGX	2	0.0214821005	1.193334	1.172293
		(0.008479)	(1.263626)	(1.235721)
MIX MGX	3	0.2217117777	1.305529	1.273163
SPAT MGX	1	0.0002683631	65682.933354	1.257527
SPAT MGX	2	0.002867771	1.350454	1.292601
		(0.0003084)	(49280)	(1.412943)
SPAT MGX	3	0.0005826151	2.46194	1.376843

Table B.3: Parameter estimates from the logistic model fits and final fitness averages. Estimates in parentheses are from data including additional replicate fitness measures.

Assay Env	Dep Var	Df	Sum Sq	Mean Sq	F	P
a) Mannose	S	4	1.0965	0.2741	24.396	< 0.0001 *
	T	1	0.1171	0.1171	6.1301	0.0164 *
	S × T	4	0.4074	0.1019	5.3319	0.0011 *
b) Glucose	S	4	1.7595	0.4399	27.930	< 0.0001 *
	T	1	0.0717	0.0717	2.1406	0.1491
	S × T	4	0.1811	0.0453	1.3526	0.2623
c) Xylose	S	4	2.0552	0.5138	6.0483	0.0097 *
	T	1	0.7047	0.7047	16.286	0.0002 *
	S × T	4	0.4393	0.1098	2.5382	0.0501

Table B.4: Results from repeated measures ANOVAs (RM-ANOVAs) testing the effects of selection environment (S), time in generations (T), and their interaction (S × T), on the three measured traits: a) fitness assayed in mannose, b) fitness assayed in glucose, and c) fitness assayed in xylose, within each population repeated over time. * indicates significance with $\alpha < 0.05$

Assay Env	Dep Var	Df	Sum Sq	Mean Sq	F	P
a) Mannose	S	2	1.5014	0.75069	6.5817	0.002978 *
b) Glucose	S	2	1.3804	0.69020	9.5633	0.0003194 *
c) Xylose	S	2	0.0741	0.037048	0.2209	0.803

Table B.5: Results from ANOVAs testing the effects of selection environment (S) on the extent adaptation to a) mannose, b) glucose, and c) xylose at generation 1000. Multi-resource environments (MIX and SPAT) and the corresponding single-resource environment are compared for each assay environment. * indicates significance with $\alpha < 0.05$.

Ind Var	Dep Var	Df	Sum Sq	Mean Sq	F	P
Log(degree of specialization)	S	4	61.718	15.4296	6.7947	0.0066 *
	T	1	4.112	4.1123	3.3581	0.0723
	S × T	4	4.776	1.1940	0.9750	0.4287

Table B.6: Results from a RM-ANOVA testing the effects of selection environment (S), time in generations (T), and their interaction (S × T), on the degree of specialization, within each population repeated over time. Degree of specialization data was log-transformed to meet ANOVA assumptions of homogeneity of variance. * indicates significance with $\alpha < 0.05$.

Ind Var	Dep Var	Df	Sum Sq	Mean Sq	F	P
G × E	S	4	0.012597	0.0031493	2.7353	0.0897
	T	1	0.000793	0.00079332	0.8075	0.3728
	S × T	4	0.006783	0.00169575	1.7261	0.1574

Table B.7: Results from a RM-ANOVA testing the effects of selection environment (S), time in generations (T), and their interaction (S × T), on a) the genotype by environment interaction (G × E).

Appendix C

Appendix to Chapter 5

C.1 Supplemental figures and tables

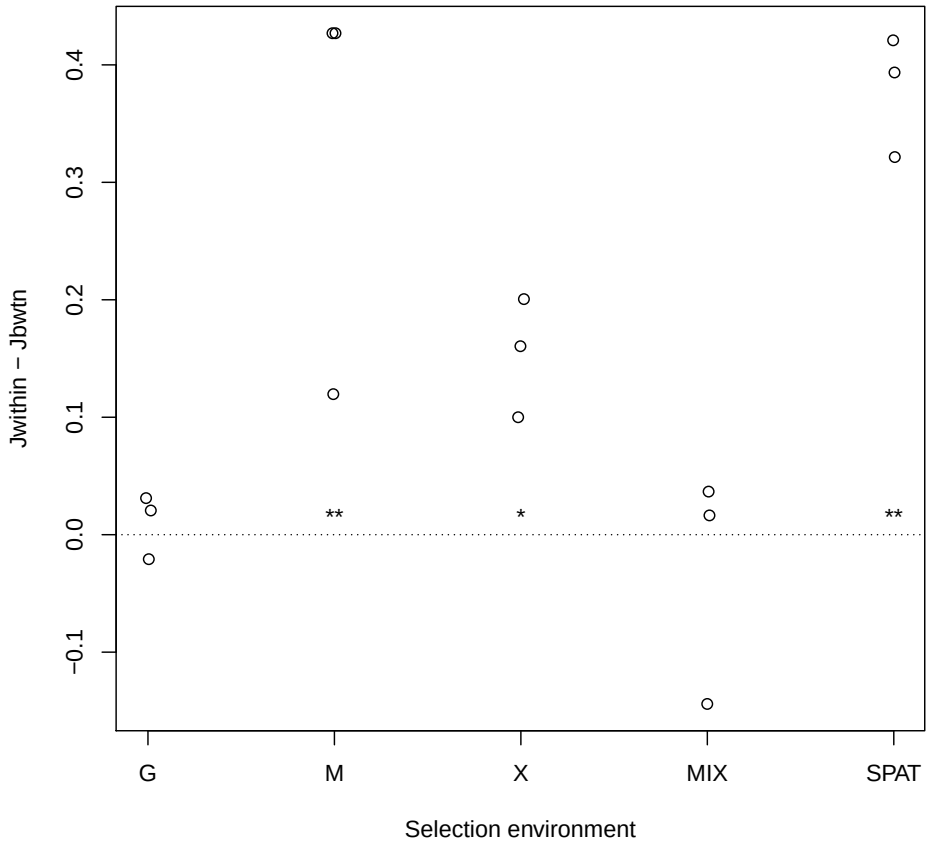


Figure C.1: Difference between within-environment and between-environment similarity, measured as the mean within-environment J minus the mean between-environment J for each replicate population, plotted by selection environment. Positive values represent populations that are more similar on average to populations selected in the same selection environment, while negative values represent populations that are more similar on average to populations selected in different selection environments. Selection environments with significantly positive values are indicated by * and ** ($P < 0.05$ and $P < 0.01$ respectively).

Selection environment	Replicate	Position	Gene	Mutation	Change type	Effect	Old AA/ new AA	Functional category	Motility related	Proportion of reads
X	2	194140	PFLU0172	C→T	SNP	NON_SYNONYMOUS_CODING	T/I	Function unknown	n	1.00
SPAT	2	448467	PFLU0405	A→C	SNP	NON_SYNONYMOUS_CODING	T/P	Energy production and conversion	n	0.38
G	3	449940	PFLU0406	G→A	SNP	STOP_GAINED	Q/STOP	Cell envelope biogenesis, outer membrane	n	1.00
M	3	450662	PFLU0406	-A	DEL	FRAME_SHIFT		Cell envelope biogenesis, outer membrane	n	0.29
M	1	903861	fruR	+T	INS	FRAME_SHIFT		Transcription	n	1.00
MIX	2	904637	fruR	T→G	SNP	NON_SYNONYMOUS_CODING	N/T	Transcription	n	1.00
SPAT	1	904687	fruR	Δ11 bp	DEL	FRAME_SHIFT		Transcription	n	0.68
SPAT	2	904687	fruR	Δ11 bp	DEL	FRAME_SHIFT		Transcription	n	0.74
M	2	904697	fruR	11 bp x 2	INS	FRAME_SHIFT		Transcription	n	0.60
SPAT	3	904689	fruR	Δ4 bp	DEL	FRAME_SHIFT		Transcription	n	0.53
MIX	2	1315026	PFLU1183	A→G	SNP	NON_SYNONYMOUS_CODING	D/G	Function unknown / Phage protein	n	0.53
MIX	3	1439177	PFLU1301	C→T	SNP	STOP_GAINED	Q/STOP	Cell envelope biogenesis, outer membrane	n	1.00
SPAT	1	1439177	PFLU1301	C→T	SNP	STOP_GAINED	Q/STOP	Cell envelope biogenesis, outer membrane	n	0.78
SPAT	2	1439177	PFLU1301	C→T	SNP	STOP_GAINED	Q/STOP	Cell envelope biogenesis, outer membrane	n	1.00
M	3	2372830	gacA	-G	DEL	FRAME_SHIFT		Signal transduction mechanisms / Transcription	n	0.41
X	3	2577036	[PFLU2365],	Δ2,864 bp	DEL	LOSS OF GENES			n	1.00
SPAT	2	2577444	PFLU2366	12 bp x 2	INS	CODON_INSERTION		Transcription	n	0.42
SPAT	1	2577572	PFLU2366	T→G	SNP	NON_SYNONYMOUS_CODING	T/P	Transcription	n	1.00
MIX	1	2577585	PFLU2366	Δ162 bp	DEL	FRAME_SHIFT		Transcription	n	1.00
X	2	2577714	PFLU2366	Δ70 bp	DEL	FRAME_SHIFT		Transcription	n	1.00
SPAT	3	2577913	PFLU2366	C→T	SNP	NON_SYNONYMOUS_CODING	R/H	Transcription	n	0.52
X	1	2578023	PFLU2366	+C	INS	FRAME_SHIFT		Transcription	n	1.00
SPAT	3	2578052	PFLU2366	C→A	SNP	STOP_GAINED	Q/STOP	Transcription	n	0.32
SPAT	1	2644930	PFLU2428	+A	INS	FRAME_SHIFT		Function unknown	n	0.75
MIX	2	2840233	[PFLU2574],	Δ3,175 bp	DEL	LOSS OF GENES		Function unknown	n	1.00
MIX	3	2840233	[PFLU2574],	Δ3,175 bp	DEL	LOSS OF GENES		Function unknown	n	1.00
X	3	3524329	PFLU3217	Δ5 bp	DEL	FRAME_SHIFT		Amino acid transport and metabolism	n	1.00
M	3	4174835	PFLU3777	A→C	SNP	NON_SYNONYMOUS_CODING	K/N	Signal transduction mechanisms	n	0.52
MIX	2	4891052	fliP	G→A	SNP	STOP_GAINED	Q/STOP	Cell motility and secretion / Intracellular trafficking and secretion	y	0.28
G	2	4898340	fliJ, fliI, fliH, fl	Δ35,110 bp	DEL	LOSS OF GENES		Cell motility and secretion / Intracellular trafficking and secretion	y	1.00
MIX	3	4902498	fliF	Δ6 bp	DEL	CODON_DELETION		Cell motility and secretion / Intracellular trafficking and secretion	y	1.00
X	1	4903514	fliF	-GGAA	DEL	FRAME_SHIFT		Cell motility and secretion / Intracellular trafficking and secretion	y	0.17
X	2	4903873	fliE	Δ17 bp	DEL	FRAME_SHIFT		Cell motility and secretion / Intracellular trafficking and secretion	y	1.00
M	1	4907168	PFLU4443	+G	INS	FRAME_SHIFT		Signal transduction mechanisms, likely flagellar protein regulation	y	1.00
SPAT	3	4907173	PFLU4443	G→A	SNP	NON_SYNONYMOUS_CODING	A/V	Signal transduction mechanisms, likely flagellar protein regulation	y	0.57
SPAT	1	4907425	PFLU4443	G→A	SNP	NON_SYNONYMOUS_CODING	P/L	Signal transduction mechanisms, likely flagellar protein regulation	y	1.00
G	1	4907597	PFLU4443	G→A	SNP	NON_SYNONYMOUS_CODING	R/C	Signal transduction mechanisms, likely flagellar protein regulation	y	0.65
SPAT	2	4908083	PFLU4443	G→A	SNP	STOP_GAINED	Q/STOP	Signal transduction mechanisms, likely flagellar protein regulation	y	1.00
M	3	4908167	PFLU4443	G→A	SNP	STOP_GAINED	Q/STOP	Signal transduction mechanisms, likely flagellar protein regulation	y	0.60
G	1	4908340	PFLU4443	-T	DEL	FRAME_SHIFT		Signal transduction mechanisms, likely flagellar protein regulation	y	0.32
M	2	4908484	PFLU4443	Δ7 bp	DEL	FRAME_SHIFT		Signal transduction mechanisms, likely flagellar protein regulation	y	1.00
X	3	4919495	flgI	Δ15 bp	DEL	FRAME_SHIFT		Cell motility and secretion / Intracellular trafficking and secretion	y	1.00
MIX	1	4919948	flgI	Δ6 bp	DEL	CODON_DELETION		Cell motility and secretion / Intracellular trafficking and secretion	y	0.24
X	1	4920009	flgI	G→A	SNP	NON_SYNONYMOUS_CODING	A/T	Cell motility and secretion / Intracellular trafficking and secretion	y	0.30
X	1	4920642	flgH	+C	INS	FRAME_SHIFT		Cell motility and secretion / Intracellular trafficking and secretion	y	0.14
MIX	1	5209116	flgD	17 bp x 2	INS	FRAME_SHIFT		Cell motility and secretion / Intracellular trafficking and secretion	y	1.00
MIX	1	5323400	PFLU4845	G→A	SNP	NON_SYNONYMOUS_CODING	A/V	Carbohydrate transport and metabolism	n	1.00
G	2	5324011	PFLU4845	G→A	SNP	SYNONYMOUS_CODING	G/G	Carbohydrate transport and metabolism	n	0.82
G	2	5324080	PFLU4845	A→G	SNP	SYNONYMOUS_CODING	A/A	Carbohydrate transport and metabolism	n	0.21
G	1	5324097	PFLU4845	C→T	SNP	NON_SYNONYMOUS_CODING	A/T	Carbohydrate transport and metabolism	n	1.00
SPAT	1	6377588	PFLU5822	A→C	SNP	NON_SYNONYMOUS_CODING	S/A	Function unknown	n	1.00
X	2	6518494	PFLU5960	-CAG	DEL	CODON_DELETION		Signal transduction mechanisms	n	0.18

Table C.1: Summary of all detected mutations.

Appendix D

Appendix to Chapter 6

D.1 Supplemental figures and tables

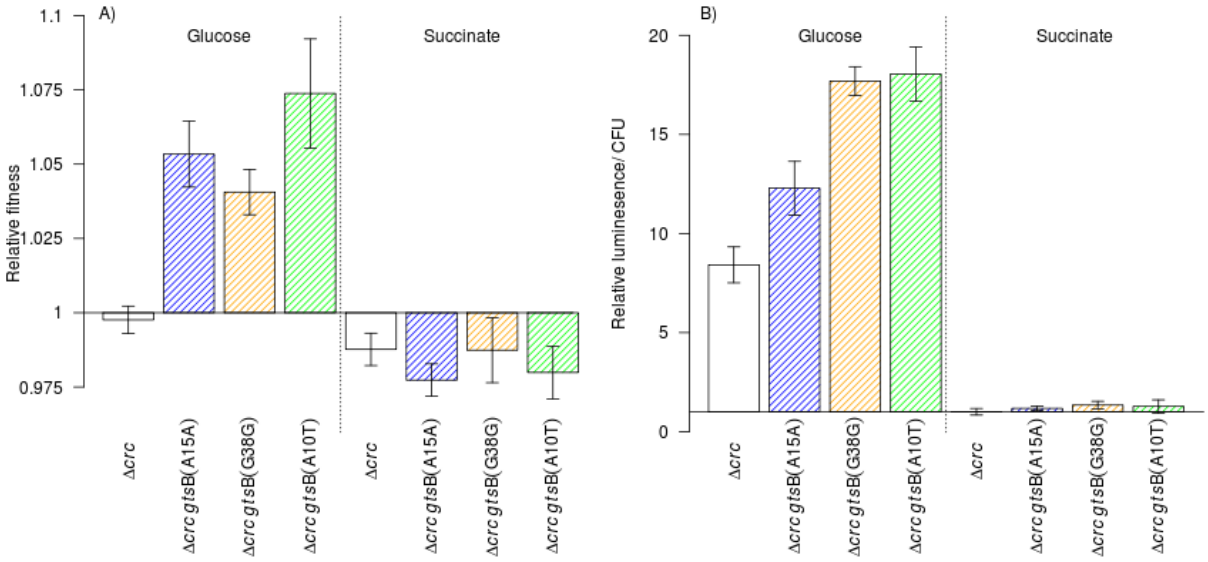
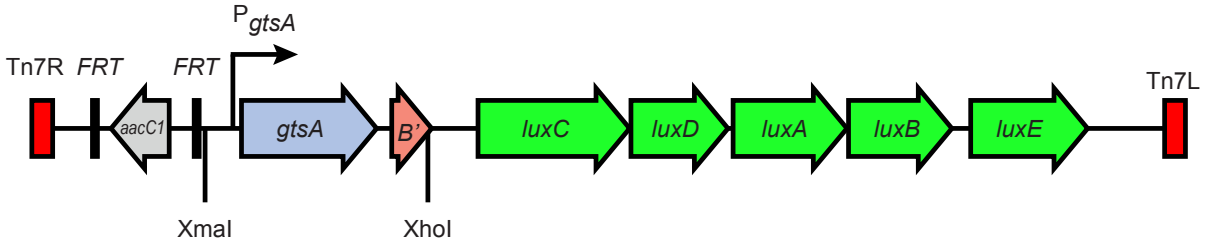


Figure D.1: The increased fitness and gene expression caused by the *gtsB* substitutions do not require the *crc* catabolite repressor gene. (A) Fitness effects of the two synonymous mutations, *gtsB*(A15A) and *gtsB*(G38G), the non-synonymous mutation, *gts*B(A10T) in the Δ*crc* background. The competitor strain was SBW25-*lacZ*. The mean ± s.e. for each treatment is shown. (B) Effect of *gtsB* substitutions on gene expression (luminescence) in the Δ*crc* background. The mean ± s.e. for each treatment is shown.

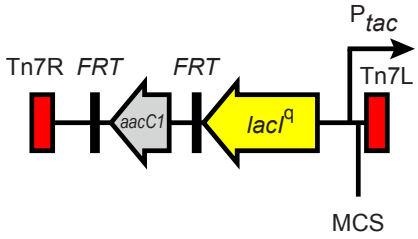
	Change in CAI compared to *gtsB*(WT)		Change in *gtsB* mRNA structure free energy compared to *gtsB*(WT) (kcal/mole)	
Mutant	Codon	Gene	First predicted structure	Mean of first 10 predicted structures
gtsB(A15A)	-0.085	-0.000562	-0.30	-0.30
gtsB(G38G)	-0.170	-0.000432	2.40*	2.18*
gtsB(A10T)	0.226*	0.000595*	3.40*	3.17*

Table D.1: The effect of *gtsB* mutations on codon adaptation index (CAI) and mRNA folding stability measured as free energy. CAI was calculated by comparing sequence data to a reference set of highly expressed genes using R (see Materials and Methods in Chapter 6). mRNA free energy (kcal/mole) was calculated for the *gtsB* sequence using 'mfold' (Zuker, 2003). *indicates a change in the direction expected to increased gene expression.

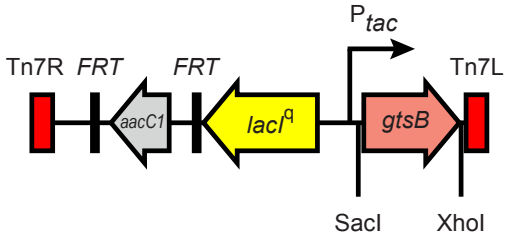
mini-Tn7T-Gm-P$_{gtsA}$-*gtsAB'-lux*



mini-Tn7T-LAC



mini-Tn7T-LAC-*gtsB*



mini-Tn7T-LAC-*gtsABCD-oprB*

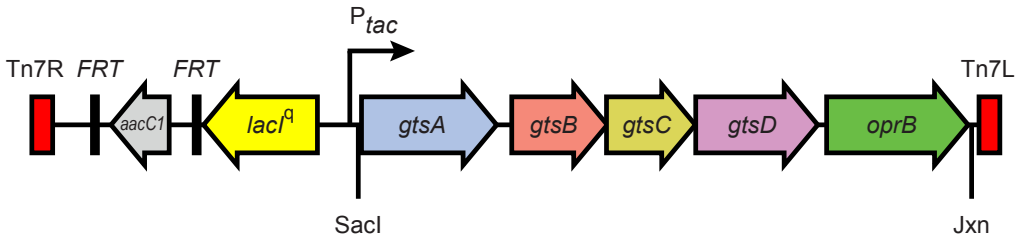


Figure D.2: Continued on following page.

Figure D.2: Transposon constructs used for transcriptional luciferase fusions and P_{tac}-regulated gene expression. The transposons were derived from mini-Tn7T-Gm-*lux* and mini-Tn7T-LAC (Choi and Schweizer, 2006). Each transposon is bounded by Tn7-specific sequences (Tn7R and Tn7L) and contains a gentamicin resistance gene (*aacC1*) flanked by Flp-recombinase targets (*FRT*) near the multiple cloning site (MCS). The transcriptional reporter (top) contains the *gtsA* promoter region and open reading frame and truncated *gtsB* open reading frame (B') upstream of the *Photorhabdus luminescens lux* operon. Assuming independent translation of the luciferase genes, the luciferase activity (luminescence) is indicative of *gtsAB'-luxCDABE* transcript abundance. P_{tac}-regulated gene expression involved the miniTn7T-LAC transposon, which carries the Lac repressor gene with a promoter-up mutation ($lacI^q$), P_{tac} promoter, and lac operator. In the absence of inducer, P_{tac}-mediated transcription of the inserted genes (*gtsB* or *gtsABCD-oprB*) is repressed by LacI. Addition of inducer (IPTG) relieves the repression, leading to transcriptional induction.

Strain	**Genotype and/or relevant features**	**Source**
Pseudomonas fluorescens		
SBW25	Wild type	Bailey et al 1995
SBW25-*lacZ*	Contains neutral *lacZ* marker at a prophage locus	Zhang & Rainey 2007
AHF305	SBW25-*lacZ* Δ(*PFLU4435-PFLU4466*) *gtsB*(A15A); generation-1000 isolate from evolved population	This study
AHF306	SBW25-*lacZ* Δ(*PFLU4435-PFLU4466*) *gtsB*(G38G); generation-1000 isolate from evolved population	This study
AHF307	SBW25-*lacZ PFLU4443*(T72fs) *gtsB*(A10T); generation-1000 isolate from evolved population	This study
AHF303	SBW25 *gtsB*(A15A); unmarked allelic replacement using pUIC3-A15A	This study
AHF304	SBW25 *gtsB*(G38G); unmarked allelic replacement using pUIC3-G38G	This study
AHF316	SBW25 *gtsB*(A10T); unmarked allelic replacement using pAH79-A10T	This study
AHF201	SBW25 Δ*gtsB;* unmarked deletion constructed using pAH79-Δ*gtsB*	This study
AHF336	SBW25 Δ*crc;* unmarked deletion constructed using pAH79-Δ*crc*	This study
AHF340	SBW25 Δ*crc gtsB*(A15A); deletion of *crc* gene from AHF303	This study
AHF342	SBW25 Δ*crc gtsB*(G38G); deletion of *crc* gene from AHF304	This study
AHF344	SBW25 Δ*crc gtsB*(A10T); deletion of *crc* gene from AHF316	This study
AHF356	SBW25 mini-Tn7T-Gm-P_{gtsA}-*gtsAB*'-*lux;* single-copy *gtsB*(WT)-*lux* transcriptional reporter from pAH241	This study
AHF372	SBW25 mini-Tn7T-Gm-P_{gtsA}-*gtsAB*'(A10T)-*lux*; single-copy *gtsB*(A10T)-*lux* transcriptional reporter from pAH242	This study
AHF376	SBW25 mini-Tn7T-Gm-P_{gtsA}-*gtsAB*'(A15A)-*lux*; single-copy *gtsB*(A15A)-*lux* transcriptional reporter from pAH245	This study
AHF365	SBW25 mini-Tn7T-Gm-P_{gtsA}-*gtsAB*'(G38G)-*lux*; single-copy *gtsB*(G38G)-*lux* transcriptional reporter from pAH247	This study
AHF408	SBW25 Δ*crc* mini-Tn7T-Gm-P_{gtsA}-*gtsAB*'-*lux;* single-copy *gtsB*(WT)-*lux* transcriptional reporter from pAH241 in AHF336	This study

Table D.2: Bacterial strains and plasmids used in Chapter 6.

AHF412	SBW25 Δ*crc* mini-Tn7T-Gm-P_{gtsA}-*gtsAB*'(A10T)-*lux*; single-copy *gtsB*(A10T)-*lux* transcriptional reporter from pAH242 in AHF336	This study
AHF414	SBW25 Δ*crc* mini-Tn7T-Gm-P_{gtsA}-*gtsAB*'(A15A)-*lux*; single-copy *gtsB*(A15A)-*lux* transcriptional reporter from pAH245 in AHF336	This study
AHF417	SBW25 Δ*crc* mini-Tn7T-Gm-P_{gtsA}-*gtsAB*'(G38G)-*lux*; single-copy *gtsB*(G38G)-*lux* transcriptional reporter from pAH247 in AHF336	This study
AHF425	SBW25 mini-Tn7T-LAC; single-copy P_{tac} expression construct control in SBW25	This study
AHF421	SBW25-*lacZ* mini-Tn7T-LAC; single-copy P_{tac} expression construct control in SBW25-*lacZ*	This study
AHF437	SBW25 mini-Tn7T-LAC-*gtsB*; single-copy inducible P_{tac}-*gtsB* construct (pAH308)	This study
AHF433	SBW25 mini-Tn7T-LAC-*gtsABCD-oprB*; single-copy inducible P_{tac}-*gtsABCD-oprB* construct (pAH305)	This study
Escherichia coli		
DH5α	F–, Δ*lacU169*(φ80 *lacZ*ΔM15), *recA1*, *endA1*, *hsdR17*, *gyrA96*, *thi-1*, *relA1*	Hanahan 1983
DH5αλpir	F–, Δ*lacU169*(φ80 *lacZ*ΔM15), *recA1*, *endA1*, *hsdR17*, *gyrA96*, *thi-1*, *relA1*, λpir lysogen	Rainey 1999
Plasmids		
pUIC3	*P. fluorescens* allelic replacement vector; Ap^r, Tc^r, *lacZY*, R6K replicon	Rainey 1999
pAH79	pUIC3 containing the *sacB* counterselection gene in place of *lacZY*; Ap^r, Tc^r, *sacB*, R6K replicon	This study
pRK2013	Mobilization helper plasmid; Km^r, Tra, ColE1 replicon	Figurski & Helinski 1979
pUIC3-A15A	Vector for allelic transfer of A15A mutation; pUIC3 with 2-kb insert containing *gtsB*(A15A) allele amplified from AHF305	This study
pUIC3-G38G	Vector for allelic transfer of G38G mutation; pUIC3 with 2-kb insert containing *gtsB*(G38G) allele amplified from AHF306	This study
pAH79-A10T	Vector for allelic transfer of A10T mutation; pAH79 with 2.3-kb insert containing *gtsB*(A10T) allele amplified from AHF307	This study
pAH79-Δ*gtsB*	Vector for generating *gtsB* deletion; pAH79 with 2-kb fused PCR fragment carrying a *gtsB* allele deleted from codon 8 to codon 274	This study
pAH79-Δ*crc*	Vector for generating *crc* deletion; pAH79 with	This study

Table D.2: (continued)

	2-kb fused PCR fragment carrying a *crc* allele deleted from codon 31 to codon 216	
pUC18-mini-Tn7T-Gm-*lux*	Luciferase transcriptional fusion vector; Ap^r, Gm^r, *luxCDABE*, ColE1 replicon	Choi et al 2006
pAH241	*gtsB*(WT)-*lux* transcriptional reporter; pUC18-mini-Tn7T-Gm-*lux* containing 2.1-kb insert (P_{gtsA}-*gtsAB*') amplified from SBW25	This study
pAH242	*gtsB*(A10T)-*lux* transcriptional reporter; pUC18-mini-Tn7T-Gm-*lux* containing 2.1-kb insert (P_{gtsA}-*gtsAB*') amplified from AHF307	This study
pAH245	*gtsB*(A15A)-*lux* transcriptional reporter; pUC18-mini-Tn7T-Gm-*lux* containing 2.1-kb insert (P_{gtsA}-*gtsAB*') amplified from AH305	This study
pAH247	*gtsB*(G38G)-*lux* transcriptional reporter; pUC18-mini-Tn7T-Gm-*lux* containing 2.1-kb insert (P_{gtsA}-*gtsAB*') amplified from AHF306	This study
pUC18-miniTn7T-LAC	Inducible gene expression vector; Ap^r, Gm^r, *lacI*^{q}-P_{tac}, ColE1 replicon	Choi et al 2006
pAH308	Inducible P_{tac}-*gtsB* expression construct; pUC18-miniTn7T-LAC containing 1-kb insert (*gtsB* gene) amplified from SBW25	This study
pAH305	Inducible P_{tac}-*gtsABCD-oprB* expression construct; pUC18-miniTn7T-LAC containing 5.8-kb insert (*gtsABCD-oprB* genes) amplified from SBW25	This study
pTNS2	Helper plasmid for mini-Tn7 delivery; Ap^r, TnsABCD, R6K replicon	Choi et al 2006

Table D.2: (continued)

Primer name	Sequence (5' to 3')	Restriction site	Function
4845-BglII-F	G**AGATCT**GAAAGCCGGTATCACCAAGA	BglII	A15A and G38G constructs
4845-AvrII-R	G**CCTAGG**GCCAGAACGACAGCACATAA	AvrII	A15A and G38G constructs
22F-5324097	ACTGCG**AGATCT**GCCAGAACGACAGCACATAA	BglII	A10T construct
22R-5324097	ACTGCG**TCTAGA**AGACGTCGCCAAAGAAGAAA	XbaI	A10T construct
4845-del-F1	ACTGCG**GGATCC**GAAAGCCGGTATCACCAAGA	BamHI	*gtsB* deletion
4845-del-R1	ACTGCG**GAGCTC**TGCTGAACACAGCAACAGAA	SacI	*gtsB* deletion
4845-del-F2	ACTGCG**GAGCTC**CCAGTGCAATCCTGATGCT	SacI	*gtsB* deletion
4845-del-R2	ACTGCG**TCTAGA**CATTGCGAAGTTCAAGCGTA	XbaI	*gtsB* deletion
crc-del-F1	ACTGCG**AGATCT**GCGTTTAACTTGAGCCTTGC	BglII	*crc* deletion
crc-del-R1	ACTGCG**GAGCTC**AGATGACGTCGGCATTCTG	SacI	*crc* deletion
crc-del-F3	ACTGCG**GAGCTC**CTGGCGTTTCGACTACCAGT	SacI	*crc* deletion
crc-del-R3	ACTGCG**ACTAGT**GAGCGTAACCAGCGTGAAG	SpeI	*crc* deletion
SacB-EcoRI	GTGA**GAATTC**AGACGGTCACAGCTTGTCTG	EcoRI	pAH79 construction
SacB-MfeI	GTGA**CAATTG**GGCGAAGATCGACTCTAGCT	MfeI	pAH79 construction
4845-lacZ F1	ACTGCG**CCCGGG**GCGAGTTGCATTACGAAACC	XmaI	Luciferase fusion
4845-lacZ R2	ACTGCG**CTCGAG**GGTGCGGATAAAGCCTTCAC	XhoI	Luciferase fusion
SacI-F2-Tac	ACTGCG**GAGCTC**AACACACTGGCTCCGACAG	SacI	*gtsB* overexpression
XhoI-R1-Tac	ACTGCG**CTCGAG**CAGGTTTGGAGGCGAGACTA	XhoI	*gtsB* overexpression
SacI-F1-Tac	ACTGCG**GAGCTC**TGCATGCATAACAACAAGAAAA	SacI	*gtsABCD-oprB* overexpression
NheI-R3-Tac	ACTGCG**GCTAGC**CGCACAGAGAGAAGGAGCTT	NheI	*gtsABCD-oprB* overexpression
PglmS-up-SBW25	GGAAATGGCATCACCTTCAA		Tn7 validation
PglmS-down-SBW25	GCACATCAATGACACCCTGT		Tn7 validation
PTn7R	CACAGCATAACTGGACTGATTTC		Tn7 validation
PTn7L	ATTAGCTTACGACGCTACACCC		Tn7 validation

Table D.3: Oligonucleotide primers used in Chapter 6.

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