Adaptive diversification in experimental populations of *Escherichia coli*

by

Jabus Tyerman

B.Sc., McMaster University, 1997

M.Sc., Simon Fraser University, 2001

A THESIS SUBMITTED IN PARTIAL FULFILLMENT
OF THE REQUIREMENTS FOR THE DEGREE OF

Doctor of Philosophy

in

THE FACULTY OF GRADUATE STUDIES

(Zoology)

The University Of British Columbia

(Vancouver)

December 2009

Abstract

What processes contribute to the origin and maintenance of biological diversity? When populations occupy different environments, that have divergent ecological characteristics, natural selection can cause each population to adapt to its environment, resulting in phenotypic divergence between populations. But can natural selection cause a single population to diverge? Adaptive dynamics theory predicts that ecological interactions between individuals in a population can result in negative frequency-dependent selection, and the branching of the population into phenotypically distinct subpopulations. Here, I tested predictions from adaptive dynamics theory. To do this, I experimentally evolved populations of the clonal bacterium *Escherichia coli* in the lab. First, using replicate populations of *E. coli* that had diversified in parallel, I tested whether convergent ecotypes among replicate populations competed in a likewise manner. I found they do not, suggesting that the genetic underpinnings of the convergent ecotypes among populations were different. The ecological interaction that has received the most attention from adaptive dynamics theory is resource competition, yet there are few direct tests that competition for resources does cause phenotypic distributions to evolve. I used diversified populations of bacteria, and experimentally demonstrated that competition can cause ecological character displacement. Subsequently, I explored whether mutational bias for novelty can regulate adaptive radiation when ecological opportunities and selection were similar. I found an asymmetry in the extent and range of diversification among replicate populations initiated from different genotypes, suggesting that mutational bias can regulate adaptive diversification. Finally, I tested for resource specialization trade-offs by measuring the extent of variation in populations evolved in environments that range in complexity from one to three

resources and found that diversity was not related to environmental complexity. Additionally, I explored the role of growth rate vs. yield trade-offs within and among populations that have diversified, and found little support for the hypothesis that these trade-offs underlie adaptive diversification in our bacterial populations. Together, these chapters test the predictions of adaptive diversification, so that we may better understand adaptive diversification in nature.

Table of contents

List of tables

List of figures

Acknowledgments

The completion of this thesis would not have been possible without the guidance and direction of my supervisor, Michael Doebeli. The long-term evolution experiments required the help of Chrissy Spencer and Mel Bertrand, and numerous undergraduate assistants. Without you all, this work would not have been possible. My committee provided me with feedback that strengthened the work presented here. Alistair Blachford provided intellectual stimulation and, along with the Quake gang, some much needed entertainment and stress relief. My friends and family outside of academia were always supportive. Finally, my wife, Dilara Ally, was patient and supportive, challenging and honest, and above all she believed in me.

Co-authorship statement

In chapter 2, G. Saxer, M. Doebeli, and M. Travisano designed the initial longterm evolution experiment. G. Saxer conducted the initial long term evolution experiment that created the bacterial strains used as source strains in the competition experiments described in chapter 2. J. Tyerman and M. Doebeli designed the competition experiments. J. Tyerman and N. Havard executed the competition experiments. J. Tyerman analysed the data. J. Tyerman and M. Doebeli prepared the manuscript; J. Tyerman wrote the manuscript and M. Doebeli edited the manuscript.

In chapter 3, J. Tyerman, C. Spencer and M. Doebeli conceived the longterm evolution experiment that generated the diversified strains used as source populations in this study. J. Tyerman, C. Spencer, and M. Bertrand evolved the source strains. J. Tyerman and M. Doebeli designed the character displacement experiments. J. Tyerman executed the experiments and conducted the statistical analysis. J. Tyerman and M. Doebeli prepared the manuscript; J. Tyerman wrote the manuscript, and M. Doebeli edited the manuscript.

In chapter 4, J. Tyerman and M. Doebeli designed the experiment. J. Tyerman executed the experiment and conceived of and conducted the statistical analysis. J. Tyerman and M. Doebeli prepared the manuscript; J. Tyerman wrote the manuscript and M. Doebeli edited the manuscript and conceived of the complex pairwise invasion plot in the discussion.

In chapter 5, J. Tyerman, C. Spencer, and M. Doebeli designed the experiment that generated the source strains. The design of the experiment to characterize those strains was by J. Tyerman, C. Spencer, and M. Doebeli. J. Tyerman directed, and conducted the execution of the phenotypic assays. J. Tyerman conducted the statistical analysis. J. Tyerman, C. Spencer and M. Doebeli prepared

the manuscript; J. Tyerman wrote the manuscript, and C. Spencer and M. Doebeli edited the manuscript.

Dedication

. . . to my loving wife, Dilara Ally.

Chapter 1

Introduction

1.1 Overview

The processes that contribute to the origin and maintenance of biological diversity are of fundamental interest to evolutionists. For more than fifty years, however, it has remained a contentious issue as to whether the process of natural selection can cause a population to diverge into phenotypically differentiated subpopulations, i.e., to adaptively diversify (Mayr, 1942, Dieckmann et al., 2004). The consequence of natural selection causing ecological diversification has implications for related processes in evolution and ecology, namely ecological speciation, parallel evolution, ecological character displacement, and adaptive radiation (Schluter, 2000), so developing and testing theory is of great importance.

Over the past decade, theory has established that frequency-dependent ecological interactions – usually intraspecific competition for resources – can cause populations to undergo evolutionary branching and speciation (Geritz et al., 1998, Dieckmann and Doebeli, 1999, Doebeli et al., 2005). Yet without empirical validation or testing, a theory can remain peripheral to scientific advancement. Thus, whether and how the theory of adaptive diversification can explain or predict phenotypic and ecological divergence in real biological systems is now an important focus of evolutionary research (e.g., Friesen et al., 2004).

There are two main challenges facing evolutionists trying to validate and test the predictions of adaptive diversification. First, adaptive diversification, like any

evolutionary process, requires the study of populations over more than one generation; studying long lived species makes this task difficult. To side-step this difficulty, evolutionists often infer evolutionary process from biogeographic pattern. However, methodologies without manipulation or without controls, for example, observational or correlational studies, do not provide the critical tests that allow novel theories to pass muster in the scientific community. Second, studying evolution in nature can be problematic because many uncontrolled factors (e.g., heterogeneous or fluctuating environments, spatial distribution and dispersal, ecological interactions within and beyond the focal species, etc.) may mitigate, exacerbate or obscure patterns making the interpretation of results difficult. Thus, conclusions from natural studies often have caveats that make them less than ideal for establishing an empirical foundation for a novel theoretical framework.

Both of these challenges have been overcome by researchers employing evolution experiments using microbial populations (reviewed in Kassen, 2009, MacLean, 2005, Kassen and Rainey, 2004, Elena and Lenski, 2003, Kassen, 2002, Travisano and Rainey, 2000). First, microbes evolve over timescales that can be readily studied by evolutionists. Thus, evolutionary processes can be studied directly, rather than indirectly through inference. Second, under lab conditions, evolutionary factors can be isolated and directly tested. For example, Kassen et al. (2000) clearly showed how diversity peaked at intermediate levels of disturbance in evolved populations of *Pseudomonas fluorescens*, and Tyerman et al. (2008) clearly demonstrated a role for resource competition in causing ecological character displacement using *E. coli*. Thus, microbial evolution experiments are useful model systems that allow us to determine how and under what circumstances a particular process may operate. The knowledge gained about an evolutionary process under controlled lab conditions is a proof of principle; once it is established that a process *can* work, researchers can focus on determining whether the process *does* work in other circumstances. Thus, evolution experiments with microbes can bridge the gap between the development of theory and the validation of theory in nature.

Here, I present four experimental evolution studies that contribute to understanding the adaptive origin and maintenance of biological diversity. In a lab setting, I studied evolving populations of the bacterium *E. coli*. I experimentally tested several factors believed to be important in generating and maintaining diversity in

nature. These factors included the nature of selection, evolutionary environment, genetic background, and degree of environmental complexity (i.e., number of carbon resources). Below, I outline the questions that motivated each chapter in my dissertation. However, before I outline these questions, I explain why I use the term adaptive radiation.

1.2 What is adaptive radiation?

Adaptive radiation is an evolutionary process characterized by rapid phenotypic diversification and speciation within a lineage (Schluter, 2000). Adaptive radiation is often used as a noun (i.e., the Galapagos finches is a celebrated example of an adaptive radiation), but it is also possible to conceive of adaptive radiation as a process, or more correctly, as a series of processes: rapid phenotypic divergence, and rapid speciation. While bacterial systems may be excellent model systems for studying the first part of adaptive radiation (i.e., phenotypic divergence driven by natural selection and ecological opportunity), they may be less than ideal for studying the second part of adaptive radiation (i.e., speciation). Does this limitation preclude the use of "adaptive radiation" in describing the relevance of a model system to a process that is integral to adaptive radiation?

In this thesis, I considered the mechanisms that underlie the rapid phenotypic diversification in adaptive radiation to be analogous to the mechanisms that underlie adaptive divergence (or diversification). Technically, I may be studying the more narrow case of adaptive diversification, yet I may speak to the more broad case of adaptive radiation. This is because, in each case, what is important is the fact that natural selection has driven phenotypic divergence. Thus, I elected to use the terms adaptive divergence and adaptive radiation interchangeably.

1.3 Scientific questions

1.3.1 Are the genetic mechanisms that underlie recurrent phenotypes the same?

If natural selection is involved in the evolution of phenotypes, then we would predict convergence in phenotypic form in populations facing similar environmen-

tal challenges (Schluter, 2000). By extension, if adaptive diversification occurs, i.e., natural selection drives population divergence, then phenotypes evolved within populations should recur among lineages undergoing analogous adaptive radiation. However, because there is not a one-to-one mapping between genotype and phenotype (West-Eberhard, 2003), it is an open question whether convergence extends to the level of genotype (Kassen, 2009).

A direct answer to this question would involve sequencing the DNA of convergently evolved phenotypes. As the costs for DNA sequencing become less prohibitive, this direct method will more often be used. An alternative approach is to indirectly test for parallelism at the genetic level by testing for correlated responses to selection at the phenotypic level (Travisano and Lenski, 1996). If genetic causes of recurrent phenotypes are different, then we might detect these differences when these genotypes are subjected to phenotypic characterization in novel (i.e., unselective) environments. In Chapter 2, I used competition experiments between recurrent ecotypes, i.e., evolved among populations, to test for correlated responses to selection and thus test for parallelism in the underlying genetics.

This work is important and relevant because it builds on our knowledge about the degree of repeatability at the genetic level, given recurrent phenotypes. These results were published in *The Royal Society of London, Proceedings B.* (Tyerman et al., 2005).

1.3.2 Does competition cause ecological character displacement?

Ecological character displacement is a process that shifts or maintains phenotypic distributions (Losos, 2000, Schluter, 2000). It results from ecological interactions between species. The ecological interaction that has received the most attention is resource competition (Schluter, 2000, 2003), although other ecological interactions are also capable of shifting phenotypic distributions (Doebeli and Dieckmann, 2000). There are few direct tests of the role of resource competition causing ecological character displacement (but see Taper, 1990), yet the idea that competition causes phenotypes to diverge ((or to converge upon competitive release, Losos and De Queiroz, 1997)) is central to models of adaptive speciation and ecological coexistence (Taper and Case, 1992, Dieckmann and Doebeli, 1999, Doebeli

and Dieckmann, 2003). In Chapter 3, I explicitly tested the role of resource competition in maintaining and shifting resource related phenotypes. First, I evolved replicate populations of *E. coli* in mixtures of glucose and acetate and found that the populations diverged repeatedly into glucose specialists and acetate specialists. Next, I isolated these specialists and released them from competition, and allowed them to evolve with the prediction that they would undergo phenotypic convergence. Finally, I competed "convergent" types with the prediction that they would diverge due to the competitive interactions. These results were published in *BMC Evolutionary Biology* (Tyerman et al., 2008).

1.3.3 Are mutational constraints important for understanding adaptive radiation?

What regulates the extent of ecological diversification under adaptive radiation (Kassen, 2009)? Broadly speaking, factors that regulate the extent of ecological diversity fall under two categories. Extrinsic factors – those factors that are external to the organism – include ecological opportunity and divergent selection (Schluter, 2000). Intrinsic factors – those factors that are internal to the organism – include mutation and development, processes that determine the input of novel phenotypic variation in an evolving lineage (West-Eberhard, 2003, Brakefield, 2006). There is broad acceptance that external factors regulate the diversity observed in adaptive radiation (Schluter, 2000). As such, when diversity evolved in adaptive radiation varies among lineages, it is explained by differences in available niche space or differences in divergent selection among lineages. Yet, variation in the extent of diversity evolved in adaptive radiation might also result from variation in the input of phenotypic novelty, and there are examples of adaptive radiations where different lineages facing similar ecological opportunities undergo different degrees of diversification (Kassen, 2009).

In Chapter 4, I explored this issue by characterizing the derived ecotypes (described in Chapter 3) that evolved under competitive release from populations founded by different genotypes. Because the extrinsic factors facing these ecotypes were similar, I argued that the subsequent variation in diversity evolved under adaptive radiation was due to differences in mutational input. Specifically, one ecotype diversified to a greater extent than another ecotype when evolved in similar

environments. This result is important because it emphasizes how intrinsic factors can regulate the degree of diversity evolved during adaptive radiation and thus may be useful for extending the ecological theory of adaptive radiation (Schluter, 2000).

1.3.4 Are trade-offs in carbon resource specialization related to diversity?

Trade-offs arise when increases in fitness in one phenotypic dimension are accompanied by fitness decreases in other phenotypic dimensions (Roff and Fairbairn, 2007). For example, the evolution of specialization may enhance the ability of a species to consume one resource yet diminish its ability to consume other resouces. This may allow species to coexist, because they differ in their carbon utilization profiles, i.e., each species specializes on a subset of resources (Barrett et al., 2005, Barrett and Bell, 2006). Because no single phenotype can fill all possible niches, trade-offs are fundamentally important to theories explaining adaptive diversity.

In Chapter 5, I tested two predictions that follow directly from the concept that trade-offs in carbon use are important for the levels of diversity observed in evolved populations of *E. coli*. First, I tested whether diversity among populations is directly proportional to the number of resources, or environmental complexity. This idea has been studied in other systems (for example, Barrett et al., 2005, Barrett and Bell, 2006, Habets et al., 2007), however several of these studies did not control for productivity (Hall and Colegrave, 2007). In studies that did not control for productivity, the authors often found that diversity increased with increasing environmental complexity, however these results are confounded because the increase in diversity may have reflected the concomitant increase in productivity in the complex environment rather than actual complexity of environment. Controlling for productivity, I evolved populations of *E. coli* in one, two, and three carbon resource environments. Because I controlled for productivity, my research filled a gap in the literature in determining whether simple trade-offs in carbon usage were important in predicting levels of diversity among populations.

Second, I tested whether trade-offs between growth rate and yield could explain diversity within and among evolved populations of *E. coli*. The hypothesis that rate vs. yield trade-offs exist is based on first principles of thermodynamics (Pfeiffer et al., 2001, Helling, 2002), and has been identified in several experimental evo-

lution studies (Novak et al., 2006, Maharjan et al., 2007). Briefly, individuals or populations that maximize their rate of using a resource may be inefficient at using that resource and vice versa. I isolated clones from evolved populations and tested whether there was variation in rate vs. yield traits and whether there was a negative correlation between growth rate and yield in populations that had diversified.

This work is important because it tested the idea that trade-offs in carbon resource specialization were generally involved in the evolution of diversity within evolved populations of *E. coli*.

Bibliography

Barrett, R. D. H. and Bell, G. (2006). The dynamics of diversification in evolving pseudomonas populations. *Evolution*, 60(3):484–490.

Barrett, R. D. H., MacLean, R. C., and Bell, G. (2005). Experimental evolution of Pseudomonas fluorescens in simple and complex environments. *Am Nat*, 166(4):470–480.

Brakefield, P. (2006). Evo-devo and constraints on selection. *TREE*, 21:362–368.

Dieckmann, U. and Doebeli, M. (1999). On the origin of species by sympatric speciation. *Nature*, 400:354–357.

Dieckmann, U., Metz, J., Doebeli, M., and Tautz, D. (2004). *Introduction*. Adaptive speciation. Cambridge University Press, Cambridge.

Doebeli, M. and Dieckmann, U. (2000). Evolutionary branching and sympatric speciation caused by different types of ecological interactions. *Am Nat*, 156:S77–101.

Doebeli, M. and Dieckmann, U. (2003). Speciation along environmental gradients. *Nature*, 421:259–264.

Doebeli, M., Dieckmann, U., Metz, J., and Tautz, D. (2005). What we have also learned: Adaptive speciation is theoretically plausible. *Evolution*, 59:691–695.

Elena, S. and Lenski, R. (2003). Evolutionary experiments with microorganisms: the dynamics and genetics of adaptation. *Nature reviews: Genetics*, 4:457–69.

Friesen, M., Saxer, G., Travisano, M., and Doebeli, M. (2004). Experimental evidence for sympatric ecological diversification due to frequency dependent competition in *Escherichia coli*. *Evolution*, 58:245–60.

Geritz, S., Kisdi, E., Meszena, G., and Metz, J. (1998). Evolutionary singular strategies and the adaptive growth and branching of the evolutionary tree. *Evol Ecol Res*, 12:35–57.

Habets, M., Cuthill, I. C., Hoekstra, R., and de Visser, J. A. G. M. (2007). Spatial structure inhibits the rate of invasion of beneficial mutations in asexual populations. *Proc.R.Soc.Lond.B*, 274:2139–43.

Hall, A. R. and Colegrave, N. (2007). How does resource supply affect evolutionary diversification? *Proc R Soc Lond.B*, 274:73–78.

Helling, R. B. (2002). Speed versus efficiency in microbial growth and the role of parallel pathways. *J.Bacteriol.*, 184(4):1041–1045.

Kassen, R. (2002). The experimental evolution of specialists, generalists, and the maintenance of diversity. *J Evolution Biol*, 15:173–190.

Kassen, R. (2009). Towards a general theory of adaptive radiation: insights from microbial experimental evolution. *Proc NY Acad Sci*, 1168:3–22.

Kassen, R., Buckling, A., Bell, G., and Rainey, P. (2000). Diversity peaks at intermediate productivity in a laboratory microcosm. *Nature*, 406:508–12.

Kassen, R. and Rainey, P. (2004). The ecology and genetics of microbial diversity. *Ann Rev Microbiol*, 58:207–231.

Losos, J. (2000). Ecological character displacement and the study of adaptation. *PNAS*, 97:5693–5695.

Losos, J. and De Queiroz, K. (1997). Evolutionary consequences of ecological release in caribbean *Anolis* lizards. *Biol. J. Linn. Soc. Lond.*, 61:459–483.

MacLean, R. C. (2005). Adaptive radiation in microbial microcosms. *J.Evol Biol*, 18:1376–1386.

Maharjan, R. P., Seeto, S., and Ferenci, T. (2007). Divergence and redundancy of transport and metabolic rate-yield strategies in a single Escherichia coli population. *The Journal of Bacteriology*, 189(6):2350–2358.

Mayr, E. (1942). *Systematics and the origin of species*. Columbia University Press, New York.

Novak, M., Pfeiffer, T., Lenski, R., Sauer, U., and Bonhoeffer, S. (2006). Experimental tests for an evolutionary trade-off between growth rate and yield in Escherichia coli. *Am Nat*, 168:242–251.

Pfeiffer, T., Schuster, S., and Bonhoeffer, S. (2001). Cooperation and competition in the evolution of atp-producing pathways. *Science*, 292(5516):504–507.

Roff, D. and Fairbairn, D. (2007). The evolution of trade-offs: where are we? *Journal of Evolutionary Biology*, 20:443–447.

Schluter, D. (2000). *The ecology of adaptive radiation*. Oxford University Press, Oxford.

Schluter, D. (2003). Frequency dependent natural selection during character displacement in sticklebacks. *Evolution*, 57:1142–1150.

Taper, M. (1990). *Experimental character displacement in the adzuki bean weevil, Callosobruchus chinensis*. Bruchids and Legumes: economics, ecology and coevolution. Kluwer.

Taper, M. and Case, T. (1992). Models of character displacement and the theoretical robustness of taxon cycles. *Evolution*, 46:317–333.

Travisano, M. and Lenski, R. (1996). Long-term experimental evolution in Escherichia coli. iv. targets of selection and specificity of adaptation. *Genetics*, 143:15–26.

Travisano, M. and Rainey, P. (2000). Studies of adaptive radiation using model microbial systems. *Am Nat*, 156:S35–44.

Tyerman, J., Bertrand, M., Spencer, C., and Doebeli, M. (2008). Experimental demonstration of ecological character displacement. *BMC Evol Biol*, 8(1):34.

Tyerman, J. G., Havard, N., Saxer, G., Travisano, M., and Doebeli, M. (2005). Unparallel diversification in bacterial microcosms. *Proc R Soc B*, 272:1393–1398.

West-Eberhard, M. (2003). *Developmental plasticity and evolution*. Oxford University Press, Oxford, UK.

Chapter 2

Unparallel diversification in bacterial microcosms[1]

2.1 Introduction

In theory, disruptive selection arising as a consequence of frequency-dependent processes (e.g. competition for limited resources) can result in adaptive diversification, that is, the splitting of an ancestral lineage into distinct descendent lineages as a consequence of natural selection acting on individuals within the population (Dieckmann and Doebeli, 1999, Friesen et al., 2004). This idea highlights the central role of ecology in the speciation process. Empirical support for adaptive speciation is accumulating (Via, 2001, Dieckmann et al., 2004) although experimental support has, in general, been lacking (but see Rainey and Travisano, 1998, Friesen et al., 2004). Evolution experiments using microbial systems are useful for testing ideas of adaptive diversification because microbes have large population sizes, short generation times and are easy to culture in the laboratory (Lenski et al., 1991, Rosenzweig et al., 1994, Travisano et al., 1995, Travisano and Lenski, 1996, Rainey and Travisano, 1998, Treves et al., 1998, Rainey et al., 2000, Rainey and Rainey, 2003). In an attempt to study diversification experimentally, 36 lines of *Escherichia coli* B were evolved under seasonal conditions (i.e. daily serial

[1] A version of this chapter has been published. Tyerman, J.G., N. Havard, G. Saxer, M. Travisano, and M. Doebeli (2005) Unparallel diversification in bacterial microcosms *Proc R Soc B* **272:** 1393-8.

batch transfer into fresh media) for 1,000 generations in different nutritional environments (Friesen et al., 2004). One of the main findings of these experiments was that the ancestor had repeatedly diversified into (at least) two types that can be identified by the size of the colony formed on agar plates. One type formed large colonies (L), which were visible after 24h, and the second type formed small colonies (S), which were visible after 48h. This polymorphism was observed in lines that evolved in glucose as well as in lines that evolved in a mixture of glucose and acetate, two environmental conditions that are actually very similar (see below). In both environments, the LS polymorphism, which we refer to as a diversified pair, appeared to be stable over both evolutionary and ecological time-scales. Friesen et al. (2004) suggested that the L and S colonies corresponded to two ecological types, a glucose specialist and a fast-switcher (or acetate) specialist (named after their differential growth performance in glucose-acetate environments as detailed below), which were maintained by negative frequency-dependent selection (Levin, 1988); that is, each type had the advantage when rare.

Being a consequence of natural selection, adaptive diversification is a deterministic process and is therefore often associated with "repeatability". For example, parallel diversification is often recognized as evidence for adaptive diversification (or ecological speciation; Schluter, 2000) because parallelism implies the action of natural selection. However, does adaptive diversification imply parallelism? In other words, if one sees adaptive diversification repeatedly under similar ecological conditions, does this imply parallel diversification? Or can subtle differences in the environment lead to qualitatively different pairs of diversified types? We address this question by using a biological system– the L-S polymorphism – for which we are confident that adaptive processes have generated diversification (Friesen et al., 2004). Saxer et al. (in preparation) argue that there is good evidence for parallel diversification among all replicate microcosms that evolved in the glucose-acetate mixture. Here we ask whether diversification in glucose alone, a very similar environment, occurred in parallel with diversification in the glucose-acetate mixture. To do this, we conducted competition experiments between L and S strains in order to determine if the outcome of competition between L and S strains from diversified pairs that evolved in different environments was analogous to the outcome of competition between L and S strains that evolved in the same environment.

2.1.1 Background: evolved strains

All bacterial lines were initiated by Saxer et al. (in preparation) from, alternately, Ara- or Ara+ variants of a common ancestral strain of *E. coli* B that differed from one another only in their ability to metabolize l-arabinose. This difference served as a useful cross-line contamination check (Lenski et al., 1991). We briefly review their experimental protocol and results (see also Friesen et al., 2004). The bacterial strains used reproduce asexually and contain no plasmids. Replicate cultures were grown in 10mL of Davis Minimal (DM) media and supplemented with either 410μg/mL glucose (DM_{glu}), 410μg/mL acetate (DM_{ace}), or 205μg/mL glucose and 205μg/mL acetate (DM_{mix}) as the additional source(s) of carbon in 50mL flasks at 37° and 120 r.p.m. Each culture underwent a 100-fold dilution during transfer to fresh media once every 24h, resulting in 6.6 binary divisions per day.

By 1,000 generations (ca. 150 days), a stable polymorphism had appeared in 6 of 12 DM_{glu} replicates and 6 of 12 DM_{mix} replicates. In both environments, the ancestral type had diversified into two types with respect to colony morphology. One type formed large colonies (L) within 24h after plating on arabinose-free agar and a second type formed small colonies (S) between 24 and 48h after plating. L and S types also differed in their growth characteristics in liquid media (Friesen et al., 2004). Similar diversity has previously been described (Helling et al., 1987, Rosenzweig et al., 1994, Turner et al., 1996, Rozen and Lenski, 2000).

When bacteria are grown in liquid media containing two resources, they exhibit diauxie, whereby an initial growth phase is correlated with use of the preferred metabolite (e.g. glucose) and a subsequent growth phase is correlated with use of the less preferred metabolite (e.g. acetate). This diauxic growth pattern is therefore expected in the DM_{mix} environment, as two resources are present at the onset of the transfer period. However, diauxie also occurred in DM_{glu} replicates, for reasons that can be found in the metabolic details: the aerobic breakdown of glucose in glycolysis generates acetate, which is available for uptake and catabolism by *E. coli* once the glucose in the media has been exhausted. Because aerobic metabolism of glucose yields acetate, the DM_{glu} environment progressively becomes more like the DM_{mix} environment during a single season (batch) and differs only in the temporal availability of acetate and the absolute and relative amounts of glucose and acetate

throughout the course of the season. We exploited this similarity of nutritional environments to test the repeatability of parallel diversification.

Friesen et al. (2004) proposed that diauxic growth is the basis for the L and S polymorphism and that the polymorphism is maintained by frequency-dependent competition for limited resources and a trade-off between initial growth on glucose and subsequent growth on acetate (see also Turner et al., 1996). Within the course of one transfer period, L appears to grow faster on glucose (i.e., L is a glucose specialist) but takes longer to switch to acetate metabolism than S (i.e., S is a fast switching [or acetate] specialist). Frequency-dependence restores the balance between the two types whenever one type becomes too common; that is, whenever competition for the resource on which the common type specializes becomes intense. In the glucose-only environment, the observed polymorphism appears to be related to the crossfeeding polymorphisms that have been found in chemostat cultures of *E. coli* (Rosenzweig et al., 1994, Turner et al., 1996, Treves et al., 1998).

2.1.2 Why competition experiments?

Preliminary investigations revealed that a diversified pair mixed in different starting proportions of L and S and observed over ecological time (up to 14 days or about 100 generations) consistently reached a stable, intermediate frequency (Friesen et al., 2004). When rare, each type had an advantage and increased in frequency until a stable frequency was obtained. Thus, it appeared that frequency-dependent selection played an important role in generating and maintaining the stable polymorphism within replicates. We use this result as a basis of comparison to determine if diversified types were functionally equivalent (in their "competitive ability") across replicates within and between nutrient environments. We use the term competitive ability as a composite term encompassing all traits required to extract and metabolize resources (glucose and acetate) from the environment in the face of competition with the complementary partner of a diversified pair. We employ competition experiments as an ecological assay for parallelism because of the importance of competition for generating frequency-dependent selection and, ultimately, for the maintenance of diversity.

2.2 Methods

All colonies in this experiment were selected from cultures evolved by Saxer et al. (in preparation). We assayed diversity on arabinose-free plates, and isolated single L and S genotypes from DM_{glu}-derived population 10 and DM_{mix}-derived populations 31 and 33 by plating the diversified strains on arabinose-free agar plates and picking single colonies as representatives of each diversified pair (identified by characteristic colony morphology). We suspended and grew these in 10mL of DM_{glu} or DM_{mix} for 24h (37° at 250 r.p.m.) and then mixed L and S in three different proportions to initiate our competition experiments. Starting proportions of L were 90, 50 and 10% (with S making up the complementary proportion) and were mixed by correcting proportionate volumes for cell density (determined by optical density using a spectrophotometer). We added 100μL of this mixture to 10mL of liquid media (DM_{mix} or DM_{glu}) and subsequently transferred 100μL of stationary phase culture (24h) to new media for 8, 10 or 12 days. At each transfer, we assayed cultures by plating on four to six arabinose-free plates (providing us with 500-1000 colonies per assay) to determine the density of L and S. S and L ecotypes were identified as detailed above.

We replicated each treatment three or four times and recorded the means of proportion L ($\pm$s.e.m.). In addition to the mixed cultures, we initiated pure cultures of L or S to examine the possibility that the complementary type in a diversified pair (S or L, respectively) might arise by mutation and become established in cultures within the time-frame of our study. We periodically checked for cross-contamination between cultures.

In our first assay, we repeated earlier work (Friesen et al., 2004) to establish a baseline of comparison for our subsequent assays. Thus, we mixed L and S from a single diversified pair in different starting proportions to confirm that mixed cultures would equilibrate to an intermediate frequency of L due to negative frequency dependence (with each type having an advantage when rare). The evolutionary environment for each player and the ecological environment of the assay were the same: DM_{mix}. Our second assay involved mixing an L from one diversified pair in DM_{mix} with an S from a different diversified pair in DM_{mix} (and vice versa). Therefore, both players evolved in and competed against one another in the DM_{mix}

environment; however, the players came from different replicate microcosms. Finally, we mixed one partner from a diversified pair that evolved in DM_{mix} with its complementary partner from another diversified pair that evolved in DM_{glu}, and vice versa. Comparing results from this last set of assays with results from the other assays would therefore shed light on whether diversified pairs that evolved in different environments underwent parallel diversification. Specifically, we would conclude that diversification of the phenotypes underlying competitive ability had occurred in parallel if the dynamics of competition between L and S, when L and S come from different diversified pairs that evolved in different environments (assay 3 above), are similar to the dynamics when L and S come from either the same diversified pair (assay 1) or from different diversified pairs that evolved in the same environment (assay 2). However, if those dynamics were different we would conclude that diversification had not occurred in a parallel manner.

2.3 Results

Our first assay involved competing L and S from diversified pairs of Populations 31 and 33 which were initiated at different starting proportions and evolved and competed in the DM_{mix} environment. Regardless of starting proportions, all trials between strains from population 31 diversified pairs levelled off at a stable, intermediate frequency by day 8. If we define the frequency at the end of each trial as the equilibrium frequency, then the equilibrium frequencies from different trials were indistinguishable from one another (proportion large=0.49, F_2,6=0.7647, p=0.506; Figure 2.1). Likewise, diversified pairs from population 33 reached intermediate equilibrium frequencies by day 8 that were also indistinguishable from one another (proportion large=0.80, F_2,6=0.1552, p=0.860; Figure 2.1b). Combining all trials involving population 31 and 33 diversified pairs, we tested whether the equilibrium frequency was similar between populations 31 and 33. We determined that the equilibrium frequencies were in fact different from one another (t=8.76, p $<$0.0001). Treatments initiated with only L or S types in both population 31 and 33 remained pure throughout the entire study (results not shown).

Our second assay involved competing complementary partners (L versus S) from two different diversified pairs, albeit that both evolved in the DM_{mix} environ-

ment. Figure 2.2a illustrates the dynamics of competition between L_{mix-31} versus S_{mix-33}. Again, regardless of starting proportion, all trials converged to an intermediate equilibrium frequency by day 10 (proportion large =0.79, $F_{2,6}$=2.074, p=0.2067). Similarly, when we competed L_{mix-33} versus S_{mix-31}, we observed convergence to an intermediate equilibrium frequency within 8 days (proportion large =0.37, $F_{2,6}$=0.6047, p=0.5764; Figure 2.2b). Because our first assay revealed that sets of diversified pairs were fine-tuned to different intermediate equilibrium frequencies (see above), we had little reason to expect that the equilibrium frequencies attained in our mixed-partner trials would be equal and, indeed, they were not (t=7.845, p $<$0.0001).

Third, complementary partners that had evolved in different environments were placed in a competitive environment. Figures 2.3a,b and 2.4a,b cogently summarize the results of these mixed partner/mixed evolutionary environment assays: the competitive dynamics are qualitatively different from those attained above and from one another, with outcomes depending on both the partners and the environments.

Figure 2.3a illustrates the competitive dynamics between L_{mix-33} and S_{glu-10} in the DM_{mix} environment. Though the dynamics appeared to converge to similar frequency dynamics for different initial conditions, the frequencies tend to oscillate over the 12-day duration of the assay rather than level off at some intermediate frequency. In contrast, when the partners came from reciprocal environments (L_{glu-10} versus S_{mix-33}), L almost vanished in all trials, regardless of initial conditions (Figure 2.3b). Because one of the partners had evolved in DM_{glu}, we set up analogous competition assays in the DM_{glu} environment. When we placed L_{mix-33} and S_{glu-10} in a competitive environment, we observed a convergence in frequency dynamics for different initial conditions but oscillatory dynamics over time (Figure 2.4a). Finally, when we placed L_{glu-10} and S_{mix-33} in DM_{glu}, we observed an initial decline in the proportion L and then a slow recovery over the duration of the assay (Figure 2.4b). Overall, the dynamics observed when L and S from diversified pairs that evolved in different environments were placed in competition are qualitatively different from the equilibrium dynamics that result when L and S from diversified pairs that evolved in the same environment were placed in competition.

2.4 Discussion

We competed diversified strains of *E. coli* with each other to examine the degree of parallel diversification that had occurred in replicate populations, within and between evolutionary environments. Our results (first assay) agree with earlier studies (Helling et al., 1987, Rosenzweig et al., 1994, Turner et al., 1996, Friesen et al., 2004) that have demonstrated that diversified types can be maintained by negative frequency-dependent selection. This empirical result formed the basis of comparison for our next two experiments. Evidence for parallel diversification would consist of a similar convergence to intermediate frequency when complementary partners selected from different replicates were pitted against one another.

In our second set of assays, we competed complementary partners from different replicates, each derived in identical evolutionary environments (L_{mix-31} versus S_{mix-33} and L_{mix-33} versus S_{mix-31}, both derived in DM_{mix}). Because L and S converged to intermediate frequencies, we conclude that parallel diversification has occurred. However, when we selected complementary partners from different evolutionary environments for competition, our results were significantly different. In one outcome, we observed the near elimination of one partner; in three others, we observed oscillatory dynamics with no evidence of convergence to a stable intermediate frequency.

No simple pattern emerged to help explain the range of competitive outcomes we observed when we competed complementary partners from disparate evolutionary environments. Although S_{mix-33} was clearly superior to L_{glu-10} in the DM_{mix} environment, the advantage was not so clear in the DM_{glu} environment, suggesting perhaps that home turf (i.e. competition occurring in the environment that one partner had evolved in) confers some competitive advantage – or at least restored some semblance of balance to the system. There were no other indications that one type was absolutely superior in competition, or that home turf afforded any advantages.

It is important to note that the two environments in which the microcosms evolved and diversified are actually quite similar. In the glucose-acetate mixture (DM_{mix}), the acetate is present from the start and bacteria use the two resources sequentially. In the glucose-only environment (DM_{glu}), only glucose is present at the start of each batch culture, but acetate is produced during glucose metabolism and

is available for uptake and catabolism once the glucose has been exhausted. Thus, the DM_{glu} environment differs from the DM_{mix} environment only in the absolute and relative amounts of glucose and acetate throughout the course of the season.

The similarity of the environments is reflected in the fact that our diversified populations have apparently evolved in parallel with respect to colony morphology (diameter and form). Because we observed similar phenotypic patterns of diversification in the two environments, and because in both environments, diversification appears to be a result of similar mechanisms (owing to the existence of a trade-off between metabolic efficiency on glucose and acetate), one might expect that diversification has occurred in parallel. However, our results suggest that only replicates that shared identical environments diversified in parallel with respect to competitive ability. Thus, in general, whether we find evidence of parallel diversification may be contingent on the traits selected for experimentation. It would be interesting to quantify why some traits are indifferent to the details of the disruptive selection (e.g. colony morphology), while other traits appear to be more sensitive (e.g. competitive ability).

Functional variation (in competitive ability) within each type suggests that there is more than one way to diversify as an L or S partner within a diversified pair. We find this result somewhat sobering, as researchers using this system (and others, e.g., *Pseudomonas fluorescens*, Rainey and Travisano, 1998) often rely on colony morphology to identify diversified types within their cultures. We suspect that employing colony phenotype to estimate diversification underestimates the variation present in diversified cultures.

It is interesting to consider the unstable dynamics observed in Figures 2.3a and 2.4a, and speculate about the cause of such patterns. Ultimately, we need to account for the observation of a change in the trajectory of population composition (increasing versus decreasing proportion L) given the same initial frequencies of L within the population, differing only in the time period in which they were observed. Hypotheses to explain this variation fall into two broad categories: genetic variation and phenotypic variation.

2.4.1 Genetic hypotheses

This category of hypotheses depends on genetic variation (for competitive ability) existing within the competitive microcosms that is masked by the characteristic morphological traits. This requires that genetic variation has arisen within the time-frame of the experiment, as L and S types were isolated from single (presumably genetically uniform) colonies. Imagine there are two types of L, L1 and L2. Then one could envisage that competition within the L category (L1 versus L2), as well as competition among the L and S categories, drives the observed dynamics. Thus, when L is rare (relative to S), L1 out-competes L2; however, when L is common, L2 out-competes L1. We might expect, then, for the population to consist of different types of L at different times throughout the competitive experiment. If we isolated L colonies at different times, and competed them against the S from their reciprocal times, then we would expect to see a reversal in the trajectory of population composition. One difficulty with testing hypotheses of this nature is that they rely on no mutations arising within the course of the test, yet the hypothesis of genetic variation requires variation to have arisen within the time-frame of the original experiment in the first place (since L and S cultures were isolated from a single colony).

2.4.2 Phenotypic hypotheses

This category of hypotheses depends on phenotypic variation driving the observed cyclical dynamics. One possibility is that gene expression is sensitive to extreme frequencies of L. Crossing such a threshold alters gene expression, shifting the dynamics between L and S. No doubt variations on this hypothesis exist. They await future formulation and testing.

This study adds to the body of work that highlights the importance of frequency dependence for generating and maintaining diversity (reviewed in Levin, 1988, Rainey et al., 2000). In our system, negative frequency dependence maintains the balance between different types specializing on different resources. However, one general implication of our study is that polymorphisms maintained by negative frequency dependence may be highly sensitive to the environmental conditions and evolutionary histories of the players involved. Thus, even slight changes in

conditions may shift the system from a state of stability to one of periodicity or extinction. For example, other forms of frequency dependence involving spatial structure may also be sensitive to subtle shifts in the environment (Rainey and Travisano, 1998). Our results also have implications for conservation management plans. Reintroductions of "similar" species (ecotypes) in restoration projects after the loss of a species (e.g. swapping one type for another) could result in similar patterns of instability or even system collapse.

Further, our results suggest that while diversification in one trait (e.g. colony morphology) may be robust across environments, diversification in other traits may not be (e.g. competitive ability), because we observed parallelism with respect to colony morphology, but not with respect to competitive ability. Parallelism has often been used as a test of the role of ecology in speciation (or adaptation in diversification; Schluter, 2000). We are confident that our microcosms have diversified due to similar adaptive processes (Friesen et al., 2004), yet we failed to see parallel diversification when we used competitive ability as our assay of parallelism. Thus, studies that observe non-parallel patterns should not necessarily conclude that ecology has played little role in the diversification process. Unparallel diversification may simply reflect subtle differences between otherwise similar environments.

2.5 Acknowledgements

We thank G. Takhar, J. Briscoe and S. Merali for laboratory assistance, R. Redfield, C. Spencer, M. Bertrand and S. Kahlon for technical advice and members of the Doebeli and SOWD laboratory groups for fruitful discussions and feedback on early versions of this manuscript. The James S. McDonnell Foundation and NSERC provided financial assistance to M.D.

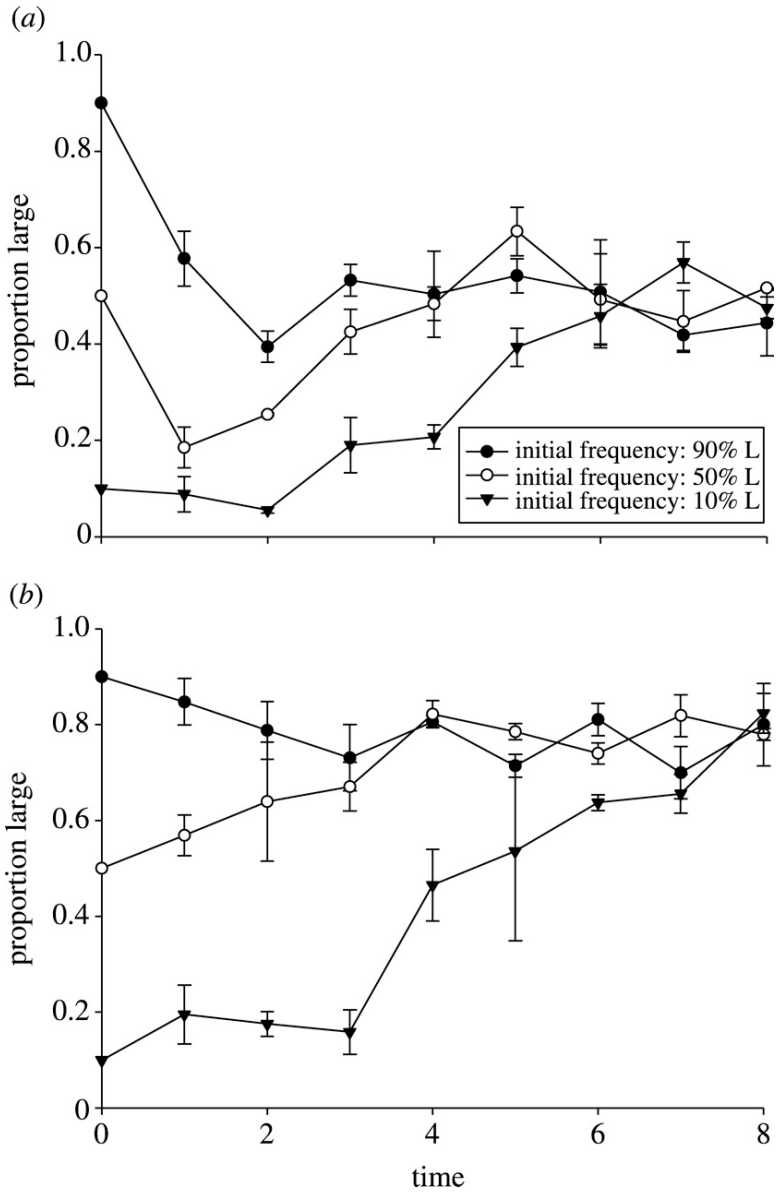

Figure 2.1: The means of proportion Large (±s.e.m.) for three starting mixtures of (a) L_{mix-31} and S_{mix-31} versus time (days) in DM_{mix} and (b) L_{mix-33} and S_{mix-33} versus time (days) in DM_{mix}. In both cases, the proportions stabilized at an intermediate frequency by day eight in all treatments.

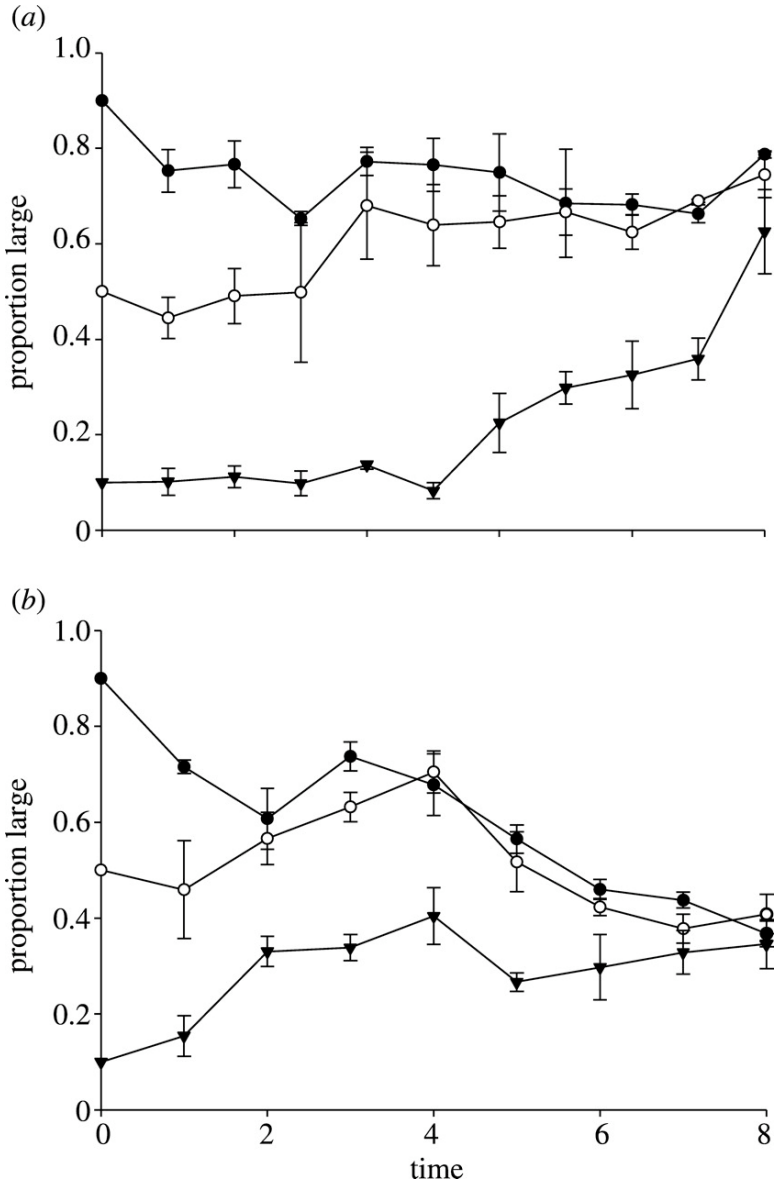

Figure 2.2: The means of proportion Large (±s.e.m.) for three starting mixtures of (a) L_{mix-31} and S_{mix-33} versus time (days) in DM_{mix} and (b) L_{mix-33} and S_{mix-31} versus time (days) in DM_{mix}. The proportions stabilized at an intermediate frequency within eightten days of competition. Symbols are the same as for Figure 2.1.

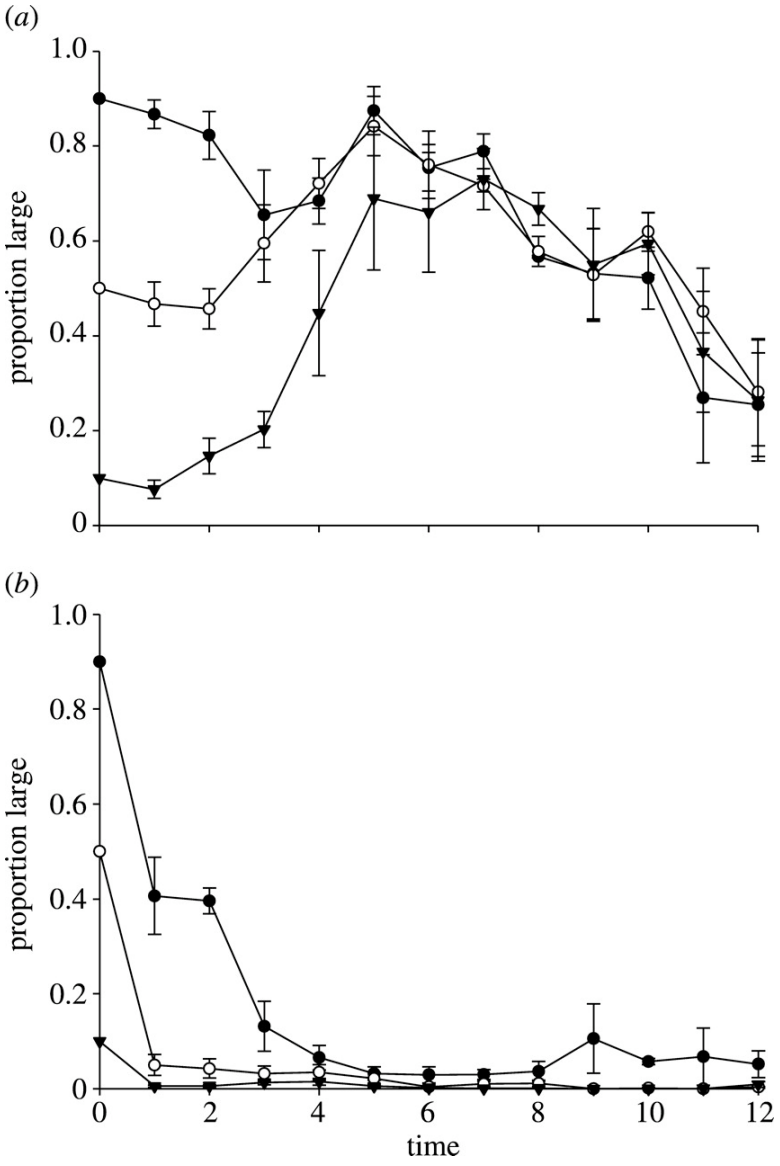

Figure 2.3: The means of proportion Large (±s.e.m.) for three starting mixtures of (a) L_{mix-33} and S_{glu-10} versus time (days) and (b) L_{glu-10} and S_{mix-33} versus time (days) in DM_{mix}. Symbols are the same as for Figure 2.1.

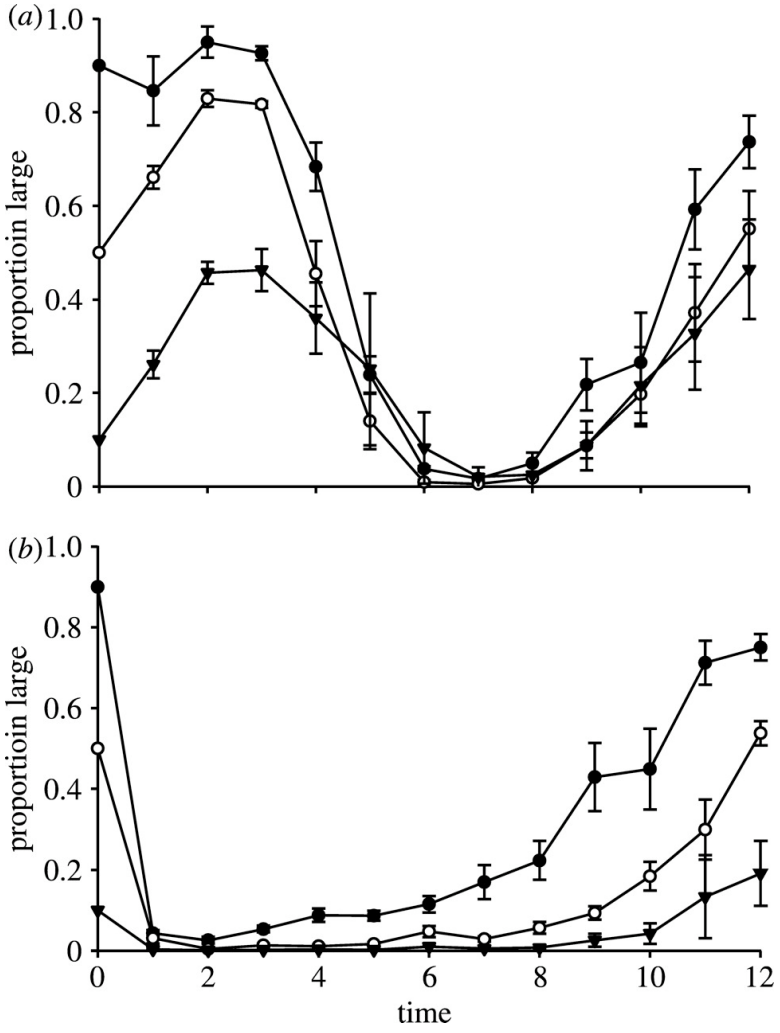

Figure 2.4: The means of proportion Large (±s.e.m.) for three starting mixtures of (a) L_{mix-33} and S_{glu-10} versus time (days) and (b) L_{glu-10} and S_{mix-33} versus time (days) in DM_{glu}. The proportions did not stabilize within the duration of the experiment. Symbols are the same as for Figure 2.1.

Bibliography

Dieckmann, U. and Doebeli, M. (1999). On the origin of species by sympatric speciation. *Nature*, 400:354–357.

Dieckmann, U., Metz, J., Doebeli, M., and Tautz, D. (2004). *Introduction*. Adaptive speciation. Cambridge University Press, Cambridge.

Friesen, M., Saxer, G., Travisano, M., and Doebeli, M. (2004). Experimental evidence for sympatric ecological diversification due to frequency dependent competition in *Escherichia coli*. *Evolution*, 58:245–60.

Helling, R., Vargas, C., and Adams, J. (1987). Evolution of Escherichia coli during growth in a constant environment. *Genetics*, 116:349–358.

Lenski, R., Rose, M., Simpson, S., and Tadler, S. (1991). Long-term experimental evolution in *Escherichia coli*. I. Adaptation and divergence during 2,000 generations. *Am Nat*, 138:1315–1341.

Levin, B. (1988). Frequency-dependent selection in bacterial populations. *Phil.Trans.R.Soc.Lond.B*, 319:459–472.

Rainey, P., Buckling, A., Kassen, R., and Travisano, M. (2000). The emergence and maintenance of diversity: insights from experimental bacterial populations. *TREE*, 15:243–247.

Rainey, P. and Travisano, M. (1998). Adaptive radiation in a heterogeneous environment. *Nature*, 394:69–72.

Rainey, P. B. and Rainey, K. (2003). Evolution of cooperation and conflict in experimental bacterial populations. *Nature*, 425:72–74.

Rosenzweig, R., Sharp, R., Treves, D., and Adams, J. (1994). Microbial evolution in a simple unstructured environment: Genetic differentiation in *Escherichia coli*. *Genetics*, 137:903–917.

Rozen, D. and Lenski, R. (2000). Long-term experimental evolution in Escherichia coli viii: Dynamics of a balanced polymorphism. *Am Nat*, 155:24–35.

Schluter, D. (2000). *The ecology of adaptive radiation*. Oxford University Press, Oxford.

Travisano, M. and Lenski, R. (1996). Long-term experimental evolution in Escherichia coli. iv. targets of selection and specificity of adaptation. *Genetics*, 143:15–26.

Travisano, M., Mongold, J., Bennett, A., and Lenski, R. (1995). Experimental tests of the roles of adaptation, chance, and history in evolution. *Science*, 267:87–90.

Treves, D., Manning, S., and Adams, J. (1998). Repeated evolution of an acetate-crossfeeding polymorphism in long-term populations of *Escherichia coli*. *Mol Biol Evol*, 15:787–797.

Turner, P., Souza, V., and Lenski, R. (1996). Tests of ecological mechanisms promoting the stable coexistence of two bacterial genotypes. *Ecology*, 77:2119–2129.

Via, S. (2001). Sympatric speciation in animals: the ugly duckling grows up. *TREE*, 16:381–390.

Chapter 3

Experimental demonstration of ecological character displacement[1]

3.1 Introduction

When populations of different species occur in sympatry (together), they often have trait values that are more extreme than the values occurring in allopatric (isolated) populations (Brown and Wilson, 1956). For traits associated with resource acquisition or metabolism, this phenomenon is called ecological character displacement, to distinguish it from reproductive character displacement, which describes shifts in traits associated with reproduction. Ecological character displacement is observed in Galapagos finches (Schluter, 2000b, Grant and Grant, 2006, Schluter et al., 1985), plethodontid salamanders (Adams and Rohlf, 2000), sticklebacks (Schluter, 2000b), Anolis lizards (Losos, 1994), and spadefoot toads (Pfennig and Murphy, 2003, Pfennig et al., 2007), and is generally believed to be caused by resource competition. Theory (Doebeli, 1996, Schluter, 2000a, Slatkin, 1980, Taper and Chase, 1985) predicts that character displacement will result from competition

[1] A version of this chapter has been published. Tyerman, J.G., M. Bertrand, C.C. Spencer and M. Doebeli (2008) Experimental demonstration of ecological character displacement *BMC Evol Biol* **8** 34p.

selecting and maintaining extreme phenotypes to minimize phenotypic overlap and thus minimize interspecific competition.

Experiments also support the hypothesis that competition can select for divergence in resource-related traits. Schluter (1994) measured selection in sticklebacks and demonstrated that growth rates and survival were depressed in the presence of competitors, and that selection was frequency dependent (Schluter, 2003); and Bolnick (2004) showed that competition could generate disruptive selection regimes in natural populations of sticklebacks. However, selection is not evolution, and few studies have shown that interspecific competition for resources leads to evolutionary shifts in phenotypic distributions of resource-related traits (Taper, 1990). Taper (1990) demonstrated character shifts using bean weevils, however he failed to detect trade-offs associated with the observed shifts, thus the divergence may have evolved for reasons other than interspecific resource competition. Microbes have been employed to great advantage in studying the generation and sorting of adaptive variation (Rainey and Travisano, 1998, MacLean et al., 2005, Hall and Colegrave, 2007, Barrett et al., 2005, Meyer and Kassen, 2007). Using microbes to test evolutionary hypotheses is possible because microbes evolve quickly in response to environmental conditions set and controlled by the researcher. Additionally, replicate populations can be studied in order to determine the repeatability of evolutionary response, and microbes can be stored indefinitely at -80° so that assays between ancestors and descendants can be conducted (reviewed in Elena and Lenski, 2003). MacLean et al. (2005) used biolog plates to characterize diversification of *Pseudomonas* bacteria in response to resource competition. This study also demonstrated how diverse genotypes were maintained by frequency dependent interactions likely resulting from competition for resources. Similarly, Barrett et al. (2005) showed that diversification of *Pseudomonas* generated imperfect generalists in response to competition for substitutable resources. These studies nicely illustrate how metabolic diversification occurs in the face of resource competition. While they show divergence in phenotype space, the phenotypes measured are not functionally linked to competitive performance in the environment experienced during evolution. Therefore, the importance of the phenotype for competition remains unclear. For example, it is not clear whether in experimental populations seeded with only two phenotypes, competition would lead to divergence, i.e. to

an increase in the phenotypic distance between these two strains. Similarly, it is unclear what the effects of removing competition from other strains would be on a single focal phenotype.

Using diversified *Escherichia coli* B populations, we show in this paper that competition for resources can lead to phenotypic divergence of competing strains, i.e., to ecological character displacement, and that absence of competition can lead to phenotypic convergence. We evolved *E. coli* for 1,000 generations in liquid batch cultures with glucose and acetate as sole carbon resources. Ten replicate populations diversified into cultures consisting of two ecotypes that specialized on glucose or acetate (Figures 3.1b and 3.2). When *E. coli* grows in batch culture, glucose is consumed first, followed by acetate (Mahadevan et al., 2002). This generates a two-phase (i.e., diauxic) growth profile within a single 24 h batch cycle (Figure 3.2).

Diauxic growth profiles reveal how bacteria consume one resource (e.g., glucose) and switch to a second resource (e.g., acetate) only when the first is exhausted. Resource exploitation can thus be described as a metabolic reaction norm (Friesen et al., 2004), and different metabolic reaction norms correspond to different 24 h growth profiles.

Our evolved cultures had diversified into two ecotypes, identifiable by different 24 h growth profiles (Figure 3.2a). These growth profiles were assayed in the absence of competitors of the opposite ecotype and hence are not a plastic response to the presence of a competitor. Instead, they reflect genetically distinct metabolic reaction norms, because offspring clones generate similar 24 h growth profiles as parental clones from which they descend (Friesen et al., 2004). We named the two distinct ecotypes Slow-Switchers (SS) and Fast-Switchers (FS) after differences in their relative switching lags (lag_{ace}) between diauxic growth phases (Figure 3.2b). We extracted lag_{ace} and nine additional quantifiable traits from diauxic growth curve profiles. These phenotypic traits carry the signatures of different strategies for metabolizing resources and have been shaped and maintained by competition for resources (Friesen et al., 2004, , Figure 3.3).

3.2 Methods

3.2.1 Description of evolved strains

Ten replicate populations of *E. coli* B were alternately initiated from two isogenic lines (Lenski et al., 1991), which differed with respect to a neutral marker. The isogenic lines differed in their ability to utilize arabinose (ara+/-), which we exploited to discriminate between lineages in mixed cultures (see "Fitness assays" and "Competition induced" sections below). We followed the protocols of Lenski et al. (1991) with minor variations. We used large, loosely-covered test tubes, filled with 10 mL of Davis Minimal Salts media (DM) supplemented with 250 μg/mL glucose and 575 μg/mL acetate as the sole carbon sources. These resources were selected because diversification in their presence has been shown previously (Friesen et al., 2004, Spencer et al., 2007a, Tyerman et al., 2005). Cultures were incubated at 37° and vigorously shaken (250 r.p.m.) for 24 h. Each day (i.e., after 24 1 h of growth), 100 μL of culture was transferred to 10 mL of fresh media (1/100 dilution) and thus the seasonal cycle was reset. Each batch cycle yielded on average $\log_2 100 = 6.6$ generations.

To test whether adaptation had occurred, we competed three populations (dst1018, dst1019, dst1020) against the ancestor of opposite marker type, and calculated relative fitness as done previously (Lenski et al., 1991, , see below). These three populations were selected from the initial ten populations because there was a high correlation between colony morphology variation (large vs. small) and ecotype (SS vs. FS), which we exploited for purposes of identification in mixed culture assays. Fitness increased by 14% (Figure 3.1a) in all three populations. This suggests that adaptive evolution occurred over the course of 1,000 generations.

By generation 1,000 two discernible *E. coli* ecotypes, Fast-switching (FS) and Slow-switching (SS), were identified in all ten replicate populations (Figure 3.1b), and there was extensive variation in frequency of the two ecotypes. We view the parallel emergence of diversity in each population as an indication that the divergence was adaptive (Schluter, 2000a).

To show that there was a functional (i.e., adaptive) explanation for the divergence in our *E. coli* populations, we assessed whether trade-offs in resource usage

were detectable between SS and FS strains. From previous work (Friesen et al., 2004, Spencer et al., 2007a, Tyerman et al., 2005) and this study, it appears that SS was functionally similar to the ancestor, while FS had diverged to exploit acetate earlier in the 24 h growth cycle (indicated by reduced lag_{ace}, Figure 3.2b).

Presumably this enhanced performance on acetate is associated with reduced performance on glucose. Such a trade-off has previously been found in diversified strains that have evolved under similar conditions (Friesen et al., 2004, Spencer et al., 2007b). To test for trade-offs in resource use, we competed SS and FS in environments that were skewed to having either more glucose or more acetate (see "Fitness Assays" below). A trade-off would imply that in a glucose-enhanced/acetate-reduced environment, SS – having a metabolic profile geared towards efficient glucose use – would have higher fitness, while in an acetate-enhanced/glucose-reduced environment, FS – having a metabolic profile geared towards enhanced acetate use – would have higher fitness. Indeed, we found support for the hypothesis that trade-offs in resource use underlie the maintenance of diversity in metabolic profiles ($t = 4.305$, $p < 0.0005$, Figure 3.3). This trade-off in resource use strongly supports the hypothesis that resource competition was the selective cause for the divergence into SS and FS ecotypes.

3.2.2 Asexual nature of our lines

E. coli exchange DNA via conjugation, passing plasmids between donor and recipient cells. However, *E. coli* B has no plasmids and can thus be considered asexual (Lenski et al., 1991). We ensured that the ancestral lines (rel606 and rel607) and evolved lines used in this study had no plasmids with a standard mini preparation of genomic DNA isolated from cells grown from each culture (Sigma GenElute Plasmid Miniprep Kit). No plasmids were detected in the ancestors or evolved cells.

3.2.3 Fitness assays

Fitness of each evolved line was determined relative to the ancestor using competition experiments as described in Lenski et al. (1991). Briefly, evolved cultures (mixed sample of SS and FS) from the endpoint of our evolution experiment (gen-

eration 1,000) and cultures from the ancestors (both marker types) were inoculated from frozen stock into evolutionary media, and grown for 24 h. Evolved culture and ancestor (of opposite marker type) were mixed in equal proportions (by volume) and inoculated into fresh medium (1/100 dilution) in ten replicates; plated on Tetrazolium agar with arabinose to determine densities at inoculation (T0), and then grown and transferred for two days before being plated to yield T2 densities. Relative fitness was calculated as ln(EVT2/EVT0)/ln(ANCT2/ANCT0) (modified from Lenski et al. (1991)), where EV is the density of evolved culture and ANC is the density of the ancestor (at times T0 and T2). To determine fitness of SS and FS in skewed resource environments (i.e., 90% [glucose]-10% [acetate] or 10% [glucose]-90% [acetate]), we isolated 10 SS and 10 FS genotypes from diversfied strain dst1018, inoculated them individually into fresh medium (50% [glucose] - 50% [acetate]) for 24 h, and then arbitrarily selected pairs of SS and FS to mix in equal proportions (by volume). We inoculated ten pairs into both extreme environments. We plated T0 and T2 on Tetrazolium agar plates (without arabinose) and used colony morphology (large or small colonies, Friesen et al., 2004) to aid us in determining the densities of both SS and FS ecotypes at each time point. We calculated relative fitness as above, substituting SS and FS for EV and ANC.

3.2.4 Growth parameter extraction

Growth curves were obtained by inoculating 1.5 μL of conditioned culture into 150 μL of fresh evolutionary medium (see above) in individual wells of a 96-well microplate. Microplate cultures were grown in a Biotek 808UI Optical Density reader, under similar conditions to the original evolutionary environment (37°, well shaken). Measurements consisted of optical densities (OD, 600 nm) obtained every 10 min over the course of 24 h. Data files were converted to a usable format using Microsoft Excel, and growth curve parameters were extracted with a program written in object oriented C++.

Table 3.1 summarizes the parameters extracted from growth curves. These were modified from (Friesen et al., 2004). The parameter StartTime was extracted but not used directly in the analysis – it was used indirectly in the calculation of other variables (see Table 3.1) – and was the time where the OD (600 nm) of the

growing culture first reached 0.08. Slopes were extracted using a moving window algorithm (i.e., linear regression through nine successive time points), and were used for the calculation of $r_{max-glu}$, switching OD, and $r_{max-ace}$. OD_{max} and OD_{final} were the maximum and final optical densities during the 24 h growth period. Means for each ecotype in Table 3.1 were calculated from twenty SS and twenty FS clones isolated from population dst1018.

3.2.5 Character displacement experiments

Competitive release

We selected three of ten diversified populations for this experiment (dst1018, dst1019, dst1020). From the 1,000-generation mark (maintained at -80°) we conditioned these populations in fresh evolutionary media for 24 h, and plated on Tetrazolium agar to isolate genotypes. We selected 20 SS and FS genotypes (initially by colony morphology and confirmed by growth profile) from each population, and used these genotypes to initiate allopatric cultures (i.e., no interspecific competition). 1.5 μL of each culture was inoculated into a single well containing 150 μL evolutionary media of a 96-well microtitre plate (1/100 dilution). Growth conditions and protocols mirrored the evolutionary conditions, with the exception of differences in volume between test tubes (10 mL in the original evolution experiment) and microplate wells (150 μL in this experiment). Separate microtitre plates were used for SS and FS cultures to prevent the possibility of cross-contamination between ecotypes. Although growth curves were measured on ecotypes grown in isolation (i.e., no interspecific competition), we assumed that initial growth parameter values (T1) had no mutations, and thus reflected the evolutionary signal of each ecotype under sympatry. This assumption is conservative, because we are actually measuring the parameters in isolation for the sympatric values to compare to later measures in allopatry. All cultures were propagated in isolation (allopatry) for 200 generations by transferring 1/100 of the culture to fresh media in a new microplate every 24 h for 30 days. After 30 days of evolution, the values obtained from growth curves were assumed to reflect the mean evolutionary response for each replicate to the treatment of allopatry. A detailed analysis of individual genotypes is described

in Chapter 4. Growth parameters from all derived cultures were log transformed.

Competition induced

From T30 cultures generated in the first phase of this study (see above) we noted that all cultures were genetically heterogeneous (as determined by variation in growth curve profiles from isolated clones). Generally, there were between two to three genotypes in FS_{ALLO} (i.e., cultures derived from FS) and between two to five genotypes in SS_{ALLO} (i.e., cultures derived from SS). In many SS_{ALLO} cultures, we noted one particular recurring genotype that had a decreased lag_{ace}, here labeled SS' (Figure 3.6a).

Similarly, in FS_{ALLO} cultures, we noted one particular genotype with reduced maximum yields (OD_{MAX}) in each phase of diauxic growth (relative to the ancestral FS genotype), here labeled FS' (Figure 3.6b).

We considered these novel genotypes as intermediate between SS and FS ecotypes, and relatively convergent towards the opposite ecotype, when compared with the ecotype from which they were descended (SS' derived from SS and FS' derived from FS). A single SS-derived genotype (SS') was selected from one of the twenty dst1018 replicates, and a single FS-derived genotype (FS') was isolated from one of the dst1019 replicates. We used single genotypes for each novel ecotype because we wanted to focus on the role of competition (as opposed to extant genetic makeup of initially variable populations) in ecological character displacement. Additionally, a fully replicated design with all possible complimentary pairs of isolated novel genotypes in competition would be impractical.

We initiated ten mixed cultures of SS' vs. FS' (1:1, by volume) and inoculated these treatments into microplate wells (as above). We also inoculated pure SS' or FS' culture to determine ΔZ_{ALLO} for each ecotype. We propagated the mixed cultures for 30 days (200 generations) to determine if competition would cause the SS' and FS' to diverge in resource-related phenotype space. Because the frequency of FS'-derived clones $<0.1\%$ by T30, we assayed our populations at T15. We plated all replicate populations onto Tetrazolium agar (with arabinose) and identified descendent clones by their ara +/- status. Fourteen clones for each of SS'-derived and FS'-derived subpopulations from each replicate mixture were iso-

lated and conditioned for 24 h before being assayed for growth curve parameters. We then calculated the mean parameter value from descendants from each ecotype from each competition replicate for statistical analysis (see below).

3.2.6 Statistical analysis

In both phases of the experiment, we tested the hypothesis that competition caused character displacement such that ΔZ_{SYM} - $\Delta Z_{ALLO} > 0$.

Competitive release

We calculated the evolutionary response to competitive release (i.e., sympatry to allopatry) for each of ten traits by taking the difference in log-transformed trait values between T1 and T30. We pooled the evolutionary responses for all 120 replicates (3 source populations x 2 ecotypes x 20 replicates/population/ecotype). We conducted a PCA using the correlation matrix of the pooled response data (Dillon and Goldstein, 1984). We used only the first four principle components as they had eigenvalues > 1 (Dillon and Goldstein, 1984), and accounted for $> 81\%$ of the variation in response to allopatry (Figure 3.4 and Table 3.2). We used the loadings from these four components and the difference data to generate independent (orthogonal) composite trait values. Thus, our ecotypes are described as points in four-dimensional phenotype space. From T1, we calculated:

$$\Delta Z_{SYM} = Z_{FS-SYM} - Z_{SS-SYM} \tag{3.1}$$

and from T30, we calculated:

$$\Delta Z_{ALLO} = Z_{FS-ALLO} - Z_{SS-ALLO} \tag{3.2}$$

where Z is a vector in four dimensional trait space reflecting mean population values for FS or SS ecotypes in sympatry or allopatry. We analyzed the three source populations separately. We determined ΔZ_{SYM} and ΔZ_{ALLO} (and 95% C.I.) by randomly sampling 20 distances 1,000 times from the fully permuted distance data set. We used a randomization test procedure to determine the probability of obtaining a test statistic (ΔZ_{SYM} - ΔZ_{ALLO}) that was $\geq$ observed data (Manly, 1997).

P values in the main text indicate the proportion of 100,000 analogous datasets created having (ΔZ_{SYM} - $\Delta Z_{ALLO} >$ observed data), after randomly reclassifying all distances into ΔZ_{SYM} or ΔZ_{ALLO} datasets.

Competition induced

Our competition replicates comprised pairs (n = 10) of SS' and FS' derived genotypes. Thus, we used a paired t-test to determine whether Ha: ΔZ_{SYM} - $\Delta Z_{ALLO} > 0$. This allowed us to quantify evolutionary response (i.e., divergence) in each replicate (i.e., ΔZ_{SYM}) separately, so that divergence across replicates could arise even if ecotypes made different contributions to divergence in different replicates.

3.3 Results & discussion

We envisage ecotypes occupying different regions of multidimensional phenotype space, characterized by particular values of resource-related traits, Z. We can measure the distance between ecotypes, ΔZ, under transitions from sympatry to allopatry (or vice versa), and ask whether that distance changes due to character displacement as theory predicts (Doebeli, 1996, Slatkin, 1980, Taper and Chase, 1985, Abrams, 1986). Under competitive release, i.e., moving from sympatry to allopatry, phenotypic distributions should evolve towards intermediate values and thus appear closer in phenotype space, so that the distance measured in sympatry, ΔZ_{SYM} is larger than the distance in allopatry, ΔZ_{ALLO} (i.e., ΔZ_{SYM} - $\Delta Z_{ALLO} >$ 0). We tested this prediction by evolving FS and SS ecotypes (from three populations) in isolation (i.e., under competitive release) for 200 generations. Growth curve parameters were extracted at T1 (generation 0), corresponding to sympatry, and at T30 (generation 200), corresponding to allopatry. We measured evolutionary response as the difference in trait value (T1-T30) for each ecotype from each population. We reduced the number of traits by conducting a PCA (Figure 3.4) and characterized SS and FS ecotypes in composite phenotype space (Figure 3.2a).

We calculated the distances ΔZ_{ALLO} and ΔZ_{SYM}, and tested whether ΔZ_{SYM} - $\Delta Z_{ALLO} > 0$. Under competitive release, we found strong support for phenotypic convergence (Figure 3.5b) between ecotypes from all three populations (randomization test, dst1018: P = $1.0\text{x}10^{-6}$, dst1019: P = $1.0\text{x}10^{-6}$ and dst1020: P =

$1.0\text{x}10^{-6}$). Convergence occurred primarily along the first principal component axis, with parallel shifts occurring on the remaining axes. Patterns of evolutionary response differed among populations. For example, convergence in two populations (dst1019 and dst1020) consisted of both ecotypes moving towards one another in phenotype space, but in population dst1018, convergence was due to a shift of both ecotypes in the same direction, but with SS changing to a larger extent (Figure 3.2a). We suspect that initial differences in position in phenotype space (dst1018 vs. dst1019 and dst1020) accounted for differences in evolutionary response of these ecotypes when released from competition.

Next, we investigated whether adding competition would induce phenotypic divergence. For this we selected intermediate, convergent genotypes (SS' and FS'), which we isolated from T30 cultures (Figure 3.6). We competed SS' vs. FS' for 200 generations, after which the frequency of FS'-derived genotypes was ¡0.1% in 4 of the 10 competition replicates, suggesting that SS'-derived ecotypes were often able to competitively exclude FS'-derived ecotypes. Thus, we isolated genotypes derived from SS' (SS_{SYM}) or FS' (FS_{SYM}) from an earlier time point (generation 100, when FS was still present in an appreciable frequency in all cultures), and calculated the mean growth-curve parameters for SS_{SYM} and FS_{SYM}. We projected these parameters using the same composite trait space characterized during competitive release (Figure 3.7a). Thus, we explicitly tested whether competition could induce evolutionary divergence by directly reversing the changes that occurred during competitive release. Indeed we found that competition induced divergence ($t = 2.73$, $df = 9$, $p < 0.02$, Figure 3.7b).

Interestingly, divergence did not exactly retrace the evolutionary trajectory of convergence (Figure 3.7a vs. Figure 3.5a). Both convergence under competitive release, and subsequent divergence due to competition, occurred along the first composite trait axis. However, under competitive release, both ecotypes contributed to convergence, whereas only the SS' phenotype contributed to divergence. Moreover, the magnitude of the evolutionary response during the divergent phase was smaller than during the convergent phase (Figure 3.7b vs. Figure 3.5b). This difference in magnitude may be because we assayed character displacement after 200 generations in the first phase and only 100 generations in the second phase, allowing less time for evolution. However, the difference in magnitude of evolutionary

response may also have ecological reasons. Schluter (2000b) argued that the speed of divergence during character displacement is greatest when phenotypic distance (i.e., degree of similarity) between competing species is intermediate. In particular, while very similar species experience intense competition, the speed of divergence is not expected to be high, because an increase in phenotypic distance may not substantially decrease competitive intensity. Instead, divergence becomes faster only after it has progressed considerably (See Figure 6.1 in Schluter, 2000b). Since the phenotypes we competed were rather similar (Figure 3.6), this effect may have delayed the response in our divergence treatments.

Finally, the evolutionary response under competition may be different because divergence may have occurred in phenotypic dimensions not captured by the composite trait space defined by the PCA analysis of the competitive release experiment. We conducted an independent PCA analysis on the data from only the divergence phase of our experiment, which yielded a different composite trait space. In this new trait space divergence was also significant, but the response was of similar magnitude to the evolutionary response initially identified (data not shown).

3.4 Conclusions

Ecologists (Schluter, 2000b, Doebeli, 1996, Slatkin, 1980, Taper and Case, 1992) continue to emphasize a causal role for competition in ecological character displacement. However, other factors, such as predation (Meyer and Kassen, 2007, Rundle et al., 2003, Nosil and Crespi, 2006) can also affect adaptive processes of diversification. Grant and Grant (2006) have therefore recently called for a definitive demonstration of competition's causal role in ecological character displacement. Here, we answer this call using experimental tests in bacterial populations. Our evidence for character convergence after competitive release is particularly compelling, and our work supports the trust that ecological theory (Schluter, 2000b, Slatkin, 1980, Dieckmann and Doebeli, 1999, Nosil and Crespi, 2006) has placed on competition for resources as an important driver of character divergence.

Our study demonstrates that interspecific competition for resources can cause resource-related phenotypes to shift as expected in response to competition. The initial adaptive diversification generating SS and FS ecotypes, followed by our

manipulations of interspecific competition (by removing and subsequently adding competitors) reveals competition's role in driving accordion-like shifts on distributions of resource-related phenotypes: divergence followed by convergence followed by divergence. Coexistence in the face of interspecific competition for shared resources may demand such an evolutionary response, with the exclusion of the inferior ecotype as an alternative outcome (Hardin, 1960).

3.5 Acknowledgements

Financial support provided by NSERC to J.G.T. and M.D., and by the James S. McDonnell Foundation to M.D. Lab assistance was provided by M. Ciou, M. Chan, G. Khaira, R. McBride, and R. Suprin. R.E. Lenski kindly provided our lab with *E. coli* B strains (rel606 and rel607) that served as the ancestors in our evolution experiment. M. Friesen helped develop protocols to extract parameters from growth curves. Discussions with D. Ally, A. Blachford, R. Blok, J. Fletcher, L. Harmon, M. Le Gac and D. Schluter aided us in preparing the study and manuscript.

Table 3.1: Description of parameters extracted from growth curves and summary data for Slow-switcher and Fast-switcher ecotypes (isolated from strain dst1018).

Parameter	Explanation	Slow-switcher mean (±95%C.I.)	Fast-switcher mean (±95%C.I.)
StartTime	Time where optical density (OD) can be easily detected (OD = 0.08 at 600 nm) not directly included in the analysis, but included in calculation of $r_{max-glu}$TP, time$Tor_{max-glu}$, SP, $r_{max-ace}$TP, and OD_{max}TP	–	–
$r_{max-glu}$	Maximum growth rate during "glucose phase" of diauxie.	0.081 (0.076-0.086)	0.083 (0.077-0.089)
$r_{max-glu}$TP	$r_{max-glu}$ time point - StartTime.	23.5 (22.9-24.2)	27.6 (26.7-28.5)
SP	Switching (Time) Point from glucose to acetate phase - StartTime.	32.2 (31.8-32.7)	31.9 (31.4-32.4)
OD_{SP}	OD of switching point.	0.24 (0.23-0.25)	0.25 (0.24-0.26)
Lag_{ace}	Switching lag (time) from glucose to acetate growth	82.7 (76.3-89.0)	17.2 (10.4-24.0)
$r_{max-ace}$	Maximum growth rate during "acetate phase" of diauxie.	0.0020 (0.0015-0.0024)	0.028 (0.023-0.034)
$r_{max-ace}$TP	$r_{max-ace}$ time point StartTime	114.9 (108.7-121.2)	49.0 (42.2-55.9)
OD_{max}	Maximum OD.	0.24 (0.23-0.25)	0.36 (0.34-0.38)
OD_{max}TP	OD_{max} timepoint - StartTime	49.2 (35.2-63.2)	80.5 (72.6-88.4)
OD_{final}	Yield or OD at the end of the 24 h.	0.19 (0.19-0.20)	0.32 (0.31-0.33)

Table 3.2: Summary of PCA conducted on correlation matrix of the difference data during competitive release. See Table 3.1 for explanation of parameters.

	PC1	PC2	PC3	PC4
Variation explained	31.3%	21.6%	16.2%	12.0%
Eigenvalue	1.77	1.47	1.27	1.09
Parameter	PC Loadings			
$r_{max-glu}$	0.393	-0.411	-	-
$r_{max-glu}$TP	-0.262	0.261	0.307	0.468
SP	-	0.285	0.195	0.634
OD_{SP}	0.197	-0.373	-0.382	0.448
lag_{ace}	0.447	-	0.380	-
$r_{max-ace}$	-0.420	-0.140	0.224	-0.107
$r_{max-ace}$TP	0.384	0.153	0.398	0.248
OD_{max}	-0.114	0.609	-	0.149
OD_{max}TP	0.298	-	0.508	-
OD_{final}	-0.330	-0.357	-0.329	0.269

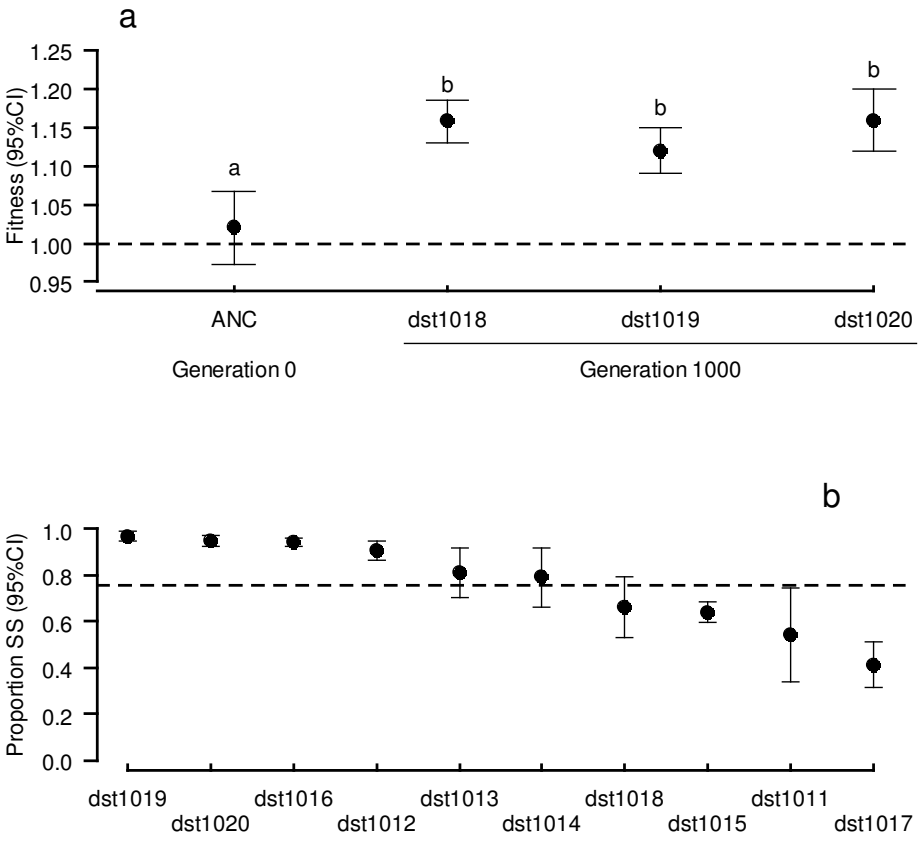

Figure 3.1: (a) Relative fitness of the ancestor (generation 0) and three populations (at generation 1,000) versus the ancestor of opposite marker type (ara+/-). The dashed horizontal line is equivalent fitness, error bars indicate 95% confidence intervals, and letters above error bars denote significantly different groups. (b) The proportion of SS (95% CI) in ten replicate populations evolved in glucose-acetate environment (populations in rank order). The dashed horizontal line represents the grand mean for all populations.

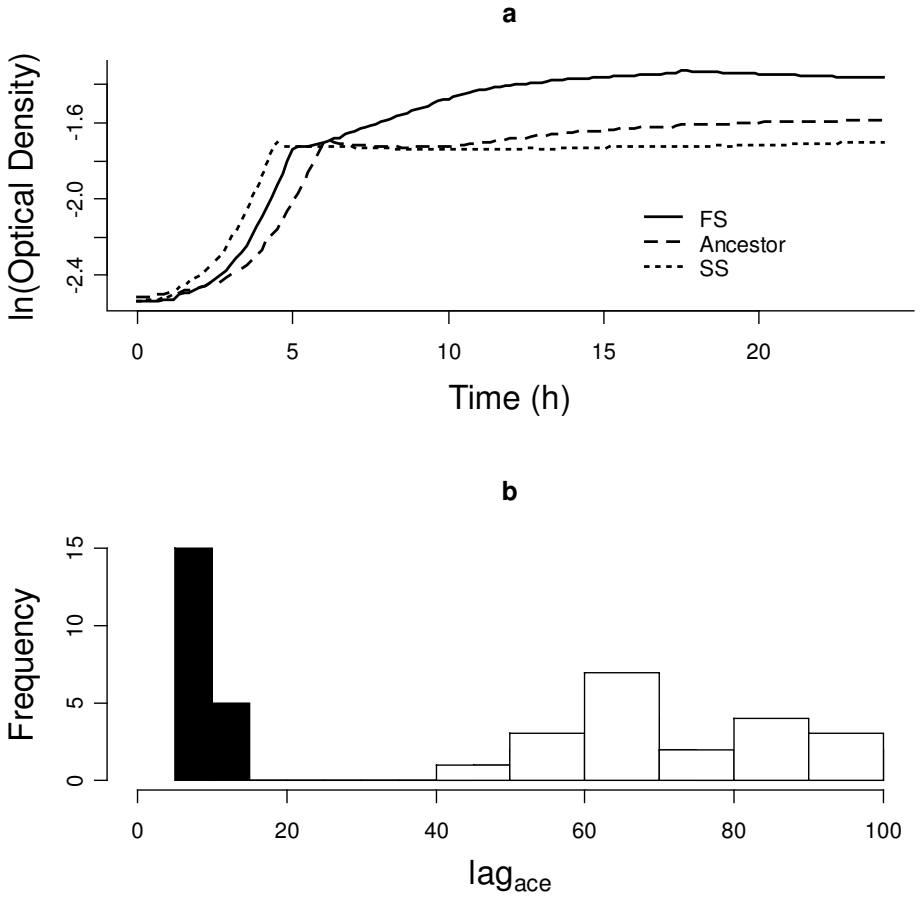

Figure 3.2: 24 h growth curves reveal resource usage differences between ecotypes. (a) Examples of 24 h growth curves for the ancestor and derived ecotypes (Slow-switchers and Fast-switchers) from strain dst1018 after 1,000 generations of evolution. (b) Histogram of lag_{ace} reveals two phenotypic clusters (Fast-switchers = black and Slow-switchers = white).

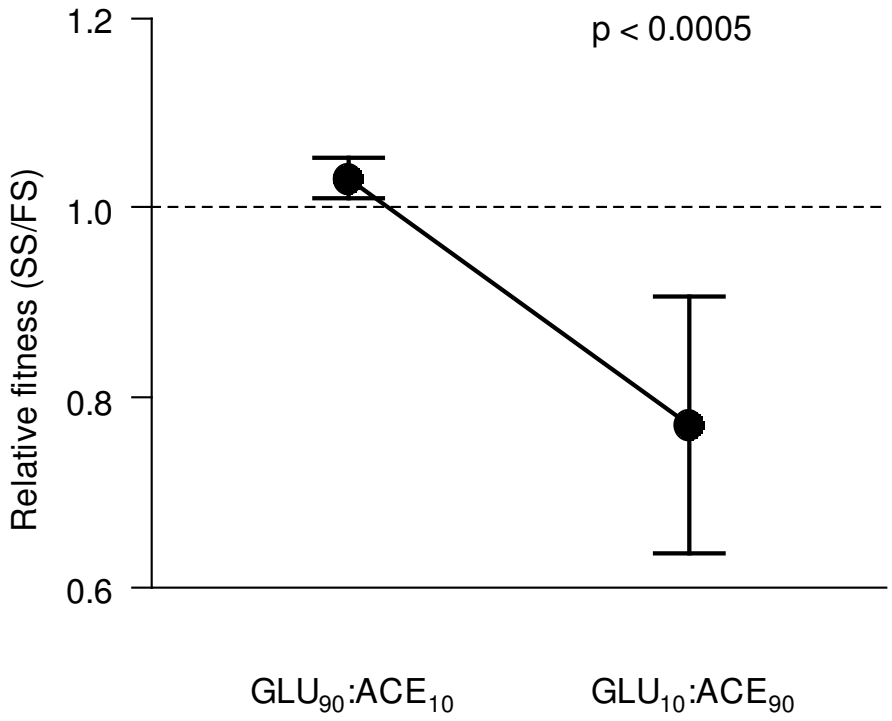

Figure 3.3: Competition experiments in skewed resource environments reveal that mean SS fitness is greater than mean FS fitness when [glucose] is enhanced (from 50% to 90%) and [acetate] reduced (from 50% to 10%) (left) and that mean SS fitness is lower than mean FS fitness when [glucose] is reduced and [acetate] enhanced (right). The horizontal line indicates equal fitness, and the error bars indicate 95% CI.

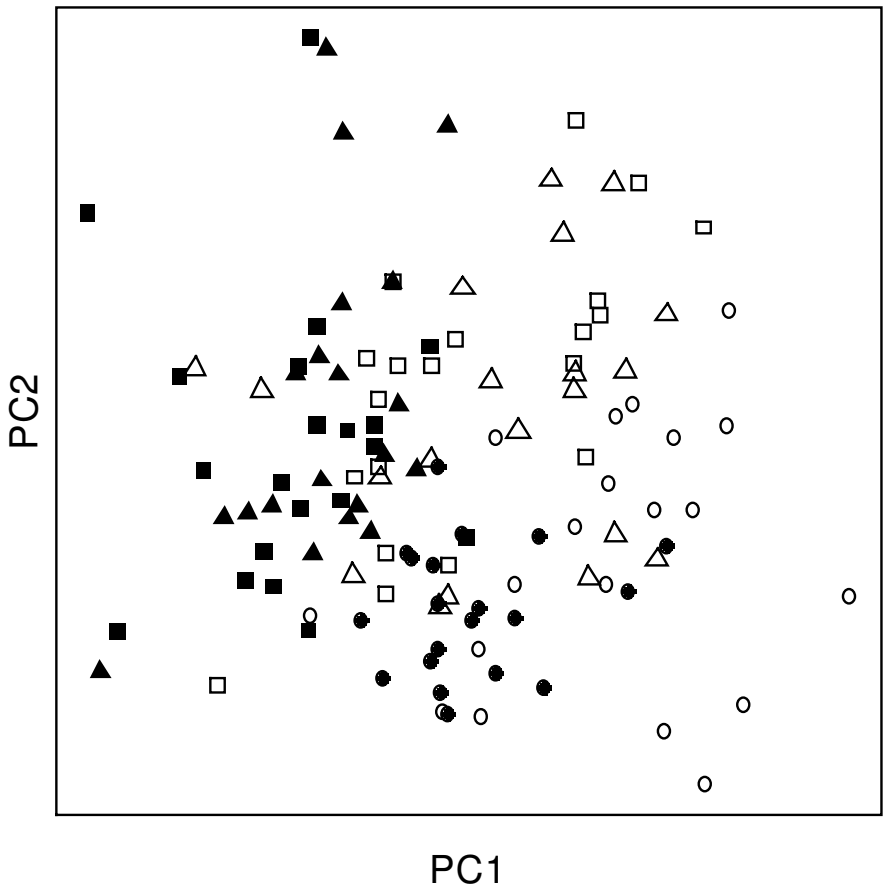

Figure 3.4: Principle component analysis (PC1 vs. PC2) on differences between sympatric and allopatric trait values for Slow-switchers (white) and Fast-switchers (black) from replicates initiated from three populations (dst1018 = circles, dst1019 = triangles, dst1020 = squares).

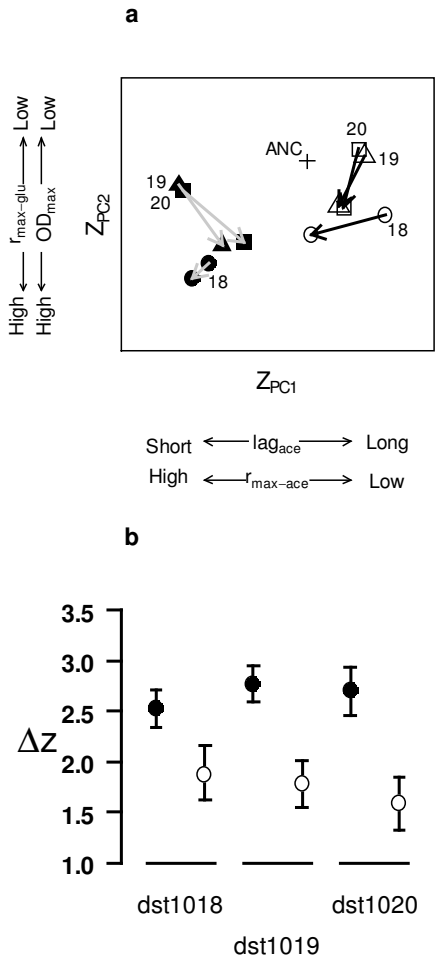

Figure 3.5: Character displacement under competitive release. (a) Symbols reflect mean ecotype evolutionary response from replicates (n = 20) evolved from each of three source populations (dst1018 = circles; dst1019 = triangles; dst1020 = squares), and arrows show evolutionary trajectories from sympatry to allopatry. Black symbols are FS ecotypes, white symbols are SS ecotypes. The ancestor (+) to the original evolution experiment is illustrated for comparison. Phenotypes are projected into two dimensions using the loadings from PC1 and PC2. (b) Mean distance in trait space, ΔZ, between ecotypes in sympatry (black) and allopatry (white) during competitive release, for replicates (n = 20) from three populations. Error bars are 95% confidence intervals.

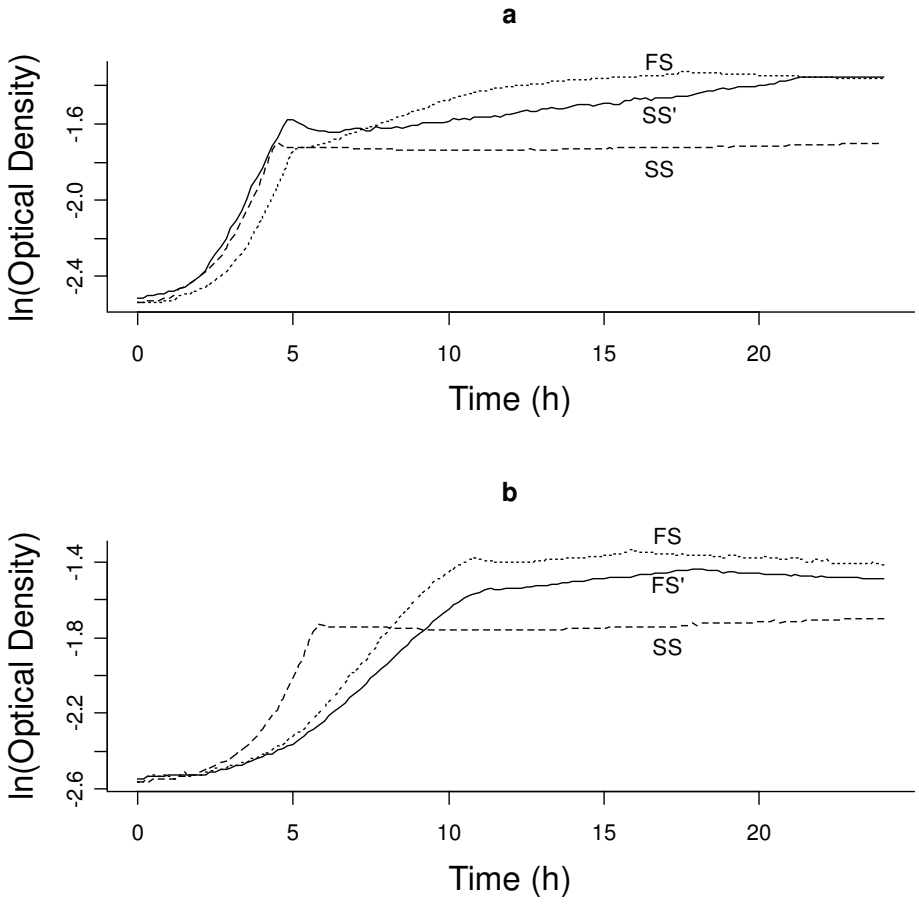

Figure 3.6: After 200 generations (T30) of isolated evolution, "convergent" cultures (SS_{ALLO} and FS_{ALLO}) were assayed for intermediate genotypes. (a) SS' ecotype derived in an ara- culture (dst1018), with FS (dotted) and SS (dashed) ecotypes shown for comparison and (b) FS' ecotype derived from an ara+ culture (dst1019) with FS (dotted) and SS (dashed) ecotypes shown for comparison.

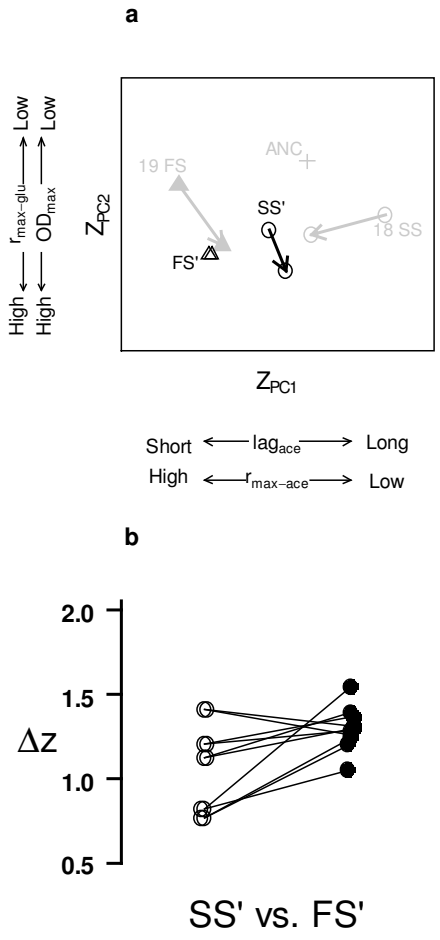

Figure 3.7: Character displacement after competition was induced between intermediate ecotypes (SS vs. FS). (a) Phenotypes are projected and scaled as in Figure 3.5, and gray symbols and arrows illustrate the evolutionary trajectories that occurred during "competitive release" (first phase of study) in the relevant populations for comparison (see Figure 3.5a). Mean ecotype trajectories for SS' (circles) and FS' (triangles) from allopatry to sympatry. The black arrow shows the mean evolutionary trajectory of SS'-derived genotypes during competition, while the FS'-derived genotypes did not change substantially. (b) Distance in trait space, ΔZ, between pairs of SS' and FS' competitors in allopatry (white) and sympatry (black).

Bibliography

Abrams, P. A. (1986). Character displacement and niche shift analyzed using consumer-resource models of competition. *Theor Pop Biol*, 29:107–160.

Adams, D. and Rohlf, R. (2000). Ecological character displacement in plethodon: biomechanical differences found from geometric morphometric study. *PNAS*, 97:4106–4111.

Barrett, R. D. H., MacLean, R. C., and Bell, G. (2005). Experimental evolution of Pseudomonas fluorescens in simple and complex environments. *Am Nat*, 166(4):470–480.

Bolnick, D. (2004). Can intraspecific competition drive disruptive selection? an experimental test in natural populations of sticklebacks. *Evolution*, 58:608–618.

Brown, W. L. and Wilson, E. O. (1956). Character displacement. *Syst Zool*, 5:49–64.

Dieckmann, U. and Doebeli, M. (1999). On the origin of species by sympatric speciation. *Nature*, 400:354–357.

Dillon, W. and Goldstein, M. (1984). *Multivariate analysis: methods and applications*. John Wiley & Sons, New York, NY.

Doebeli, M. (1996). An explicit genetic model for ecological character displacement. *Ecology*, 77:510–520.

Elena, S. and Lenski, R. (2003). Evolutionary experiments with microorganisms: the dynamics and genetics of adaptation. *Nature reviews: Genetics*, 4:457–69.

Friesen, M., Saxer, G., Travisano, M., and Doebeli, M. (2004). Experimental evidence for sympatric ecological diversification due to frequency dependent competition in *Escherichia coli*. *Evolution*, 58:245–60.

Grant, P. and Grant, B. (2006). Evolution of character displacement in Darwin's finches. *Science*, 313:224–226.

Hall, A. R. and Colegrave, N. (2007). How does resource supply affect evolutionary diversification? *Proc R Soc Lond.B*, 274:73–78.

Hardin, G. (1960). The competitive exclusion principle. *Science*, 131:1292–1297.

Lenski, R., Rose, M., Simpson, S., and Tadler, S. (1991). Long-term experimental evolution in *Escherichia coli*. I. Adaptation and divergence during 2,000 generations. *Am Nat*, 138:1315–1341.

Losos, J. (1994). Integrative approaches to evolutionary ecology: Anolis lizards as model systems. *Ann Review of Ecol and Systematics*, 25:467–493. ID: 754.

MacLean, R. C., Dickson, A., and Bell, G. (2005). Resource competition and adaptive radiation in a microbial microcosm. *Ecol Lett*, 8:38–46.

Mahadevan, R., Edwards, J., and Doyle, I. F. (2002). Dynamic flux balance analysis of diauxic growth in Escherichia coli. *Biophysical J*, 83:1331–1340.

Manly, B. F. (1997). *Randomization, bootstrap and Monte Carlo methods in biology*. Chapman & Hall, London.

Meyer, J. and Kassen, R. (2007). The effects of competition and predation on diversification in a model adaptive radiation. *Nature*, 446:432–435.

Nosil, P. and Crespi, B. (2006). Experimental evidence that predation promotes divergence in adaptive radiation. *PNAS*, 103:9090–9095.

Pfennig, D. and Murphy, P. (2003). A test of alternative hypotheses for character divergence between coexisting species. *Ecology*, 84:1288–1297.

Pfennig, D., Rice, M., and Martin, R. (2007). Field and experimental evidence for competition's role in phenotypic divergence. *Evolution*, 61:257–271.

Rainey, P. and Travisano, M. (1998). Adaptive radiation in a heterogeneous environment. *Nature*, 394:69–72.

Rundle, H., Vamosi, S., and Schluter, D. (2003). Experimental test of predation's effect on divergent selection during character displacement in sticklebacks. *PNAS*, 100:14943–14948.

Schluter, D. (1994). Experimental evidence that competition promotes divergence in adaptive radiation. *Science*, 266:798–801.

Schluter, D. (2000a). Ecological character displacement in adaptive radiation. *Am Nat*, 156:S4–S16. ID: 669.

Schluter, D. (2000b). *The ecology of adaptive radiation*. Oxford University Press, Oxford.

Schluter, D. (2003). Frequency dependent natural selection during character displacement in sticklebacks. *Evolution*, 57:1142–1150.

Schluter, D., Price, T., and Grant, P. (1985). Ecological character displacement in darwin's finches. *Science*, 227:1056–1059.

Slatkin, M. (1980). Ecological character displacement. *Ecology*, 61:163–177.

Spencer, C. C., Bertrand, M., Travisano, M., and Doebeli, M. (2007a). Adaptive diversification in genes regulating resource use in *Escherichia coli*. *PLoS Genetics*, 3:83–88.

Spencer, C. C., Saxer, G., Travisano, M., and Doebeli, M. (2007b). Seasonal resource oscillations maintain diversity in bacterial microcosms. *Evol Ecol Res*, 9:775–787.

Taper, M. (1990). *Experimental character displacement in the adzuki bean weevil,* Callosobruchus chinensis. Bruchids and Legumes: economics, ecology and coevolution. Kluwer.

Taper, M. and Case, T. (1992). Models of character displacement and the theoretical robustness of taxon cycles. *Evolution*, 46:317–333.

Taper, M. and Chase, T. (1985). Quantitative genetic models for the coevolution of character displacement. *Ecology*, 66:355–371.

Tyerman, J. G., Havard, N., Saxer, G., Travisano, M., and Doebeli, M. (2005). Unparallel diversification in bacterial microcosms. *Proc R Soc B*, 272:1393–1398.

Chapter 4

Variation in the propensity to diversify in experimental populations of *Escherichia coli*: Consequences for adaptive radiation[1]

> [U]nless profitable variations do occur, natural selection can do nothing. (Darwin, 1859)

4.1 Introduction

Adaptive radiation is an evolutionary process that transforms one species into an array of species each having a distinct phenotype (Simpson, 1953, Schluter, 2000). Extrinsic factors, like divergent selection and ecological opportunity, have been identified via natural and experimental studies as important drivers of adaptive radiation (reviewed in Schluter (2000)). This emphasis on extrinsic factors – those

[1] A version of this chapter has been submitted for publication. Tyerman, J.G. and M. Doebeli. Variation in the propensity to diversify in experimental populations of *Escherichia coli*: Consequences for adaptive radiation.

factors that are external to the individual organism – has culminated in a successful "ecological theory" (Schluter, 2000) to explain the maintenance of diversity during adaptive radiation. In contrast, intrinsic factors, like mutation and development (or "mutation-as-altered-development" (Stoltzfus, 2006)) have received less attention from evolutionary ecologists studying adaptive evolution (but see West-Eberhard (2003), Brakefield (2006)). There are at least two reasons for considering intrinsic factors in adaptive radiation. First, a consideration of intrinsic factors may explain *what* forms arise under adaptive radiation, and serve to complement explanations for *why* those forms persist. Second, mutation is the only evolutionary force that introduces novel adaptive phenotypes to a population (i.e., increases the frequency of an allele from zero to >zero). Thus, differences in mutation rates among species may result in the asymmetric introduction of novel phenotypes; this may cause divergent outcomes among evolving lineages even in the face of convergent opportunities and convergent patterns of selection. Ultimately, asymmetries in intrinsic factors may explain why some species leave more descendent species than others when ecological opportunity and selection are similar (Kassen, 2009).

In this paper, we characterize variation in the propensity to diversify and its consequences to adaptive radiation. Using a model adaptive radiation of *Escherichia coli* (Le Gac et al., 2008, Spencer et al., 2008, Tyerman et al., 2008), we tested whether there was variation between ecotypes (i.e., species) in propensity to diversify with respect to metabolic traits. Specifically, we isolated two phenotypically divergent ecotypes from each of three diversified populations. Next, we initiated new populations from each ecotype and propagated them for 200 generations under the same ecological (i.e., extrinsic) conditions that caused adaptive radiation in their recent ancestor. Previously, we have analysed changes to mean metabolic traits within these newly derived populations (Tyerman et al., 2008). Here, we characterize variation in metabolic phenotypes within these populations and then contrast the level of variation among populations.

By isolating divergent ecotypes, we reduced interspecific competition for resources (Tyerman et al., 2008) and thereby increased the ecological opportunity (Schluter, 2000). This is analogous to "competitive release" that is expected to occur when species arrive on islands that do not contain interspecific competitors that are normally experienced in their native habitats. Our expectation was that this

would promote continued adaptive radiation. Additionally, because the isolated ecotypes experienced analogous ecological conditions as their ancestor, we had an *a priori* expectation for the extent and form of variation possible in our experimental system that we used as a baseline hypothesis. Thus, in controlling extrinsic factors (e.g., ecological opportunity), we could experimentally test whether intrinsic factors could affect the extent and form of diversity evolved during subsequent adaptive radiation.

Variation in mutational input, resulting from intrinsic variation (e.g., in genetic background), may exacerbate the role of historical contingency (i.e., the series of [mutational] events experienced by different species). Contingency is often described as a stochastic element in evolutionary scenarios, in contrast to deterministic processes (i.e., selection) (Gould, 2002, Losos et al., 1998, Travisano et al., 1995). Yet contingency reflects a dependence on initial (or prior) conditions, not whether a process is stochastic or deterministic. Thus, mutational contingency may be biased (and thus predictable), with the result that some lineages may be predisposed to access regions of phenotypic space not available (or, with reduced availability) to other lineages. Whether variation in accessibility contributes to variation in patterns of diversity under adaptive radiation is not traditionally considered by theory. Rather, theory often assumes that the production of phenotypic variation is isotropic (i.e., equivalent in all directions). However, when theory has explicitly allowed variation in genetic opportunity, it has been shown to direct adaptive evolution (Yampolsky and Stoltzfus, 2001).

If mutational contingency does not play a role in the evolution of diversity under adaptive radiation, then we expect the outcome of adaptive radiation – when we have controlled for extrinsic factors – to be convergent, regardless of initial conditions. Departures from this expectation suggest that contingency has played an important role during adaptive radiation.

The proliferation of phenotypes during adaptive radiation is an important topic in evolutionary biology, yet most research has focused on ecological opportunities and patterns of selection that sort phenotypic variants in diverging populations. In this study, we test for mutational bias (an intrinsic factor), and its impact on the diversity produced under adaptive radiation. Our data show that ecotypes vary in the propensity to diversify, leading to significant differences in diversity produced

under adaptive radiation. These results suggest that mutational constraints may be an important factor in shaping evolutionary outcomes.

4.2 Methods

4.2.1 Background

The bacterial strains used in this study were isolated from the 1,000-generation time point of a long-term evolution experiment (Le Gac et al., 2008, Spencer et al., 2008, Tyerman et al., 2008). In that long-term evolution experiment, we propagated *Escherichia coli* B following a seasonal (or "batch") protocol (see Lenski et al., 1991, Spencer et al., 2007b). We supplemented the medium with glucose and acetate as the sole carbon sources (after Friesen et al., 2004). In ten of ten replicate populations, we observed parallel patterns of within-culture (i.e., sympatric) diversification with respect to metabolic function (Tyerman et al., 2008).

To understand this metabolic diversity, we briefly review resource consumption in *E. coli*. When *E. coli* is propagated in batch culture with mixed resources, these resources are consumed sequentially (reviewed in Harder and Dijkhuizen, 1982). Bacteria supplemented with glucose and acetate typically (i.e., ancestral condition) consume glucose exclusively until exhausted from the medium, and then switch to consuming acetate. Metabolism is thus developmentally flexible (i.e., phenotypically plastic), as the expression of acetate metabolizing genes are repressed in environments with sufficient concentrations of glucose. The ancestor is therefore sensitive to the (glucose) environment. This environmental sensitivity that takes the form of a metabolic inhibition is called catabolite repression. Catabolite repression likely evolved because of functional trade-offs between metabolizing glucose and other carbon sources (e.g., acetate). This pattern of metabolic regulation results in a two-phase, or diauxic, growth profile (Figure 4.1). Glucose is consumed during the first phase, and acetate is consumed during the second phase. Previous studies have demonstrated that genetic changes can modify metabolism such that growth profiles are shifted (Spencer et al., 2007a). Thus, we measure shifts in growth profile as a quantitative measure of metabolic evolution (Tyerman et al., 2008).

After 1000 generations of evolution, we commonly observed two coexisting

ecotypes in our evolved populations. An ecotype is a phenotype that correlates with some aspect of ecology experienced by that phenotype (in our case, resource availability and metabolic strategy). One ecotype — the slow switcher — was characterized as having a long diauxic lag in transitioning from glucose to acetate growth. A second ecotype — the fast-switcher — had a negligible diauxic lag in transitioning from glucose to acetate consumption (Figure 4.1). Slow-switchers have evolved stronger catabolite repression then the ancestor (Spencer et al., 2008). Thus, slow-switchers are highly sensitive to the glucose environment. Fast-switchers, on the other hand, appear to be free from catabolite repression and may consume acetate constitutively (Spencer et al., 2007a). Using a microarray study, Le Gac et al. (2008) described how expression profiles changed during the course of the long-term evolution experiment, indicating that both the slow- and fast-switcher ecotypes had changed relative to their ancestor, and relative to each other.

4.2.2 Isolation treatment

We have previously reported on the changes in mean trait levels that occurred in populations evolved from each ecotype in isolation (Tyerman et al., 2008). Here, we focus on the diversity evolved within these populations by characterizing individual clones isolated from a subset of these populations.

First, we provide an overview of our experimental design and expand on the details below. From the 1000-generation point of our long-term evolution experiment, we selected three of ten diversified populations (strains: dst1018, dst1019, and dst1020, hereafter referred to as 18, 19 and 20 respectively) to serve as "Source populations". From each source population, we isolated and selected five slow-switcher and five fast-switcher clones (hereafter referred to as "Source ecotypes") to serve as founding populations in this study. Thus we had three diversified source populations x two source ecotypes x five clones per source ecotype per source population = 30 founding populations. We evolved the founding populations for 200 generations. After 200 generations, we referred to the 30 populations as "Derived populations" and to individual clones isolated from derived population as "Derived ecotypes." We elaborate these details below.

We maintained our source populations at -80° in glycerol. Our selection of

three source populations (from a possible ten diversified populations) in this study was based on preliminary analyses of diversity in these strains (Tyerman et al., 2008). From frozen stock, we inoculated each source population into fresh medium and conditioned for 24 h at 37° and 250 rpm, following the conditions used in the long-term evolution experiment (Tyerman et al., 2008). We plated these cultures on agar, and arbitrarily selected five clones of each ecotype based on differences in colony morphology (slow-switchers having large colonies; fast-switchers having small colonies, Friesen et al. (2004)). We verified that the selected clones had appropriate growth curve characteristics for the anticipated source ecotype (slow- or fast-switcher) and stored these clones at -80° in glycerol.

We initiated 30 founding populations by inoculating frozen stock of each clone into fresh medium for 24 h (as above), which gave rise to isogenic cultures (assuming no mutation). These cultures served as the sources for our founding populations. We inoculated 1.5 μL of stationary phase founding population into 150 μL of media into individual wells of a 96-well microplate. We propagated these populations daily (transferring 1/100 of population once every 24 h into fresh media) for 30 days. This yielded approximately 200 generations of evolution for each population after isolation from its diversified source population. Otherwise, conditions were analogous to the original long-term evolution experiment.

After 30 days, derived populations were plated on agar and we randomly selected $\sim$ 92 clones per derived population to characterize evolved phenotypic variation.

4.2.3 Growth curve profiles

Following Tyerman et al. (2008), we characterized the metabolic phenotypes of the derived clones. Briefly, we conditioned culture from each derived clone and inoculated 1.5 μL into 150 μL of fresh medium into an individual well of a 96-well plate, and incubated using Biotek shaking incubators that periodically measured optical density. Growth curves were created by measuring optical density (600 nm) at ten minute intervals over 24 h of incubation. We used a computer program to extract relevant growth curve parameters for each clone. Further details are provided in (Tyerman et al., 2008).

4.2.4 Statistical analysis

All analyses were conducted with R, version 2.6.2 (R Development Core Team, 2008). For each growth curve, ten growth parameters were extracted (see Tyerman et al. (2008)) and log-transformed to correct for scaling. For each derived population, we conducted a Principle Components Analysis (PCA) on the correlation matrix of its derived clones, and generated 10 composite traits (Tyerman et al., 2008). Using these composite traits, we conducted a hierarchical cluster analysis with *hclust* (Euclidean distance, 'ward' method) detailed in the package *cluster* (Maechler et al., 2005). We conducted bootstrap analyses using *pvclust* (Suzuki and Shimodaira, 2006) to determine P-values for nodes of the branching points for all dendrograms (nboot=10,000 randomizations). We used a threshold of 5 distance units to discriminate between clusters within derived populations. This allowed us to compare diversity across derived populations. The criterion of 5 units reflected a balance between choosing clusters that had bootstrap support with a probability $\geq 80\%$ and having ≥ 5 individuals. We called these clusters "ecotypes" and hereafter refer to them as "Derived ecotypes" to distinguish them from the source ecotypes (i.e., slow- or fast-switcher) that were used as the progenitors to the founding populations. To analyse the range of phenotypic space accessed by derived populations, we conducted a hierarchical cluster analysis on the set of derived ecotypes from all derived populations. For derived ecotypes, we used the mean derived ecotype, rather than the collection of individuals. As above, we used a threshold of 5 distance units to discriminate between clusters. We conducted Analyses of Variance (ANOVA) to determine if variation in two response variables, extent and range of radiation, could be explained by variation in source population, source ecotype and the interaction term. Extent of radiation was a comparison of the number of ecotypes evolved in each derived population. Range of radiation was the amount of phenotypic space accessed by each derived population, using a common projection (see description in Results).

Results

We assayed the metabolic variation among clones in thirty derived populations (3 source populations x 2 source ecotypes x 5 clones per source ecotype per source

population). From each population, we assessed 92 derived clones. For each derived clone, we extracted growth curve parameters (i.e., metabolic phenotypes). On the collection of derived clones, we conducted several analyses to assess the form and extent of diversity under adaptive radiation. We used source population (population 18, 19, or 20) and source ecotype (slow- or fast-switcher) to explain sources of variation in our analyses.

4.2.5 Extent of radiation

First, we define the extent of radiation as the number of ecotypes in each derived population following 200 generations (i.e., 30 days) of evolution. The extent of radiation allows us to compare how many ecotypes evolved among derived populations, allowing for independent avenues of phenotypic diversification. Thus, founding populations could vary in how they diversified into different clusters. Below, we consider how populations diversified using a common phenotypic space (see Range of radiation).

For each derived population, we conducted PCA on the growth curve parameters using the collection of clones isolated from that population after it was allowed to evolve for 30 days. We derive composite traits that describe independent (and orthogonal) dimensions of diversification for each derived population. Next, we performed a hierarchical cluster analysis on the distance matrix of clones isolated from each derived population. We determined the number of significant clusters (e.g., "derived ecotypes") in each population (see Methods). Next we conducted ANOVA to determine whether source population (Pop 18, 19, and 20) and source ecotype (slow-switcher and fast-switcher) explained variation in numbers of derived ecotypes among derived populations (Table 1). Source population had a marginal, though insignificant role in explaining the observed variation ($F=2.836$, $P=0.08$), however, source ecotype (slow-switcher or fast-switcher) strongly affected the subsequent extent of radiation ($F=12.291$, $P<0.0018$). As there was no significant interaction between source population and source ecotype in explaining variation in extent of radiation ($F=1.38$, $P=0.27$), we grouped data from different source populations, and calculated the mean number of derived ecotypes in slow-switcher- and fast-switcher derived populations. On average, slow-switcher pop-

ulations diversified into 4.9 (95%CI: 4.0-5.8) derived ecotypes and fast-switcher populations diversified into 3.1 (95%CI: 2.4-3.9) derived ecotypes.

4.2.6 Range of radiation

We define range of radiation as the range of phenotypic space accessed during adaptive radiation. To this end, we transformed all derived ecotypes from all derived populations using a common phenotypic mapping, first described in Tyerman et al. (2008). This mapping illustrated phenotypic convergence along (Z_{PC1}) (and parallel phenotypic shifts along Z_{PC2}) that occurred as a result of release from interspecific competition (Figure 4.2). We note that Z_{PC1} was a composite trait that reflects to a large degree the lag_{ACE} trait (while Z_{PC2} largely reflects the maximum growth rate on glucose trait, $r_{MAX-GLU}$). We determined the mean trait values (i.e., Z_{PC1}, Z_{PC2}) for each derived ecotype in each derived population. We collected these data from all derived populations (plus the slow- and fast-switcher source ecotypes from each source population) into a single dataset [120 derived ecotypes + (3 source population x 2 source ecotypes) = 126 total ecotypes], and conducted PCA (as above), again to generate composite trait descriptions that are independent (and orthogonal) in phenotypic space. We conducted a hierarchical cluster analysis on the 126 ecotypes and differentiated between clusters using a threshold of 5 distance units. This resulted in nine clusters (Figure 4.3). Examination of the identities revealed that clustered ecotypes were from different derived populations. Thus, parallelism was apparent in our experiment. Figure 4.4 illustrates the "mean" growth curve of each cluster, in comparison to the growth curves of the progenitor slow-switcher and fast-switcher founding ecotypes. We mapped the nine clusters identified in Figure 4.3 onto Z_{PC1} and Z_{PC2}, and ranked them according to their mean Z_{PC1} score (Figure 4.5). We scored all the derived ecotypes using the rank of the cluster to which it belonged, and conducted the subsequent analyses using these ranks. Using ranks, we calculated the range of radiation as the range separating the maximum and minimum cluster rank for each replicate radiation. We then determined whether the variation in range could be explained by source population or source ecotype (Table 2). We found that source ecotype (F=7.0314, p=0.01397) but not source population (F=2.4036, p=0.11182) explained variation in range of ra-

diation, with slow-switcher derived populations accessing more phenotypic space than fast-switcher derived populations (Figure 4.5).

4.3 Discussion

The propensity to diversify has the potential to determine the extent and form of diversity evolved under adaptive radiation. Here, using bacterial ecotypes isolated from experimental populations that had undergone adaptive radiation, we tested whether there were intrinsic differences between source ecotypes to undergo subsequent adaptive radiation. We found that slow- and fast-switcher *E. coli* ecotypes differed in their propensities to diversify, resulting in variation in the extent and form of variants derived under subsequent adaptive radiation. Populations derived from slow-switchers accessed more regions of metabolic phenotypic space than populations derived from fast-switchers. As a consequence, slow-switchers diversified more than fast-switchers. These differences culminated in many slow-switcher populations evolving a fast-switcher ecotype within 200 generations; however, fast-switcher populations never evolved the slow-switcher ecotype. Thus, the original adaptive radiation recurred only when initiated from populations founded by the slow-switcher, indicating that diversity in our experimental system is sensitive to the initial (genetic) conditions.

Our data support the contention that, under some demographic conditions (i.e., isogenic founding cultures), variation in mutational input can result in variation in patterns of diversity evolved under adaptive radiation, even when extrinsic factors – selection and ecological opportunity – are similar.

Other studies have observed differential ability to diversify among bacterial ecotypes (Spiers et al., 2002, Buckling et al., 2003, MacLean et al., 2005, Spencer et al., 2008). Using wrinkly-spreader ecotypes from a *Pseudomonas fluorescens* system, Spiers et al. (2002) compared the ability of various wrinkly-spreader^{-} (i.e., "smooth" revertants) to undergo subsequent diversification, in comparison to the ancestral ("smooth") ecotype. The authors observed variation among revertants in ability to diversify. Using the same experimental system, Buckling et al. (2003) evolved *Pseudomonas* in static broth microcosms, selecting the dominant genotype after each bout of diversification and monitoring the ability to diversify.

The authors found that the dominant genotype had reduced ability to diversify as the experiment progressed and argued that adaptation limited the ability to diversify (but see Spencer et al. (2008)). Finally, MacLean et al. (2005) evolved *Pseudomonas*, initiated from mixtures of *pan+* and *pan-* isogenic lines. While the pan marker had no initial effect on fitness, the authors found that the degree of subsequent divergence under adaptive radiation was dependent on the initial marker state, suggesting an important role for intrinsic factors in adaptive radiation.

Our findings extend these findings in two important ways. First, our results on *E. coli*, along with those of (Spencer et al., 2008) extend the findings and observations on differential variation production beyond *Pseudomonas*. Second, our results can be placed against a well-characterized ecological context. In previous studies, we have developed a detailed understanding of the utility and functional consequences of the phenotypic variation evolved in our experimental populations (Friesen et al., 2004, Tyerman et al., 2005, Spencer et al., 2007a, Le Gac et al., 2008, Spencer et al., 2008, Tyerman et al., 2008). Additionally, because we have characterized the diversity that evolved in the original populations, we have an expectation for patterns of diversity expected under subsequent bouts of adaptive radiation in the same environment.

This current study complements two earlier studies from our research group. As noted above, we have previously characterized the mean phenotypic changes that occurred in these derived populations (Tyerman et al., 2008). Those findings indicated that, during convergence due to release from competition for limited carbon resources, the slow-switcher derived populations "closed the gap" to a much larger degree than did the fast-switcher derived populations. Our findings in this study reveal that slow-switcher derived populations simply contained more variants than did fast-switcher derived populations, with more variants oriented towards the fast-switcher region of phenotypic space. The second paper, by Spencer et al. (2008), characterized the propensity to diversify using "fossil" populations of these strains prior to their evolutionary diversification. The authors found that the propensity to diversify increased as the fossil sample point approached the actual point of diversification, and attributed this result to a shifting adaptive landscape – due to frequency-dependent selection – that was becoming ever more permissive to invasion by the fast-switcher ecotype. Thus, Spencer et al. (2008) focused

on the extrinsic factors that favour diversification in the ancestors that gave rise to both the slow- and fast-switcher ecotypes. The authors rejected a role for intrinsic factors ("genetic constraints") because proto-fast-switchers were occasionally produced by the pre-diversified fossil populations. Here, we start with full fledged fast-switchers and argue that their inability to produce a slow-switcher is likely due to genetic constraints (see below).

The importance of intrinsic factors to explaining the origin and maintenance of phenotypic diversity may extend beyond our findings in the lab, to microbes in nature, and to suites of phenotypic traits beyond metabolism (e.g., antibiotic resistance). Additionally, these findings may be of relevance to the study of adaptive radiation in general; our results suggest that a greater emphasis be given to the generation of novel phenotypic variation when explaining patterns of evolved diversity.

4.3.1 Caveats

Our interpretation of the data requires several caveats. First, in testing for variation in propensity to diversify, we required an experimental system that controlled for extrinsic factors, i.e., ecological opportunity and selection. Failing this, we could not argue that variation in propensity to diversify was due to variation in intrinsic factors per se (e.g., variation in propensity to diversify could be confounded by variation in opportunity or selection).

Because the vacant niches available to populations founded by different ecotypes were complementary, they were not exactly equivalent, and therefore could reflect differences in ecological opportunity. If the opportunity for a fast-switcher founded population to diversify to fill the niche previously occupied by the slow-switcher was in some way diminished, then this reduced opportunity could result in the observed lower levels of diversity in fast-switcher derived populations. We offer three arguments against this line of reasoning. First, Tyerman et al. (2008) noted that the proportion of slow-switchers in the original diversified source populations was substantial (about 78%). Thus, it is not immediately apparent that the niche is fundamentally limited or intrinsically marginal. Second, from a physiological perspective, the hypothesized role filled by slow-switchers in this model adaptive

radiation is as a fast-growth-on-glucose specialist, having reduced performance on acetate (Friesen et al., 2004, Tyerman et al., 2008). As acetate specialists, fast-switchers – having traded off growth performance on glucose for enhanced growth performance on acetate – still grow on glucose at the outset of diauxic growth (Tyerman et al., 2008). Thus it is not logically consistent that the ample glucose made available in the daily batch environment – which is consumed by the fast-switchers – should be considered as negligible or absent as an opportunity by the fast-switcher ecotype. Third, Spencer et al. (2008) showed, in the evolution of the diversified populations prior to diversification, there was evolution towards the slow-switcher ecotype. We interpret this to indicate that the ancestor had been selected to evolve towards being a slow-switcher and to fill the opportunity of the slow-switcher niche. For these reasons, we feel that the reduced diversity seen in the fast-switcher derived populations was not a direct result of reduced ecological opportunity.

A second criticism to our interpretation of the results is that the nature of selection acting on slow- and fast-switcher populations may have differed. To understand this criticism, it helps to first consider our data in light of theory developed for sympatric ecological speciation (Dieckmann and Doebeli, 1999, Doebeli and Dieckmann, 2003). From adaptive dynamics theory (Geritz et al., 1998), competition for resources can generate evolutionary dynamics characterised by two phases. In the first phase, directional selection causes a population to evolve towards an evolutionary branching point in phenotypic space. Upon occupying the evolutionary branching point, negative frequency-dependent selection leads to a shift in the nature of selection. Here, the selection regime turns from directional to disruptive and causes the population to undergo evolutionary branching into two phenotypic clusters, which can be illustrated using pairwise invasibility plots (Geritz et al., 1998), as shown in Figure 4.6. Note that according to the evolutionary dynamics resulting from the situation shown in Figure 4.6, evolutionary branching and diversification should occur irrespective of the starting phenotype. In particular, evolutionary branching should occur even if the starting phenotype is a fast-switcher, contrary to our observations. If we assume that the invasion conditions outlined in the evolutionary model presented in Figure 4.6 are indeed met in our experimental system (Friesen et al., 2004), we conclude that our results diverge from

the predictions of the model because of mutational constraints in populations initiated by fast-switchers. However, instead of the differences in variational constraint facing slow- and fast-switcher derived populations, one could also envisage that the conditions for invasion and evolutionary branching do not hold for populations initiated by fast-switcher ecotypes. This could happen if the pairwise invasibility plot looks different from the one shown in Figure 4.6. For example, Figure 4.7 shows a more complicated pairwise invasibility plot, according to which evolutionary branching and diversification would still occur when the starting phenotype is a slow-switcher, but would not occur, or would be less likely to occur, with a fast-switcher as starting phenotype. This is because according to the pairwise invasibility plot shown in Figure 4.7, an initial population of fast-switchers would not evolve to the same branching point as a population of slow-switchers, and instead would evolve to a local ESS consisting of fast-switchers that cannot be invaded by nearby mutations. Effectively, due to the more complicated nature of the pairwise invasibility plot shown in Figure 4.7, the corresponding evolutionary dynamics has more than one possible equilibrium point.

Thus, selection of the type reflected in Figure 4.7, rather than mutational constraint, could account for asymmetries in divergence. Our response to this criticism is twofold. First, while we can imagine complex ecological scenarios involving, for example, combinations of competition and facilitation leading to diversification (Friesen et al., 2004, Tyerman et al., 2005, Saxer et al., 2009), we have no evidence that such complexity is operating in our system (i.e., whereby intermediate levels of slow-switching have negative invasion fitness, yet extreme levels of slow-switching have positive invasion fitness). We acknowledge that complex invasion scenarios are possible, and thus we remain open to a role for extrinsic factors in explaining our data. Indeed, in contrast to the possibility of selection against slow-switchers in resident populations of fast-switchers, competition experiments have confirmed that there is strong selection for slow-switchers to invade resident populations of fast-switchers. Previously (Friesen et al., 2004, Tyerman et al., 2005), we have shown that, when rare, slow-switcher ecotypes can invade populations of fast-switchers. Additionally , when we competed intermediate slow-switcher and intermediate fast-switcher populations to initiate ecological character divergence (Tyerman et al., 2008), we found that the intermediate slow-switcher lineage ini-

tially increased in frequency prior to the generation of novel, divergent phenotypes. Thus, our empirical measures of selection from competition experiments find that there is selection for slow-switcher (or intermediate slow-switcher) phenotypes in populations founded by fast-switchers.

4.3.2 Possible mechanisms

The apparent genetic constraints that limit fast-switchers from subsequent metabolic diversification have not yet been identified. A previous study involving analogous slow- and fast-switcher *E. coli* ecotypes identified an IS5 insertion that disrupted *iclR* function (Spencer et al., 2007a). This mutation caused the constitutive expression of acetate metabolizing genes (i.e., *aceBAK*), though other mutations leading to similar fast-switcher phenotypes were evident. We suspect that mutations that target regulatory systems responsible for catabolite-repression may be involved in the genetic changes leading from the ancestral *E. coli* (Le Gac et al., 2008, Spencer et al., 2008, Tyerman et al., 2008) or slow-switcher ecotype (this study) to the fast-switcher ecotype. Indeed, there is now evidence that at least some fast-switchers carry a mutation in the *arcA* gene that affects the activity of this important regulator of glucose and acetate pathways (M. Le Gac and M. Doebeli, unpublished data). Mutations that disrupt function via deletion, inversion, or other complex genetic rearrangements may not be easily reversed to reconstitute the original phenotypic function (although compensatory mutations may aid in reconstitution). If the probability of disrupting regulation is greater than the probability of reconstituting regulatory function, then we might expect variation in the propensity to diversify to be common.

"Deregulation" (reviewed by Kassen and Rainey (2004)) has been commonly identified in studies focused on the genetic mechanisms underlying diversification in other model adaptive radiations, including the wrinkley-spreader morphs in *Pseudomonas fluorescens*, the GASP forms in *E. coli* (Finkel and Kolter, 1999, Zinser and Kolter, 2004), and cross-feeding ecotypes in *E. coli* (Rosenzweig et al., 1994, Treves et al., 1998). We speculate that deregulation may often be involved in the generation of novel phenotypes in adaptive radiation with microbes. Deregulation leading to constitutive expression is a clear example of genetic assimilation.

Genetic assimilation is the evolutionary reduction of trait sensitivity to an environmental stimulus (Waddington, 1953, West-Eberhard, 2003). In the case of catabolite repression, the ancestor and slow-switcher ecotypes are highly sensitive to the glucose environment, corresponding to the regulated inhibition of acetate metabolizing genes. Through genetic assimilation, the fast-switcher may have evolved reduced sensitivity to the glucose environment, via mutations that disrupted the regulation associated with catabolite repression (Spencer et al., 2007a). If true, our findings of genetic assimilation may be of relevance to medically important strains where genetic assimilation has been found. Recently, Hoboth et al. (2009) found evidence for evolution towards constitutive expression (i.e., genetic assimilation) of metabolic traits in highly mutable strains of *Pseudomonas aeruginosa* isolated from patients with chronic lung disease associated with cystic fibrosis.

The ecological theory of adaptive radiation pertains to the external factors that drive phenotypic and ecological diversification. Internal factors, like the mutational bias suspected in this study, provide additional information about constraints that may be operating in the system. While often under-appreciated, these constraints can explain divergent outcomes under adaptive radiation. Our study provides greater motivation to include internal factors in a more general theory of adaptive radiation.

4.4 Acknowledgements

We wish to thank C. Spencer and M. Bertrand for lab assistance, R. Fitzjohn for statistical assistance, D. Ally and A. Blachford for invaluable and thoughtful discussion, and members of the Doebeli and SOWD research groups at the University of British Columbia and members of the Harmon, Forney, Rosenblum and Top lab groups at the University of Idaho for critical feedback. Financial assistance was provided by NSERC to J.T. and M.D. and the James S. McDonnell Foundation to M.D.

Table 4.1: Summary of analysis of variance conducted on extent of adaptive radiation (i.e., number of derived ecotypes) after 200 generations of evolution under competitive release.

Source of variation	Df	Sum Sq	Mean Sq	F	Pr(>F)
Population	2	10.4	5.2	2.84	0.078
Ecotype	1	22.5	22.5	12.29	**0.0018**
Population x Ecotype	2	5.1	2.5	1.38	0.27
Residuals	24	44.0	1.8		

Table 4.2: Summary of analysis of variance conducted on range of adaptive radiation (i.e., breadth of diversification along Z_{PC1}) after 200 generations of evolution under competitive release.

Source of variation	Df	Sum Sq	Mean Sq	F	Pr(>F)
Population	2	17.867	8.933	2.4036	0.11182
Ecotype	1	26.133	6.133	7.0314	**0.01397**
Population x Ecotype	2	0.267	0.133	0.0359	0.96481
Residuals	24	89.200	3.717		

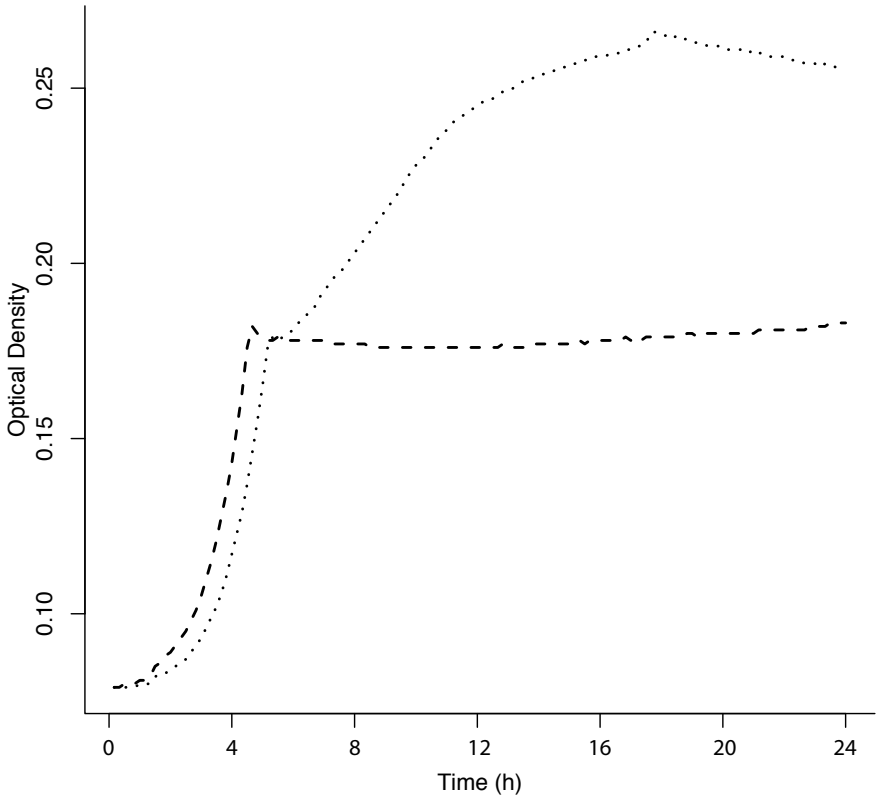

Figure 4.1: 24 h growth curves for source ecotypes from source population 18 (strain dst1018). Slow- and fast-switcher ecotypes are shown by dashed and dotted lines, respectively.

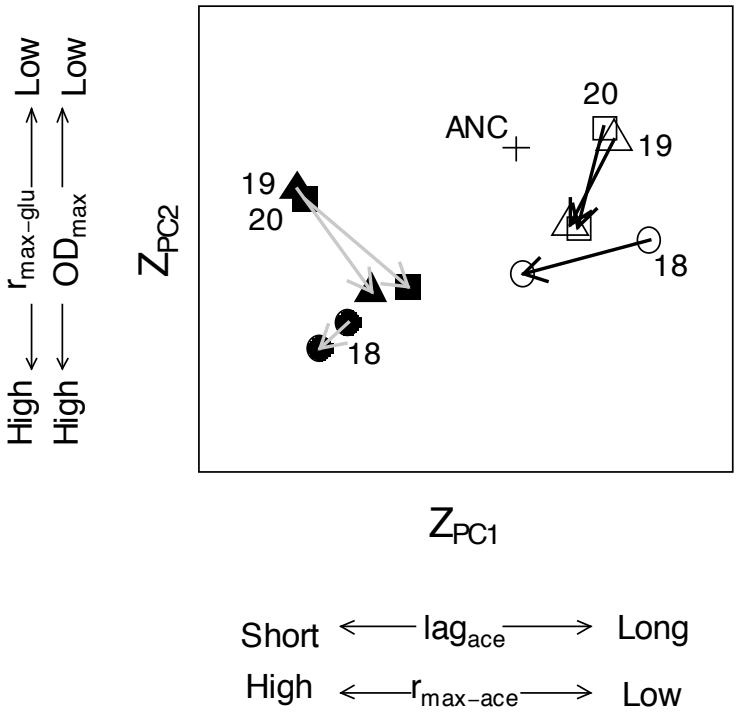

Figure 4.2: Upon competitive release, mean phenotypes for slow-switcher (open symbols) and fast-switcher (filled symbols) populations generally converged in Z_{PC1} and shifted down in Z_{PC2}. Arrows depict evolutionary trajectories of mean trait values, connecting source populations (generation 0, arrow tail) and derived populations (generation 200, arrow head). $\circ$ is population 18, $\triangle$ is population 19, and $\bigtriangledown$ is population 20. For reference, the ancestor (ANC) that gave rise to the source ecotypes used in this study is indicated with the +. For details, see Tyerman et al. (2008).

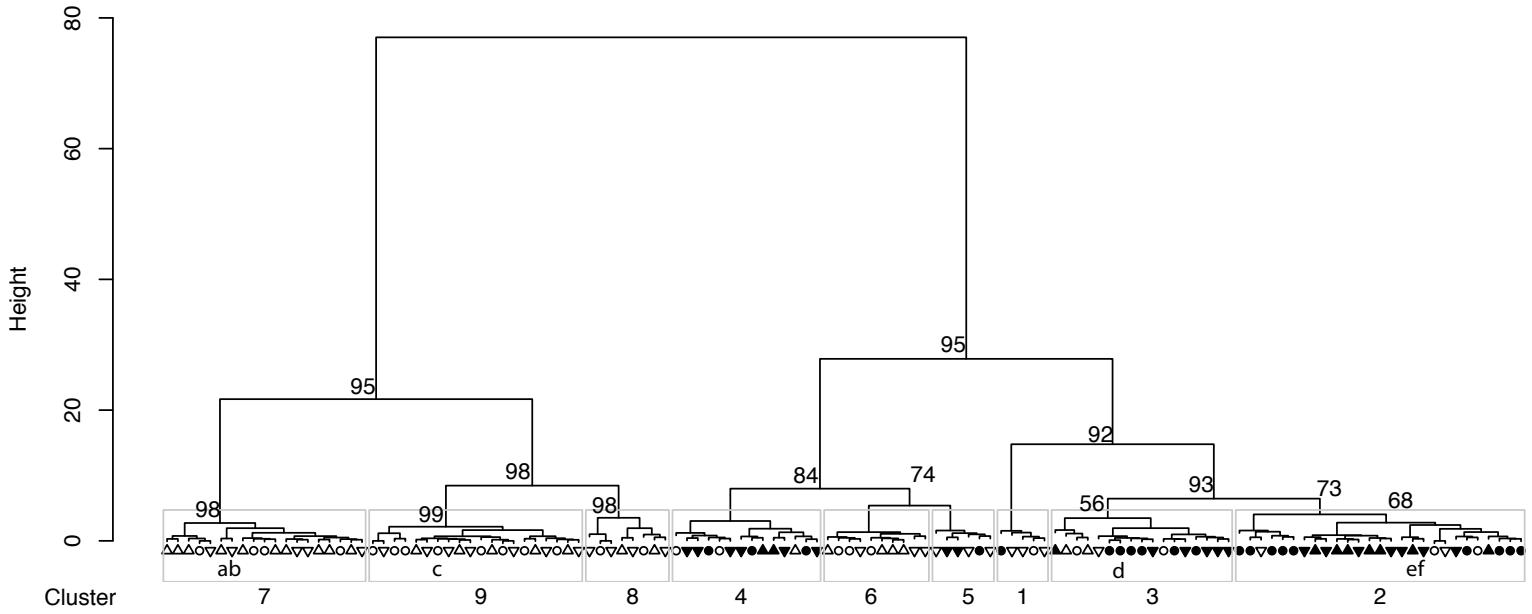

Figure 4.3: Cluster analysis of 126 ecotypes from 30 derived populations and founding populations. Branch lengths (heights) represent the euclidean distance in (log transformed) phenotypic space. Derived ecotypes are labeled by shape, reflecting source population (○, 18; △, 19; ▽, 20), and source ecotype (open symbols, slow-switcher; closed symbols, fast-switcher). Ancestral populations that served as the source ecotypes (slow- and fast-switchers) are indicated by letters: a, 19-SS; b, 20-SS; c, 18-SS; d, 18-FS; e, 19-FS; and f, 20-FS. Grey boxes outline clusters of "convergent ecotypes" that are closer than 5 units in phenotypic space. Large numbers under the grey boxes denote cluster identity (see text). The numbers above selected branch nodes represent the bootstrap support for this cluster hypothesis.

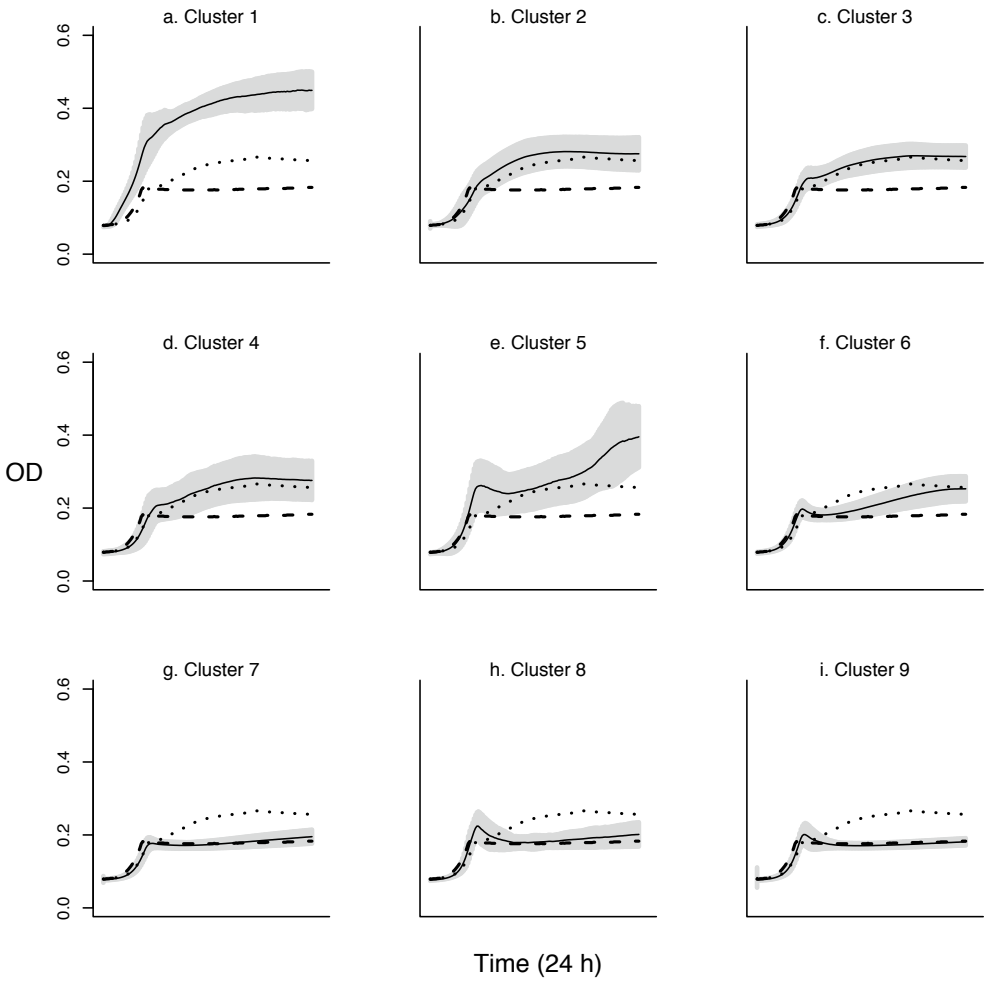

Figure 4.4: Growth curves of derived ecotypes from the nine clusters (a-i) identified in cluster analysis (Figure 4.3). The X axis (time) and Y axis (Optical density, 600 nm) is the same in each panel. The dashed and dotted lines are slow- and fast-switcher source ecotypes (see Figure 4.1), shown in each panel for comparison. The solid line illustrates the mean growth curve for that cluster, and the grey region denotes growth curve values within $\pm$ 1 standard deviation of the mean growth curve, calculated for each time point.

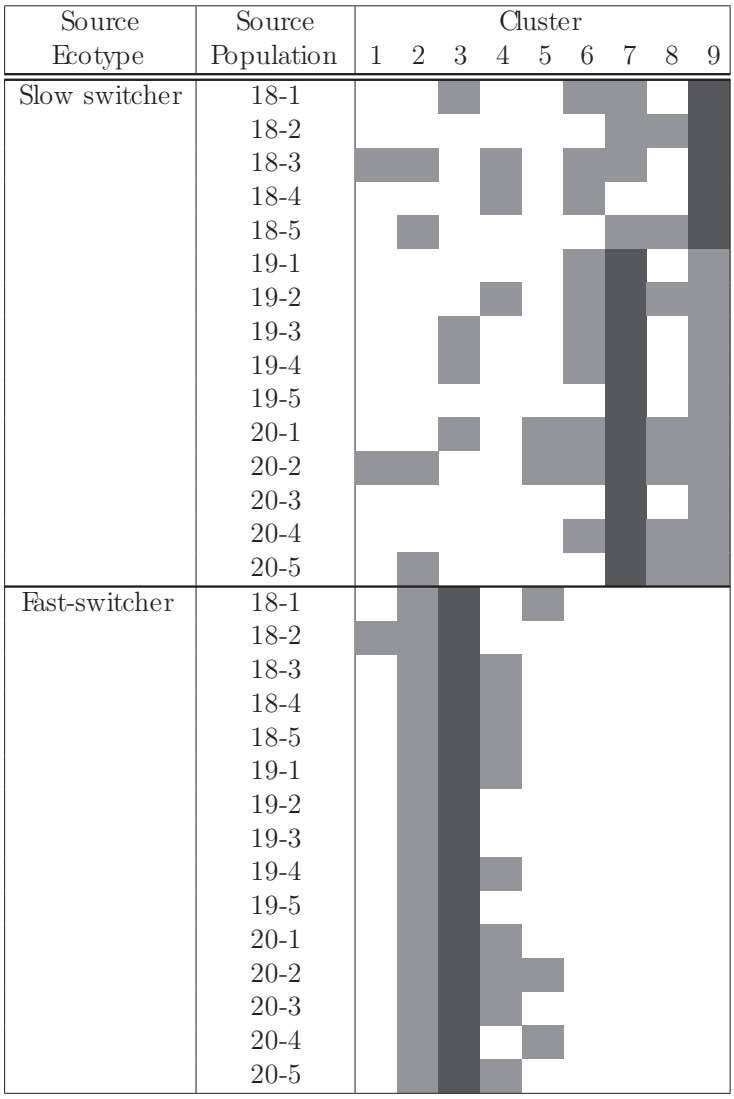

Figure 4.5: Derived ecotypes isolated from founding population (rows) were classified according to one of nine clusters (columns). Grey and white boxes indicate the presence and absence of a particular cluster respectively within each population. Dark grey boxes indicate the cluster of the ancestor that founded each population. The range of adaptive radiation was calculated as the range (in cluster-ranks) spanned by a derived population.

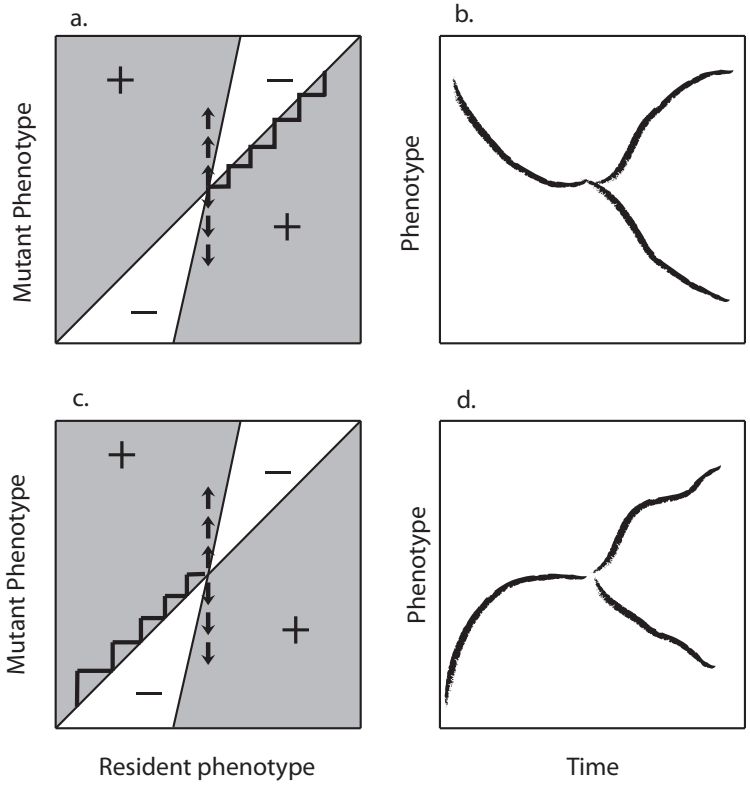
a.
b.
c.
d.
Mutant Phenotype
Phenotype
Mutant Phenotype
Phenotype
+
−
+
−
+
−
+
−
Resident phenotype
Time

Figure 4.6:

Figure 4.6 Pairwise invasibility plots (a and c) and corresponding evolutionary trajectories (b and d) illustrating scenarios of adaptive diversification. The pairwise invasibility plots show regions of positive invasion fitness (grey) and negative invasion fitness (white) for all combinations of resident (X axis) and mutant phenotype (Y axis). To derive the evolutionary trajectory, it is assumed that if a mutant can invade a resident population (i.e., if the corresponding resident-mutant pair lies in the grey region), then the mutant replaces the resident and becomes the new resident. This substitution is indicated by the thick black line segments in a) and c). Note that along the diagonal, mutant phenotypes are equal to resident phenotypes, and hence invasion fitness is 0 (i.e., mutant and resident have the same fitness). Evolutionary equilibrium points, or evolutionary singularities, are given as intersection points of the diagonal with the 0-isocline of the invasion fitness function (Geritz et al. 1998). The slope of this 0-isocline at the evolutionary singularity determines the evolutionary dynamics. In the situation shown in Figure 4.6, the singularity is an evolutionary branching point: irrespective of the starting phenotype the resident population first evolves towards the singularity, which is therefore an evolutionary attractor. At the branching point, selection becomes disruptive due to negative frequency dependence. As a consequence, every nearby mutant can invade, and hence the population diversifies into two separate phenotypic clusters (black arrows in a) and c)), resulting in the evolutionary trajectories shown in b) and d).

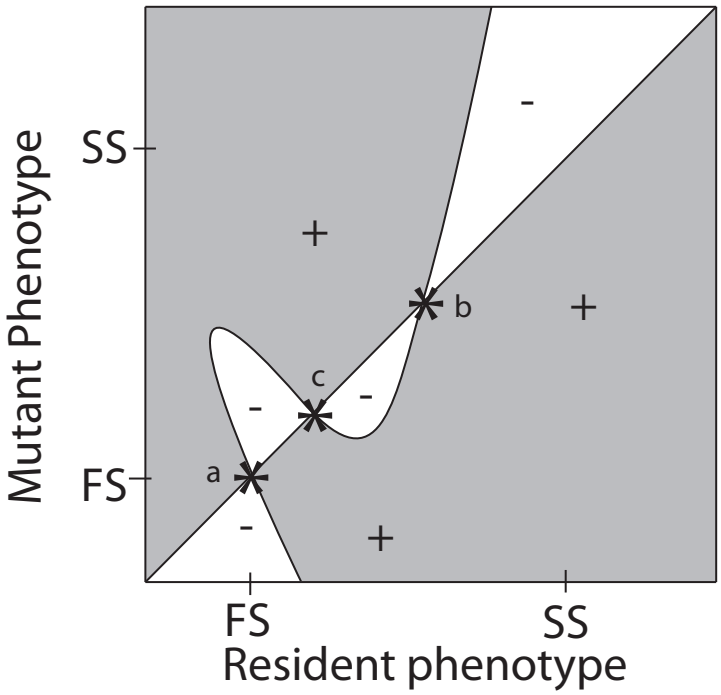

Figure 4.7: Hypothetical pairwise invasibility plot showing multiple equilibria, i.e., multiple intersection points between the 0- isocline of the invasion fitness and the diagonal (*). In this case, the evolutionary dynamics depend on initial conditions. Populations initiated from slow-switchers (SS) would evolve to the branching point (b) and diversify as in Figure 4.6. However, populations initiated from fast-switchers (FS), i.e., with small phenotypic values on the X-axis, would evolve towards a local evolutionarily stable strategy (ESS; a), i.e., to a phenotype that is non-invadable by nearby mutants. In this hypothetical example, the branching point corresponding to slow-switchers and the ESS corresponding to fast-switchers are separated by an evolutionary repellor (c), the existence of which leads to dependence of the evolutionary dynamics on initial conditions.

Bibliography

Brakefield, P. (2006). Evo-devo and constraints on selection. *TREE*, 21:362–368.

Buckling, A., Wills, M. A., and Colegrave, N. (2003). Adaptation limits diversification of experimental bacterial populations. *Science*, 302:2107–2109.

Darwin, C. (1859). *The origin of species*. Penguin books, London.

Dieckmann, U. and Doebeli, M. (1999). On the origin of species by sympatric speciation. *Nature*, 400:354–357.

Doebeli, M. and Dieckmann, U. (2003). Speciation along environmental gradients. *Nature*, 421:259–264.

Finkel, S. E. and Kolter, R. (1999). Evolution of microbial diversity during prolonged starvation. *PNAS*, 96(7):4023–4027.

Friesen, M., Saxer, G., Travisano, M., and Doebeli, M. (2004). Experimental evidence for sympatric ecological diversification due to frequency dependent competition in *Escherichia coli*. *Evolution*, 58:245–60.

Geritz, S., Kisdi, E., Meszena, G., and Metz, J. (1998). Evolutionary singular strategies and the adaptive growth and branching of the evolutionary tree. *Evol Ecol Res*, 12:35–57.

Gould, S. (2002). *The structure of evolutionary theory*. Harvard University Press, Cambridge, MA.

Harder, W. and Dijkhuizen, L. (1982). Strategies of mixed substrate utilization in microorganisms. *Philos T R Soc B*, 297:459–480.

Hoboth, C., Hoffmann, R., Eichner, A., Henke, C., Schmoldt, S., Imhof, A., Heesemann, J., and Hogardt, M. (2009). Dynamics of adaptive microevolution of hypermutable *Pseudomonas aeruginosa* during chronic pulmonary infection in patients with cystic fibrosis. *J Infect Dis*, 200(1):118–130.

Kassen, R. (2009). Towards a general theory of adaptive radiation: insights from microbial experimental evolution. *Proc NY Acad Sci*, 1168:3–22.

Kassen, R. and Rainey, P. (2004). The ecology and genetics of microbial diversity. *Ann Rev Microbiol*, 58:207–231.

Le Gac, M., Brazas, M. D., Bertrand, M., Tyerman, J. G., Spencer, C. C., Hancock, R. E. W., and Doebeli, M. (2008). Metabolic changes associated with adaptive diversification in *Escherichia coli*. *Genetics*, 178(2):1049–1060.

Lenski, R., Rose, M., Simpson, S., and Tadler, S. (1991). Long-term experimental evolution in *Escherichia coli*. I. Adaptation and divergence during 2,000 generations. *Am Nat*, 138:1315–1341.

Losos, J., Jackman, T., Larson, A., d. Queiroz, K., and Rodriguez-Schettino, L. (1998). Contingency and determinism in replicated adaptive radiations of island lizards. *Science*, 279:2115–2118.

MacLean, R. C., Dickson, A., and Bell, G. (2005). Resource competition and adaptive radiation in a microbial microcosm. *Ecol Lett*, 8:38–46.

Maechler, M., Rousseeuw, P., Struyf, A., and Hubert, M. (2005). *Cluster Analysis Basics and Extensions*.

R Development Core Team (2008). *R: A Language and Environment for Statistical Computing*. R Foundation for Statistical Computing, Vienna, Austria. ISBN 3-900051-07-0.

Rosenzweig, R., Sharp, R., Treves, D., and Adams, J. (1994). Microbial evolution in a simple unstructured environment: Genetic differentiation in *Escherichia coli*. *Genetics*, 137:903–917.

Saxer, G., Doebeli, M., and Travisano, M. (2009). Spatial structure leads to ecological breakdown and loss of diversity. *Proc Roy Soc B*, 276(1664):2065–2070.

Schluter, D. (2000). *The ecology of adaptive radiation*. Oxford University Press, Oxford.

Simpson, G. G. (1953). *The major features of evolution*. Columbia University Press, New York.

Spencer, C. C., Bertrand, M., Travisano, M., and Doebeli, M. (2007a). Adaptive diversification in genes regulating resource use in *Escherichia coli*. *PLoS Genetics*, 3:83–88.

Spencer, C. C., Saxer, G., Travisano, M., and Doebeli, M. (2007b). Seasonal resource oscillations maintain diversity in bacterial microcosms. *Evol Ecol Res*, 9:775–787.

Spencer, C. C., Tyerman, J., Bertrand, M., and Doebeli, M. (2008). Adaptation increases the likelihood of diversification in an experimental bacterial lineage. *PNAS*, 105(5):1585–1589.

Spiers, A., Kahn, S., Bohannan, J., Travisano, M., and Rainey, P. (2002). Adaptive divergence in experimental populations of *Pseudomonas fluorescens*. I. Genetic and phenotypic bases of wrinkly spreader fitness. *Genetics*, 161:33–46.

Stoltzfus, A. (2006). Mutationism and the dual causuation of evolutionary change. *Evol Dev*, 8:304–317.

Suzuki, R. and Shimodaira, H. (2006). *pvclust: Hierarchical Clustering with P-Values via Multiscale Bootstrap Resampling*. R package version 1.2-0.

Travisano, M., Mongold, J., Bennett, A., and Lenski, R. (1995). Experimental tests of the roles of adaptation, chance, and history in evolution. *Science*, 267:87–90.

Treves, D., Manning, S., and Adams, J. (1998). Repeated evolution of an acetate-crossfeeding polymorphism in long-term populations of *Escherichia coli*. *Mol Biol Evol*, 15:787–797.

Tyerman, J., Bertrand, M., Spencer, C., and Doebeli, M. (2008). Experimental demonstration of ecological character displacement. *BMC Evol Biol*, 8(1):34.

Tyerman, J. G., Havard, N., Saxer, G., Travisano, M., and Doebeli, M. (2005). Unparallel diversification in bacterial microcosms. *Proc R Soc B*, 272:1393–1398.

Waddington, C. (1953). Genetic assimilation of an acquired character. *Evolution*, 7:118–126.

West-Eberhard, M. (2003). *Developmental plasticity and evolution*. Oxford University Press, Oxford, UK.

Yampolsky, L. and Stoltzfus, A. (2001). Bias in the introduction of variation as an orienting factor in evolution. *Evol Dev*, 3:73–83.

Zinser, E. R. and Kolter, R. (2004). *Escherichia coli* evolution during stationary phase. *Res Microbiol*, 155(5):328–336.

Chapter 5

Diversification in experimental populations of *E. coli* not generally explained by resource diversity or rate-yield trade-offs[1]

5.1 Introduction

Evolutionists explain adaptive phenotypic diversity as a consequence of genetic trade-offs. Trade-offs arise when increases in fitness in one phenotypic dimension are accompanied by decreases in fitness in a second phenotypic dimension. Because no single phenotype can maximally exploit all possible resources and fill all possible niches we observe phenotypic diversity (Roff and Fairbairn, 2007). Trade-offs are necessary for the origin and maintenance of ecologically relevant phenotypic diversity, and are essential to understanding the rules that underlie adaptive diversification. Therefore, identifying and detecting trade-offs has become an integral part of understanding how adaptive diversity evolves.

Trade-offs are often detected in two ways. First, when a variety of divergent

[1] A version of this chapter will be submitted for publication. Tyerman, J.G., C.C. Spencer and M. Doebeli. Diversification in experimental populations of *E. coli* not generally explained by resource diversity or rate-yield trade-offs

types have evolved under different environments (or in a single heterogeneous environment), the performance of each type is measured across environments (or across subsets of the components in a heterogeneous environment) and evidence is sought for local adaptation (or specialization). Where trade-offs exist, it is expected that locally adapted "specialists" will have higher fitness in native environments than in novel environments. Thus, trade-offs are identified when species or populations have a high fitness in one environment (or one subset of components of the heterogeneous environment), but a low fitness in other environments (or other subsets of the heterogeneous environment) (for example, see Tyerman et al., 2008).

Alternatively, when functional relationships between traits are known or suspected, trade-offs can be identified via negative bivariate correlations between realized combinations of trait values, either among individuals in a population or among populations within a community (Roff and Fairbairn, 2007). The strength of this approach is that mechanisms underlying the trade-off are explicitly considered.

The evolution of resource specialization depends on trade-offs between the ability to exploit substitutable resources to explain diversity in resource related traits (MacLean et al., 2004, MacLean and Bell, 2002, MacLean, 2005a, Barrett and Bell, 2006, Barrett et al., 2005). When resource specialization depends on the supply of exogenous resources, it may manifest during adaptive radiation as diversity in specialized feeding phenotypes. The beaks of finches in the Galapagos and the mouth parts of African cichlid fishes exemplify this process. Thus, a population of generalists that encounters a series of substitutable resources may evolve to specialize on a subset of those resources and as a consequence have reduced performance on the remaining resources (Barrett et al., 2005, Barrett and Bell, 2006). Competition for limited resources, and the existence of trade-offs between the abilities to exploit different types of resources, lead to the hypothesis that simple environments (i.e., environments having few substitutable resources) should harbour less biological diversity than complex environments (i.e., environments having more substitutable resources).

Alternatively, when resource specialization involves the internal flux of cellular metabolites, it may manifest during adaptive radiation as variation in metabolic pathways. This variation in metabolism may culminate in variation in maximum

growth rates and yield, as described in several microbial models of adaptive radiation (King et al., 2004, Maharjan et al., 2006, Novak et al., 2006, MacLean, 2005b). For example, organisms may maximize the rate (i.e., energy per unit time) at which they exploit a resource or the efficiency (i.e., energy per unit resource) of their resource use, but not both (Pfeiffer et al., 2001, Helling, 2002, Novak et al., 2006). Under this scenario, the resource specialization hypothesis employs a rate vs. yield trade-off: individuals that maximize growth rate are predicted to have compromised efficiency (and therefore diminished yield), or vice versa; this scenario predicts a negative correlation between growth rate and growth yield among populations in different environments, or among individuals within an environment (Pfeiffer et al., 2001).

Interestingly, the rate-yield hypothesis can predict levels of biological diversity in excess of the levels of diversity predicted by the number of resources found in the external environment because each external resource can potentially be used in a variety of metabolic processes, e.g., aerobic respiration and fermentation of glucose. Thus, superficially it appears that the rate-yield hypothesis is an alternative hypothesis to the resource specialization hypothesis. Here, however, we take the view that the rate-yield hypothesis is a subset of the more general resource specialization hypothesis, with the focus on variation in intracellular resources brought about by the use of alternative metabolic pathways, rather than variation in extracellular resources. Regardless of whether resource specialization occurs due to external or internal resources, it is predicted to depend on the presence of genetic trade-offs between the exploitation of different resources.

To explore the role of genetic trade-offs in resource specialization, we have evolved *Escherichia coli* B in a series of environments ranging in complexity from one to three substitutable carbon sources (glucose, glycerol, acetate). Our goal was to determine whether diversity scaled with changes in environmental complexity, using environments where follow-up studies on the particular forms of resource specialization could be conducted. This requirement precluded the inclusion of highly complex environments, where a functional characterization of all the diversity would be exceedingly difficult.

Similar long-term evolution studies, using *Pseudomonas* bacteria, found that diversity increased with the complexity of the environment (i.e., number of re-

sources)(Barrett et al., 2005, Barrett and Bell, 2006). However, these studies did not control for total productivity; thus, diversity may reflect changes in productivity rather than environmental complexity (Barrett and Bell, 2006, Hall and Colegrave, 2007). Here, we controlled for productivity and rigorously tested the hypothesis that environmental complexity determines the degree of diversity. In general, we found no support for the hypothesis that diversity is determined by environmental complexity.

Because we detected high levels of colony size variation within single resource environments (i.e., environments of low complexity), we also conducted several tests to confirm that our measure of diversity – colony size variation – actually reflected ecologically relevant variation, i.e., variation under selection.

Next, we tested whether there was evidence for rate vs. yield trade-offs operating within our evolved populations. While we detected genetic variation in rate and yield traits, we rarely encountered negative correlations, suggesting that rate-yield trade-offs are not responsible for the diversity within and among our evolved populations.

The evolution of resource specialization in response to competition for resources has often been considered an important outcome of the process of adaptive radiation (Schluter, 2000). Yet, while resource specialization may have occurred during the adaptive radiations that lead to Galapagos finches and cichlid fishes, whether and how it operates in simple bacterial systems, may not be so simple. Our study questions several important assumptions about how trade-offs may affect adaptive diversity.

5.2 Methods

5.2.1 Evolution experiment

We evolved *E. coli* B following a batch culture protocol for approximately 1,000 generations (150 days). From a common ancestor, we initiated ten lines in each of seven environments, for a total of 70 populations. We used two strains to found our populations, rel606 and rel607 kindly provided by R.E. Lenski, that differed at the arabinose (marker) locus. The juxtaposition of alternately marked lines (i.e.,

ara+, ara-, ara+,..., etc.) provided us the ability to monitor for cross-contamination between experimental lines (i.e., periodically screening for opposite marker clones using arabinose indicator plates, after Lenski et al., 1991). The media consisted of Davis Minimal media supplemented with glucose, glycerol and/or acetate (Table 5.1). The concentration of each carbon source in each medium was set to balance the numerical yield (i.e., productivity) achieved by the ancestor on that carbon source after 24 h of growth. For example, the yield achieved by the ancestor in 24 h on 500 μg/ml of glucose was equivalent to the yield achieved by the ancestor in 24h on 1150 μg/ml on acetate. Hence, a glucose-acetate culture contained 250 μg/ml glucose and 575 μg/ml acetate in an attempt to balance (50:50) the yield achieved on each resource. Thus, our protocol controlled for (ancestral) productivity among treatments. Previous studies have employed other protocols, i.e., maintaining the concentrations of each component resource across media of differing complexity (failing to control for productivity, e.g., Barrett and Bell, 2006), or maintained a balance of the number of carbon molecules (e.g., Friesen et al., 2004), which assumes that all carbon molecules are equal in the eyes of a cell, regardless of how they are packaged or how they must be processed.

Each 24 ($\pm$ 1) h, we transferred 0.1 ml of mature culture into 10 ml fresh media, yielding $\log_2 100$ (approximately 6.6) generations per day. The populations were evolved in 20mm (diam) test-tubes and were shaken in an orbital shaker at 250 rpm, at 37°. We screened for contamination following Lenski et al. (1991) once per seven days, and froze the populations at -80° once every three or four days. If a culture failed to grow, we re-initiated the culture from a day-old sample stored at 4°. If cross-contamination was detected, we re-initiated the contaminated culture from a -80° sample from a previous time point known to be free from contamination.

The strains at 1,000 generations are labeled dst10XX (10=1,000 generations, and XX = 01 to 70 to reflect the identity of the population).

5.2.2 Colony morphology

Because of the high number of evolved populations in our study, we decided to use morphological variation in colony size – which is relatively easy to assess – as our measure of ecological variation within populations. Preliminary studies using

populations evolved on glucose-acetate revealed that variation in colony morphology often reflected variation in growth parameters and competitive ability (Friesen et al., 2004, Tyerman et al., 2008). Additionally, because we found high levels of colony size diversity for populations evolved in single resource environments, we conducted several follow-up studies to confirm that colony size variation was under selection and not neutral (described below).

We spread 1,000 generation cultures on a standard Tetrazolium (i.e., a rich medium) agar plates and incubated for 24 h at 37°. We selected plates diluted to have 40-80 colonies per plate, as pilot studies had revealed that colony area was not significantly affected by colony density within this range. (Higher colony densities on plates resulted in smaller colony areas.) We digitally photographed 6-8 plates from a fixed height (25 cm) and processed the images using ImageJ software available for download from N.I.H. (http://rsbweb.nih.gov/ij/) (Abramoff et al., 2004). We converted all images to black and white using a constant binary threshold setting. We visually inspected each photograph and excluded colonies that had fused or overlapped. We calculated the area of colonies (in pixels). We removed data having an area $<$25 pixels; these data were often artifacts caused by imperfections on the agar surface. Finally, we standardized colony area by dividing each colony by the mean colony area of the plate, as pilot studies have shown an effect of individual plate causing variation in colony area between replicates of the same dilution and culture.

We calculated the variance in colony areas for each population. We log transformed the data and conducted analysis of variance to test for a relationship between number of resources and diversity (i.e., variance in standardized colony area). All statistics were conducted with R (version 2.6.2) (R Development Core Team, 2008).

5.2.3 Colony size variation in acetate-evolved populations

Because we found high levels of variation in colony size in acetate-evolved populations (see Results), we conducted two follow-up tests to determine whether the colony size variation was related to ecologically relevant variation. We looked at how colony morphology varied with growth curve parameters, and whether colony

size variation correlated with competitive ability.

Growth parameters of clones isolated from populations evolved in acetate

Preliminary studies indicated that variation in colony size was not clearly correlated with growth curve parameters when clones were grown in acetate (data not shown). However, for many acetate evolved populations, we could distinguish clones using growth parameters from cultures grown in different media, i.e., glucose and acetate (DM_{GA}). Here, we analysed clones isolated from acetate-evolved population dst1025 in DM_{GA} to test whether colony size variation was related to growth parameter variation, as done previously (Friesen et al., 2004, Tyerman et al., 2008).

Invasion experiments in acetate-evolved populations

As a direct test of the ecological relevance of colony size variation, we conducted invasion experiments between large and small colony variants to determine whether colony size variation was related to competitive ability in these populations. For two diversified populations (dst1023 and dst1024), we selected three large and three small colony clones, and conditioned them for 24 h in acetate. We pooled equal volumes of conditioned cultures from large clones (corrected for density), and pooled equal volumes of conditioned cultures from small clones (again, corrected for density). From these large and small pools, we initiated competition experiments (in duplicate) in the following proportions of large and small types: 99:1, 90:10, 10:90 and 1:99. We plated the initial mixture (T0), and the cultures following one day of batch culture growth (T1) in the evolutionary media. We determined relative fitness following standard protocols, i.e., the ratio of Malthusian growth rates, (Lenski et al., 1991, Tyerman et al., 2008), and regressed the relative fitness of large colony clones against the initial frequency of large clones in the mixed populations.

5.2.4 Growth curve parameters: rate vs. yield

We selected a subset of populations from each treatment ($\sim$6) to test for trade-offs between maximum growth rate and yield. These populations were selected

as they had the lowest number of freezer "re-start" days due to suspected cross-contamination. In total, we analysed individual colonies selected from 45 evolved populations. From each population, we isolated 30-60 genotypes (by randomly selecting colonies on agar plates) and conditioned them for 24 h in their evolutionary media. If a population had diversified into large- and small-sized colonies, we attempted to sample an equal number of colonies from each size class colony. When colony size variation was bimodal we were able to easily distinguish whether a colony was large or small by eye. When colony size variation was unimodal, we treated the population as a single size class; furthermore we did not attempt to quantify colony size variation within each size class. We tested for the heritability of colony size variation by picking colonies of each size class (large or small) and conditioning for 24 h in liquid media and plating on agar. The repeatability (heritability) of large clones was high (99-99.9%) and the repeatability of small clones was typically not as high (95-99.9%). We randomly sampled colonies of each size class by selecting colonies closest to an arbitrary point on agar plates. Using individual wells of a 96-well microtitre plate, we inoculated 200 μl of evolutionary media with 2 μl of conditioned culture for each genotype. We incubated the cultures for 24 h using a Biotek optical density reader (Tyerman et al., 2008).

The optical density (OD) at 600 nm was measured every 10 minutes over 24 h. From the OD vs. time data, we calculated the maximum growth rate, r_{max}, by selecting the maximum slope as determined by sliding-window analysis across 9 data points (Tyerman et al., 2008). When there were multiple resources in the medium, the growth curves would have several growth phases. This followed our expectations from our previous characterization of growth curves of clones isolated from populations evolved in mixtures of glucose and acetate medium (Friesen et al., 2004, Tyerman et al., 2008). In general, these clones were diauxic – having two growth phases, one corresponding to growth on glucose, and one corresponding to growth on acetate – with the overall highest growth rate, r_{max} occurring during the first growth phase on glucose, and the maximum optical density (OD_{max}, or yield) during the second phase of growth on acetate. These traits, in addition to the acetate lag (lag_{ace}) trait, suggested that populations had diversified such that clones within these populations either exploited glucose via fast growth, or exploited acetate via a short transition to acetate, leading to a reduced acetate lag and a higher yield.

Based on these patterns, here we assumed that the growth curve of a clone, if it had multiple growth phases, would have similar characteristics. That is, a clone would have its fastest rate during the first phase and its yield would be highest following growth in the final growth phase. This assumption appeared to be true for cultures grown in glucose and acetate (Tyerman et al., 2008, Friesen et al., 2004), and for all genotypes we tested in this study.

We conducted bivariate correlation analyses (r_{max} vs. yield) within and between populations. If discrete size classes (i.e., large and small colony) types occurred within populations, we also conducted correlation analyses on the separate size classes that were present (Novak et al., 2006), as well as the entire population. For the among population analysis, we used the mean trait values from all populations, or the mean trait values from each size class in diversified populations. We analysed the populations for each media separately. Because of the large number of analyses, we summarized all the statistics in tabular format.

5.3 Results

5.3.1 Colony size morphology

To determine whether there was variation among *E. coli* populations to diversify, we estimated the degree of diversity using variation in colony morphology. We detected variation in diversity that was explained by type of media ($F=4.92$, $p<0.0005$), but not ancestral marker state, i.e., ara+/- ($F=0.17$, $p<0.28$, Table 5.2, but see acetate evolved populations below). Interestingly, populations evolved in acetate alone had surprisingly high levels of diversity (Figure 5.1).

We grouped the data by environmental complexity, i.e., number of exogenously supplied resources (1=A, G, Y; 2= GA, YA, YG; or 3= GAY) to test whether number of resources could explain variation in diversity in colony area. There was significant variation in colony morphology variation with number of resources ($F=5.83$, $df=2$, $p<0.00476$, Figure 5.2). However, variation in colony morphology did not increase with environmental complexity. Rather, variation in colony morphology was depressed when the number of resources was 2, relative to the variation when the number of resources was 1 or 3.

Because colony size variation was high in single resource treatments, we decided to confirm that colony size actually conveyed information about ecological variation. Notably, because we did not anticipate colony size variation in acetate evolved populations, we conducted follow-up tests with several acetate-evolved populations to determine whether colony morphology variation in acetate reflected ecologically relevant diversity. Additionally, we have previously characterised how colony morphology variation correlates with growth curve parameters in populations that evolved in the glucose-acetate environment (Friesen et al., 2004, Tyerman et al., 2008).

First, we analysed growth curves for clones isolated from various acetate evolved populations. In acetate, there was continuous variation in many growth curve parameters (i.e., maximum growth rate, r_{max}; and maximum yield, OD_{max}), however there was not a clear correspondence between variation in these parameters and colony size variation (data not shown). However, when we assessed the growth curves of these clones in different media (i.e., a mixture of glucose and acetate, DM_{GA}), in many instances, we were able to discriminate clusters of clones based on parameters extracted from their growth curves, using colony size variation. For example, clones of similar class size (large or small) from one acetate population, dst1025, clustered with respect to their maximum growth rate in glucose (r_{max}) and maximum yield (OD_{max}) (Figure 5.3). The correspondence between colony size and growth parameters was not as clear-cut as the correspondence in other studies (Friesen et al., 2004, Tyerman et al., 2008). Further, while this correspondence was found in other acetate evolved populations, it was not found in all populations. Thus, colony size variation may reflect growth parameter variation in acetate populations, but not always in the same way.

Next, using two diversified populations evolved in acetate (dst1023 and dst1024), we conducted invasion competition experiments. We created pools of small or large colony clones, and mixed these pools in different starting proportions of large and small types. From extreme invasion conditions (i.e., each invading the other, from an initially rare frequency), we detected negative frequency-dependent fitness in both populations (Figure 5.4). This suggested that ecological interactions between large and small-sized class variants, and thus the frequency of large and small variants, determined the fitness of large and small clones.

5.3.2 Trade-offs: rate vs. yield

Within populations

If rate vs. yield trade-offs were operating within populations, then we would expect to see negative bivariate correlations between realized traits.

For nine of the populations evolved in acetate, we conducted a correlation analysis on the maximum growth rate on acetate (r_{max}) and the numerical yield (OD_{max}). We observed significant positive correlations for most data subsets in eight of nine populations (Figure 5.5, Table 5.3). In a single population (dst1029) we detected a negative correlation between rate and yield in the large colony subset, which was no longer significant when all the data was included in the analysis. In eight of nine populations where clear colony morphology variation was present (i.e., large and small colony size classes), there was no clear differentiation between large and small colony genotypes when viewed in rate-yield phenotype space. This suggested that colony morphology variation reflected diversification with respect to a suite of traits different from growth rate and yield.

For eight of eight glucose-evolved populations that we studied, we observed positive correlations in all data subsets with the exception of a single subset of large colony variants isolated from strain dst1004 (Figure 5.6, Table 5.4). This subset had a significant negative correlation. In one of eight populations (dst1005), colony morphology captured the natural clustering of the data in rate-yield space. In the remaining populations, colony morphology did not clearly discriminate between rate-yield strategies. As above, this indicated that variation in colony morphology was correlated with a suite of traits different from growth rate and yield.

We analysed four glycerol-evolved populations (Figure 5.7, Table 5.5). One population (dst1041) had not diversified with respect to colony morphology, and exhibited a positive correlation between rate and yield. The three remaining populations, which had diversified with respect to colony morphology, showed significant positive correlations in all data subsets.

Previously, we have reported that all ten glucose-acetate populations have diversified with respect to colony morphology (Tyerman et al., 2008). Here, we describe six of those ten populations. For three populations, there was no apparent

clustering in rate-yield space (dst1011, dst1012, dst1013; Figure 5.8, Table 5.6), although there was colony morphology variation. This suggests that diversification with respect to colony morphology was not correlated with rate-yield diversification. However, for the other three populations (dst1015, dst1017, dst1019), there were clear clusters in rate-yield space that correlated with colony morphology. Interestingly, we detected positive correlations between rate and yield in the first set of three populations, and several negative correlations between rate and yield in the second set of three populations. In strain dst1015, where colony morphology clearly discriminated between clones with different rate-yield strategies, there was a positive correlation within the large colony data subset and a negative correlation when all the data was included. This suggested that the major axis of variation within the large subset of clones (having a negative slope), was orthogonal to the axis of variation that discriminated between types (having a positive slope). Finally, in population dst1019, there were significant negative correlations with colony size subsets (large and small) and when all the data was considered. Thus, in contrast to the structure of variation in population dst1015, the major axis of variation within colony size classes (large and small, respectively) was oriented in a similarly direction as the major axis of variation that discriminated between large- and small-colony clones.

There was variation in colony morphology in four of six glucose-glycerol evolved populations, although these types overlapped in rate-yield space. In three of the populations, we detected significant positive correlations (Figure 5.9, Table 5.7).

We noted colony morphology variation in five of six populations evolved in glycerol-acetate (Figure 5.10). However, in population dst1031, which had not diversified, there was a positive correlation between rate and yield. In the diversified populations, there was some clustering in rate-yield space that appeared to be correlated with colony size. Interestingly, however, the pattern of clustering was not consistent across replicate populations. In three populations (dst1032, dst1034, dst1038), large colony types had relatively higher yields than small colony types, but in one population (dst1037), the pattern was reversed with the large colony types having lower yields than the small colony types. This suggested that colony variation may often evolve in a correlated manner with rate-yield strategy, but not always in a consistent fashion. There were significant positive correlations in sev-

eral colony-size subgroups in three populations and a single instance of a significant negative correlation within the small data subset in one population (Figure 5.10, Table 5.8).

Finally, in populations evolved in glucose-glycerol-acetate, we observed colony morphology variation in five of six populations (Figure 5.11, Table 5.9). We observed positive correlations between rate and yield in four of these populations. For the five populations that had diversified with respect to colony size, we noted that colony-size variants did tend to cluster in rate-yield space in four of the populations, with large types having higher rates or yields (or both) in these four populations.

Between populations

We used the mean rate and mean yield for each population, or subpopulation based on colony size, to assess whether we could detect trade-offs between rate and yield among populations (Figure 5.12, Table 5.10). We noted a positive correlation between rate and yield in populations evolved in glycerol (Figure 5.12c), but no significant relationships were identified among populations evolved in the other types of media.

5.4 Discussion

Trade-offs are generally believed to be important for understanding the evolution of ecologically relevant diversity (Roff and Fairbairn, 2007). Using evolved populations of *E. coli*, we looked for evidence of resource specialization trade-offs by testing whether evolved diversity varied directly with degree of environmental complexity (i.e, number of resources). We found no direct relationship between environmental complexity and diversity. These findings suggest that even in simple experimental systems having few resources, that diversity does not directly scale with the complexity of the environment.

Because we found high levels of colony size variation within single resource populations, where we thought variation would be minimal, we selected several populations evolved in acetate to confirm that colony size variation reflected ecologically relevant variation. Most compelling, invasion competition experiments

between large and small colony clones revealed the operation of negative frequency-dependent selection in both acetate evolved populations that we tested. Additionally, colony size variation predicted clusters in rate-yield space, suggesting that colony size variation in the tested populations was under the influence of selection, and not simply neutral.

Next, we tested whether trade-offs between growth rate and yield was involved in the origin and maintenance of diversity in our experimental populations. In contrast to the expectation of negative bivariate correlations between rate and yield, we found positive correlations within and among most populations. While our data does not obviate a role for trade-offs in resource specialization in adaptive diversification, it does question the general belief that levels of diversity in evolved populations of bacteria can be predicted by the complexity of the environment manipulated in the experiment. In general, we found little support for the simple predictions of resource specialization theory in predicting levels of diversity in our experimental populations.

5.4.1 Colony morphology variation and resource specialization

A diversity of resources may provide a diversity of niches for organisms to exploit. This hypothesis is an extension of Gause's Law which states that two species competing for the same resource cannot coexist (Hardin, 1960). If resources are substitutable, and metabolic trade-offs exist where increased performance on one resource only comes with diminished performance on others, then we would expect environments with higher resource diversity to contain higher levels of biological diversity. We evolved populations of *E. coli* in different resource environments, from simple to complex, in order to test this intuition in the lab.

While we did detect variation in colony morphology variation across environments, it was not predicted by degree of environmental complexity provided by the experimental design. Specifically, populations evolved in environments with a single resource had levels of diversity as high as populations evolved in environments with multiple resources. One explanation for this result is that the metabolic activity of some bacteria provides new opportunities (i.e., resources) for others to exploit (e.g., "cross-feeding"). That diversity may not be easily predicted from the

number of resources (i.e., niches) provided by the experimenter has been noted by others (Maharjan et al., 2006, 2007). Facilitation or cross-feeding may operate in glucose or glycerol environments (Saxer et al., 2009, Rosenzweig et al., 1994) to explain the high levels of phenotypic diversity. Yet, interestingly, populations evolved on solely acetate, where metabolic byproducts are not expected, also had high levels of diversity. This suggested that opportunities for diversification beyond metabolic facilitation may be involved in driving and supporting adaptive diversification. At this time, we do not understand why or how acetate-evolved populations diversified. One possible avenue for diversification that is unrelated to cross-feeding may involve trade-offs between growth rate and growth yield within cultures (see below).

Likely, variation in colony morphology underestimates the total phenotypic variation within our populations (Tyerman et al., 2005). As our follow-up tests of acetate evolved populations (in glucose-acetate environments) confirm, however, this underestimate does not mean that colony size variation is irrelevant. We acknowledge that variation in colony morphology may quickly saturate with increasing number of resources and thus be inadequate for quantifying diversity when environments are complex. Although colony morphology has been used extensively to define ecological variants in other experimental systems (Rainey and Travisano, 1998, Friesen et al., 2004, Tyerman et al., 2005), our results suggest that caution should be taken when using colony size variation as an overall quantitative measure of diversity. If colony size variation is not an adequate indicator of ecologically relevant variation, then our study, which finds no clear relationship between colony-size variation and environmental complexity, would not be a definitive test of the hypothesis about ecologically relevant variation and environmental complexity.

Another factor that may have impacted our study was the resource supply rate in our evolutionary environments. Hall and Colegrave (2007) found that supply rates, in addition to environmental complexity, regulated diversity in a bacterial system. Specifically, the authors found that diversity in *Pseudomonas fluorescens* peaked at intermediate resource supply rates for several different resources. While we controlled for productivity in our experiment, we did not vary overall supply rates. As the supply rates in our experiment were high (see Methods), the bacteria in our experiment may not in fact have been limited by the availability of carbon,

but perhaps by other factors, e.g., electron acceptors (oxygen) or nitrogen or phosphorous. Varying the supply rate in our complexity treatments may have altered the diversity in our system, and thus revealed a role for trade-offs involved in resource specialization at other supply rates. However, at the supply rates of carbon resources in our experimental system, we were unable to detect a role for trade-offs and environmental complexity affecting degree of diversification.

5.4.2 Trade-offs: rate vs. yield

Metabolism may be constrained by a fundamental trade-off between maximum growth rate and yield (Novak et al., 2006, Helling, 2002). There is little support for this hypothesis in our dataset, which included 45 evolved populations from seven environments. We evaluated rate-yield trade-offs within and between populations of *E. coli* evolved in environments with a single resource, or mixtures of two or three resources. While we occasionally observed a negative correlation between rate and yield, we generally failed to detect a relationship or in fact found positive relationships between r_{max} and OD_{max} (yield). Again, our data do not generally refute a role for rate-yield trade-offs during the course of adaptive evolution and diversification, but do refute the hypothesis that rate-yield trade-offs underlie adaptive diversity within our evolved populations of *E. coli*.

Others have studied rate-yield trade-offs using experimental evolution (Novak et al., 2006, Maharjan et al., 2007). The work of Novak et al. (2006) is perhaps the best comparison for our study because we used the same ancestral strains, yet observed contrasting results. Like our study, Novak et al. failed to detect trade-offs between rate and yield (i.e., a negative correlation) using a between-population analysis. However, in contrast to our study, Novak et al. observed negative correlations between rate and yield using within population analyses in three of four populations assessed. Additionally, in the fourth population studied, which had diversified, they observed negative correlations within type, but not across types. As noted, we almost never detected evidence for rate-yield trade-offs within our study populations. There are several explanations for this discrepancy. First, Novak et al. evolved strains of *E. coli* for 20,000 generations while our strains were evolved for only 1,000 generations. Novak et al. note that in order to detect trade-

offs, the populations in question must have evolved for a "sufficiently long time." Thus, a census of phenotypes taken too early may catch an evolving population still far removed from the trade-off curve, where mutations that confer advantages to both rate and yield improvements remain possible (Novak et al., 2006). On the other hand, we note that populations that have evolved for "too long" may have evolved compensatory mutations that act to erode genetic constraints that cause the trade-offs and may be present when the experiment is initiated. Finally, because we sought an explanation for the diversity in our evolved populations, we were not testing whether rate-yield trade-offs exist, but only whether they explain the diversity we already know to exist.

Next, Novak et al. only considered populations evolved on a single resource (glucose). We postulate that rate vs. yield trade-offs are most likely to be detected in this simply one resource situation, as complex environments, having multiple resources, may complicate the analysis because there are multiple dimensions available for trade-offs to arise and potentially cause adaptive diversification that is not related to growth rate and yield (e.g., transition lags between consuming subsequent resources, e.g., diauxie, (Friesen et al., 2004)). Three of our treatments, however, entailed a single resource, including the same resource studied by Novak et al, so environmental complexity does not fully explain why we did not see the rate-yield trade-offs detected by Novak et al. In particular, for glucose-evolved populations in this study, we did not find the negative correlations between clones within populations that were found by Novak et al. We suspect that the difference in resource concentrations used by Novak et al. and in our study may contribute to our contrasting results. To illustrate, the concentration of glucose was ten-fold greater in our experiment. Higher concentrations may ultimately result in greater opportunity for metabolic byproducts to accumulate to biologically meaningful levels and thus provide opportunities for subsequent adaptation and diversification, via facilitation or cross-feeding (Saxer et al., 2009). This may marginalize the role of rate vs. yield trade-offs in our study system.

Previously, using populations of *E. coli* that diversified in a glucose-acetate environment, we have identified and described a trade-off between fast rate of growth on glucose and the time to switch to consuming acetate (Friesen et al., 2004, Tyerman et al., 2005, 2008). These studies suggest two caveats to the approach taken

in this study. First, in the previous studies, we relied on a trade-off involving a demographic trait (i.e., lag_{ace}) that we did not consider in this study. This was because intermediate lag phases were not observed across all replicate populations. For three glucose-acetate populations (one of which is included in this study), we found a high correlation between lag_{ace} and OD_{max} (data not shown), however the correlation could not be calculated for populations growing in other types of media that did not have an intermediate lag trait, i.e., lag_{ace}. Presumably, we could measure the lag_{ace} for clones in a common glucose-acetate environment. This would allow us to look for alternative trade-offs that may be operating within our evolved populations.

Presumably, other demographic traits (i.e., initial lag, or survival in stationary phase) may be traded off against growth rate or yield, just as lag_{ace} was traded off against growth rate (Tyerman et al., 2008). The form of the trade-off may be specific for each type of resource and may require in-depth study in that resource environment. Second, the trade-off between glucose and acetate use was detected in competition experiments between the glucose specialist and the acetate fast-switching specialist (Tyerman et al., 2008). The growth rate and yield in this study, as in many studies, was measured for each clone in isolation from its population. If trade-offs are more pronounced in the presence of competitors, or are partially contingent on the presence of competitors, then our failure to detect them in this study may not reflect their importance to the underlying adaptive diversity. Developing marked types within populations would allow for the characterization of demographic traits (rate and yield) when they are cultured in the presence of other genotypes from their native population.

5.5 Conclusions

Trade offs are believed to be of fundamental importance in explaining biological diversity. We tested the hypothesis that number of resources would drive diversification in *E. coli* and thus result in higher levels of diversity. Instead, we found that diversity was high for single resource and three resource environments, and lowest for two-resource environments. We confirmed that the colony size diversity within populations evolved in acetate – a simple, single resource environment – was eco-

logically relevant. Our results suggest that trade-offs between exogenous resource exploitation strategies may not explain adaptive diversification in our experimental populations. We also tested our populations for the presence of a growth rate (r_{max}) vs. yield (OD_{max}) trade-off. We did not find the predicted negative correlation between rate and yield. In general, we found no relationship or a positive relationship between rate and yield. These data suggest that rate-yield trade-offs may not be an important factor during the evolution of adaptive diversity in our experimental populations.

5.6 Acknowledgements

We wish to thank M. Bertrand, M. Chiu, G. Khaira, and R. Suprin for lab assistance, and D. Ally, R. Fitzjohn, and C. Jordan for statistical assistance. Members of the Doebeli and SOWD research groups provided useful feedback during discussions. Financial assistance was provided by NSERC and the James F. McDonnell Foundation to M.D.

Table 5.1: Summary of types and concentrations of carbon sources added to Davis Minimal media in the 1,000 generation evolution experiment.

		Carbon Source (μg/mL)		
Populations	Media Abbrev.	Glucose	Acetate	Glycerol
1-10	G	500	-	-
11-20	GA	250	575	-
21-30	A	-	1150	-
31-40	YA	-	575	200
41-50	Y	-	-	400
51-60	YG	250	-	200
61-70	GAY	166.7	383.3	133.3

Table 5.2: Summary of analysis of variance conducted on variation in colony size among populations in evolution experiment.

Source of variation	Df	Sum Sq	Mean Sq	F	Pr(>F)
Media	6	13.83	2.31	4.93	**0.0005**
Ancestor (Ara+/-)	1	0.55	0.55	1.17	0.28
Media x Anc (Ara+/-)	6	3.11	0.52	1.11	0.37
Residuals	52	24.33	0.47		

Table 5.3: Summary of correlation analyses between growth rate (r_{max}) and yield (OD_{max}) conducted on clones isolated from acetate populations.

Strain	Colony size subset	r	DF	p-value
dst1021	All	0.66	82	<0.0001
	Large	0.68	43	<0.0001
	Small	0.49	37	0.0017
dst1022	All	0.54	93	<0.0001
	Large	0.75	18	0.0002
	Small	0.47	73	<0.0001
dst1023	All	0.57	83	<0.0001
	Large	0.62	65	<0.0001
	Small	0.32	20	0.1463
dst1024	All	0.56	94	<0.0001
	Large	0.53	45	0.0002
	Small	0.73	47	<0.0001
dst1025	All	0.58	64	<0.0001
	Large	0.20	24	0.3185
	Small	0.73	38	<0.0001
dst1026	All	0.38	91	0.0002
	Large	-	-	-
	Small	0.48	88	<0.0001
dst1028	All	0.59	68	<0.0001
	Large	0.89	30	<0.0001
	Small	0.23	36	0.1703
dst1029	All	-0.35	28	0.0582
	Large	-0.67	13	0.0060
	Small	0.33	13	0.2233
dst1030	All	0.63	28	0.0003
	Large	0.90	5	0.0063
	Small	0.43	21	0.0433

Table 5.4: Summary of correlation analyses between growth rate (r_{max}) and yield (OD_{max}) conducted on clones isolated from glucose populations.

Strain	Colony size subset	r	DF	p-value
dst1001	All	0.79	46	<0.0001
	Large	0.80	26	<0.0001
	Small	0.78	23	<0.0001
dst1002	All	0.83	48	<0.0001
	Large	0.87	23	<0.0001
	Small	0.76	23	<0.0001
dst1003	All	0.97	46	<0.0001
	Large	0.96	18	<0.0001
	Small	0.98	26	<0.0001
dst1004	All	0.14	48	0.3303
	Large	-0.42	23	0.0352
	Small	0.73	23	<0.0001
dst1005	All	0.81	48	<0.0001
	Large	0.65	23	0.0005
	Small	0.56	23	0.0033
dst1007	All	0.84	46	<0.0001
	Large	0.84	21	<0.0001
	Small	0.87	23	<0.0001
dst1008	All	0.84	48	<0.0001
	Large	0.90	23	<0.0001
	Small	0.77	23	<0.0001
dst1009	All	0.96	45	<0.0001
	Large	0.96	32	<0.0001
	Small	0.97	11	<0.0001
dst1010	All	0.37	48	0.0091
	Large	0.78	16	0.0002
	Small	0.33	30	0.0679

Table 5.5: Summary of correlation analyses between growth rate (r_{max}) and yield (OD_{max}) conducted on clones isolated from glycerol populations.

Strain	Colony size subset	r	DF	p-value
dst1041	All	0.56	28	0.0014
	Large	-	-	-
	Small	-	-	-
dst1044	All	0.65	28	0.0002
	Large	0.47	10	0.1236
	Small	0.81	16	<0.0001
dst1048	All	0.53	28	0.0024
	Large	0.67	8	0.0324
	Small	0.46	18	0.0420
dst1049	All	0.76	28	<0.0001
	Large	0.88	5	0.0084
	Small	0.49	21	0.0180

Table 5.6: Summary of correlation analyses between growth rate (r_{max}) and yield (OD_{max}) conducted on clones isolated from glucose-acetate populations.

Strain	Colony size subset	r	DF	p-value
dst1011	All	0.47	71	<0.0001
	Large	0.17	42	0.2591
	Small	0.77	27	<0.0001
dst1012	All	0.44	94	<0.0001
	Large	0.42	71	0.0003
	Small	0.38	21	0.0751
dst1013	All	0.40	86	0.0002
	Large	0.31	50	0.0231
	Small	0.49	34	0.0023
dst1015	All	-0.55	94	<0.0001
	Large	0.52	44	0.0002
	Small	-0.11	48	0.4558
dst1017	All	-0.03	94	0.757
	Large	-0.13	44	0.3787
	Small	0.40	44	0.0056
dst1019	All	-0.26	132	<0.0001
	Large	0.64	67	0.0078
	Small	-0.18	63	0.0152

Table 5.7: Summary of correlation analyses between growth rate (r_{max}) and yield (OD_{max}) conducted on clones isolated from acetate-glycerol populations.

Strain	Colony size subset	r	DF	p-value
dst1031	All	0.46	28	0.0107
	Large	-	-	-
	Small	-	-	-
dst1032	All	0.48	28	0.0070
	Large	0.42	13	0.1196
	Small	0.74	13	0.0016
dst1033	All	0.36	28	0.0536
	Large	0.28	8	0.4307
	Small	0.53	18	0.0168
dst1034	All	0.25	27	0.1907
	Large	-0.34	8	0.3237
	Small	-0.004	17	0.9866
dst1037	All	-0.33	28	0.0791
	Large	0.08	18	0.7311
	Small	-0.34	8	0.3301
dst1038	All	-0.28	28	0.1285
	Large	0.84	8	0.0025
	Small	-0.47	18	0.0377

Table 5.8: Summary of correlation analyses between growth rate (r_{max}) and yield (OD_{max}) conducted on clones isolated from glucose-glycerol populations.

Strain	Colony size subset	r	DF	p-value
dst1051	All	0.51	28	0.0038
	Large	-	-	-
	Small	-	-	-
dst1052	All	0.36	28	0.0529
	Large	-		-
	Small	-		-
dst1053	All	0.0064	28	0.9734
	Large	0.14	8	0.7031
	Small	0.084	18	0.7246
dst1054	All	0.79	28	<0.0001
	Large	0.81	8	0.0044
	Small	0.87	18	<0.0001
dst1057	All	0.28	28	0.1351
	Large	-0.30	13	0.2820
	Small	0.48	13	0.0682
dst1058	All	0.70	28	<0.0001
	Large	0.88	13	<0.0001
	Small	0.21	13	0.4626

Table 5.9: Summary of correlation analyses between growth rate (r_{max}) and yield (OD_{max}) conducted on clones isolated from glucose-acetate-glycerol populations.

Strain	Colony size subset	r	DF	p-value
dst1061	All	0.44	28	0.0145
	Large	0.55	13	0.0341
	Small	0.11	13	0.708
dst1062	All	0.36	28	0.0221
	Large	-	-	-
	Small	-	-	-
dst1064	All	0.24	28	0.1926
	Large	0.28	13	0.3048
	Small	0.47	13	0.0787
dst1065	All	0.14	28	0.4759
	Large	-0.38	13	0.1598
	Small	0.06	13	0.8244
dst1066	All	0.41	28	0.0249
	Large	0.40	13	0.1346
	Small	0.48	13	0.0686
dst1067	All	0.62	28	0.0003
	Large	0.72	13	0.0027
	Small	0.64	13	0.0102

Table 5.10: Summary of between-population correlation analyses, between mean growth rates (r_{max}) and mean yields (OD_{max}).

Media	r	DF	p-value
Acetate (A)	0.025	16	0.923
Glucose (G)	0.38	14	0.1521
Glycerol (Y)	0.95	5	0.0012
Glucose-acetate (GA)	0.39	10	0.2143
Glucose-gycerol (GY)	0.60	8	0.0666
Acetate-glycerol (AY)	0.02	10	0.9494
Glucose-acetate-glycerol (GAY)	0.55	10	0.0638

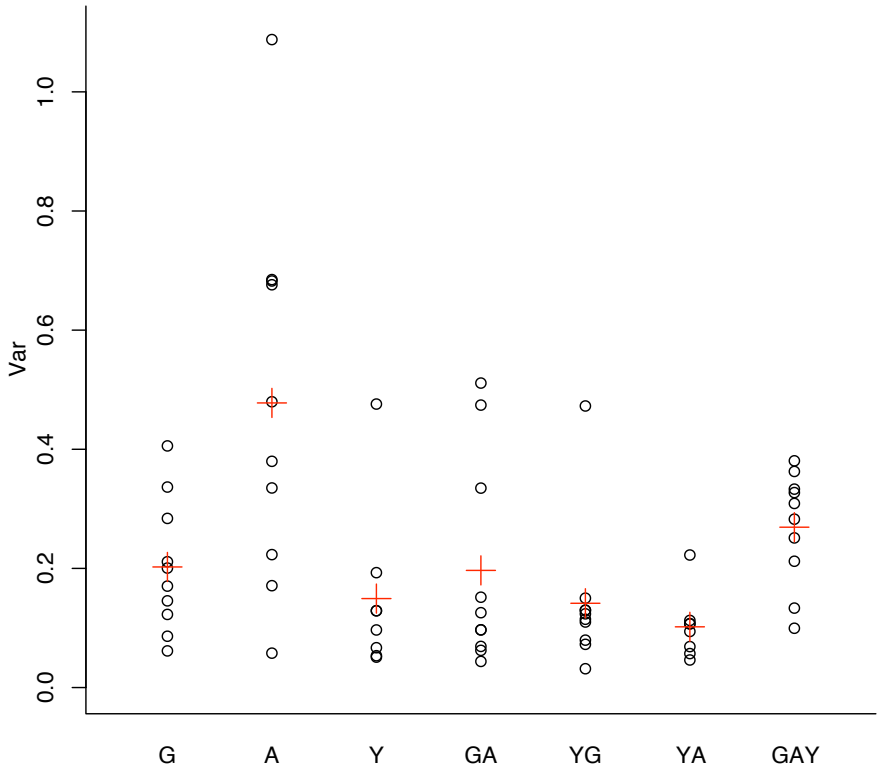

Figure 5.1: Variance in colony morphology (area) for populations evolved in different media (G=glucose, A=acetate, Y=glycerol). A red cross indicates mean variance for a given treatment.

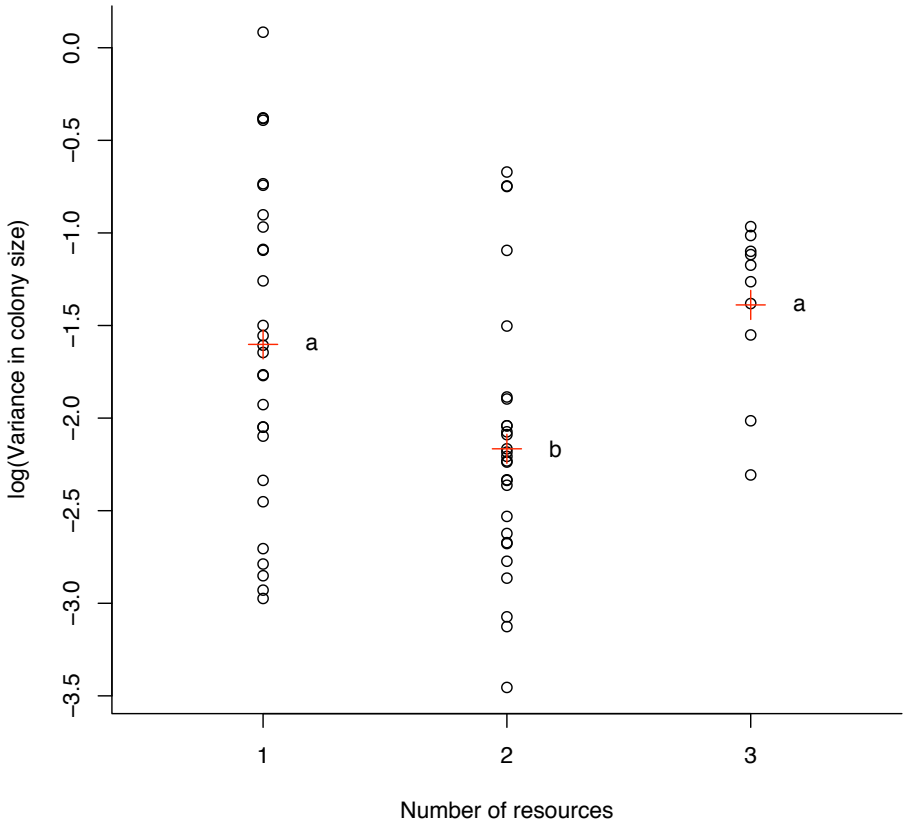

Figure 5.2: Variance in colony morphology (area) for populations supplemented with 1, 2 or 3 resources (Glucose, acetate and/or glycerol). A red cross indicates mean variance for a given treatment, and identical letters beside crosses indicate treatments having means that are not significantly different from each other.

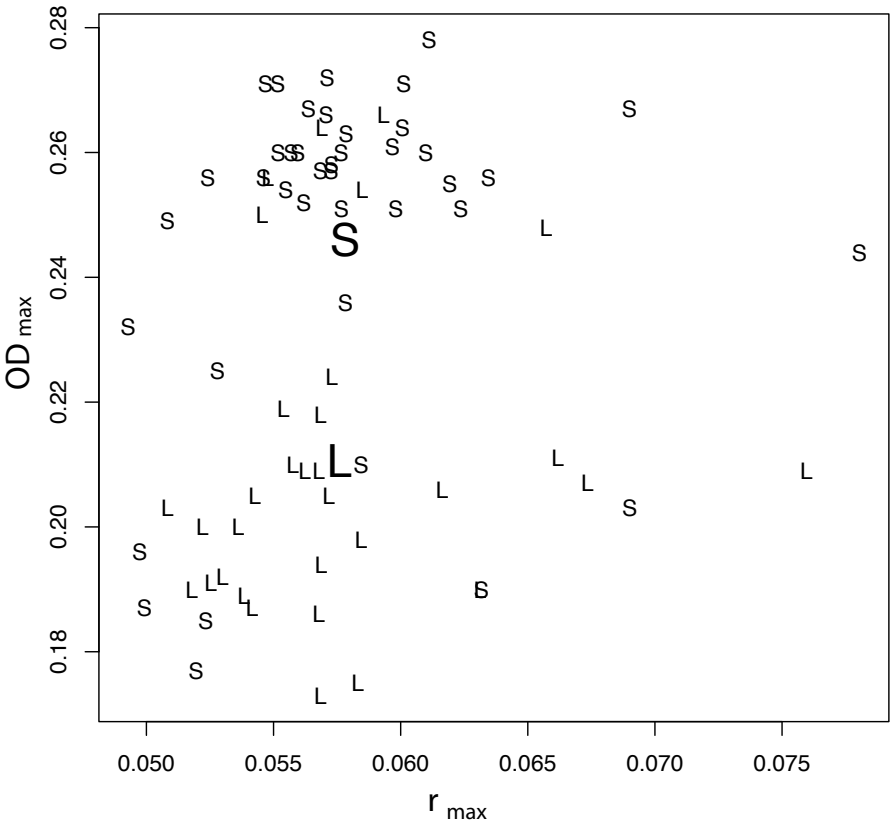

Figure 5.3: Population dst1025, evolved in acetate, diversified into large (L) and small (S) colony types, which can discriminate between two clusters in r_{max} vs. OD_{max} space. These data were extracted from growth curves from individual clones grown in DM_{GA}. The large letters indicate the means of both clusters.

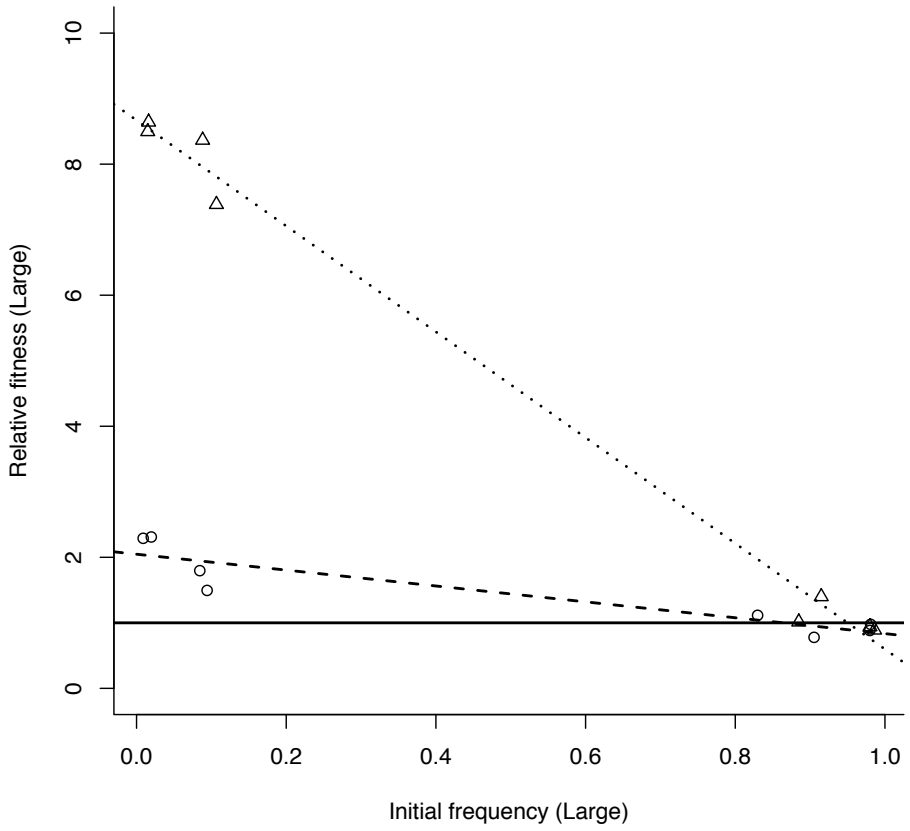

Figure 5.4: Invasion experiments between large and small colony ecotypes in two populations (dst1023, dashed line, fitness(L) = 2.0 - 1.21(initial freq(large)), P-value for slope = 0.001; dst1024, dotted line, fitness(L) = 8.7 - 8.1 (initial freq(large)), P-value for slope <0.0001) indicate that fitness is negative frequency-dependent. The solid horizontal line at Y=1 shows equivalent relative fitness between large and small ecotypes.

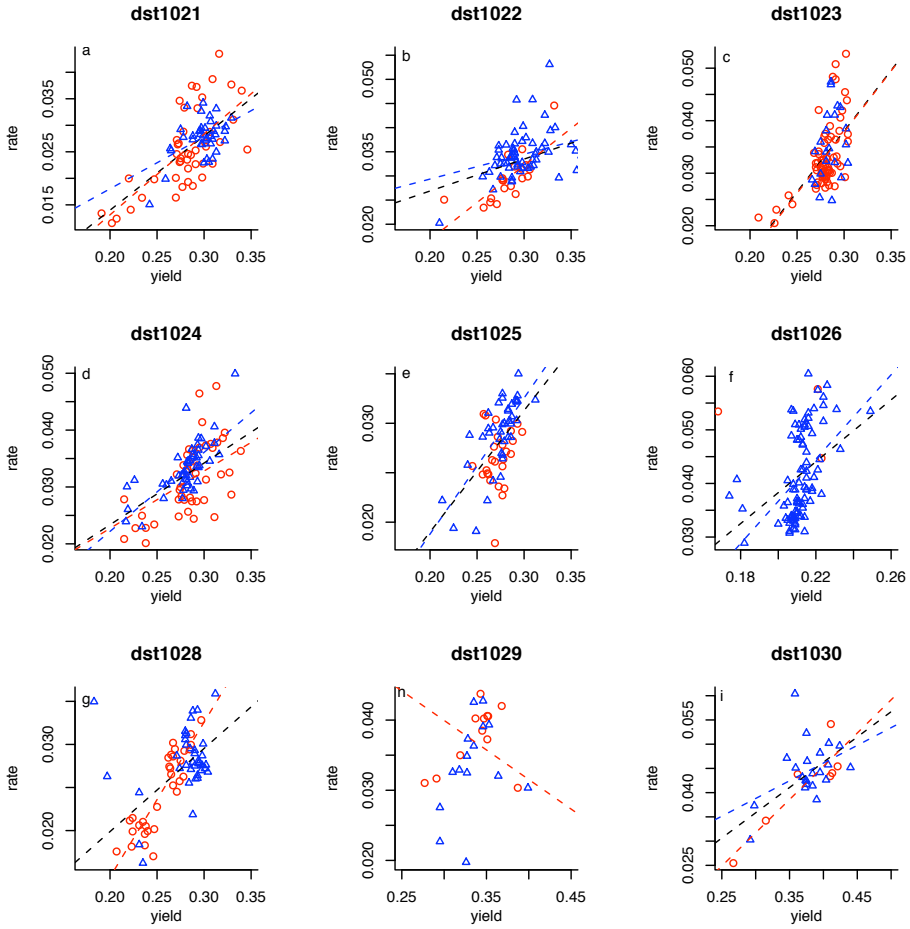

Figure 5.5: Correlation analysis for maximum growth rate (h^{-1}) and yield (optical density units) for clones isolated from nine populations (a-h) evolved in acetate batch culture for 1,000 generations. Red circles and blue triangles are clones having large and small colony morphologies, respectively. Dashed lines indicate significant correlations (red: large colony clones, blue: small colony clones).

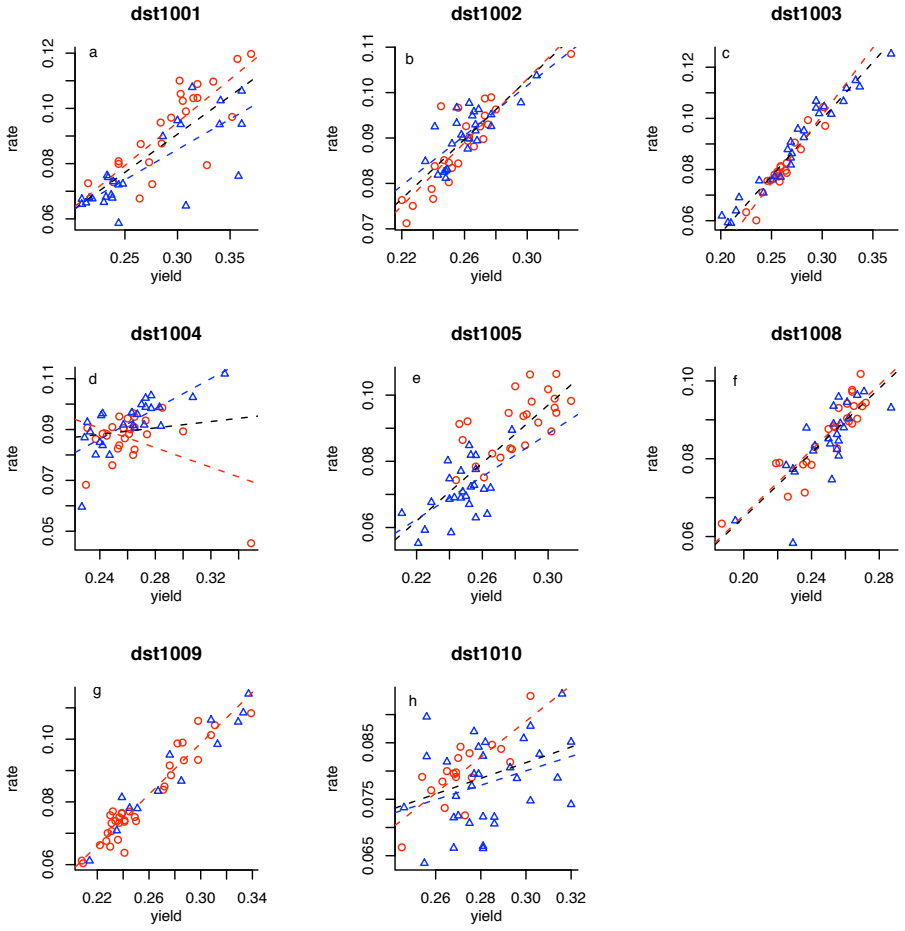

Figure 5.6: Correlation analysis for maximum growth rate (h^{-1}) and yield (optical density units) for clones isolated from nine populations (a-h) evolved in glucose batch culture for 1,000 generations. Symbols as in Figure 5.5.

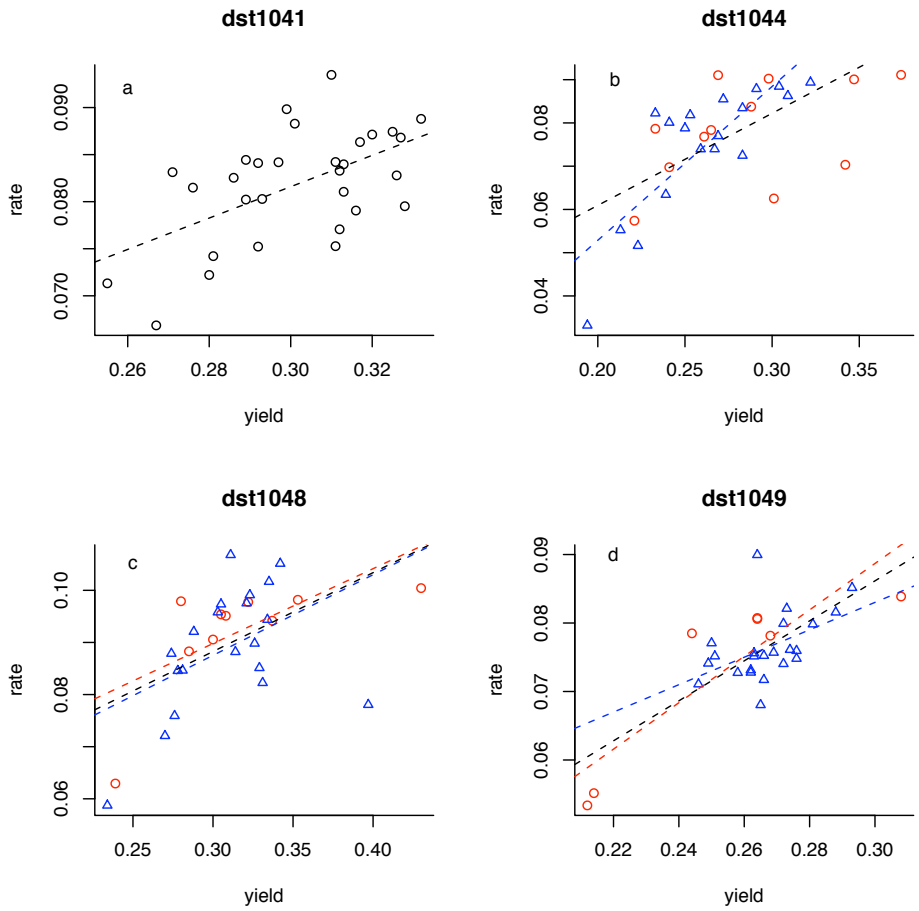

Figure 5.7: Correlation analysis for maximum growth rate (h^{-1}) and yield (optical density units) for clones isolated from nine populations (a-h) evolved in glycerol batch culture for 1,000 generations. Symbols as in Figure 5.5, and black circles indicate populations undiversified with respect to colony size.

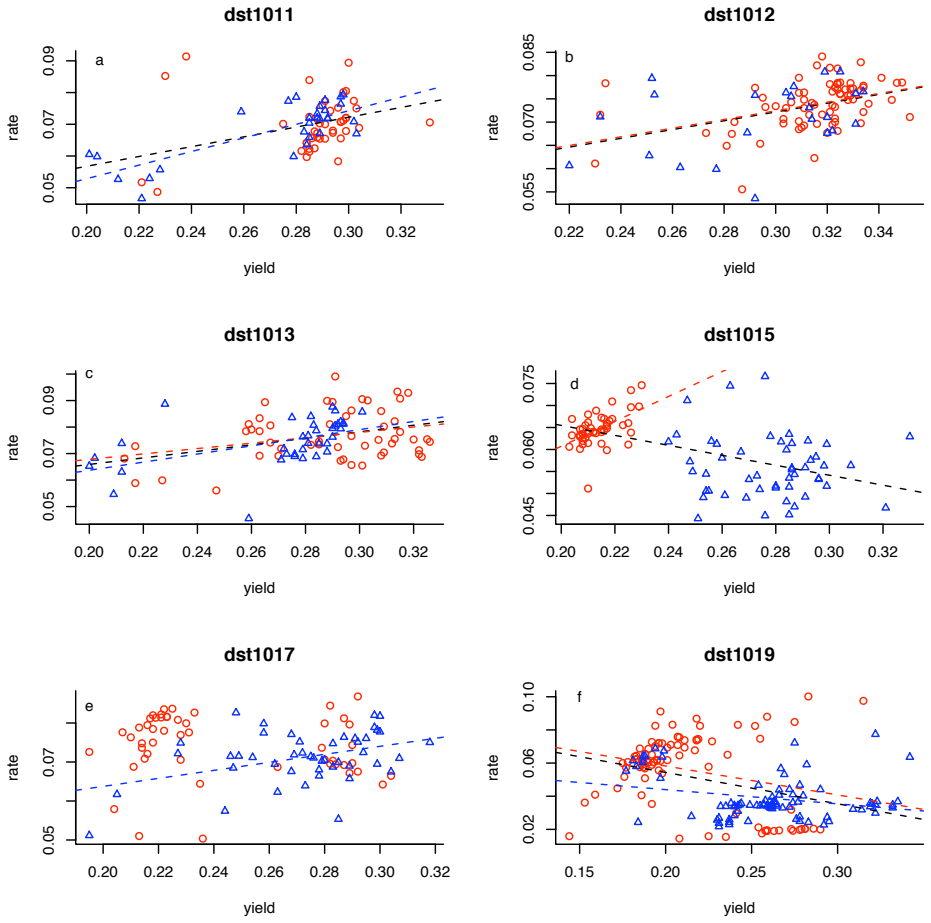

Figure 5.8: Correlation analysis for maximum growth rate (h^{-1}) and yield (optical density units) for clones isolated from nine populations (a-h) evolved in glucose-acetate batch culture for 1,000 generations. Symbols as in Figure 5.7.

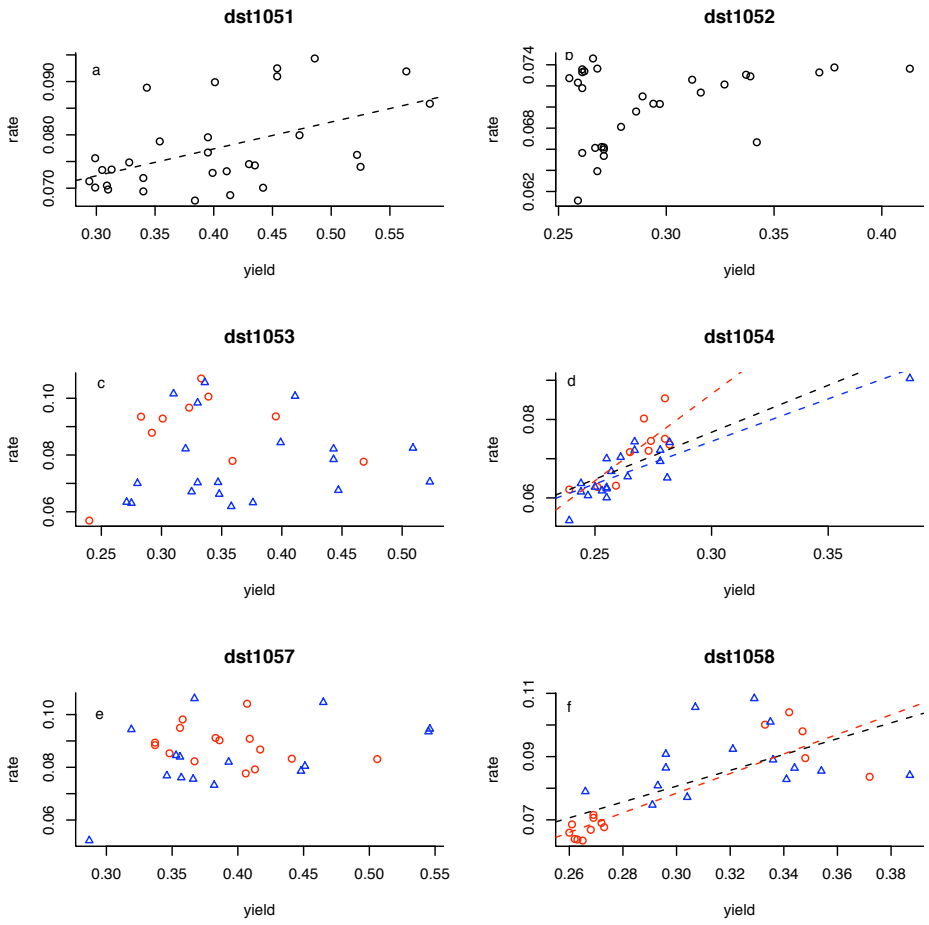

Figure 5.9: Correlation analysis for maximum growth rate (h^{-1}) and yield (optical density units) for clones isolated from nine populations (a-h) evolved in glucose-glycerol batch culture for 1,000 generations. Symbols as in Figure 5.7.

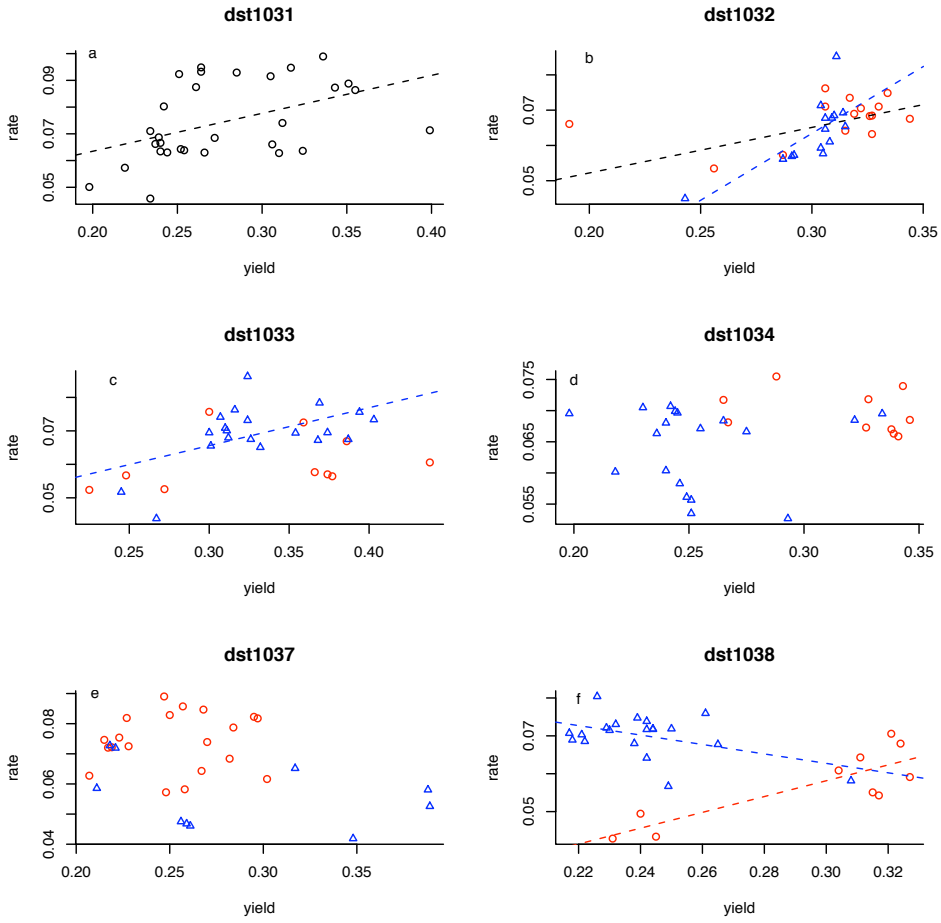

Figure 5.10: Correlation analysis for maximum growth rate (h^{-1}) and yield (optical density units) for clones isolated from nine populations (a-h) evolved in glycerol-acetate batch culture for 1,000 generations. Symbols as in Figure 5.7.

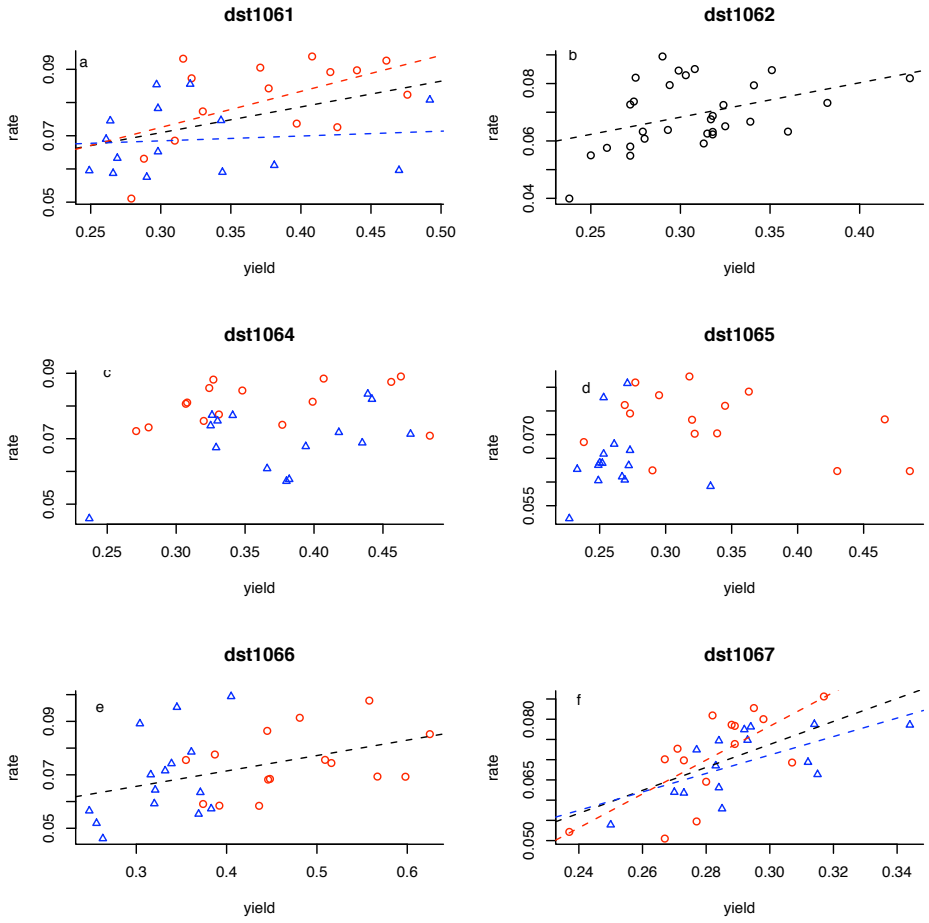

Figure 5.11: Correlation analysis for maximum growth rate (h^{-1}) and yield (optical density units) for clones isolated from nine populations (a-h) evolved in glucose-glycerol-acetate batch culture for 1,000 generations. Symbols as in Figure 5.7.

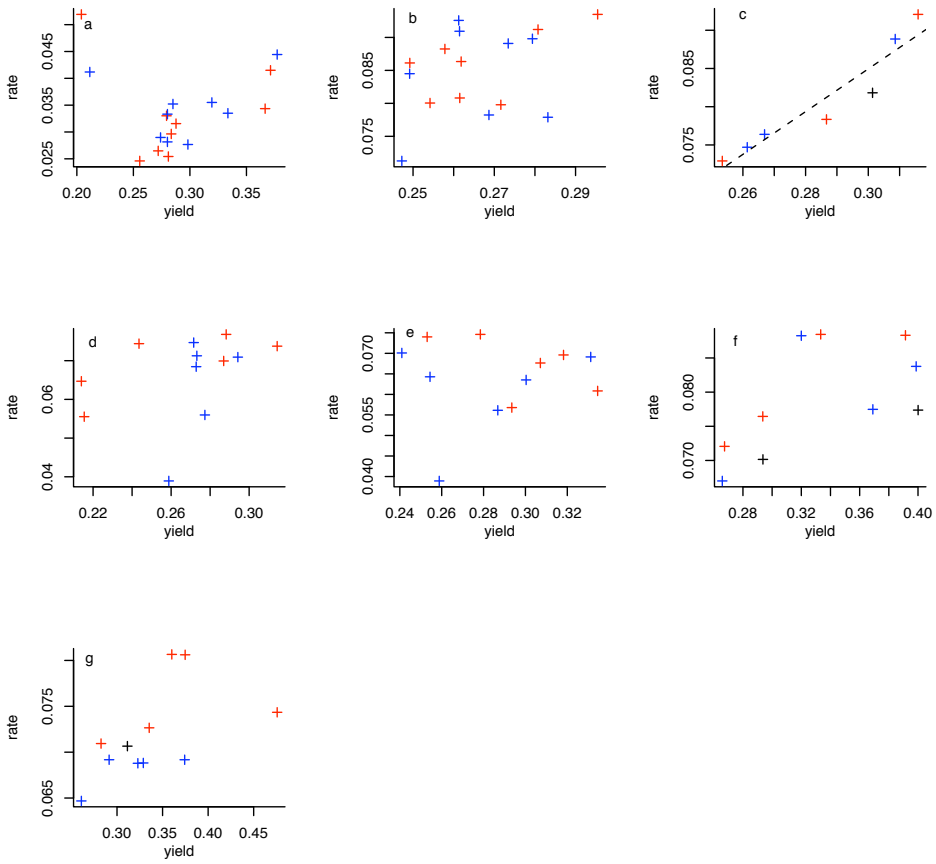

Figure 5.12: Between population correlation analysis for maximum growth rate (h^{-1}) and yield (optical density units) for populations evolved in a) acetate, b) glucose, c) glycerol, d) glucose-acetate, e) glucose-glycerol, f) glycerol-acetate, and g) glucose-acetate-glycerol. Red, blue and black crosses indicate mean values for clones with large colony morphologies, small colony morphologies, or undifferentiated colony morphologies respectively. Dashed lines indicate significant correlations.

Bibliography

Abramoff, M., Magelhaues, P., and Ram, S. (2004). Image processing with imagej. *Biophotonics International*, 11:36–42. http://rsb.info.nih.gov/ij/.

Barrett, R. D. H. and Bell, G. (2006). The dynamics of diversification in evolving pseudomonas populations. *Evolution*, 60(3):484–490.

Barrett, R. D. H., MacLean, R. C., and Bell, G. (2005). Experimental evolution of Pseudomonas fluorescens in simple and complex environments. *Am Nat*, 166(4):470–480.

Friesen, M., Saxer, G., Travisano, M., and Doebeli, M. (2004). Experimental evidence for sympatric ecological diversification due to frequency dependent competition in *Escherichia coli*. *Evolution*, 58:245–60.

Hall, A. R. and Colegrave, N. (2007). How does resource supply affect evolutionary diversification? *Proc R Soc Lond.B*, 274:73–78.

Hardin, G. (1960). The competitive exclusion principle. *Science*, 131:1292–1297.

Helling, R. B. (2002). Speed versus efficiency in microbial growth and the role of parallel pathways. *J.Bacteriol.*, 184(4):1041–1045.

King, T., Ishihama, A., Kori, A., and Ferenci, T. (2004). A Regulatory Trade-Off as a Source of Strain Variation in the Species Escherichia coli. *J. Bacteriol.*, 186(17):5614–5620.

Lenski, R., Rose, M., Simpson, S., and Tadler, S. (1991). Long-term experimental evolution in *Escherichia coli*. I. Adaptation and divergence during 2,000 generations. *Am Nat*, 138:1315–1341.

MacLean, R. C. (2005a). Adaptive radiation in microbial microcosms. *J.Evol Biol*, 18:1376–1386.

MacLean, R. C. (2005b). The decline and fall of a metabolic pathway in yeast. *Heredity*, 94:278–279.

MacLean, R. C. and Bell, G. (2002). Experimental adaptive radiation in pseudomonas. *Am Nat*, 160(5):569–581.

MacLean, R. C., Bell, G., and Rainey, P. (2004). The evolution of a pleitropic fitness tradeoff in Pseudomonas fluorescens. *PNAS*, 101:8072–8077.

Maharjan, R., Seeto, S., Notley-McRobb, L., and Ferenci, T. (2006). Clonal adaptive radiation in a constant environment. *Science*, 313:514–517.

Maharjan, R. P., Seeto, S., and Ferenci, T. (2007). Divergence and redundancy of transport and metabolic rate-yield strategies in a single Escherichia coli population. *The Journal of Bacteriology*, 189(6):2350–2358.

Novak, M., Pfeiffer, T., Lenski, R., Sauer, U., and Bonhoeffer, S. (2006). Experimental tests for an evolutionary trade-off between growth rate and yield in e. coli. *Am Nat*, 168:242–251.

Pfeiffer, T., Schuster, S., and Bonhoeffer, S. (2001). Cooperation and competition in the evolution of atp-producing pathways 10.1126/science.1058079. *Science*, 292(5516):504–507.

R Development Core Team (2008). *R: A Language and Environment for Statistical Computing*. R Foundation for Statistical Computing, Vienna, Austria. ISBN 3-900051-07-0.

Rainey, P. and Travisano, M. (1998). Adaptive radiation in a heterogeneous environment. *Nature*, 394:69–72.

Roff, D. and Fairbairn, D. (2007). The evolution of trade-offs: where are we? *Journal of Evolutionary Biology*, 20:443–447.

Rosenzweig, R., Sharp, R., Treves, D., and Adams, J. (1994). Microbial evolution in a simple unstructured environment: Genetic differentiation in *Escherichia coli*. *Genetics*, 137:903–917.

Saxer, G., Doebeli, M., and Travisano, M. (2009). Spatial structure leads to ecological breakdown and loss of diversity. *Proc Roy Soc B*, 276(1664):2065–2070.

Schluter, D. (2000). *The ecology of adaptive radiation*. Oxford University Press, Oxford.

Tyerman, J., Bertrand, M., Spencer, C., and Doebeli, M. (2008). Experimental demonstration of ecological character displacement. *BMC Evol Biol*, 8(1):34.

Tyerman, J. G., Havard, N., Saxer, G., Travisano, M., and Doebeli, M. (2005). Unparallel diversification in bacterial microcosms. *Proc R Soc B*, 272:1393–1398.

Chapter 6

Conclusion

What processes cause populations to diversify? And are these processes responsible for the maintenance of diversity within populations? These questions set the stage for the specific questions and ideas explored in this thesis. In general, I endeavored to test whether and how natural selection caused populations of the bacterium *Escherichia coli* to diversify during evolution in the lab. Specifically, I asked whether adaptive diversification was repeatable across replicate populations (Chapter 2), whether competition for resources resulted in ecological character displacement (Chapter 3), whether variation in intrinsic factors – namely the propensity to diversify – could result in asymmetrical patterns of diversity during adaptive radiation (Chapter 4), and finally, whether I could detect trade-offs in resource specialization within and among replicate experimental populations (Chapter 5). Together, these chapters contribute to understanding adaptive diversification in the lab, and, perhaps by extension, how adaptive diversification may contribute to the origin and maintenance of diversity in nature. Below, I summarize my findings.

In Chapter 2, I asked whether ecotypes evolved in experimental populations and appearing convergent in colony morphology and diauxic growth profile were also convergent in terms of competitive ability. Essentially, I used competitive ability against an alternative competitor to the co-evolved competitor as a correlated response, or indirect measure of natural selection. Whether parallel instances of selection result in convergence at one phenotypic level or many, is unknown. I found that the dynamics of competition were not the same between apparently con-

vergent ecotypes when the competition was between ecotypes evolved in slightly different environments (i.e., mixtures of glucose and acetate vs. glucose). This result demonstrated indirectly that convergence at the level of colony morphology and diauxic growth profile did not extend to competitive ability; from this I inferred that the underlying genetic basis to the apparently convergent ecotypes was different (Travisano and Lenski, 1996, Travisano, 2001). Additionally, I observed a pattern that indicated that cryptic variation may exist within our focal ecotypes. In this study, I had used colony morphology to distinguish between large and small (analogous to slow- and fast-switcher) ecotypes. Early in the competition assay, one ecotype was ascending (in terms of ecological dynamics) when at a particular frequency in the population. Later in the same competition assay, however, that same ecotype, occurring at the same frequency, was in decline. Cryptic variation within ecotypes (i.e., indistinguishable by colony morphology) is one explanation for shifts in the dynamics of competition. This suggests that colony morphology may not always be an appropriate marker for ecotypic variation within populations, and anticipates some results in Chapter 5 (see below). Recently, Saxer et al. (2009) demonstrated that facilitation was operating in the diversified populations evolved on glucose. Thus, the large (or slow-switcher) ecotype provided a resource (acetate) to the small (or fast-switcher) ecotype, and thus facilitated as well as competed with the small ecotype. The added interaction (facilitation), in addition to competition, may provide a functional explanation for why I observed subtle differences in competitive ability between the apparently convergent ecotypes.

In Chapter 3, I tested whether competition for resources, one of the most common ecological interactions, could cause ecological character displacement. Ecological character displacement is a process that shifts or maintains ecologically relevant phenotypes (Schluter, 2000b). Traditionally, ecological character displacement is believed to occur when co-occurring species compete for similar resources. If the species have partially overlapping phenotypic distributions for exploiting the common resource base (e.g., different sized beaks for exploiting a continuum of different sized seeds), then resource competition should select against overlapping phenotypes and ultimately cause the species to diverge (Brown and Wilson, 1956, Schluter et al., 1985, Schluter, 2000a, 2003). If ecological character displacement is ongoing in sympatry, then the removal of one species (say, from a two species

community) should release the remaining species from competition, and result in the evolutionary shift ("convergence") of its phenotypic distribution towards the phenotypes most efficient on the underexploited resources (Schluter, 2000b). Finally, if competition for resources is negative frequency dependent, then disruptive selection against intermediate phenotypes can arise and cause the branching of a single species into two separate species (Geritz et al., 1998, Dieckmann and Doebeli, 1999, Dieckmann et al., 2004, Doebeli and Dieckmann, 2003). Thus, ecological character displacement not only plays a role in the maintenance of diversity, but in its origin as well.

Using experimental populations of *E. coli*, and along with other studies (Friesen et al., 2004, Tyerman et al., 2005, Spencer et al., 2007b), I showed that competition for resources caused the initial diversification of populations of bacteria evolved in mixtures of glucose and acetate into (two) discrete ecotypes. Subsequently, I demonstrated that ecological character displacement was ongoing, as populations founded from one ecotype (or the other) converged, presumably due to the release from competition. This result was important as few studies have shown evolutionary convergence upon being released from competition (reviewed in Kassen, 2009). Finally, when competition was re-introduced, ecological character displacement caused the competitors to re-diverge, a result seen in other studies with microbes (e.g., Barrett and Bell, 2006).

In Chapter 4, I focused on the variation within the evolved populations that were initiated from different ecotypes and released from interspecific competition (described in Chapter 3). Because these populations faced similar extrinsic conditions, namely ecological opportunity and disruptive selection, I expected that they would subsequently diversify and recapitulate the adaptive radiation that gave rise to them. However, I found that one ecotype, the slow-switcher, diversified to a greater extent then the second ecotype, the fast-switcher. Further, while the fast-switcher ecotype recurred in the slow-switcher lineages, the slow-switcher ecotype did not recur in the fast-switcher lineages. As I argued in Chapter 4, these results suggested that the diversity attained by a lineage during adaptive radiation may depend not only on extrinsic factors, but on the genetics within the source population. In relative terms, some ecotypes may harbour key innovations that allow them to diversify and fill the "adaptive zone" (Simpson, 1953); conversely

other ecotypes lack the innovation that facilitates the evolution of novelty and this constrains their diversification. In microbes, this key innovation may involve a phenotypically plastic regulatory system that is sensitive to the resource environment. Diversification may involve the genetic assimilation, or deregulation of the environmentally induced phenotype. In general, it may be easier to erode regulatory systems (mutationally) than to construct them, so variants that have evolved diminished regulation may consequently be ineffective as evolutionary "stem species" (West-Eberhard, 2003).

In Chapter 5, I conducted a survey for the presence of resource specialization trade-offs within and among populations of *E. coli*. In the first part of the study, I tested whether increasing environmental complexity (i.e., number of resources) resulted in the evolution of greater levels of diversity. I found that diversity did not increase with environmental complexity. I used colony morphology variation as a surrogate measure of ecologically relevant variation. One reason that I did not find increasing diversity with environmental complexity could be because colony morphology variation saturates quickly – even in environments with a single, simple, carbon source (i.e., acetate), while ecologically relevant diversity accumulates cryptically (as I suspected in Chapter 2). Thus, trade-offs involving resource specialization may be involved in the evolution of diversity within my experimental populations, yet remain undetected. Other studies have used colony morphology variation as a surrogate measure of ecologically relevant phenotypic variation (e.g., Rainey and Travisano, 1998); while simple, this practise may need to be augmented with other methods.

In the second part of Chapter 5, I tested whether rate vs. yield trade-offs were involved in the maintenance of diversity within and among populations. While I found variation in growth rate and yield traits within and among experimental populations, I did not find a negative correlation between these traits, which is predicted if this trade-off is important in causing or maintaining variation in these populations. Previously, Novak et al. (2006) found evidence for rate vs. yield trade-offs within four populations of the same bacterium, albeit evolved for over 10,000 generations. The contrast in results may be a function of the duration of the experiment (Novak et al., 2006) or the resource supply rate (Hall and Colegrave, 2007).

In conclusion, the results and ideas presented here contribute to understanding how adaptive diversification occurs in the lab. Other lab studies have complemented and benefited from the results presented here (Spencer et al., 2007b,a, Le Gac et al., 2008, Saxer et al., 2009). This work bridges the gap between the theory of adaptive diversification and explanations for the origin and maintenance of diversity in nature.

Bibliography

Barrett, R. D. H. and Bell, G. (2006). The dynamics of diversification in evolving pseudomonas populations. *Evolution*, 60(3):484–490.

Brown, W. L. and Wilson, E. O. (1956). Character displacement. *Syst Zool*, 5:49–64.

Dieckmann, U. and Doebeli, M. (1999). On the origin of species by sympatric speciation. *Nature*, 400:354–357.

Dieckmann, U., Metz, J., Doebeli, M., and Tautz, D. (2004). *Introduction*. Adaptive speciation. Cambridge University Press, Cambridge.

Doebeli, M. and Dieckmann, U. (2003). Speciation along environmental gradients. *Nature*, 421:259–264.

Friesen, M., Saxer, G., Travisano, M., and Doebeli, M. (2004). Experimental evidence for sympatric ecological diversification due to frequency dependent competition in *Escherichia coli*. *Evolution*, 58:245–60.

Geritz, S., Kisdi, E., Meszena, G., and Metz, J. (1998). Evolutionary singular strategies and the adaptive growth and branching of the evolutionary tree. *Evol Ecol Res*, 12:35–57.

Hall, A. R. and Colegrave, N. (2007). How does resource supply affect evolutionary diversification? *Proc R Soc Lond.B*, 274:73–78.

Kassen, R. (2009). Towards a general theory of adaptive radiation: insights from microbial experimental evolution. *Proc NY Acad Sci*, 1168:3–22.

Le Gac, M., Brazas, M. D., Bertrand, M., Tyerman, J. G., Spencer, C. C., Hancock, R. E. W., and Doebeli, M. (2008). Metabolic changes associated with adaptive diversification in *Escherichia coli*. *Genetics*, 178(2):1049–1060.

Novak, M., Pfeiffer, T., Lenski, R., Sauer, U., and Bonhoeffer, S. (2006). Experimental tests for an evolutionary trade-off between growth rate and yield in Escherichia coli. *Am Nat*, 168:242–251.

Rainey, P. and Travisano, M. (1998). Adaptive radiation in a heterogeneous environment. *Nature*, 394:69–72.

Saxer, G., Doebeli, M., and Travisano, M. (2009). Spatial structure leads to ecological breakdown and loss of diversity. *Proc Roy Soc B*, 276(1664):2065–2070.

Schluter, D. (2000a). Ecological character displacement in adaptive radiation. *Am Nat*, 156:S4–S16. ID: 669.

Schluter, D. (2000b). *The ecology of adaptive radiation*. Oxford University Press, Oxford.

Schluter, D. (2003). Frequency dependent natural selection during character displacement in sticklebacks. *Evolution*, 57:1142–1150.

Schluter, D., Price, T., and Grant, P. (1985). Ecological character displacement in darwin's finches. *Science*, 227:1056–1059.

Simpson, G. G. (1953). *The major features of evolution*. Columbia University Press, New York.

Spencer, C. C., Bertrand, M., Travisano, M., and Doebeli, M. (2007a). Adaptive diversification in genes regulating resource use in *Escherichia coli*. *PLoS Genetics*, 3:83–88.

Spencer, C. C., Saxer, G., Travisano, M., and Doebeli, M. (2007b). Seasonal resource oscillations maintain diversity in bacterial microcosms. *Evol Ecol Res*, 9:775–787.

Travisano, M. (2001). Towards a genetical theory of adaptation. *Current Biology*, 11:R440–2.

Travisano, M. and Lenski, R. (1996). Long-term experimental evolution in Escherichia coli. iv. targets of selection and specificity of adaptation. *Genetics*, 143:15–26.

Tyerman, J. G., Havard, N., Saxer, G., Travisano, M., and Doebeli, M. (2005). Unparallel diversification in bacterial microcosms. *Proc R Soc B*, 272:1393–1398.

West-Eberhard, M. (2003). *Developmental plasticity and evolution*. Oxford University Press, Oxford, UK.