Experimental evolution of *Pseudomonas fluorescens* in simple and complex environments

Rowan Douglas Hilton Barrett

Department of Biology

McGill University

Montreal, Quebec, Canada

August 2005

A thesis submitted to McGill University in partial fulfillment of the requirements of the degree of Master of Science.

Your file Votre référence
ISBN: 978-0-494-24612-2
Our file Notre référence
ISBN: 978-0-494-24612-2

ABSTRACT

Determining the factors responsible for the origin and maintenance of diversity remains a difficult problem in evolutionary biology. There is extensive theoretical work which suggests that environmental heterogeneity plays a major role. This theory argues that diversification is ultimately due to divergent natural selection for alternative resources. In this thesis I investigate adaptation and the evolution of diversity in experimental populations of the asexual bacterium *Pseudomonas fluorescens*. In all experiments I introduce clonal isolates of *Pseudomonas* to a novel environment and allow evolution to occur through the substitution of random mutations. Adaptation can then be quantified by comparing evolved genotypes to the ancestor. These experiments show that when *Pseudomonas* is selected in a complex environment containing several resources, sympatric genotypes adapt to use different resources, leading to the evolution of genetically diverse populations. In environments containing just a single resource, most genotypes adapt to use the same resource and no such diversity is observed. Adaptation in the experimental populations is caused by the fixation of beneficial mutations of intermediate fitness effect. My results highlight the value of microbial model systems for answering evolutionary questions and provide strong evidence for the role of ecological factors in the origin of diversity.

RÉSUMÉ

L'étude des facteurs responsables de l'origine et du maintien de la biodiversité est une facette de la biologie évolutive qui demeure sans réponse. De nombreux travaux théoriques proposent l'hétérogéinité environnementale comme facteur dominant. La théorie stipule que la diversification est due aux pressions sélectives divergentes causées par la présence de ressources alternatives. Dans cette thèse, l'évolution de la biodiversité est testée sur des populations bactériennes expérimentales de *Pseudomonas fluorescens*. Dans chacune des expériences, un isolat clonal de *Pseudomonas* a été introduit à un nouvel environnement où une période d'adaptation par la substitution de mutations aléatoires a été observée. Cette adaptation a été quantifiée par la comparaison de la croissance des génotypes évolués à celle de l'isolat clonal ancestral. Ces expériences démontrent que, lorsque soumis à des pressions sélectives dans un environnement complexe présentant plusieurs ressources, différents génotypes de *Pseudomonas* développe le métabolisme nécessaire à l'utilisation de différentes ressources, ce qui mène à l'évolution sympatrique de populations génétiquement diverses. A l'inverse, dans un environnement ne présentant qu'une seule ressource, la majorité des génotypes s'adaptent à l'utilisation de cette même ressource et une diversification n'est pas observée. L'adaptation de ces populations expérimentales est causée par la fixation de mutations bénéfiques d'effets intermédiaires sur leur valeur sélective. Ces résultats démontrent la grande valeur des systèmes microbiens comme système modèle dans l'étude de la biologie évolutive et confirment le rôle important des facteurs écologiques dans l'évolution de la diversité.

TABLE OF CONTENTS

REMARKS ON STYLE AND CONTRIBUTION OF AUTHORS

This thesis consists of a collection of manuscripts that have been written for publication in peer-reviewed journals. Each chapter corresponds to a single manuscript that was prepared in accordance with the style of a given journal. Detailed citations for each manuscript can be found as a footnote on the first page of each chapter. This section details the contribution of each author to each manuscript in this thesis. Chapter 1 was planned in collaboration with Dr. G. Bell and R.C. MacLean. I performed all experiments and analyzed the data. I prepared the manuscript with Dr. G. Bell. R.C. MacLean provided useful comments on the manuscript. I conceived of and planned the experiment presented in Chapter 2. I performed all experiments and analyzed the data. I prepared the manuscript with Dr. G. Bell. The original idea for Chapter 3 was provided by Craig MacLean. The experiment was planned in collaboration with Dr. G. Bell and R.C. MacLean. I performed the experiment and the data analysis. I prepared the manuscript with Dr. Bell. R.C. MacLean provided useful comments on the manuscript.

ACKNOWLEDGEMENTS

It has been a very short and very long two years since I arrived in the Bell lab at McGill. Throughout my degree Graham has been a patient and thoughtful supervisor, putting up with an often foolish and over-ambitious student. Above all, I must thank him for making me slow down and consider problems carefully. I feel that the quality of my work has greatly improved under his supervision, although I'm still not sure how to "keep it simple". I also thank my supervisory committee for sage advice and encouragement during all too infrequent committee meetings.

I am indebted to my parents, Suzanne and Spencer Barrett, for instilling my curiosity about the natural world. I thank them for unwavering support and guidance in all aspects of my life. In particular, I thank my father for showing me where the doors were, but never opening them for me. I would also like to thank Emily Davis for her love and confidence in me.

My fellow denizens in the Bell lab deserve many thanks. Craig MacLean was an invaluable source of insight and advice. Craig went beyond the call of duty and I am deeply appreciative of his patience with me during my first year in the lab. Kathy Tallon provided the essential counterbalance to Graham and I thank her for making the lab seem like more than just a place of work. I have learned much from Gabriel Perron, Scott Smith, Sinead Collins, Taica Replansky and Erin Cameron. I thank them for helping to show a wayward Torontonian all that Montreal has to offer and for laughing at me when I deserved it.

Finally, I thank the many good friends I have made inside and outside of the department who have made my time in this city two of the best years of my

life. Special thanks go to: Mike Ross, Andrew Moeser, Rod Docking, Marie Fortin, Leslie Paine, Robin Soper, Marc Dunn, Charles Berube, Sam Bernard, everyone who made it to a Furious George party and all the members of Pete, Pete United, I'll Think About It, Bertha, Groundhog Day Squad, Frisbee, Contingency Plan and Vertigo.

GENERAL INTRODUCTION

Determining the factors responsible for the origin and maintenance of biological diversity remains one of the central goals of evolutionary biology. There has been strong support for the idea that diversification is the ultimate result of divergent natural selection for ecological specialization (Dobzhansky 1937, Simpson 1944, 1953, Mayr 1963, Schluter 2000). This view argues that diversification occurs when ecological opportunity exists in the form of underexploited or novel resources. If distinct habitats possess different resources, then divergent natural selection will result in diversification despite moderate levels of migration between habitats (Levene 1953, Strobeck 1979, Day 2000). More recently, advocates of this hypothesis have suggested that even when the distinct resources are found within the same habitat, competition for intensely exploited resources can generate divergent selection for specialization on underexploited resources (Dieckmann and Doebeli 1999, Doebeli and Dieckmann 2000). In summary, environmental heterogeneity provides the ecological opportunity which allows for diversification.

Despite extensive theoretical work on the subject, direct empirical tests of the role of ecological opportunity on diversification are rare. This is largely because experiments in evolutionary ecology are often conducted with large, long-lived eukaryotes. It is almost impossible to conduct an experiment on an evolutionary timescale using this sort of organism. A more promising approach is to use microbes, which have large population sizes and short generation times. In the case of strictly asexual microorganisms, most species can be classified only by genetic or functional divergence, so the appearance of a polymorphism in a

microbial population is an equivalent process to speciation in a sexual eukaryote (Friesen et al. 2004). In this thesis I conduct experimental evolution with microcosm populations of the asexual soil bacterium *Pseudomonas fluorescens*. By selecting populations on mixtures of resources, I test the hypothesis that the ecological opportunity afforded by environmental heterogeneity promotes diversification. Furthermore, I provide a detailed investigation of the genetic basis for adaptation in the experimental populations.

This thesis consists of a collection of manuscripts written for publication in peer-reviewed journals. Chapters 1 and 2 investigate adaptive diversification in environments of varying complexity. Both chapters provide direct experimental tests of the hypothesis that adaptive diversification is the evolutionary consequence of ecological opportunity. In chapter 1 emphasis is placed on the evolution of ecological niche width and whether specialists or generalists are more likely to evolve in complex environments. Chapter 2 focuses on the evolution of diversity and provides a dynamic perspective by tracking diversification through time. Chapter 3 considers the fundamental unit of adaptation: a single fixed beneficial mutation. It thereby investigates the underlying mechanism which drives the adaptive diversification observed in chapters 1 and 2.

LITERATURE CITED

Day, T. 2000. Competition and the effect of spatial resource heterogeneity on evolutionary diversification. American Naturalist 155: 790-803.

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

Dobzhansky, T. 1937. Genetics and the Origin of Species. Columbia University Press, New York.

Doebeli, M., and Dieckmann, U. 2000. Evolutionary branching and sympatric speciation caused by different types of ecological interactions. American Naturalist 156(Supplement): S77-101.

Friesen, M. L., Saxer, G., Travisano, M. and Doebeli, M. 2004. Experimental evidence for sympatric ecological diversification due to frequency-dependent competition in *Escherichia coli*. Evolution 58: 245-260.

Levene, H. 1953. Genetic equilibrium when more than one ecological niche is available. American Naturalist 88: 690-692.

Schluter, D. 2000. The Ecology of Adaptive Radiation. Oxford University Press, Oxford.

Simpson, G. G. 1944. Tempo and mode in evolution. Columbia University Press, New York.

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

Strobeck, C. 1979. Haploid selection with *n* alleles in *m* niches. American Naturalist 113: 439-444.

Chapter 1: Experimental evolution of niche width in simple and complex environments[1]

In this chapter I present a selection experiment that had the goal of elucidating the role of environmental complexity in the evolution of niche width. Environmental complexity can be thought of as a specific type of environmental heterogeneity that occurs when an environment contains several resources that are everywhere present. This is distinct from environmental structure, which has received far more experimental attention. In a complex environment, a population may evolve into generalists able to consume most of the resources present, or alternatively into specialists able to consume a small number of the resources present. My experiment was designed to determine which of these two outcomes was more likely. I introduced two isogenic clones to environments which varied in complexity. After almost 1000 generations of selection the populations growing on complex media were composed of several metabolically differentiated generalists that were each able to use most substrates available but not all of them.

[1]Barrett, R.D.H., R.C. MacLean and G. Bell. 2005. Experimental evolution of *Pseudomonas fluorescens* in simple and complex environments. *American Naturalist*. 160(4): 470-480.

Abstract

In complex environments that contain several substitutable resources, lineages may become specialized to consume only one or a few of them. Here we investigate the importance of environmental complexity in determining the evolution of niche width over ~900 generations in a chemically defined experimental system. We propagated 120 replicate lines of the bacterium *Pseudomonas fluorescens* in environments of different complexity by using between one and eight carbon substrates in each environment. Genotypes from populations selected in complex environments evolved greater mean and variance in fitness than those from populations selected in simple environments. Thus, lineages were able to adapt to several substrates simultaneously, without any appreciable loss of function with respect to other substrates present in the media. There was greater genetic and genotype-by-environment interaction variance for fitness within populations selected in complex environments. It is likely that genetic variance in populations grown on complex media was maintained because the identity of the fittest genotype varied among carbon substrates. Our results suggest that evolution in complex environments will result neither in narrow specialists nor in complete generalists but instead in overlapping imperfect generalists, each of which has become adapted to a certain range of substrates but not to all.

Introduction

All organisms require supplies of certain essential resources, the simplest of which are elements such as carbon and nitrogen, whose rate of supply limits the rate of growth. An ideal simple environment contains a single limiting substrate, providing one of these resources, that is freely available to all individuals. As this substrate is depleted, types able to grow on lower concentrations will be favored, until at last only the single most frugal type remains (Tilman 1977). Organisms may differ in the ratio of resources that they require, however, and two or more different kinds may then coexist within a certain range of intermediate resource supply rates (Brzezinski and Nelson 1988; Dykhuizen and Davies 1980; Gottschal et al. 1979; Gottschal et al. 1981; Grover 1988; Laanbroek et al. 1979; Legan et al. 1987; Tilman 1977). Resources of this kind cannot be substituted for one another: carbon, say, cannot be made to take the place of nitrogen. There is a wide range of substrates, however, that will supply either: for example, most microbes are able to use a variety of sugars, organic acids and other compounds to provide energy or carbon skeletons. Such substrates are said to be substitutable, because growth can be supported by any one of them, or any mixture of them, and is not prevented by the absence of any one of them. Consequently, organisms may evolve as generalists that are able to consume a large proportion of the substitutable substrates in a given category, or as specialists that are able to grow on only one substrate, or a very few substrates. Communities might thus comprise a diversity of specialists, at one extreme, or a single universal generalist, at the other.

The outcome of competition between generalists and specialists will

depend in part on how the available substrates are distributed. If they occur patchily in time, with only a single substrate being available in each period of time, the single generalist type with the greatest geometric mean fitness should exclude all others (Bell and Reboud 1997; Bennett et al. 1992; Kassen and Bell 1998; Weaver et al. 1999). If they are distributed patchily in space, then a variety of specialists may co-exist, depending on the relative extent of the different kinds of patch and the rate of migration between them (Bell 1996; Dykhuizen and Davies 1980; Garcia-Dorado et al. 1991; Joshi and Thompson 1997; Silver and Mateles 1969; Taplitz and Coffin 1997; Verdonck 1987; Wasserman and Futuyma 1981). If the patches are completely separated, adaptive radiation leads quickly to the evolution of a wide range of specialists (MacLean and Bell 2002). Although temporal and spatial structure are important attributes of natural environments, most communities grow under conditions such that several or many substitutable resources are simultaneously available. We may then ask whether specialists or generalists will tend to evolve in mixtures of substitutable resources.

A single type of microbe inoculated into growth medium containing a mixture of carbon substrates usually uses them in sequence; typically consuming glucose first and switching to other sugars only when glucose has been reduced to very low concentration (see Harder and Dijkhuizen 1982). When growing in mixtures of substrates, bacteria are capable of tuning their metabolism quite precisely through mechanisms such as catabolite suppression (Paigen and Williams 1970). If the growth medium is initially very dilute, however, all substrates may be utilized simultaneously; for example, Lindenmann et al. (1996) have shown that *Escherichia coli* is capable of utilizing at least six substrates

simultaneously, without establishing any upper bound on the number that might be used in more complex mixtures. The response of organisms to mixtures of substrates thus depends on the conditions of culture: sequential utilization of substrates is more likely to occur in batch culture, and simultaneous utilization in chemostats (Harder and Dijkhuizen 1976). The behavior of mixed cultures of microbes on mixtures of substrates is considerably more complicated. In many cases, a generalist able to use both (or all) of the substrates present eliminated a specialist able to use only one (for example, Gottschal et al. 1979), but more complex outcomes have also been observed, perhaps as the result of less direct interactions between the competing types. The field has been reviewed by Harder and Dijkhuizen (1976) and Gottschal (1986).

Almost all the experiments reported in the literature have been ecological, in the sense that the types used to inoculate the system are assumed to retain their properties unchanged. In this study, we ask whether an initially isogenic population will adapt to a defined mixture of substrates, and if so whether the evolved population will be dominated by generalists or by specialists. We transferred replicate lines of *Pseudomonas fluorescens* in 15 environments containing between one and eight carbon substrates for ~900 generations in batch culture. All lines were founded from clones, so that all variance and covariance expressed by evolved genotypes arose through novel mutations during the selection experiment. At the end of the selection experiment we measured the fitness of genotypes from each line on every single substrate in the experiment. This allowed us to test whether populations had adapted to the substrates in which they had been cultured, and also whether they had adapted to other substrates. In

particular, we tested whether specialists or generalists evolve in complex mixtures of substitutable resources. We expected that the outcome would depend on whether there were tradeoffs involved in utilizing different substrates. This experiment follows previous work in which we have documented adaptive radiation in undefined mixtures of substrates (MacLean et al. 2005) and in spatially structured environments (MacLean and Bell 2002).

Materials and Methods

Ancestral Strain

We used clonal isolates of *P. fluorescens* strains SBW25 and SBW25Δ*panB* to found 15 selection lines, each replicated 8 times. SBW25 was isolated from the leaf of a sugar beet plant at the University Farm, Wytham, Oxford, in 1989 (Rainey and Bailey 1996). SBW25Δ*panB* is an isogenic strain of SBW25 containing a complete deletion of the *panB* gene. The *panB* gene is used to synthesize the vitamin pantothenate and when plated on indicator plates with a low concentration of pantothenate (2.4×10^{-6} %), SBW25Δ*panB* grows noticeably smaller colonies than SBW25. This marker is selectively neutral and has no effect when pantothenate is present in high concentrations (MacLean et al. 2004; Rainey 1999). The two strains were mixed in equal proportions to form a common pool to start the experimental populations (fig. 1). The ancestral clones were kept frozen at -80°C during the experiment in a mixture of 50% glycerol: 50% water (v:v).

Selection experiment

We chose eight carbon substrates involved in different pathways of *Pseudomonas* metabolism. These were used to set up eight culture media each with a single carbon substrate, four with two substrates, two with four substrates and one with all eight substrates (table 1). Lines selected in single carbon environments will hereafter be referred to as "simple" lines and lines selected in two, four or eight carbon environments will be referred to as "complex" lines. This is a somewhat artificial division, but is necessary to simplify the analysis. We grew populations in 96-well plates with each well containing an M9 salt solution (NH_4Cl 1g/L,

Na_2HPO_4 6 g/L, KH_2PO_4 3 g/L, NaCl 0.5 g/L) supplemented with a high concentration of pantothenate (2.4×10^{-3} %) and a mixture of carbon substrates. We maintained a constant concentration for each substrate (0.3 g/L per substrate), rather than maintaining a constant total concentration for each environment. This decision was made so that the rewards of specializing on a specific substrate were equal at all levels of complexity. Every 24 hours we transferred selection lines by using a 96-pin replicator to "print" the populations grown on a selection plate onto a fresh selection plate. The replicator transfers 0.06-0.07 μl of culture (~10^5 cells) on each pin to give a dilution factor of approximately 3000 fold per transfer. After each transfer we diluted and plated one replicate line from each selection environment on indicator plates containing a low concentration of pantothenate. For each of these lines we counted the ratio of colonies from each marker state using a ProtoCOL SR/HR counting system (Synoptics Ltd., Cambridge, UK). A sudden deviation in marker frequency would indicate that either a marked or unmarked genotype was linked to a beneficial mutation on its way to fixation. Monitoring marker frequencies allowed us to confirm that adaptation was occurring in the selection experiment. Due to the large population sizes and large number of colonies counted (~100), it is very unlikely that deviations would be caused by drift. We continued the selection experiment for 80 transfers, which is equal to ~900 generations. After the final transfer, we plated ~100 colonies from all replicate lines to record the proportion of lines from each selection environment that had fixed for one of the marker states. Fixation was declared when one of the marker states reached a proportion greater than 98%.

Assay

At the end of the selection experiment, we froze all of our evolved lines at -80°C in a mixture of 50% glycerol: 50% water (v:v). Before our assay, we reconditioned cultures in 96-well microplates containing dilute M9KB medium (NH_4Cl 0.1g/L, Na_2HPO_4 0.6 g/L, KH_2PO_4 0.3 g/L, NaCl 0.05 g/L, glycerol 1 g/L, proteose peptone 2 g/L) at 28°C for 2 days. Two replicates of the ancestral clones of SBW25 and SBW25Δ*panB* were also reconditioned with the evolved lines. We serially diluted and plated out cultures on indicator plates containing a very low concentration of pantothenate. We then randomly picked two (for one and two carbon-selected lines), four (for four carbon-selected lines) or seven (for the eight carbon-selected line) colonies from two lines of each selection environment. We only used lines that showed marker fixation to ensure that adaptation had occurred within the populations. A greater number of colonies were picked from environments with more carbon sources to facilitate finding genotypes that had specialized on different carbon substrates. We grew the selected colonies in dilute M9KB medium at 28°C for 2 days. We diluted and starved cultures in M9 salt solution for at least two hours before the assay began. We then added 20 μl of starved cells (10^6 viable cells) from each culture to 96-well plates. Each well on each plate contained 180 μl M9 solution plus 0.3 g/L of one of the carbon substrates used in the selection experiment. We scored three replicates of each genotype in every assay environment (3 replicates x 78 genotypes x 8 assay environments).

We measured optical density at 600 nm using a Synergy HT narrow beam plate reader (Biotek Instruments, Winooski, Vt.) at 24 hours ± 15 minutes of incubation at 28°C, so that the assay conditions were identical to those experienced by each population during one transfer in the selection experiment. The optical density score of any given well reflects the scattering of light by bacterial cells. Optical density increases asymptotically with cell density, becoming saturated at very high cell density (R.C. MacLean, unpublished results). We have used optical density as a measure of growth because it can be measured much more rapidly and accurately than cell density. We corrected optical densities by subtracting control well scores from each absolute score. We calculated the direct response to selection of each replicate of a genotype as the difference in corrected optical density between the evolved genotype and the ancestral clone, on the substrates that the genotype was selected on. We measured the correlated response to selection as the difference between the evolved genotype and the ancestral clone, on substrates that the genotype was not selected on. The responses to selection for each line were calculated as the mean of the replicates from all genotypes in a line.

Statistical analysis

The variance of the selection response within and between replicate lines was calculated using ANOVA with JMP 4.0 software (SAS Institute, Cary, NC). In forming expected mean squares, lines and genotypes have been taken as random while assay and selection environments have been taken as fixed. The justification

is that the specific lines used in the assay were chosen randomly from the subset of lines that had fixed for one marker state. The likelihood that one replicate line will achieve marker fixation before another from the same selection environment is assumed to be random. Therefore each can be regarded as a representative *Pseudomonas* population. Similarly, the genotypes used from each line were picked randomly from the populations growing on agar plates and can be considered a representative sample from the population. In contrast, the selection and assay environments were predefined and cannot be taken to be representative of natural habitats.

The variance within populations was further analyzed by decomposition into genetic, environmental and genotype-by-environment (GxE) interaction components. Finally, GxE variance was partitioned into "inconsistency" and "responsiveness" components. Robertson (1959) showed that the GxE variance of a collection of genotypes tested in two environments can be expressed as:

$$\sigma^2_{GE} = \frac{(\sigma_{G1} - \sigma_{G2})^2}{2} + \sigma_{G1}\sigma_{G2}(1 - \rho_{G1G2}) \qquad (1)$$

where σ_{G1} and σ_{G2} are the genetic standard deviations of a character expressed in environments 1 and 2, respectively, and ρ_{G1G2} is the genetic correlation of that character across environments 1 and 2. This equation can be rearranged to interpret GxE for a slightly different scenario (Bell 1990; Cockerham 1963; Cooper and Delacy 1994; Wu and Stettler 1997). If we instead test two genotypes over a range of environments, then Robertson's equation becomes:

$$\sigma^2_{GE} = \frac{(\sigma_{E1} - \sigma_{E2})^2}{2} + \sigma_{E1}\sigma_{E2}(1 - \rho_{E1E2}) \quad (2)$$

In this case σ_{E1} and σ_{E2} are the environmental standard deviations of a character expressed by genotypes 1 and 2, respectively, and ρ_{E1E2} is the environmental correlation of that character across the two genotypes. Thus GxE can be partitioned into two components. The first is responsiveness, due to differences between environmental variances among genotypes, $½(\sigma_{E1} - \sigma_{E2})^2$. The second is inconsistency, due to lack of complete correlation between genotypes over environments, $\sigma_{E1}\sigma_{E2}(1 - \rho_{E1E2})$. In order to estimate GxE for a population comprising several genotypes over a range of environments, we must take the means of these components over all pairwise combinations of genotypes:

$$\sigma^2_{GE} = \sum \frac{(\sigma_{Ei} - \sigma_{Ej})^2}{2G(G-1)} + \sum \frac{\sigma_{Ei}\sigma_{Ej}(1 - \rho_{EiEj})}{G(G-1)} \quad (3)$$

where G is the number of genotypes chosen from the population. We calculated the proportion of responsiveness versus inconsistency in the GxE of each population, and then compared populations selected in simple versus complex environments.

Results

Experimental evolution

We found evidence for periodic selection: after 900 generations of selection 68% of replicate lines had fixed for one of the two marker states (fig. 2). Fixation was equally likely in simple and complex media ($\chi^2 = 0.04$, df = 1, $P = 0.84$). However, fixation was more likely to occur in simple or complex media containing serine than in other media ($\chi^2 = 10.00$, df = 1, $P = 0.002$), showing that some of the beneficial mutations that became fixed had effects specific to a particular substrate.

Adaptation in simple media

The ancestor showed relatively poor growth in all environments (table 2). The lines cultured in simple media became adapted to them: the mean direct response was 0.015, (SE = 0.002), representing an increase of 150% over the ancestor. The response varied substantially over lines (SD = 0.008; fig. 3), with an observed range of –0.005 to 0.047. The direct response to selection on a given substrate was negatively correlated with the performance of the ancestor on that substrate ($P < 0.001$, $r^2 = 0.22$), so that there was more progress in more stressful environments.

The correlated response to selection was positive (mean = 0.012, SE = 0.001): lines cultured on simple media grew better than the ancestor on substrates neither had previously encountered. The direct response exceeded the correlated response (paired $t = 2.26$, df = 27, $P = 0.032$), so that adaptation was to some extent specific to the substrate on which a line was cultured. This is reflected in

the significant Selection x Assay environment interaction in the overall analysis of variance (table 3). The difference between the direct and correlated responses was quite small, however (mean difference across lines = 0.003, SD = 0.006), equivalent to 16% of the mean growth of the lines. There was no significant correlation between direct and correlated responses ($P > 0.05$, $r^2 = 0.13$).

Adaptation in complex media

Lines cultured on complex media had greater average growth, when assayed on each substrate separately, than lines cultured on simple media (fig. 4). This superiority was shown by the growth of the lines themselves ($t = 2.07$, df = 27.5, $P = 0.048$) and by the mean growth of genotypes isolated from the lines and assayed separately (pooled $t = 2.53$, df = 76, $P = 0.014$). Moreover, lines cultured on a complex medium (with composition ABCD, say) had on average the same growth on any of its constituents (say, A) as the lines cultured on simple media containing this substrate alone (paired $t = -0.69$, df = 7, $P = 0.51$; fig. 4).

The fitness rank curve

For any given genotype, substrates can be ranked in order of growth, from most to least. We call this the fitness rank curve. Combining these curves for several genotypes gives a useful visual summary of the differences between lines or treatments (fig. 5). Note that the identity of the substrate corresponding to a particular rank may, and usually will, differ from genotype to genotype. The curve for each genotype, and the band of curves from a group of genotypes, necessarily slopes downward, from the highest-ranking to the lowest-ranking substrate. The

slope of the curve, or band of curves, represents environmental variance: a steeper slope reflects a greater difference between the highest-ranked and the lowest-ranked substrates, and thus greater environmental variance. The width of the band of curves reflects genetic variance among genotypes.

Plasticity and genetic diversity

One possible outcome of this experiment was that lines cultured on complex media would evolve greater plasticity (similar growth on all or most substrates) than lines cultured on simple media (good growth on the substrate of selection but poor growth on others). If this were the case, it would be reflected by the fitness rank curves, which would slope down more steeply for simple than complex environments. This is not supported by our results (fig. 5), which show that environmental variance was marginally greater among genotypes isolated from lines cultured in complex media than among those from simple media ($t = 2.14$, df = 65.9, $P = 0.036$). There is substantial genetic variance within the lines, however, even though they had been fixed for a single genotype through a selective sweep in the recent past (table 3). Estimates of the genetic variance component are greater for lines selected in complex media than for those selected in simple media ($t = 2.37$, df = 28, $P = 0.025$; fig. 6). This is reflected by the greater thickness of the band of fitness rank curves for the complex environments (fig. 5). There is a modest but significant GxE (table 3). This is much larger in lines cultured on complex media; it is almost absent from lines cultured on simple media (fig. 6). The estimate of inconsistency was greater than zero in 14/14 lines from complex media, but in only 5/16 lines from simple media. Consequently, the

fraction of GxE attributable to inconsistency was much greater in lines from complex media (65%) than in lines from simple media (24%) (Mann-Whitney $U = 156$, $P = 0.02$; fig. 7).

Discussion

The theory of co-existence in mixtures of substitutable substrates has been exhaustively analyzed in the ecological literature, especially from the point of view of how many species can be maintained on a given number of substrates (MacArthur 1969; MacArthur and Levins 1964; Stewart and Levin 1973). The most general result is an extension of Gause's exclusion principle, that the number of species stably coexisting cannot exceed the number of distinct substrates. This holds for competition in chemostats (Taylor and Williams 1975), for non-substitutable resources (Tilman 1977) and for spatially structured environments (Strobeck 1975). Thus, when a single genotype is cultured in a complex medium, we might expect to observe either the evolution of a single broadly adapted generalist or an adaptive radiation involving a diversity of specialized types. What we actually found lay between these two extremes.

The fixation of the neutral marker in most of the lines bore witness to the passage of at least one beneficial mutation. Despite the fact that the substrates we used support populations of different size we do not believe that fixation was in any instance caused by genetic drift, because mean population size was ~3 x 10^8, the population size in the poorest substrate (serine) was ~3 x 10^7, and the number of cells directly transferred was ~10^5. Once the marker had become fixed, it was not possible to identify further change, and the number of substitutions occurring in any given line is not known. It is clear, nonetheless, that evolution in many of the lines proceeded through a series of selective sweeps (periodic selection) and that increased fitness is therefore attributable to adaptive genetic change. Although this is not unexpected, it has seldom been documented in complex

media. Dykhuizen and Davies (1980) used two genotypes of *E. coli*, one of which was deleted for *lac*, to study competition in mixtures of lactose and maltose. During the course of the experiment, a novel mutation to constitutive expression occurred in the lac^+ generalist, conferring the ability to grow at low levels of lactose. This was able to coexist with the ancestral strain, apparently because of functional interference between the mechanisms responsible for uptake of the two substrates. In very complex undefined mixtures, MacLean et al (2005) found that metabolic diversity evolved within experimental lines of *Pseudomonas* and was maintained by negative frequency-dependent selection, but observed no selective sweeps. Thus, adaptation to mixtures of substrates may or may not be accompanied by the periodic fixation of a single genotype. In many cases the passage of a beneficial mutation may be halted at intermediate frequencies, causing irregular fluctuations in marker frequency.

Lines cultured on simple media became specifically adapted to a single carbon substrate. Responses tended to be greater in stressful environments, to which the ancestor was poorly adapted. This finding is consistent with Fisher's geometric analogy of adaptation (Fisher 1930; Orr 2005), in which the expected fitness effect of beneficial mutations is greater in populations further from the optimal phenotype. The correlated response was also positive, on average, so that growth tended to increase on substrates that had not previously been encountered. This constitutes synclinal selection (Bell 1997; Bell and Reboud 1997), which occurs when the response is positive in both selection and novel environments (fig. 8). It is important to recognise that it is still possible to have specific adaptation when selection is synclinal. In this case, although the responses in

selection and novel environments are both positive, the response was greater in the environment of selection. Synclinal selection must be fuelled by beneficial mutations that are capable of enhancing growth on several substrates at the same time or to more general conditions of growth. It is not difficult to imagine that genes affecting transport or regulation might be responsible, although we have not attempted to identify them. In any case, we can distinguish between two categories of beneficial mutation, one conferring specific adaptation to a single substrate, whereas the other confers broader adaptation to a range of substrates. Adaptation to simple media is in part attributable to mutations of specific effect, but the positive correlated response shows that it is also in part attributable to mutations of broad effect. The magnitude of the correlated response relative to the direct response suggests that mutations of broad effect have been responsible for most of the observed response to selection. These results are consistent with previous work in single carbon environments (MacLean and Bell 2002).

In complex media the lines were able to adapt simultaneously to several substrates, to about the same extent that lines cultured in simple media adapt to each. This finding is consistent with recent work in viruses, which has shown that concurrent adaptation to several hosts does not limit ability to grow on each host separately (Novella et al. 1999; Turner and Elena 2000; Weaver et al. 1999). As with the positive correlated responses in simple media, the direct responses in complex media suggest selection of mutations of broad effect. The concentration of each substrate was the same in simple and complex media, creating the opportunity for the evolution of a community of specialists, each growing well only on one of the constituent substrates. Instead, the outcome of selection in

complex media was the evolution of generalists. Despite this, the presence of substrate-specific effects, such as the increased likelihood of fixation occurring in environments containing serine, suggests that specific adaptation to a single substitutable resource is possible for certain substrates. Thus, as in simple media, adaptation in complex media is due in part to mutations of specific effect.

The mean fitness over all substrates was greater for genotypes isolated from the complex media. The average selection response for complex-selected lines is determined through a varying proportion of direct and correlated responses, depending on the level of complexity in the specific line. In contrast, the average selection response for simple-selected lines is largely due to correlated responses. Thus, the direct and correlated responses to selection on the complex media exceeded the correlated response to selection in the simple media. This might be attributable to the selection of mutations specific to each of a number of the substrates present in a complex medium. One objection to this interpretation is that such mutations, spreading through an asexual population, would interfere with one another, as well as with any mutations of broad effect (Gerrish and Lenski 1998; Miralles et al. 1999; Wilke 2004). Alternatively, conditionally neutral mutations might accumulate more readily in simple media. These are mutations that obstruct the utilization of a particular substrate, or substrates; they are neutral in media that lack the substrate, but deleterious when the substrate is present (Cooper and Lenski 2000; Fry 1996; Kawecki 1994; Kawecki et al. 1997; Whitlock 1996). In complex media they are more likely to be deleterious because they reduce growth below the level achieved when all

available substrates are utilized. Hence, they will be more effectively eliminated by selection, and will occur at lower frequency.

Despite the evolution of generalists in complex media, these populations display more genetic variance than those in simple media. A number of genotypes could be maintained, in principle, by negative frequency-dependent selection, if the enhanced ability to utilize one substrate were accompanied by a loss of ability to utilize others (Ayala and Campbell 1976; Dempster 1955; Haldane 1932) If the loss were complete, indeed, the situation would differ little from divergent selection in allopatry. We did not find such clear-cut trade-offs, however, nor did the lines cultured in complex media come to consist of narrow specialists. We did find that lines from complex environments expressed substantial GxE, and that much of this variance was attributable to inconsistency. Thus, they consisted neither of narrow specialists nor of complete generalists. They seem instead to be mixtures of overlapping imperfect generalists, each of which has become adapted to a certain range of substrates but not to all those available.

Most experiments with microbes use growth media with a single limiting carbon source, and in batch culture experiments this substrate is initially present at high concentration. In our experiment several substrates were available, but again at high concentration. Such experiments are likely to favour the evolution of specialists, either because no alternative substrate is available or because substrates will be used sequentially. In most natural environments there will be a great variety of potential substrates for growth, but all will be present at low or very low concentration. Very few microbes are exclusive specialists on a single substrate, because very few substrates will be available at concentrations capable

of supporting growth. On the other hand, none can consume all the substrates they encounter. The metabolic profiles of bacteria and yeasts show that almost all species can consume a more or less broad range of substrates, whereas the identity of these varies among species. This is, indeed, the basis of identifying microbial taxa based on their patterns of substrate utilization (Anderson et al. 2002; Dawson et al. 2002; Stanier et al. 1966; Victorio et al. 1996). Experiments using complex mixtures of many substrates, each present at low concentration, would be likely to select for broad generalization, although none have yet been reported. Our experimental design, involving unrealistically high substrate concentrations, was predisposed to select for the evolution of a diverse community of narrow specialists, each specifically adapted to a single substrate. The fact that we observed instead the evolution of overlapping imperfect generalists suggests that this may be the usual outcome of selection in complex environments.

Acknowledgements

Pseudomonas fluorecens SBW25 was a kind gift from P. Rainey. We would like to thank two anonymous reviewers for their criticisms and suggestions. M. Pearlstein provided technical assistance. This work was supported by grants from the Natural Sciences and Engineering Research Council of Canada to GB and RCM

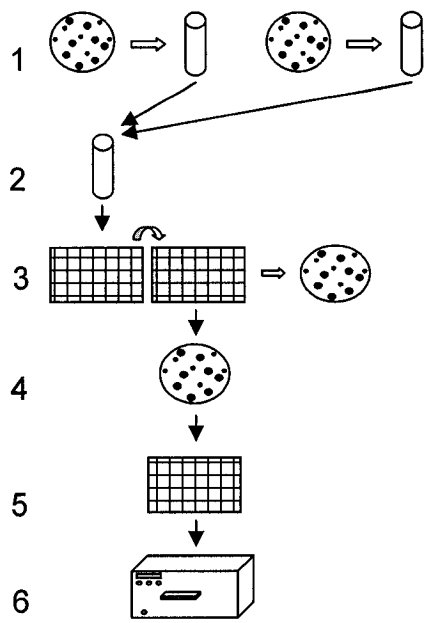

Figure 1. Flow chart of experimental design. 1. One colony from each marker state of the ancestor is inoculated into a culture. 2. Equal proportions of each marker culture are mixed. This culture is used to inoculate all selection lines. 3. Each line is grown in a different selection environment composed of between 1 and 8 substrates. Each replicate line occupies a separate well on a 96 well microplate. Plates are inoculated for 24 hours, and in this time each population undergoes approximately 11 generations. After 24 hours, a dilution of each line is plated out to record the ratios of the two different markers, and then all lines are transferred into a new plate using a pin replicator. We repeated this 24 hour cycle for 80 days. 4. Several colonies were randomly picked from two replicate lines from each selection environment. 5. Colonies were grown on every substrate in the experiment. The same is done for the ancestor, which has been frozen for the duration of the selection experiment. 6. We measured the optical density of the populations after 24 hours growth using a plate reader. The entire assay was replicated three times.

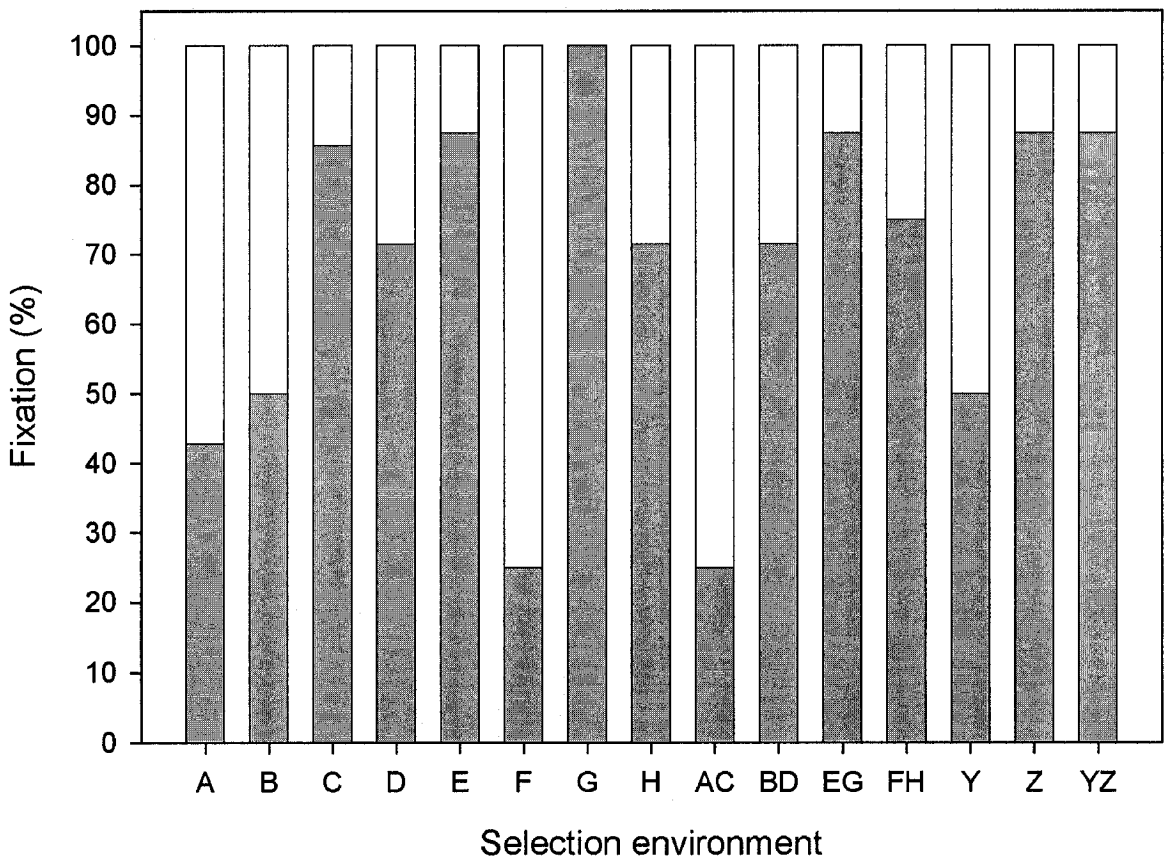

Figure 2. The proportion of replicate lines within each selection environment that achieved fixation for one of the marker states at generation 900. 68% of 110 replicate lines reached fixation. Reliable counts could not be recorded for 10 of the lines. The grey area reflects the percentage of lines from each selection environment that fixed. The substrates present in each selection environment are described in table 1, Y represents ABCD, Z represents EFGH, and YZ represents ABCDEFGH.

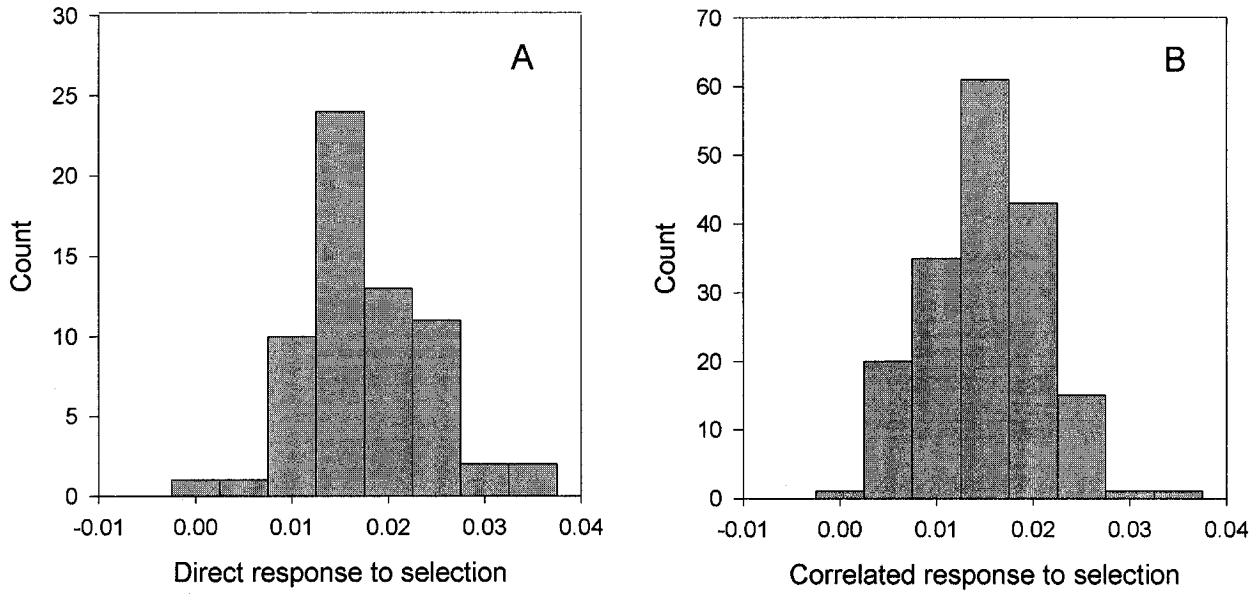

Figure 3. Frequency distribution of direct (A) and correlated (B) responses to selection for evolved lines. Almost all responses were greater than zero. The mean direct response was 0.015 (n = 64, SE = 0.002) and the mean correlated response was 0.012 (n = 177, SE = 0.001).

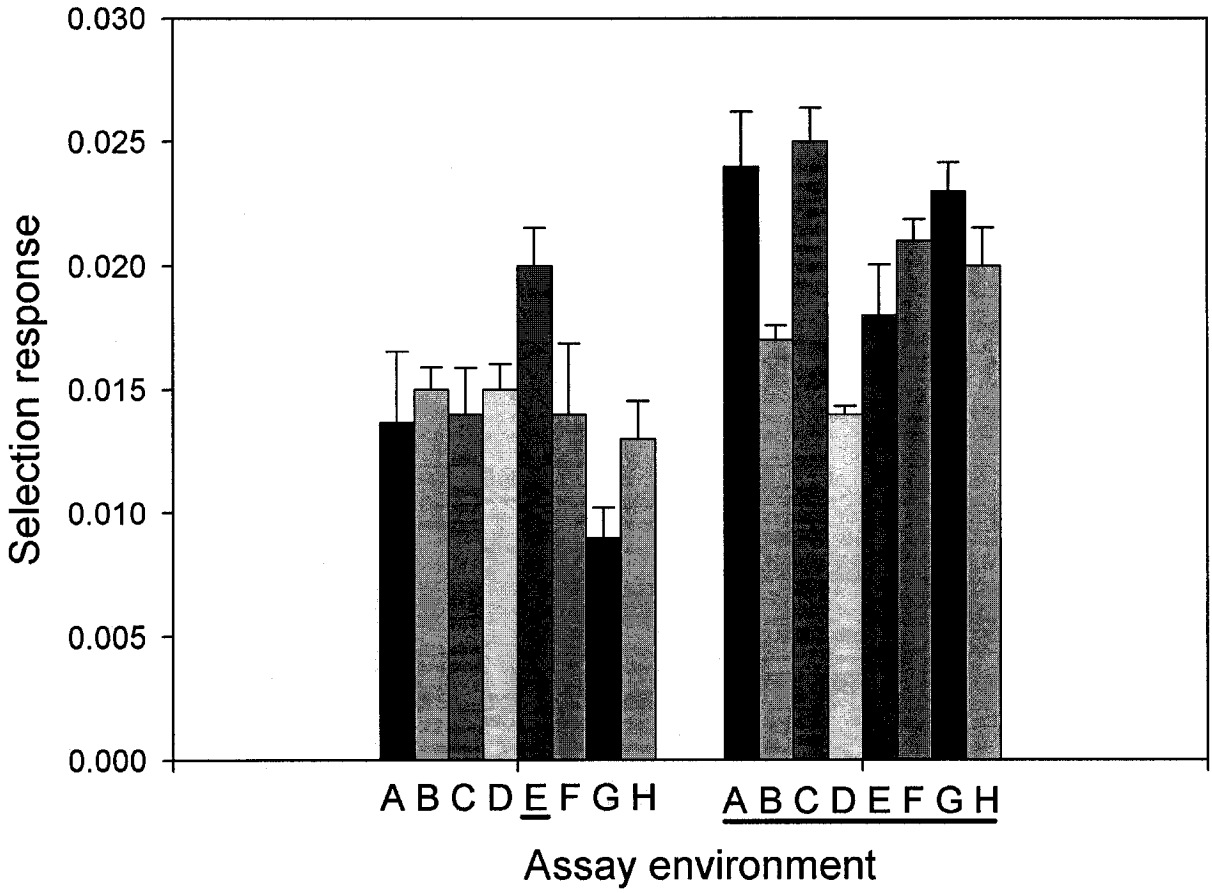

Figure 4. An example of substrate-specific performance of genotypes selected in simple and complex environments. Refer to table 1 for substrate labels. Bars represent the selection response of each genotype on a single substrate ±1 SE. The substates present in each genotype's selection environment are underlined. The simple-selected genotype selection response mean = 0.014 and SD = 0.003. The complex-selected genotype selection response mean = 0.020 and SD = 0.004.

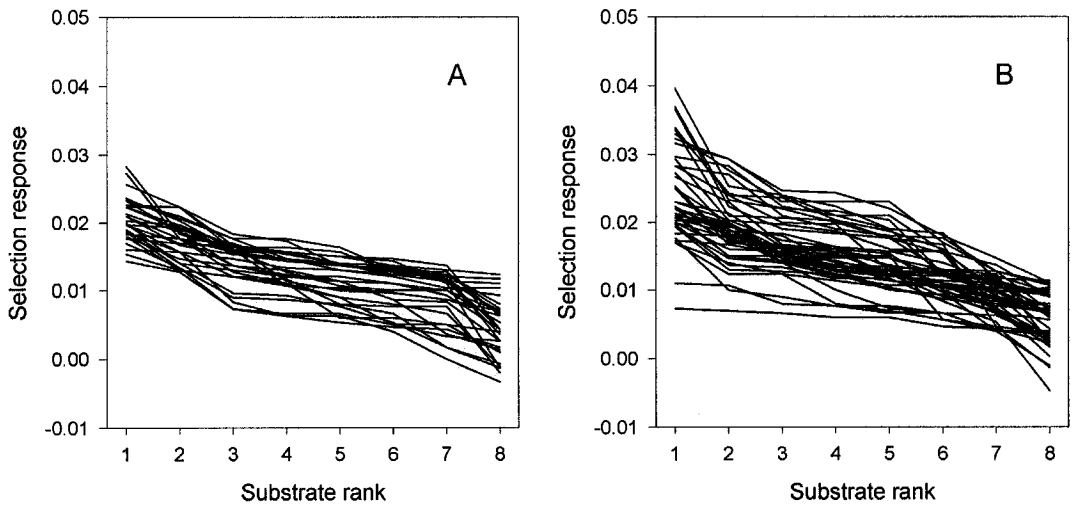

Figure 5. Ranked substrate by fitness. Each line represents the fitness of a genotype across different substrates. Substrates are ranked by decreasing fitness of each individual genotype, so that the substrate at each rank may be different for different genotypes. Increased separation between responses indicates greater genetic variance among genotypes. Increased slope indicates greater environmental variance in response. Simple-selected genotypes (A: $n = 32$) had lower mean ($t = 2.56$, df = 69.4, $P = 0.01$) and variance ($t = 2.14$ df = 65.9, $P = 0.04$) than complex-selected genotypes (B: $n = 48$).

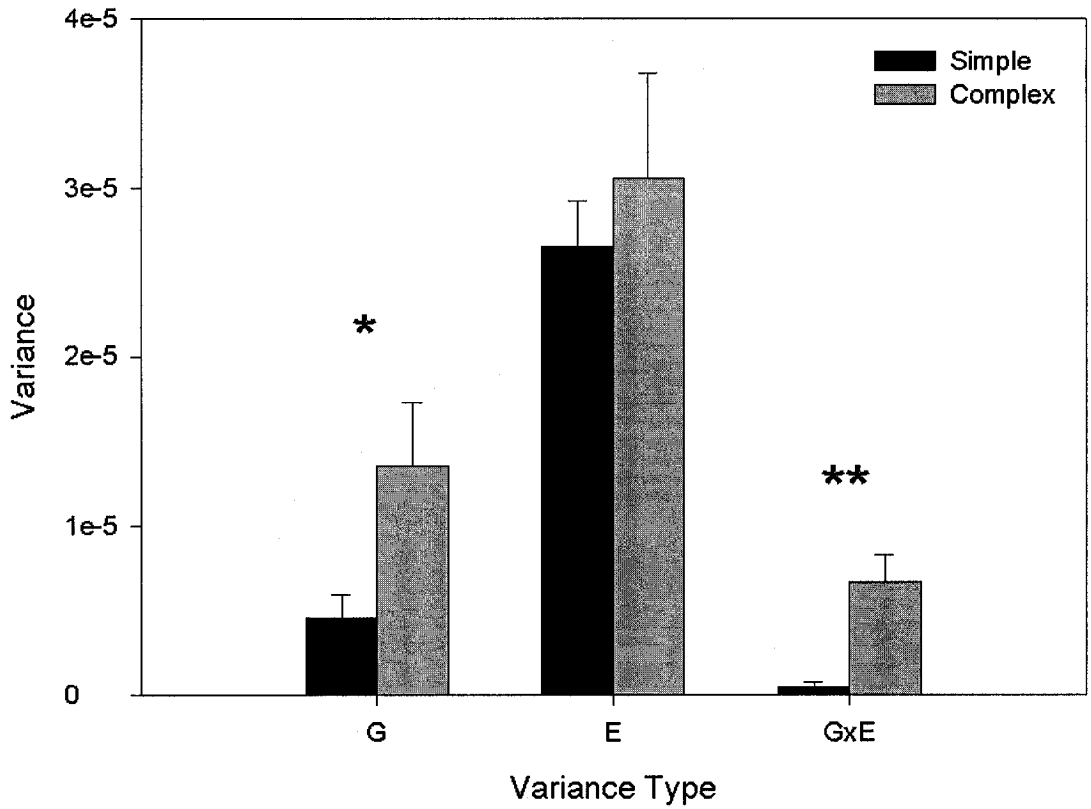

Figure 6. Within line variance components. Environmental variance contributes the largest proportion of overall variance within a selection line. Complex-selected lines have greater genetic and GxE variance than simple-selected lines. Error bars show ±1 SE. * $P<0.05$, ** $P<0.005$.

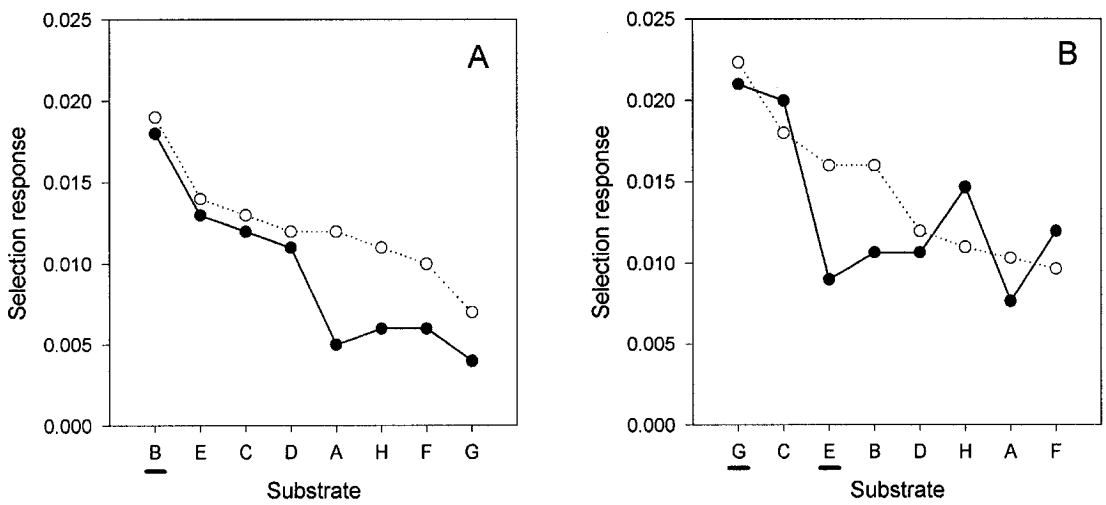

Figure 7. Inconsistency versus responsiveness in genotype-by-environment variance. Each line represents the fitness of a genotype across different substrates. A difference in slope between genotypes indicates that genotypes have unequal variances on different substrates (responsiveness). Intersecting responses indicate a lack of correlation between genotypes on different substrates (inconsistency). (A) Two simple-selected genotypes from the same population. (B) Two complex-selected genotypes from the same population. Substrates are ranked by decreasing fitness of the genotype with the highest mean out of the pair. The substrates present in the selection environments are underlined. In complex-selected populations 65% (SE = 9%) of G X E variance is generated by changes in the ranking of genotypes with respect to fitness among environments. In simple-selected populations 24% (SE = 10%) of G X E is attributable to this component.

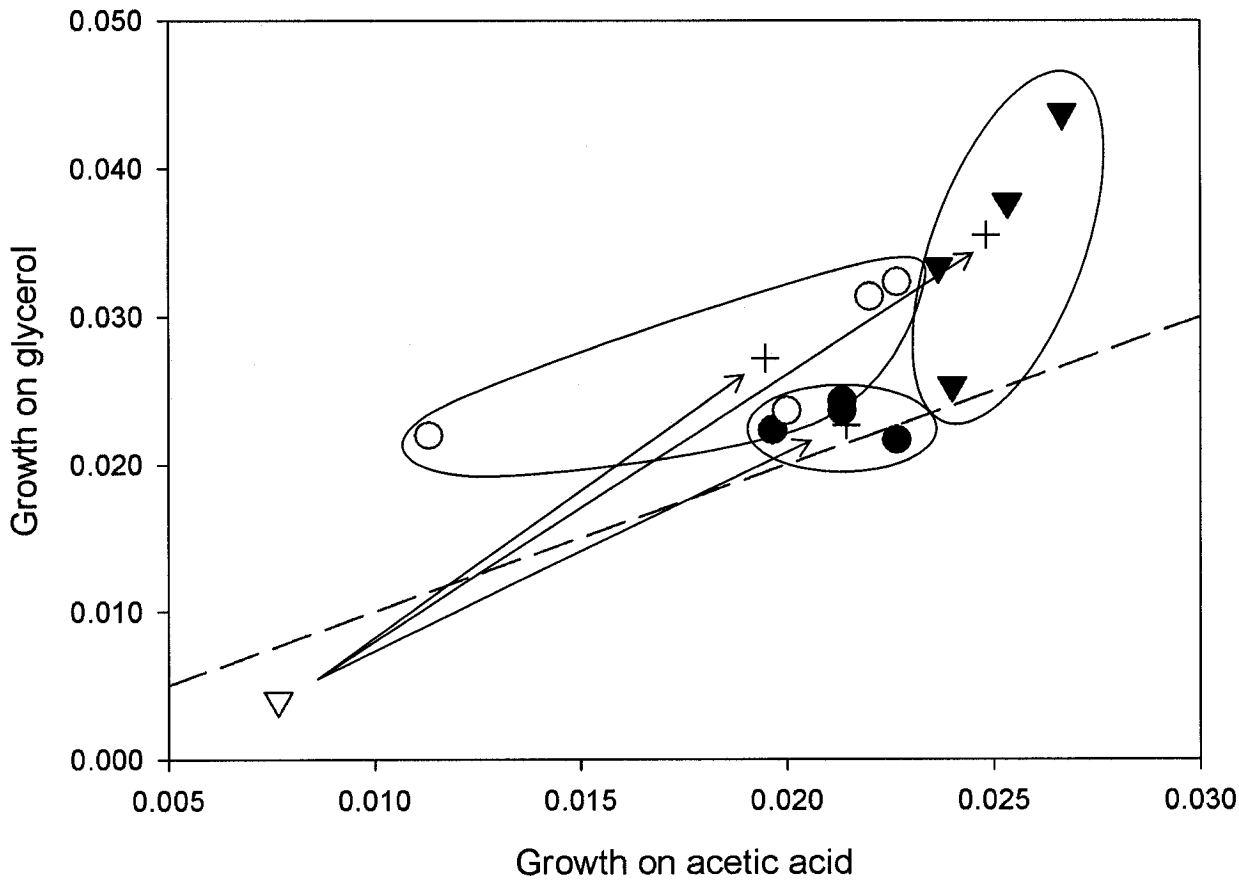

Figure 8. Example of synclinal selection. Each point represents the growth score of a genotype on glycerol and acetic acid. Solid circles represent acetic acid-selected genotypes, open circles represent glycerol-selected genotypes, solid triangles represent glycerol/acetic acid mixture-selected genotypes and the open triangle represents the ancestor. Crosses represent mean growth score for genotypes from each selection treatment. Arrows reflect the mean response to selection for genotypes from each selection treatment. The dashed line is a plot of the equation y = x.

Table 1. Carbon substrates used in selection environments.

Selection Environment	Complexity	Acetic acid	Fructose	Glycerol	Malic acid	Glucose	Ribose	Serine	Succinic acid
A	1	*							
B	1		*						
C	1			*					
D	1				*				
E	1					*			
F	1						*		
G	1							*	
H	1								*
AC	2	*		*					
BD	2		*		*				
EG	2					*		*	
FH	2						*		*
ABCD	4	*	*	*	*				
EFGH	4					*	*	*	*
ABCDEFGH	8	*	*	*	*	*	*	*	*

* Selection environment included this substrate

Table 2. Growth scores of the ancestor on all substrates. Three replicate measurements were made for two replicates of each of the ancestor genotypes: SBW25 and SBW25Δ*panB*.

Substrate	Substrate label	Mean optical density ± SE (600 nm)
Acetic acid	A	0.008 ± 0.002
Fructose	B	0.009 ± 0.001
Glycerol	C	0.004 ± 0.002
Malic acid	D	0.001 ± 0.001
Glucose	E	0.010 ± 0.004
Ribose	F	0.001 ± 0.001
Serine	G	0.000 ± 0.002
Succinic acid	H	0.014 ± 0.002
Grand mean		0.006 ± 0.001

Table 3. ANOVA for the response to selection testing effects of assay substrate (A), selection environment (S), line (L), and genotype (G). All simple and complex selection lines are included in the analysis.

Source	df	df denominator	MS ($x10^{-4}$)	*F*	*P*
S	14	15	0.404	3.78	<0.01
A	7	105	116.500	108.97	<0.001
S x A	98	105	0.752	7.03	<0.001
*L(S)	15	48	0.000	<1	ns
*L(S) x A	105	336	0.005	<1	ns
*G(L(S))	48	336	1.061	9.92	<0.001
*G(L(S)) x A	336	1243	0.148	1.39	<0.001
Replicate, r	1243		0.110		

* Random effects tested with Restricted Maximum Likelihood.

Literature Cited

Anderson, S. A., C. H. Sissons, M. J. Coleman, and L. Wong. 2002. Application of carbon utilization patterns to measure metabolic similarity of complex dental plaque biofilm microcosms. Applied and Environmental Microbiology 68:5779-5783.

Ayala, F. J., and C. A. Campbell. 1976. Frequency dependent selection. Annual review of ecology and systematics 32:115-138.

Bell, G. 1990. The ecology and genetics of fitness in *Chlamydomonas*. I. Genotype-by-environment interaction among pure strains. Proceedings of the Royal Society of London B 240:295-321.

—. 1996. Experimental evolution in *Chlamydomonas*. I. Short-term selection in uniform and diverse environments. Heredity 78:490-497.

—. 1997, Selection: The Mechanism of Evolution. New York, Chapman and Hall.

Bell, G., and X. Reboud. 1997. Experimental evolution in *Chlamydomonas*. II. Genetic variation in strongly contrasted environments. Heredity 78:498-506.

Bennett, A. F., R. E. Lenski, and J. E. Mittler. 1992. Evolutionary adaptation to temperature. I. Fitness responses *Escherichia coli* to changes in its thermal environment. Evolution 46:16-30.

Brzezinski, M. A., and D. M. Nelson. 1988. Interactions between pulsed nutrient supplies and a photocycle affect phytoplankton competition for limiting nutrients in long term culture. Journal of Phycology 24:346-356.

Cockerham, C. C. 1963. Estimation of genetic variances, Pages 164-196 *in* W. D. Hanson, and H. F. Robinson, eds. Statistical genetics and plant breeding. Washington, DC, National Academy of Sciences.

Cooper, M., and I. H. Delacy. 1994. Relationships among analytical methods used to study genotypic variation and genotype-by-environment interaction in plant breeding multi-environment experiments. Theoretical and Applied Genetics 88:561-572.

Cooper, V. S., and R. E. Lenski. 2000. The population genetics of ecological specialization in evolving *Escherichia coli* populations. Nature 407:736-739.

Dawson, S. L., J. C. Fry, and B. N. Dancer. 2002. A comparative evaluation of five typing techniques for determining the diversity of fluorescent pseudomonads. Journal of Microbiological Methods 50:9-22.

Dempster, E. R. 1955. Maintenance of genetic heterogeneity. Cold Spring Harbour Symposium of Quantitative Biology 20:25-32.

Dykhuizen, D., and M. Davies. 1980. An experimental model: bacterial specialists and generalists competing in chemostats. Ecology 61:1213-1227.

Fisher, R. A. 1930, The Genetical Theory of Natural Selection. Oxford, Oxford University Press.

Fry, J. D. 1996. The evolution of host specialization: are trade-offs overrated? American Naturalist 148:S84-S107.

Garcia-Dorado, A., P. Martin, and N. Garcia. 1991. Soft selection and quantitative genetic variation: a laboratory experiment. Heredity 66:313-323.

Gerrish, P. J., and R. E. Lenski. 1998. The fate of competing beneficial mutations in an asexual population. Genetica 102/103:127-144.

Gottschal, J. C. 1986. Mixed substrate utilization by mixed cultures *in* J. S. Poindexter, and E. R. Leadbetter, eds. Bacteria in Nature. New York, Plenum Press.

Gottschal, J. C., S. de Vries, and J. G. Kuenen. 1979. Competition between the facultatively chemolithotrophic Thiobacillus A2, an obligately chemolithtrophic Thibacillus and a heterotrophic spirillum for inorganic and organic substrates. Arch. Microbiology 121:85-86.

Gottschal, J. C., H. J. Nanninga, and J. G. Kuenen. 1981. Growth of Thiobacilus A2 under alternating growth conditions in the chemostat. Journal of General Microbiology 126:85-96.

Grover, J. P. 1988. Dynamics of competition in a variable environment: experiments with two diatom species. Ecology 69:408-417.

Haldane, J. B. S. 1932, The causes of evolution, Longmans and Green.

Harder, W., and L. Dijkhuizen. 1976, Mixed Substrate Utilization: Continuous culture 6: Applications and new fields. International symposium on continuous culture of microorganisms. Oxford, UK, Halsted Press.

—. 1982. Strategies of mixed substrate utilization in microorganisms. Philosophical Transactions of the Royal Society of London B 297:459-480.

Joshi, A., and J. N. Thompson. 1997. Adaptation and specialization in a two-resource environment in *Drosophila* species. Evolution 51:846-855.

Kassen, R., and G. Bell. 1998. Experimental evolution in *Chlamydomonas*. IV. Selection in environments that vary through time at different scales. Heredity 80:425-432.

Kawecki, T. J. 1994. Accumulation of deleterious mutations and the cost of being a generalist. American Naturalist 144:833-838.

Kawecki, T. J., N. H. Barton, and J. D. Fry. 1997. Mutational collapse of fitness in marginal habitats and the evolution of ecological specialization. Journal of Evolutionary Biology 10:407-429.

Laanbroek, H. J., A. J. Smit, G. Klein Nulend, and H. Veldkamp. 1979. Competition for L-glutamate between specialized and versatile *Clostridium* species. Arch. Microbiology 120:61-66.

Legan, J. D., J. D. Owens, and G. A. Chilvers. 1987. Competition between specialist and generalist methylotrophic bacteria for an intermittent supply of methylamine. Journal of General Microbiology 133:1061-1073.

Lindenmann, U., M. Snozzi, and T. Egli. 1996. Kinetics of the simultaneous utilization of sugar mixtures by *Escherichia coli* in continuous culture. Applied and Environmental Microbiology 62:1493-1499.

MacArthur, R. 1969. Species packing, and what interspecies competition minimizes. Proceedings of the National Academy of Science USA 64:1369-1371.

MacArthur, R., and R. Levins. 1964. Competition, habitat selection, and character displacement in a patchy environment. Proceedings of the National Academy of Science USA 51:1207-1210.

MacLean, R. C., A. Dickson, and G. Bell. 2005. Resource competition and adaptive radiation in a microbial microcosm. Ecology Letters 8:38-46.

MacLean, R. C., and G. Bell. 2002. Experimental adaptive radiation in *Pseudomonas*. American Naturalist 160:569-581.

MacLean, R. C., G. Bell, and P. B. Rainey. 2004. The evolution of a pleiotropic fitness tradeoff in *Pseudomonas fluorescens*. Proceedings of the National Academy of Science USA 101:8072-8077.

Miralles, R., P. J. Gerrish, A. Moya, and S. F. Elena. 1999. Clonal interference and the evolution of RNA viruses. Science 285:1745-1747.

Novella, I. S., C. L. Hershey, C. Escarmis, E. Domingo, and J. J. Holland. 1999. Lack of evolutionary stasis during alternating replication of an arbovirus in insect and mammalian cells. Journal of Molecular Biology 287:459-465.

Orr, H. A. 2005. The genetic theory of adaptation. Nature Genetics 6:119-127.

Paigen, K., and B. Williams. 1970. Catabolite repression and other control mechanisms in carbohydrate utilization. Advances in Microbial Physiology 4:251-324.

Rainey, P. B. 1999. Adaptation of *Pseudomonas fluorescens* to the plant rhizosphere. Environmental Microbiology 1:243-257.

Rainey, P. B., and M. J. Bailey. 1996. Physical and genetic map of *Pseudomonas fluorescens* SBW25 chromosome. Molecular Microbiology 19:521-533.

Robertson, A. 1959. Experimental design in the measurement of heritabilities and genetic correlations., Pages 79-91 *in* O. Kempthorne, ed. Biometrical Genetics. New York, Pergamon.

Silver, R. S., and R. I. Mateles. 1969. Control of mixed-substrate utilization in continuous cultures of *Escherichia coli*. Journal of Bacteriology 97:535-543.

Stanier, R. Y., N. J. Palleroni, and M. Doudoroff. 1966. The aerobic pseudomonads: a taxonomic study. Journal of General Microbiology 43:159-271.

Stewart, F. M., and B. R. Levin. 1973. Partitioning of resources and the outcome of interspecific competition: a model and some general considerations. American Naturalist 107:171-198.

Strobeck, C. 1975. Selection in a fine-grained environment. American Naturalist 109:419-425.

Taplitz, R. A., and J. M. Coffin. 1997. Selection of an avian retrovirus mutant with extended receptor usage. Journal of Virology 71:7814-7819.

Taylor, P. A., and P. J. L. Williams. 1975. Theoretical studies on the coexistence of competing species under continuous-flow conditions. Canadian Journal of Microbiology 21:90-98.

Tilman, D. 1977. Resource competition between planktonic algae: an experimental and theoretical approach. Ecology 58:338-348.

Turner, P. E., and S. F. Elena. 2000. Cost of host radiation in an RNA virus. Genetics 156:1465-1470.

Verdonck, M. 1987. Adaptation to environmental heterogeniety in populations of *Drosophila melanogaster*. Genetics Research 49:1-10.

Victorio, L., K. A. Gilbride, D. G. Allen, and S. N. Liss. 1996. Phenotypic fingerprinting of microbial communities in wastewater treatment systems. Water Research 30:1077-1086.

Wasserman, S. S., and D. J. Futuyma. 1981. Evolution of host plant utilization in laboratory populations of the southern cowpea weevil, *Callosobruchus maculatus Fabricus* (Coleoptera-Bruchidae). Evolution 35:605-617.

Weaver, S. C., A. C. Brault, W. Kang, and J. J. Holland. 1999. Genetic and fitness changes accompanying adaptation of an arbovirus to vertebrate and invertebrate cells. Journal of Virology 73:4316-4326.

Whitlock, M. C. 1996. The red queen beats the jack-of-all-trades: limitations on the phenotypic plasticity and niche breadth. American Naturalist 148:S65-S77.

Wilke, C. O. 2004. The speed of adaptation in large asexual populations. Genetics 167:2045-2053.

Wu, R., and R. F. Stettler. 1997. Quantitative genetics of growth and development in *Populus*. II. The partioning if genotype X environment interaction in stem growth. Heredity 78:124-134.

Chapter 2: The dynamics of diversification in evolving *Pseudomonas* populations [2]

The experiment presented in chapter 1 provided an analysis of niche width in populations at a single point in time after several hundred generations of selection. In this chapter, I present an experiment which provides the temporal dynamics of adaptation in simple and complex environments. Several genotypes from each population were assayed at ~100 generation intervals throughout the selection experiment. Considerably more genotypes were examined from each selection line than in the previous experiment. This permits observation of the trajectory of diversification. I show that diversity increases linearly through time in complex environments, but remains relatively stagnant in simple environments. Genotypes that were selected in complex environments show greater metabolic differentiation to sympatric than allopatric genotypes. Thus, diversity can be attributed to ecological character displacement, whereby genotypes specialize on alternative resources in order to escape competition.

[2]Accepted for publication in 2006 at *Evolution* by R.D.H. Barrett and G. Bell as "The dynamics of diversification in evolving *Pseudomonas* populations".

ABSTRACT

Determining the mechanisms that promote the evolution of diversity is a central problem in evolutionary biology. Previous studies have demonstrated that diversification occurs in complex environments and that genotypes specialized on alternative resources can be maintained over short time scales. Here, we describe a selection experiment that has tracked the dynamics of adaptive diversification within selection lines of the asexual bacteria *Pseudomonas fluorescens* over ~900 generations. We cultured experimental populations from the same two isogenic ancestral strains in simple, single-substrate environments or in complex, four-substrate environments. Following selection we assayed the growth of genotypes from each population on each substrate individually. We estimated mutational heritability, V_m/V_E, as 1×10^{-3} per generation in simple environments and 3×10^{-3} per generation in complex environments. These values are roughly consistent with estimates reported in other systems. Populations selected in complex environments evolved into genetically diverse communities. Genotypes exhibited greater metabolic differentiation from other genotypes in their own population than to genotypes evolving in other populations, presumably as a result of resource competition. In populations selected in simple environments, little genetic diversity evolved and genotypes shared very similar phenotypes. Our findings suggest that ecological opportunity provided by environmental complexity plays a major role in the evolution and maintenance of diversity.

INTRODUCTION

If a lineage is introduced to a heterogeneous environment, it will be provided with ecological opportunity in the form of underexploited or novel resources. Divergent natural selection can then arise from differences in the resources among habitats and competition for shared resources within the same habitat (MacLean et al. 2005; Schluter 2000). Environmental heterogeneity has been implicated as a primary mechanism governing the origin of diversity since at least the "Modern Synthesis" (Dobzhansky 1951; Fisher 1930; Muller 1942). Until recently, however, there has been relatively little empirical work on the subject (Rundle and Nosil 2005; Rundle and Schluter 2004; Schluter 1996; Schluter 2000; Schluter 2001). Recent advances in the study of adaptive diversification have been made through better estimates of changes in phenotypes and resource use through time (Schluter 2000). The number of cases is small, however, and most of the evidence has been obtained by comparative methods rather than by experiments (but see Buckling et al. 2003; Kassen et al. 2004). Experimental studies are illuminating because they can document the historical selection pressures that have led to divergence (Schluter 2000). In particular, microbial microcosms are now recognized as having several advantages over performing field experiments (Jessup et al. 2004; Kassen and Rainey 2004). Short generation times and large population sizes, along with the relative ease with which both organisms and environments can be manipulated, make these systems very amenable to testing ecological questions over evolutionary timescales.

The simplest type of selection experiment involves introducing a single genotype of an asexual microbe to an environment containing just a single niche

to which it is initially not well adapted. As the population grows, random mutations will lead to genetic diversification. A very small fraction of these mutations will be beneficial, in the sense that they confer a higher fitness than the ancestor. The fittest genotype among these mutants should eventually replace all others, leading to a genetically uniform population. This is the process of periodic selection, whereby evolution is characterised by a series of clonal replacements and diversity is purged each time a beneficial mutant arises and sweeps through the population (Atwood et al. 1951; Novick and Szilard 1950).

If the environment is not uniform but instead offers several distinct niches that differ in their conditions for growth, then this ecological opportunity can allow the evolution of a diverse community of genotypes (Day 2000; Geritz et al. 1998; Meeus and Goudet 2000). Rainey and Travisano (1998) provided a critical test of this prediction by selecting a single clone of the bacterium *Pseudomonas fluorescens* in microcosms incubated either with or without spatial heterogeneity. In spatially heterogeneous microcosms the ancestral genotype diversifies into several genetically differentiated morph variants. In contrast, no diversity evolves in the spatially homogeneous microcosms, presumably because of the lack of ecological opportunity provided by distinct niches. This model system has been extensively used to test questions about the ecology and genetics of diversification (Brockhurst et al. 2004; Buckling and Rainey 2002; Buckling 2003; MacLean et al. 2004; MacLean et al. 2005; Spiers et al. 2003; Travisano and Rainey 2000). None of these studies, however, have yielded insights into the dynamics of diversification on time scales longer than 100 generations.

Pseudomonas is one of the most diverse and ecologically significant groups of bacteria (Spiers et al. 2000). Members of the genus are abundant in all of the major natural environments (terrestrial, freshwater and marine) and also form intimate associations with plants and animals (Spiers et al. 2000). This distribution suggests a remarkable degree of adaptability. Here we use *P. fluorescens* to examine the *de novo* evolution of diversity in media of differing ecological opportunity. Ecological opportunity is determined by environmental complexity, which can be thought of as a specific type of environmental heterogeneity that occurs when a spatially homogeneous environment contains several resources. In a previous experiment we examined the role of environmental complexity in the evolution of niche breadth by selecting replicate lines of *P. fluorescens* in environments that varied in the number of available substrates (Barrett et al. 2005). This experiment showed that evolution in complex environments results in a genetically diverse population of overlapping generalists, each of which is adapted to a certain range of substrates but not all. Because populations were only sampled once, at the end of ~900 generations of selection, the experiment did not provide any information about the dynamics of diversification within each population. It was not possible, for example, to determine if the evolved diversity accumulated linearly or non-linearly through time.

In this paper we describe an experiment which provides a temporal perspective on evolution in complex environments. A great advantage of experimental evolution with microbes is that it permits "time-travel" through the evolutionary history of a population. Bacterial cultures can be frozen at regular

intervals throughout a selection experiment and revived for future analysis (Lenski et al. 1998). We selected replicate lines of *P. fluorescens* in five environments containing either one or four carbon substrates for ~900 generations. Every ~100 generations we froze a sample of each line and at the end of the selection experiment we revived these samples and measured the growth of genotypes from each line on every individual substrate. Lines were founded from clones, so that all variance expressed by evolved genotypes originated through novel mutations during the selection experiment. The overall goal of our work was to test the main prediction of the ecological theory of adaptive diversification: Diversity should evolve at a greater rate in environments with more ecological opportunity, and this diversification will occur as a result of selection for specialization on different resources in order to escape competition.

METHODS

Ancestral Strain

We used clonal isolates of *Pseudomonas* strains SBW25 and SBW25ΔpanB to found 8 replicates of 5 selection lines. SBW25ΔpanB is an isogenic strain of SBW25 containing a complete deletion of the panB gene. The panB gene is used to synthesize the vitamin pantothenate and when plated on indicator plates with a low concentration of pantothenate (2.4×10^{-6} %), SBW25ΔpanB grows noticeably smaller colonies than SBW25. This marker is selectively neutral and has no effect when pantothenate is present in high concentrations (MacLean et al. 2005; Rainey 1999). The two strains were mixed in roughly equal proportions to form a common pool to start the experimental populations. The ancestral clones were kept frozen at -80°C during the experiment in a mixture of 50% glycerol: 50% water (v:v).

Selection experiment

We chose four carbon substrates involved in different pathways important for *Pseudomonas* metabolism: glucose, fructose, serine and succinic acid. Two replicate lines were selected on each of the four carbon substrates, and eight replicate lines were selected on a mixture of all four substrates. Single-substrate environments will hereafter be referred to as "simple" environments and the four-substrate environment will be referred to as a "complex" environment. We grew populations on 96-well plates with each well containing an M9 salt solution (NH_4Cl 1g/L, Na_2HPO_4 6 g/L, KH_2PO_4 3 g/L, NaCl 0.5 g/L) supplemented with a

high concentration of pantothenate (2.4×10^{-3} %) and a source of carbon. We maintained a constant concentration for each substrate (0.3 g/L per substrate), rather than maintaining a constant total concentration for each environment. This decision was made so that the rewards of specializing on a specific substrate were equal in simple and complex environments (Barrett et al. 2005). Every 24 hours we transferred selection lines by using a 96-pin replicator to "print" the populations grown on a selection plate onto a fresh selection plate. The replicator transfers 0.06-0.07 μl of culture (~1×10^5 cells) on each pin to give a dilution factor of approximately 3000 fold per transfer. We continued the selection experiment for 80 transfers, which is equal to ~900 generations. Every ten transfers (~100 generations), we froze all of our lines at -80°C in a mixture of 50% glycerol: 50% water (v:v).

Assay

Before our assay, we reconditioned cultures in 96-well microplates containing dilute M9KB medium (NH_4Cl 0.1g/L, Na_2HPO_4 0.6 g/L, KH_2PO_4 0.3 g/L, NaCl 0.05 g/L, glycerol 1g/L, protease peptone 2 g/L) at 28°C for 24 hours. We serially diluted and plated out cultures from each replicate line on solid agar plates. We then randomly picked eight colonies from each line and grew these isolates in dilute M9KB medium at 28°C for 2 days. We diluted and starved cultures in M9 salt solution for at least two hours before the assay began. We then added 20 μl of starved cells (1×10^6 viable cells) from each culture to 96-well plates. Each well on each plate contained 180 μl M9 solution plus one of the carbon substrates used

in the selection experiment. We scored two replicates of each isolate in every assay substrate (2 replicates x 8 isolates x 16 lines x 4 assay substrates x 9 time points).

We measured optical density at 660 nm using a Synergy HT narrow beam plate reader (Biotek Instruments, Winooski, Vt.) at 24 hours ± 30 minutes of incubation at 28°C, so that the assay conditions were identical to those experienced by each population during one transfer in the selection experiment. The optical density score of any given well reflects the scattering of light by bacterial cells, and can therefore be used to measure the growth achieved by the populations. We corrected optical densities by subtracting control well scores from each absolute score. The growth of each line was calculated as the mean of all measurements of isolates from that line. The response to selection was calculated as the growth of a line minus the growth of the ancestor on the same substrate.

Statistical analysis

We analyzed the variance in growth within populations by partitioning into genetic, environmental and genotype-by-environmental (GxE) interaction components. We used the genetic variance component to estimate the diversity within each population. To follow the changes in diversity through time we conducted ANCOVA with JMP 4.0 software (SAS Institute, Cary, NC). We determined the rate at which new genetic variance evolved per generation, V_m, as the slope of the regression of genetic variance versus generation (Goho and Bell

2000). In order to standardize this "mutational heritability" so that it was directly related to the response to selection, we divided by the standing environmental variance, V_E, which we estimated from the y-intercept of the fitted linear regression (Lynch 1988). Note that this is based on the assumption that with a genetically uniform ancestral population, the phenotypic variation is equal to the environmental variation at the start of the experiment. Thus, this value is not equivalent to the environmental variance component determined through partitioning of the variance in growth among substrates. The standardized mutational heritability, V_m/V_E, can be used to compare the rate of accumulation of genetic variance for different traits or different organisms.

We estimated the phenotypic similarity between a pair of evolved genotypes at generation ~900 as the Euclidean distance in a multidimensional space in which each substrate represents one dimension. The Euclidean method estimates the distance between two genotypes, A and B, as $d(A,B) = (\Sigma_i(x_i - y_i)^2)^{1/2}$, where x_i and y_i are the mean scores of genotypes A and B, respectively, on substrate i, and the summation is over all substrates. We calculated the score of a single genotype in a given environment as the mean of the two replicate measurements. We used the distance matrix of all genotypes to calculate the average distance between genotypes from either the same or different lines.

RESULTS

Adaptive diversification through time

The effect of environmental complexity on adaptation and divergence has been difficult to address because of limitations in the ability to make causal associations between genetic variation and selectively important environmental factors. By measuring genetic variation in growth on substrates present in selection environments, it is possible to determine the ecological consequences of evolved diversity. The growth of lines increased non-linearly with the number of generations under selection (Fig. 1). By the end of the experiment the mean growth was 0.023 (SE = 0.00063), representing an increase of 90% over the ancestor. The response to selection varied among lines (SD = 0.003), with an observed range of 0.007 to 0.015. In contrast to the mean growth, the genetic variance within lines showed a linear increase with the number of generations under selection (Figure 2). The rate of this increase is different in simple and complex environments, as is indicated by the significant Time x Complexity interaction in the analysis of genetic covariance (Table 1). After 900 generations of selection, estimates of the genetic variance component are significantly greater for lines selected in complex media than for those selected in simple media (t = 3.54, df = 14, P = 0.003). The slopes of the regression for genetic variance versus generation (V_m) were 3.27×10^{-9} (SE 1.14×10^{-9}) for lines selected in simple environments and 1.12×10^{-8} (SE 1.94×10^{-9}) for lines selected in complex environments. The y-intercepts of this regression (V_E) were 3.60×10^{-6} (SE 7.72×10^{-7}) and 3.71×10^{-6} (SE 1.06×10^{-6}) for simple- and complex-selected lines respectively. These estimates of V_m and V_E give mutational heritabilities of

$V_m/V_E = 1.0 \times 10^{-3}$ per generation for simple-selected lines and 3.0×10^{-3} per generation for complex-selected lines.

Character displacement

If genetic variance within populations reflects adaptive diversification driven by competition for resources, sympatric genotypes should exhibit differentiation in phenotypic characters related to resource consumption (Schluter 2000). We used Euclidean distances to determine the differences in metabolic phenotype between genotypes. Relative to simple environments, genotypes selected in complex environments had more within-line phenotypic distance than between-line phenotypic distance ($X^2_{0.05,1} = 29.80$, $P < 0.0001$). Thus, complex-selected genotypes from different lines often share a common phenotype but sympatric complex-selected genotypes show divergent phenotypes. To determine whether the genetic diversity in each complex-selected population would fit into distinct phenotypic classes we performed *t*-tests for multiple comparisons on each line (Travisano et al. 1995). The eight genotypes assayed in each line were treated as fixed, and we applied a sequential Bonferroni correction since eight *t*-tests were performed (Rice 1989). A further Bonferroni correction was incorporated because we performed four such tests (one for each substrate), so that the experiment-wise type I error rate for each substrate was 0.05/4 = 0.0125. One or more genotypes were placed in a separate phenotypic class if they differed from all other genotypes in their performance on at least one substrate (Travisano and Lenski 1996). Using this method the average number of phenotypic classes within each population was very low (mean = 1.38), suggesting that most genotypes from the

same population share a common phenotype. This is because in most populations the performance of genotypes on each substrate is characterized by a fairly continuous distribution. There are few examples of specialists which are significantly better than all other genotypes on a specific substrate. There is, however, substantially greater genotype-by-environment interaction variance in complex-selected populations than in simple-selected populations (two-tailed t = 7.80, df = 14, $P < 0.001$). Moreover, the majority of genotype-by-environment interaction variance is due to inconsistency as opposed to responsiveness (average fraction of GxE attributable to inconsistency in complex lines = 86%). Thus, differences between sympatric genotypes from complex environments are not due to large differences in performance on each substrate but rather because the rankings of the genotypes vary on different substrates (Barrett et al. 2005).

DISCUSSION

Theory predicts that competition for shared resources in a genetically uniform population generates divergent selection for adaptation to alternate resources (Dieckmann and Doebeli 1999; Doebeli and Dieckmann 2000; Geritz et al. 1998; Maynard Smith 1966). A clear prediction of this theory is that diversity will increase with the number of resources available in the environment. There is evidence from natural systems to support this prediction (Armbrecht et al. 2004; McKane et al. 2002; Murdoch et al. 1972). However, these studies have been conducted on ecological timescales, in the sense that the organisms involved retain their properties unchanged during the experiment and no diversity evolves *de novo*. Microbial model systems have provided a way to follow replicated adaptive diversification over evolutionary timescales. MacLean et al. (2005) showed that selecting *Pseudomonas fluorescens* in complex mixtures of undefined substrates results in the evolution of communities of genotypes specialized to consume different resources. However, the performance of these genotypes was assayed on different substrates than were present in the selection environments, making it difficult to assess the ecological consequences of evolved diversity. Here we followed the evolution of diversity under chemically defined selection conditions and assayed performance on the same substrates that were present in the selection environments. In addition, we provided a control for the effect of complexity by also selecting in simple environments consisting of only a single resource.

Even in simple environments it appears that some diversity can evolve and be maintained. While this was not predicted, it has been shown in previous

experiments. For example, Rozenweig et al. (1995) demonstrated that a single *E. coli* genotype selected in a glucose-limited environment partitions the primary resource through its own metabolic activities. Excreted metabolites provide ecological opportunity for mutants with an enhanced ability to grow on these metabolites. When niche specialist mutants arise, they are maintained through the operation of density-dependent processes (Rainey et al. 2000). Other studies have also shown the emergence of diversity from genetically uniform populations during the course of evolution in simple environments (Rozen and Lenski 2000; Turner et al. 1996). It is possible that similar resource partitioning occurred in the simple environments in our experiment. Theory predicts, however, that cross-feeding is unlikely to evolve when using a transfer protocol like ours (Doebeli 2002). Although some genetic variance evolved in simple environments, the amount was modest relative to the complex environments. Thus, evolution in simple environments can be characterized as a gradual turnover of progressively better-adapted genotypes. In contrast, in complex environments the ancestor diversifies into a community of metabolically differentiated genotypes. Genotypes that evolved in the same populations exhibit more phenotypic differentiation from each other than to genotypes that evolved in different populations. This pattern is consistent with ecological character displacement, in which evolutionary shifts of phenotypic traits occur in response to competition for resources between similar genotypes.

The values that we obtained for V_m/V_E, 1×10^{-3} and 3×10^{-3} per generation, are comparable with other studies investigating the accumulation of genetic variance through time (reviewed in Houle et al. 1996). In the study with

the population sizes, generation times and culture conditions closest to ours, the estimate is ~4.5 x 10^{-3} (Goho and Bell 2000). This is considerably greater than the average value obtained in our experiment, suggesting that our system has accumulated either less genetic variance or expressed greater environmental variance. Their study may give biased estimates of V_m, however, because the assay conditions were different from the selection environment. It has been argued that the genetic correlation is a declining function of environmental variance, and since our selection experiment and assay were conducted in the same conditions, our estimate of V_m can be expected to be reliable (Bell 1992). Although there is some variation in V_m/V_E among studies, the overall impression is that values are remarkably consistent. Despite the various methods used to score diverse characters on organisms including *Drosophila*, *Tribolium*, *Daphnia*, *Caenorhabditis*, *Chlamydomonas*, mice, and several crop plants, almost all V_m/V_E values are between 10^{-4} and 5 x 10^{-2}, and the majority are roughly equal to 10^{-3} (Houle et al. 1996; Lynch 1988). This suggests the intriguing possibility that there may be general rules governing the accumulation of genetic variance in evolving populations. Our experiment is the first to follow the evolution of diversity over a long period of time in environments that vary in the amount of ecological opportunity available. Complex environments provided three additional substrates over the single substrate present in the simple environments. Interestingly, the V_m/V_E in complex environments was exactly three times that found in simple environments, implying that the rate of genetic accumulation may be roughly proportional to the amount of ecological opportunity present. Further tests at other

levels of ecological complexity will be required to test this relationship rigorously.

ACKNOWLEDGEMENTS

We thank R.C. MacLean and S. Smith for insightful discussion. R. Kassen and an anonymous reviewer provided useful comments on the manuscript. This work was supported by a grant from the Natural Sciences and Engineering Research Council of Canada to G.B.

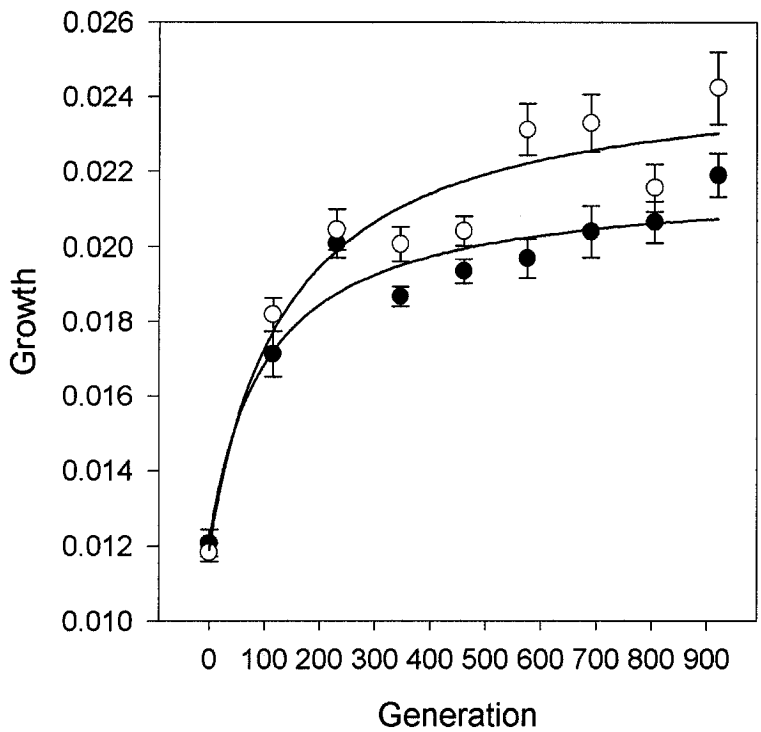

Figure 1 Adaptation in *Pseudomonas fluorescens* through time. The growth of lines cultured in simple (filled circles) and complex (open circles) environments is plotted against generation. Growth is calculated as the mean corrected growth of all simple- or complex-selected lines across all carbon substrates. Growth increased with number of generations under selection ($F = 180.98$, df = 1, $P < 0.0001$). Lines selected in complex environments had greater average growth than lines selected in simple environments ($F = 14.58$, df = 1, $P < 0.005$). The solid lines show the best fit of a hyperbolic model to the data. Error bars show ±1 SE.

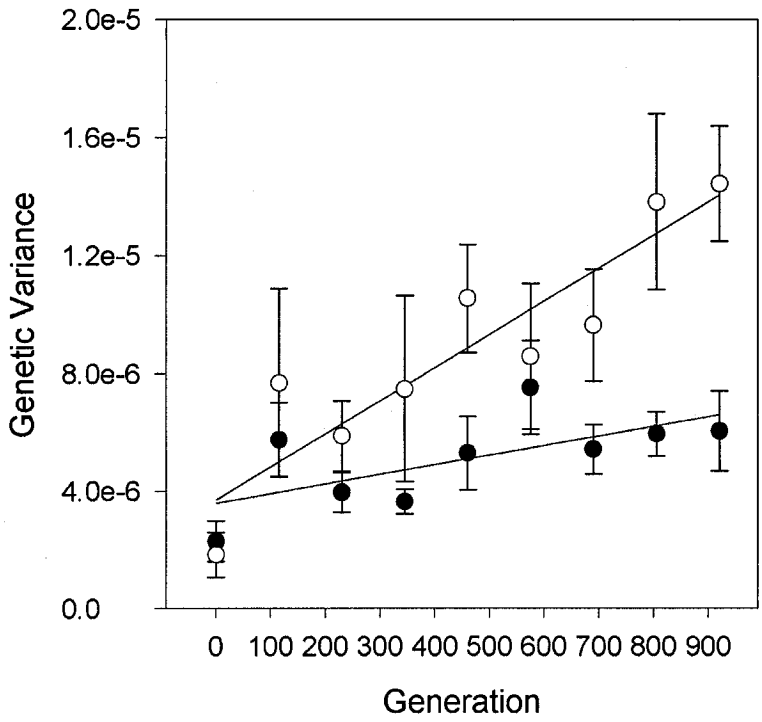

Figure 2 The evolution of genetic variance within *Pseudomonas fluorescens* populations through time. The genetic variance of lines cultured in simple (filled circles) and complex (open circles) environments is plotted against generation. Genetic variance is calculated as the mean genetic variance component within all simple- or complex-selected lines. Genetic variance increased with number of generations under selection ($F = 26.78$, df = 1, $P < 0.0001$). Lines selected in complex environments had greater genetic variance than lines selected in simple environments ($F = 20.59$, df = 1, $P < 0.0001$). Error bars show ±1 SE.

Table 1 ANCOVA for genetic variance testing effects of time and complexity. We calculated genetic variance by partitioning the growth variance in each line into genetic, environmental and genotype-by-environment interaction components. We obtained estimates of genetic variance for each level of complexity every 100 generations. The genetic variance estimate for each level of complexity is determined as the average genetic variance of all simple or complex selection lines.

Source	df	MS ($x10^{-10}$)	*F*	*P*
Time	1	6.69	26.78	<0.0001
Complexity	1	5.14	20.59	<0.0001
Time x Complexity	1	2.02	8.08	0.005
Replicate, r	140	0.25		

LITERATURE CITED

Armbrecht, I., I. Perfecto, and J. Vandermeer. 2004. Enigmatic biodiversity correlations: ant diversity responds to diverse resources. Nature 304:284-286.

Atwood, K. C., L. K. Schneider, and J. F. Ryan. 1951. Periodic selection in *Escherichia coli*. Proc. Nat. Acad. Sci. USA 37:146-155.

Barrett, R. D. H., R. C. MacLean, and G. Bell. 2005. Experimental evolution of *Pseudomonas fluorescens* in simple and complex environments. Am. Nat. in press.

Bell, G. 1992. The ecology and genetics of fitness in *Chlamydomonas*. V. The relationship between genetic correlation and environmental variance. Evolution 46:561-566.

Brockhurst, M. A., P. B. Rainey, and A. Buckling. 2004. The effect of spatial heterogeniety and parasites on the evolution of host diversity. Philos. Trans. R. Soc. Lond. ,Ser. B 271:107-111.

Buckling, A., and P. B. Rainey. 2002. The role of parasites in sympatric and allopatric host diversification. Nature 420:496-499.

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

Day, T. 2000. Competition and the effect of spatial resource heterogeneity on evolutionary diversification. Am. Nat. 155:790-803.

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

Dobzhansky, T. 1951. Genetics and the Origin of Species. Columbia Univ. Press, New York, NY.

Doebeli, M. 2002. A model for the evolutionary dynamics of cross-feeding polymorphisms in microorganisms. Popul. Ecol. 44:59-70.

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

Fisher, R. A. 1930. The Genetical Theory of Natural Selection. Oxford Univ. Press, Oxford.

Geritz, S. A. H., E. Kisdi, G. Meszena, and J. A. J. Metz. 1998. Evolutionary singular strategies and the adaptive growth and branching of the evolutionary tree. Evol. Ecol. 12:35-57.

Goho, S., and G. Bell. 2000. The ecology and genetics of fitness in Chlamydomonas. IX. The rate of accumulation of variation of fitness under selection. Evolution 54:416-424.

Houle, D., B. Morikawa, and M. Lynch. 1996. Comparing mutational heritabilities. Genetics 143:1467-1483.

Jessup, C. M., R. Kassen, S. E. Forde, B. Kerr, A. Buckling, P. B. Rainey, and J. M. Bohannan. 2004. Big questions, small worlds: microbial model systems in ecology. Trends Ecol. Evol. 19:189-197.

Kassen, R, Llewellyn, M, & Rainey, PB. 2004. Ecological constraints on diversification in a model adaptive radiation. Nature 431: 984-988.

Kassen, R., and P. B. Rainey. 2004. The ecology and genetics of microbial diversity. Ann. Rev. Microbiol. 58:207-231.

Lenski, R. E., J. A. Mongold, P. D. Sniegowski, M. Travisano, F. Vasi, P. J. Gerrish, and T. M. Schmidt. 1998. Evolution of competitive fitness in experimental populations of *E.coli*: What makes one genotype a better competitor than another? Antonie Leeuwenhoek 73:35-47.

Levene, H. 1953. Genetic equilibrium when more than one ecological niche is available. Am. Nat. 87:331-333.

Lynch, M. 1988. The rate of polygenic mutation. Genet. Res. 51:137-148.

MacLean, R. C., G. Bell, and P. B. Rainey. 2004. The evolution of a pleiotropic fitness tradeoff in *Pseudomonas fluorescens*. Proc. Nat. Acad. Sci. USA 101:8072-8077.

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

Maynard Smith, J. 1966. Sympatric speciation. Am. Nat. 100:637-650.

McKane, R. B., L. C. Johnson, G. R. Shaver, K. J. Nadelhoffer, E. B. Rastetter, B. Fry, A. E. Giblin, K. Kielland, B. L. Kwiatkowski, J. A. Laundre, and G. Murray. 2002. Resource-based niches provide a basis for plant species diversity and dominance in arctic tundra. Nature 415:68-71.

de Meeus, T. and J. Goudet. 2000. Adaptive diversity in heterogeneous environments for populations regulated by a mixture of soft and hard selection. Evolutionary Ecological Research 2: 981-995.

Muller, H. J. 1942. Isolating mechanisms, evolution and temperature. Biol. Symp. 6:71-125.

Murdoch, M. W., F. C. Evans, and C. H. Peterson. 1972. Diversity and pattern in plants and insects. Ecology 53:819-.

Novick, A., and L. Szilard. 1950. Experiments with the chemostat on spontaneous mutation of bacteria. Proc. Nat. Acad. Sci. USA 36:708-720.

Rainey, P. B. 1999. Adaptation of *Pseudomonas fluorescens* to the plant rhizosphere. Environ. Microbiol. 1:243-257.

Rainey, P. B., A. Buckling, R. Kassen, and M. Travisano. 2000. The emergence and maintenance of diversity: insights from experimental bacterial populations. Trends Ecol. and Evol. 15:243-247.

Rainey, P. B., and M. Travisano. 1998. Adaptive radiation in a heterogeneous environment. Nature 394:69-72.

Rice, W. R. 1989. Analyzing tables of statistical tests. Evolution 43:223-225.

Rosenweig, M. L. 1995. Species Diversity in Space and Time. Cambridge Univ. Press, Cambridge.

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

Rundle, H. D., and P. Nosil. 2005. Ecological speciation. Ecol. Lett. 8:336-352.

Rundle, H. D., and D. Schluter. 2004. Natural selection and ecological speciation in sticklebacks. Pp. 192-209 *in* U. Dieckmann et al. eds. Adaptive speciation. Cambridge Univ. Press, Cambridge.

Schluter, D. 1996. Ecological causes of adaptive radiation. Am. Nat. 148:S40-S64.

Schluter, D. 2000. The ecology of adaptive radiation. Oxford Univ. Press, New York.

Schluter, D. 2001. Ecology and the origin of species. Trends Ecol. and Evol. 16:372-380.

Spiers, A. J., J. M. Bohannan, S. M. Gehrig, and P. B. Rainey. 2003. Biofilm formation at the air-liquid interface by the Pseudomonas fluorescens SBW25 wrinkly spreader requires an acetylated form of cellulose. Mol. Microbiol. 50:15-27.

Spiers, A. J., A. Buckling, and P. B. Rainey. 2000. The causes of *Pseudomonas* diversity. Microbiology 146:2345-2350.

Strobeck, C. 1975. Selection in a fine-grained environment. Am. Nat. 109:419-425.

Travisano, M., and R. E. Lenski. 1996. Long-term experimental evolution in *Escherichia coli*. IV. Targets of selection and the specificity of adaptation. Genetics 143:15-26.

Travisano, M., J. A. Mongold, A. F. Bennett, and R. E. Lenski. 1995. Experimental tests of the roles of adaptation, chance, and history in evolution. Science 267:87-90.

Travisano, M., and P. B. Rainey. 2000. Studies of adaptive radiation using model microbial systems. Am. Nat. 156:S35-S44.

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

Chapter 3: Mutations of intermediate effect are responsible for adaptation in evolving *Pseudomonas fluorescens* populations[3]

In order for the diversity described in chapters 1 and 2 to evolve, it was necessary for different genotypes to specialize on different resources. This specialization occurs through adaptation, i.e. the evolution of trait values which improve fitness relative to alternative trait values. The driving force behind adaptation is the substitution of random beneficial mutations. It is clear that determining the distribution of fitness effects of beneficial mutations which achieve fixation is vitally important for understanding adaptation. Extensive theoretical work has suggested that the distribution will be bell-shaped for the first step in an adaptive walk. It is difficult to empirically test this prediction, however, because fixed beneficial mutations are extremely rare. In my previous experiments I discovered that Pseudomonas adapt rapidly to consume the substrate serine. This provides an opportunity to collect a large number of beneficial mutants in a short period of time. In my final experiment, I make use of this rapid adaptation to characterize the distribution of beneficial mutant fitness effects and show that both the average fitness effect and beneficial mutation rate are higher than previously supposed. Thus, this chapter provides a detailed examination of the mechanism which allowed the diversification reported in the previous chapters of the thesis.

[3]Submitted to *Biology Letters* by R.D.H. Barrett, R.C. MacLean and G. Bell in 2005 as "Mutations of intermediate effect are responsible for adaptation in evolving *Pseudomonas fluorescens* populations".

ABSTRACT

The fixation of a beneficial mutation represents the first step in adaptation, and the average effect of such mutations is therefore a fundamental property of evolving populations. It is nevertheless poorly characterized because the rarity of beneficial mutations makes it difficult to obtain reliable estimates of fitness. We obtained 68 genotypes each containing a single fixed beneficial mutation from experimental populations of *Pseudomonas fluorescens* evolving in medium with serine as the sole carbon source and estimated the fitness of each by competition with the ancestor. The distribution of fitness is modal and closely resembles the Weibull distribution. The average fitness effect (3.1) and beneficial mutation rate (1.0×10^{-7}) are very high relative to previous studies, possibly because the ancestral population grows poorly in serine-limited medium. Our experiment suggests that the initial stages of adaptation to stressful environments will involve the substitution of mutations with large effect on fitness.

1. INTRODUCTION

Evolution was originally conceived as an extremely gradual process, with natural selection acting on slight successive variations (Darwin 1859). R.A. Fisher developed a mathematical framework known as the infinitesimal model to describe the statistical basis of this gradualist interpretation of adaptation (Fisher 1930). This model supposes that the response to selection is fuelled by a very large number of loci each of very small effect. Fisher's influence has led to a rich body of mathematical theory on phenotypic evolution, built largely on this infinitesimal foundation. However, recent findings from the experimental study of adaptation suggest that morphological evolution often involves a modest number of genetic changes (Orr 2001). The development of quantitative trait locus (QTL) analysis has made it possible to directly map the factors underlying a response to selection. This approach has provided considerable evidence that evolution often operates through a few large changes rather than many small changes (Bradshaw et al. 1998; Shapiro & al. 2004; Wang et al. 1999). Unfortunately, it is very difficult to determine whether these findings represent the exception or the norm. The problem is that the low frequency of beneficial mutations has prevented empirical work with statistical power. Recently, experimental evolution using microbes has provided a way to increase greatly the number of beneficial mutations likely to arise during an experiment (Bull et al. 1997; Imhof & Schlotterer 2001; Rozen et al. 2002). Microbial populations facilitate the study of mutations because microbes have short generation times and large population sizes, thereby enabling the collection of large numbers of mutants.

In this study we conducted short-term experimental evolution with populations of the heterotrophic bacterium *Pseudomonas fluorescens* selected under stressful conditions. This enabled us to collect a library of genotypes, each containing a single fixed beneficial mutation. By competing these genotypes against the ancestor we obtained relative fitness values associated with each mutation. The objective of this study was to determine both the shape and scale of the distribution of these fitness effects. In doing so, we seek a more comprehensive understanding of the genetic basis of adaptive evolution and the cause of phenotypic change through time.

2. MATERIALS AND METHODS

(a) Ancestral Strain

We used clonal isolates of Pseudomonas strains SBW25 and SBW25Δ*panB* to found 96 replicate lines. SBW25Δ*panB* is an isogenic strain of SBW25 containing a complete deletion of the *panB* gene and when plated on indicator plates with a low concentration of pantothenate (2.4×10^{-6} %) grows noticeably smaller colonies than SBW25. The two strains were mixed in roughly equal proportions to form a common pool to start the experimental populations.

(b) Selection experiment

We grew populations on 96-well plates with each well containing an M9 salt solution (NH_4Cl 1g/L, Na_2HPO_4 6 g/L, KH_2PO_4 3 g/L, NaCl 0.5 g/L) supplemented with a high concentration of pantothenate (2.4×10^{-3} %) and 0.3 g/L serine. *Pseudomonas fluorescens* has very poor growth on the carbon substrate serine, but will rapidly adapt to utilize the substrate within ~100 generations of selection (Barrett et al. 2005). Every 24 hours we transferred selection lines by using a 96-pin replicator that transfers 0.06-0.07 μl of culture (~7×10^3 cells) on each pin, about a 3000-fold dilution. After each transfer we scored the marker state of half of the lines using a ProtoCOL SR/HR counting system (Synoptics Ltd., Cambridge, UK). When the frequency of a marker state exceeded 95% we froze a sample from the line, and collected samples of 70 populations in this way.

(c) Competitive fitness assays

For each line, we mixed evolved and ancestral genotypes to form a common pool that was inoculated into two replicate competition microcosms containing 200 μl of the original selection environment. Common pools were then diluted and spread on indicator plates to ascertain the initial ratio of the two competing genotypes. After incubation at 28°C for 24 hours the culture was again diluted and spread on indicator plates. Relative fitness was calculated as the ratio of the estimated growth rate of evolved genotype to the ancestor, so that equal fitness is indicated by $w = 1$ (Lenski et al. 1991).

(d) Statistical Analysis

Statistical analyses were performed using Mathematica 5.0. For the purpose of describing the distribution of fitness effects from beneficial mutations, each genotype was treated as an independent observation. We used maximum likelihood to quantify the fit of alternative probability density functions (pdf) to the observed distribution of selection coefficients and then used Akaike's Information Criterion (AIC) to compare the log likelihood of non-nested models. The model that is most consistent with the observations, while requiring the lower number of parameters, is the one with the lowest AIC.

3. RESULTS

We identified 70 genotypes with fixed mutations in 96 replicate lines. Two genotypes were discarded because their fitness was <1 and therefore they did not represent beneficial mutations. Hence, at least 68 beneficial mutations occurred over approximately 8000 generations. The substitution rate for beneficial mutations per generation is approximately $k \approx \mu N_0 g \bar{p}$, where N_0 is the cell number after transfer, g is the number of generations per transfer, and $\bar{p}$ the average fixation probability calculated using Kimura's haploid diffusion model (Crow & Kimura 1970); we then estimated the rate beneficial mutation rate as $\mu \approx k / N_0 g \bar{p}$. Given an approximate substitution rate for beneficial mutations (k) of 7.6×10^{-3} per generation, a population size after transfer (N_0) of 7.0×10^3, 11.55 generations per transfer (g), and an average probability of fixation ($\bar{p}$) of 0.93, we obtained an estimate of the beneficial mutation rate as 1.01×10^{-7} per generation. The beneficial mutations had a large effect on fitness, as shown by the size of the competitive advantage that evolved genotypes held over the ancestor (mean $w = 3.09$, $t = 16.25$, df = 67, $P < 0.0001$). The advantage varied substantially across genotypes ($F = 2.71$, df = 67, $P < 0.0001$, s.d. = 1.06), with an observed range of 1.20 to 6.19.

The measured selection coefficients of evolved genotypes are plotted as a histogram in figure 1. Among the alternative models compared with the empirical distribution, the Weibull model had the lowest AIC score (AIC = 199.379, ΔAIC to next lowest model = 4.78, Table 1). The Weibull distribution is a versatile distribution; it exhibits various shapes, depending on the coefficient of variation.

If the coefficient of variation is greater than one, the distribution is L-shaped, whereas if the coefficient of variation is less than one, it is bell-shaped. For the parameters of the Weibull distribution that maximized the likelihood of observing our data, the distribution is bell-shaped with a peak at intermediate values (mean = 2.09, CV = 0.51). In order to assess our confidence in the shape of the distribution, we calculated 95% confidence boundaries for the coefficient of variation (0.43 to 0.61) of the model. For all values of the coefficient of variation within these bounds, the Weibull distribution remains bell-shaped (Fig. 1).

4. DISCUSSION

The distribution of beneficial effects in experimental systems is poorly understood (Wilke 2004). Regardless of the specific probability distribution, however, extreme value theory predicts that the frequencies of fitness effects of beneficial mutations will be exponentially distributed (Gillespie 1984; Orr 2002). These beneficial mutations cannot be observed directly until they spread to an appreciable frequency in the population and become "contending" mutations that will be fixed unless a superior competitor arises first. Since mutations of small effect will have very low probabilities of fixation, the distribution of mutations that actually become fixed, and thus contribute to adaptation, is predicted to shift from exponential to bell-shaped among fixed mutations (Orr 1998). High selective advantages will also make it more likely for beneficial mutations to escape extinction through clonal interference or population bottlenecks (Campos & de Oliviera 2004; Gerrish & Lenski 1998; Haldane 1927; Kimura 1983; Wahl et al. 2002; Wilke 2004). Thus, although mutations of larger effect may be less frequent, they will be selected more rapidly, and in consequence mutations of intermediate effect may be the most frequent among those that are actually fixed.

There have been very few empirical studies characterizing the statistical properties of beneficial mutations. Two studies have found support for exponentially distributed beneficial fitness effects (Imhof & Schlotterer 2001; Sanjuan et al. 2004), but they involved mostly contending rather than fixed mutations. Rozen et al. (2002) reported that adaptation by *E. coli* to glucose-limited medium, in which the ancestor has high fitness, involved beneficial mutations whose frequencies were not distributed exponentially, but rather had a

peaked distribution. Our results support this finding in a more extensive sample of mutations, and suggest that a bell-shaped distribution is appropriate for describing the properties of these mutations that eventually become fixed. This pattern appears to be a robust feature of adaptation that transcends the specific features of the organism and the environment in question.

In previous studies investigating the fitness distribution of beneficial mutations (Gerrish & Lenski 1998; Imhof & Schlotterer 2001; Lenski et al. 1991; Rozen et al. 2002), estimates of both fitness effects and mutation rate were an order of magnitude lower than those reported here. The most likely cause of this discrepancy is that in these experiments *E. coli* was cultured in rich LB medium or glucose, environments in which the ancestor would have a nearly optimal phenotype. In contrast, the ancestral strain of *Pseudomonas* used in our experiment has very poor growth in carbon-limited serine environments (Barrett et al. 2005). Bull et al. (2000) found beneficial mutations of large effect ($S = 0.8$ to 13.9) when they selected a bacteriophage at very high temperatures, but they did not collect enough mutants to determine the distribution of fitness effects. Our experiment characterizes this distribution in a stressful environment, and shows that its shape remains peaked although the average fitness effect is much larger.

The rapid evolution of resistance to antibiotics, insecticides and herbicides by bacteria and other organisms has had serious consequences for human well-being. In all cases the pest populations must adapt to novel and stressful conditions. This adaptation is ultimately the result of the substitution of spontaneous beneficial mutations, which is the fundamental unit of evolutionary change. Our experiment is one of a small handful of empirical studies that have

investigated the distribution of fitness effects of beneficial mutations, and the first study to determine this distribution in an environment where the ancestor has low fitness. We found that the first mutation fixed tended to have very high relative fitness, whereas the shape of the distribution resembled that found in a previous study conducted with a different organism in more favourable conditions. Experimental work seems to be leading towards the conclusion that the mutations responsible for the initial stages of adaptive evolution may have much larger effects than is usually supposed.

Acknowledgements

Pseudomonas fluorecens SBW25 was a kind gift from P. Rainey. S. Otto provided invaluable statistical advice. This work was supported by a grant from the Natural Sciences and Engineering Research Council of Canada to G.B. R.C.M. was supported by a Postgraduate fellowship grant from the Natural Sciences and Engineering Council of Canada.

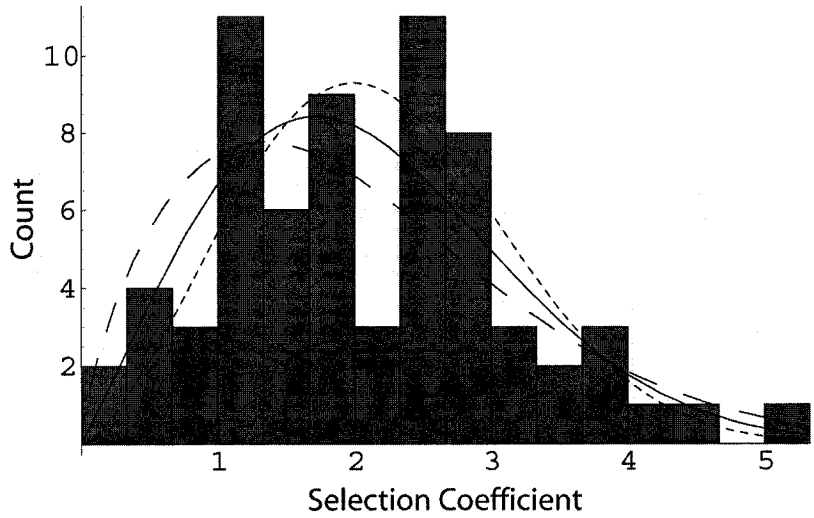

Figure 1. Distribution of fitness effects for fixed beneficial mutations. The solid line shows the Weibull distribution with parameters that maximize the likelihood of observing our data (mean = 2.09, coefficient of variation = 0.51). The dashed lines show the Weibull distribution at the 95% confidence boundaries for maximum likelihood of the coefficient of variation (short dash: mean = 2.03, coefficient of variation = 0.43, long dash: mean = 2.17, coefficient of variation = 0.61).

Table 1. Akaike's Information Criterion (AIC) for several probability density functions. The model that is most consistent with the observations, while requiring the lower number of parameters, is the one with the lowest AIC.

Model	Parameters	AIC	ΔAIC
Weibull	2	199.38	0
Normal	2	204.16	4.78
Logistic	2	206.16	6.79
Log-normal	2	215.05	15.67
Half-normal	1	216.54	17.16
Gamma	2	235.76	36.38
Exponential	1	238.46	39.08

Literature cited

Barrett, R. D. H., MacLean, R. C. & Bell, G. 2005 Experimental evolution of *Pseudomonas fluorescens* in simple and complex environments. *Am. Nat.* **166**, 470-480.

Bradshaw, H. D., Otto, K. G., Frewen, B. E., McKay, J. K. & Schemske, D. W. 1998 Quantitative trait loci affecting differences in floral morphology between two species of Monkeyflower (*Mimulus*). *Genetics* **149**, 367-382.

Bull, J. J., Badgett, M. R. & Wichman, H. A. 2000 Big-Benefit Mutations in a Bacteriophage Inhibited with Heat. *Mol. Biol. Evol.* **17**, 942-950.

Bull, J. J., Badgett, M. R., Wichman, H. A., Huelsenbeck, J. P., M., H. D., Gulati, A., Ho, C. & Molineux, I. J. 1997 Exceptional convergent evolution in a virus. *Genetics* **147**, 1497–1507.

Campos, P. R. A. & de Oliviera, V. M. 2004 Mutational effects on the clonal interference phenomenon. *Evolution* **58**, 932-937.

Crow, J. F. & Kimura, M. 1970 *An Introduction to Population Genetic Theory.* New York: Harper & Row.

Darwin, C. 1859 *On the Origin of Species by Means of Natural Selection.* London: Murray.

Fisher, R. A. 1930 *The Genetical Theory of Natural Selection.* Oxford: Oxford University Press.

Gerrish, P. J. & Lenski, R. E. 1998 The fate of competing beneficial mutations in an asexual population. *Genetica* **102/103**, 127-144.

Gillespie, J. H. 1984 Molecular evolution over the mutational landscape. *Evolution* **38**, 1116-1129.

Haldane, J. B. S. 1927 The mathmatical theory of natural and artificial selection, part V: Selection and mutation. *Proc. Camb. Phil. Soc.* **23**, 838-844.

Imhof, M. & Schlotterer, C. 2001 Fitness effects of advantageous mutations in evolving *Escherichia coli* populations. *PNAS* **98**, 1113-1117.

Kimura, M. 1983 *The Neutral Theory of Molecular Evolution.* Cambridge, UK: Cambridge University Press.

Lenski, R. E., Rose, M. R., Simpson, S. C. & Tadler, S. C. 1991 Long-term experimental evolution in *Escherichia coli* I. Adaptation and divergence during 2000 generations. *Am. Nat.* **138**, 1315-1341.

Orr, H. A. 1998 The population genetics of adaptation: the distribution of factors fixed during adaptive evolution. *Evolution* **52**, 935-949.

Orr, H. A. 2001 The genetics of species differences. *Trends Ecol. Evol.* **16**, 343-350.

Orr, H. A. 2002 The population genetics of adaptation: the adaptation of DNA sequences. *Evolution* **56**, 1317-1330.

Rozen, D. E., de Visser, J. A. G. M. & Gerrish, P. J. 2002 Fitness effects of fixed beneficial mutations in microbial populations. *Curr. Biol.* **12**, 1040-1045.

Sanjuan, R., Moya, A. & Santiago, E. 2004 The distribution of fitness effects caused by single-nucleotide substitutions in an RNA virus. *PNAS* **101**, 8396-8401.

Shapiro, M. D. & al., e. 2004 Genetic and developmental basis of evolutionary pelvic reduction in threespine stickleback. *Nature* **428**, 717-723.

Wahl, M. L., Gerrish, P. J. & Saika-Voivoda, I. 2002 Evaluating the Impact of Population Bottlenecks in Experimental Evolution. *Genetics* **162**, 961-971.

Wang, R. L., Stec, A., Hey, J., Lukens, L. & Doebley, J. 1999 The limits of selection during maize domestication. *Nature* **398**, 236-239.

Wilke, C. O. 2004 The speed of adaptation in large asexual populations. *Genetics* **167**, 2045-2053.

SUMMARY

In the introduction to this thesis I described a general theory for the origin of diversity that gives a major role to divergent adaptation to different resources available in a heterogeneous environment. In the body of this thesis I describe a series of experiments designed to better understand adaptation and the evolution of diversity in populations of *Pseudomonas fluorescens*. I summarize the primary conclusions of these experiments below.

A main factor driving the evolution of diversity is ecological opportunity provided by environmental complexity in the form of diverse resources. If a genetically uniform population is introduced into a complex environment, divergent selection for underutilized resources will result in a genetically diverse population (chapters 1 and 2). This diversity increases linearly through time (chapter 2) and after almost 1000 generations, genotypes in complex-selected populations will be neither narrow specialists or complete generalists but instead imperfect overlapping generalists (chapter 1). Although they rarely fit into distinct phenotypic classes (chapter 2), genotypes are metabolically differentiated so that the ranks of coexisting genotypes are different on different resources (chapters 1 and 2). Differentiation is greatest between genotypes from the same population, suggesting that diversification is driven by competition for resources that are uniformly distributed throughout the microcosms (chapter 2). Finally, adaptation to each substrate is underlain by the substitution of beneficial mutations that most often have intermediate fitness effects (chapter 3).