This is to certify that the

dissertation entitled

COMPARATIVE AND EXPERIMENTAL TESTS FOR A TRADEOFF IN BACTERIAL FITNESS AT LOW AND HIGH SUBSTRATE CONCENTRATION

presented by

Gregory Jon Velicer

has been accepted towards fulfillment
of the requirements for

Ph.D. degree in Genetics

Richard E. Lenski
Major professor

Date 26 February 1997

COMPARATIVE AND EXPERIMENTAL TESTS FOR A TRADEOFF IN BACTERIAL FITNESS AT LOW AND HIGH SUBSTRATE CONCENTRATION

By

Gregory Jon Velicer

A DISSERTATION

Submitted to
Michigan State University
in partial fulfillment of the requirements
for the degree of

DOCTOR OF PHILOSOPHY

Genetics Program

1997

ABSTRACT

COMPARATIVE AND EXPERIMENTAL TESTS FOR A TRADEOFF IN BACTERIAL FITNESS AT LOW AND HIGH SUBSTRATE CONCENTRATION

By

Gregory Jon Velicer

It is commonly assumed that microorganisms that are superior competitors under abundant resource conditions are relatively inferior under scarce resource conditions, and *vice versa*. The studies described in this dissertation test this tradeoff hypothesis by both comparative and experimental approaches. In the comparative study, growth rates of seven natural strains of bacteria capable of degrading the herbicide 2,4-dichlorophenoxyacetic acid (2,4-D) were measured at both high and low concentrations of 2,4-D and succinate. The results are suggestive of a positive correlation between growth rates at high and low substrate concentrations, contrary to the negative correlation predicted by the tradeoff hypothesis. For the experimental study, numerous replicate populations of 2,4-D degrading bacteria were allowed to evolve independently under both abundant (batch culture) and scarce (chemostat culture) resource regimes. The competitive performance of each evolved strain relative to its ancestor was measured in both types of selective regime. The tradeoff hypothesis predicts that adaptation to one selective regime will cause fitness loss in the alternative regime. Competition results demonstrate that this is not always the case. Numerous

evolved lines simultaneously improved their fitness in both abundant and scarce resource selective regimes, refuting the tradeoff hypothesis in its most general form. Changes in performance in the alternative regime after adaptation to a selective regime appear to be caused primarily by pleiotropic side-effects (both beneficial and harmful) of selected mutations. Also, experimental lines that adapted significantly to one substrate (either 2,4-D or succinate) in the batch regime show heterogeneous correlated performance responses on the alternative substrate, with some lines gaining and other lines losing fitness on the alternative substrate.

This dissertation is dedicated to
my parents - Dr. Leland and Janet Velicer,
my brothers - Mark and Daniel,
my wife - Yuen-tsu,
and my daughter - Irene Marie.

ACKNOWLEDGMENTS

I thank Professor Richard E. Lenski, my graduate advisor, for his many hours of guidance and encouragement over the past five years. The members of my guidance committee, Larry Forney, Tom Schmidt, Larry Snyder and Jim Tiedje, were very helpful and supportive throughout my graduate school years in numerous ways.

Several people gave scientific assistance that was important to the completion of this project, including Brendan Bohannan, John Dunbar, Ryszard Korona, Cathy McGowan, Judy Mongold, Cindy Nakatsu, Rob Sanford, and Tim Sassanella. I thank Shenandoah Oden and Elizabeth Smalley for many long hours of technical assistance and Lynette Ekunwe for helping keep the Lenski lab functional and enjoyable to work in. The friendliness and helpfulness of Paul Sniegowski, Doug Taylor, Mike Travisano, Paul Turner and Farida Vasi during my early years in the Lenski lab is much appreciated.

Thanks to my grandparents, George and Elsie Velicer, for giving generously to my education from hard-earned farm money. Thanks to my parents, Dr. Leland and Janet Velicer, for their many years of love and encouragement of my studies. And thanks to my wife and daughter, Yuen-tsu and Irene Marie, for making it a true joy to head home from the lab at the end of each day.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

INTRODUCTION

The physiological structure and function of organisms may involve tradeoffs in the allocation of limiting resources to alternative selective demands. These tradeoffs can be studied at a variety of levels, including genetic, organismal, and population levels. The studies described in this dissertation have taken population and evolutionary approaches to test for a tradeoff between competitive fitness at low *versus* high resource conditions.

To what extent can organisms optimize their ability to compete both when their primary carbon source is abundant and when it is scarce? Conventional wisdom holds that it is impossible for an organism to be a superior competitor under both of such radically different resource conditions. Holding this view, microbial ecologists have often assumed that microorganisms with high maximum growth rates under nutrient rich conditions are generally poor competitors for sparse resources, and *vice versa*.

The evidence supporting this position is largely anecdotal and comparative in nature. Here I describe research that tests the resource concentration tradeoff hypothesis, first by a systematic comparison of the growth rates of several bacterial strains, and then by evolution

experiments designed to select for competitive fitness at high and low resource concentrations.

The microbial system

The tradeoff hypothesis in question has relevance both to theoretical issues in microbial ecology and evolution and to more applied questions in bioremediation and industrial processes. Attractive organisms for testing resource concentration tradeoff theory are microbes that degrade anthropogenic compounds. Many such substances, such as 2,4-dichlorophenoxyacetic acid (2,4-D), are known to be toxic to humans and potentially disruptive to ecological processes. Effective bioremediation of toxic substrates "requires the selection of inoculant strains able to effectively degrade environmental pollutants over a wide range of concentrations" (Greer et al. 1992). Determining whether there is tradeoff in bacterial fitness at low *versus* high resource concentrations is therefore important for identifying the most useful inoculant strains.

Current bioremediation practice focuses primarily on enhancing growth conditions for indigenous microflora that are capable of growth on an unwanted substrate. If, however, a very clear tradeoff exists, such that some organisms are superior at high substrate concentrations and others at low concentrations, then it may be possible to improve the effectiveness of bioremediation by applying microbes that are appropriate for a particular site based on their kinetic

parameters. This strategy may thus enable "rapid degradation at high pollutant concentrations or thorough degradation at low pollutant concentrations" (Greer et al. 1992). 2,4-D is a herbicide used worldwide that has been subject to much research regarding its toxic effects (Brueggeman 1993, Ebasco and Herra 1993). Research on the ecology, metabolism and genetics of 2,4-D degrading bacteria is among the most extensive for microbes used in bioremediation. Therefore, 2,4-D degrading bacteria are an excellent system to use for testing the substrate concentration tradeoff hypothesis.

Knowledge of the genetics underlying 2,4-D degradative functions may allow future molecular analyses of adaptive genetic changes that have occurred during the experimental evolution portion of the studies reported in this dissertation. Bacterial strains vary in their complement of genes encoding the 2,4-D degradative pathway, which can be borne either on the chromosome or on plasmids. The degradative pathways for 2,4-D metabolism and the underlying genes have been thoroughly reviewed by Calabrese (1994). Moreover, 2,4-D degradative functions exhibit considerable diversity at the levels of gene organization, pathways, physiological traits, and bacterial classification. (Don and Pemberton 1981; Calabrese et al. 1992). Horizontal transfer and the subsequent evolution of a relatively few ancestral 2,4-D metabolic genes are believed to account for the current diversity (Don and Pemberton 1981; McGowan 1995).

The comparative study

Numerous previous studies in evolutionary biology have employed either a comparative or an experimental approach to test hypothetical tradeoffs between different performance measures. Unlike most of these previous studies, I have brought both comparative and experimental tests to bear on the substrate concentration tradeoff hypothesis, within the context of a single biological system. The comparative approach involved testing for statistical correlations between growth rates measured at high and low resource conditions using seven strains of 2,4-D degrading bacteria isolated from nature (Chapter 1).

The tradeoff hypothesis predicts a negative correlation between growth rates at high and low resource concentrations. I tested this prediction by measuring the growth rates of the seven strains at 5 µg/ml and 500 µg/ml of each of two substrates. The analysis of growth rates was performed using two different methods, one traditional and the other a modern approach that incorporates phylogenetic information. The traditional method treats each strain as an independent observation, and it is the method that has been typically employed by microbial ecologists. The modern methods incorporates information on the phylogenetic relationships of the strains whose properties were measured, thus avoiding spurious correlations that may reflect unique historical

events rather than general evolutionary trends (Harvey and Pagel 1991).

The experimental study

Evolutionary experimentation using bacteria is a powerful method for testing hypotheses in population biology. Such laboratory evolution has given important insight into numerous biological phenomena at multiple levels. At the genetic level, such studies have addressed the evolution of pleiotropy and epistasis (Lenski 1988) and the effects of mutation rate differences on fitness (Chao and Cox 1983). Experimental evolution has also addressed more ecological aspects of evolution, including the coevolution of bacteria and viruses (Lenski and Levin 1985) and the effects of antibiotic production on competitive interactions (Chao and Levin 1981). Physiologically oriented studies have addressed the relationship between metabolic flux and fitness (Dykhuizen et al. 1987; Dykhuizen and Dean 1990). Basic theoretical issues in evolutionary biology such as the roles of adaptation, history and chance in evolutionary change (Travisano et al. 1995), as well as applied corcerns such as the enhancement of competitive fitness in the biodegradation of toxic compounds (Korona et al. 1994), have been tested by the use of experimental evolution.

Rapid bacterial growth during experimental evolution allows changes to be tracked over evolutionarily significant time scales. Changes in population composition can be

accurately monitored using genetic and phenotypic markers that can be easily scored. Environmental parameters can be precisely controlled, allowing strong inferences about the causal effects of natural selection. Large population sizes ensure that a great number of spontaneous mutations occur every generation. Other important features of bacteria include the ability to freeze ancestral and experimental lines for later analyses, and to measure relative fitness in any environment by competing derived lines against their ancestors. Moreover, the general ease of culture, storage, and analysis make it feasible to obtain replicate lines for each experimental treatment. Replication of lines allows one to assess whether genetic responses to imposed selection are uniform or heterogeneous.

To complement the comparative study, I conducted a two-stage evolution experiment that allowed evolving populations of 2,4-D degrading bacteria to adapt to either high or low substrate conditions (Chapter 2). To test the tradeoff hypothesis, evolved lines were placed in competition with their ancestors to assess their performance under regimes of both resource scarcity and abundance. The tradeoff hypothesis predicts that lines that adapt significantly to one regime must lose competitive fitness in the alternative regime.

Effects of adaptation to a single substrate on performance on a different substrate

In addition to testing the tradeoff hypothesis described above, I have used the same experimentally evolved lines to test the consequences of adaptation to one substrate for competitive performance on an alternative substrate (Chapter 3). Such consequences are relevant to concerns about the evolution of genetically modified organisms following their release into the environment. In particular, it is often assumed that genetically modified organisms will perform their intended function and then "fade away," thus minimizing concerns about any unintended effects they may have. However, modified organisms used in bioremediation can be expected to adapt evolutionarily to growth on the anthropogenic substrate that they are intended to degrade. If such adaptation results in improved competitiveness for alternative, naturally occurring substrates, then this will increase the likelihood that the modified organisms will persist in the environment. Therefore, it becomes important to ask how adaptation to growth on an anthropogenic substrate, such as 2,4-D, affects competitiveness for a natural substrate, such as succinate.

Chapter 1

APPLICATION OF TRADITIONAL AND PHYLOGENETIC COMPARATIVE METHODS TO TEST FOR A TRADEOFF IN BACTERIAL GROWTH RATES AT LOW *VERSUS* HIGH SUBSTRATE CONCENTRATION

INTRODUCTION

It is widely assumed that bacterial species cannot be successful competitors at both low and high resource concentrations, but instead they must pursue one strategy or the other (Matin and Veldkamp 1978; Gerson and Chet 1981; Greer et al. 1992). This view is closely related to the distinction between *r*- and *K*-strategists in animals and plants (Pianka 1970; Mueller and Ayala 1981). When applied to microorganisms, Andrews and Harris (1986) expect *r*-strategists to have high maximum growth rates and to require abundant resources to support their rapid growth, whereas *K*-strategists should have lower maximum growth rates and require fewer resources to support their slower growth. In other words, maximum growth rate (μ_{max}) is viewed as the primary determinant of its competitiveness when resources are abundant, whereas substrate affinity (K_S) becomes more important when resources are scarce.

Understanding the nature and generality of tradeoffs in bacterial competitiveness at low versus high resource concentrations may have practical application for bioremediation of soils and groundwater contaminated by

xenobiotic compounds (Greer et al. 1992). That is, successful bioremediation may depend on identifying bacterial strains whose growth parameters are well suited to site-specific conditions in terms of resource concentration. However, prior studies that have looked for possible tradeoffs in growth rates as a function of resource concentration have yielded mixed results (Greer et al. 1992; Luckinbill 1984; Vasi et al. 1994; Zhou and Tiedje ms). Some of these studies employed an experimental approach, in which bacteria were allowed to evolve in the laboratory; the tradeoff hypothesis was then tested by comparing the growth properties of ancestral and derived genotypes. Other studies have used a comparative approach, in which growth parameters were estimated in the laboratory for bacterial strains that had been isolated from nature and correlations between these parameters tested.

In recent years, evolutionary biologists have recognized a serious problem with basing inferences about evolutionary adaptations and tradeoffs upon the traditional comparative method (Harvey and Pagel 1991). The traditional comparative method treats each strain (or species) as an independent observation for purposes of statistical analysis, but in fact certain different pairs of strains (or species) are more closely related to one another than are other pairs. Consequently, not all observations are truly independent, creating the opportunity for spurious correlations between various organismal traits. In other words, phylogenetic

relatedness tends to produce phenotypic similarity simply because of common ancestry, whether or not the resulting pattern has any particular adaptive significance. For example, consider the universe of all warm-blooded terrestrial vertebrates (i.e., birds and mammals). Treating each species as an independent observation, there is a very strong association between viviparity (as opposed to oviparity) and the possession of hairs (as opposed to feathers). But viviparity and hairiness may each have evolved only once in these combined groups, and so the association between these two traits may reflect a sort of historical accident rather than having any adaptive significance. Fortunately, new methods for comparative analysis have been developed that incorporate phylogenetic relationships between species and thereby allow reliable statistical inferences about associations between traits of interest (Clutton-Brock and Harvey 1977; Stearns 1983; Cheverud 1985; Lynch 1991; Felsenstein 1985; Grafen 1989; Harvey and Pagel 1991).

For this study, I performed comparative analyses to test the hypothesized tradeoff in bacteria between relative performance at high and low resource concentrations, using both the traditional approach (in which each strain is treated as an independent observation) and newer methods that depend on phylogenetically independent contrasts. Seven strains of bacteria isolated from nature were examined, all of which can grow on either 2,4-dichlorophenoxyacetate (2,4-

D) or succinate as a sole carbon source. Growth rates of all the strains were measured at both low and high concentrations of each substrate. I will show that the two comparative methods yield different results, although neither analysis provides compelling support for the tradeoff hypothesis. I conclude by discussing possible explanations for these results as well as considering an alternative approach to study this issue.

Materials and Methods

Bacterial strains

All of the bacteria used in this study were isolated from either soil or sludge, and all are able to catabolize 2,4-D (Tonso et al. 1995). Designations and geographical origins of the seven strains are as follows: TFD2 (Michigan), TFD3 (Oregon), TFD6 (Michigan), TFD13 (Michigan), TFD20 (Michigan), TFD41 (Michigan), and TFD43 (Australia). Based on 16S ribosomal DNA sequences (see below), these seven strains all belong to the β subgroup of the Proteobacteria (McGowan 1995). I also sought to include in our study several 2,4-D degrading strains from the α and γ subgroups of the Proteobacteria in order to increase phylogenetic diversity, but these other strains did not grow reliably in our standard culture medium.

Culture medium

Growth rates were measured in acid-washed flasks using the same base mineral medium for all strains and experiments. The base medium contained, per liter, 1.71 g K_2HPO_4, 0.3 g Na_2PO_4, 0.33 g $(NH_4)_2SO_4$, 0.246 g $MgSO_4 \bullet 7H_2O$, 0.12 g $Na_2EDTA \bullet 2H_2O$, 20 mg NaOH, 4 mg $ZnSO_4 \bullet 7H_2O$, 3 mg $MnSO_4 \bullet H_2O$, 1 mg $CuSO_4 \bullet 5H_2O$, 30 mg $FeSO_4 \bullet 7H_2O$, 52 mg Na_2SO_4, and 1 mg $NaMoO_4 \bullet 2H_2O$. Succinate or 2,4-D was added to the base medium at a concentration of either 5 or 500 μg/ml.

Estimation of growth rates

I initially sought to estimate two growth parameters for each strain: μ_{max} (maximum growth rate) and K_S (resource concentration that supports a growth rate of $\mu_{max}/2$). However, I was unable to obtain reliable estimates of K_S due to various technical difficulties (Lenski et al. 1997). For example, certain strains grew somewhat even when we added no carbon to the medium (using acid-washed flasks and double-distilled water); the cells presumably used some airborne organic material (Geller 1983). Other strains exhibited maximum growth rates at intermediate concentrations of substrate, in contrast to the hyperbolic form of the Monod model (G.J.V., unpublished observations). Therefore, I opted instead to measure each strain's growth rate at two substrate concentrations, one low (5 μg/ml) and one high (500 μg/ml), in order to test the tradeoff hypothesis.

Strains were removed from storage at -80°C and inoculated into 10 ml of medium (in 50-ml flasks) containing either 2,4-D or succinate (500 µg/ml). These initial cultures were allowed to grow for three days at 25°C while being shaken at 120 rpm. Cells from each initial culture were then transferred into fresh medium containing either 5 or 500 µg/ml of the corresponding substrate and allowed to grow for four days; this constituted a preconditioning step to ensure that cells were physiologically acclimated to the relevant medium. Cells from each preconditioning culture were transferred again into fresh medium with the same substrate and initial concentration to begin the experiment proper. Each combination of strain, substrate, and concentration was replicated three-fold. Bacterial cultures were sampled repeatedly at 3-5 hour intervals over a period of 24-30 hours. At each time point, the total biovolume (cell number × average cell size) of a culture was measured using a Coulter particle counter. Biovolume measurements were log-transformed, and a growth rate was calculated as the rate of change in biovolume during the period of exponential increase. The mean of the three independently estimated growth rates (for each combination of strain, substrate, and concentration) was used in the comparative analyses. Examples of log-transformed population volume data for strains TFD2 and TFD20 during growth on 500 µg/ml succinate are shown in Figure 1.

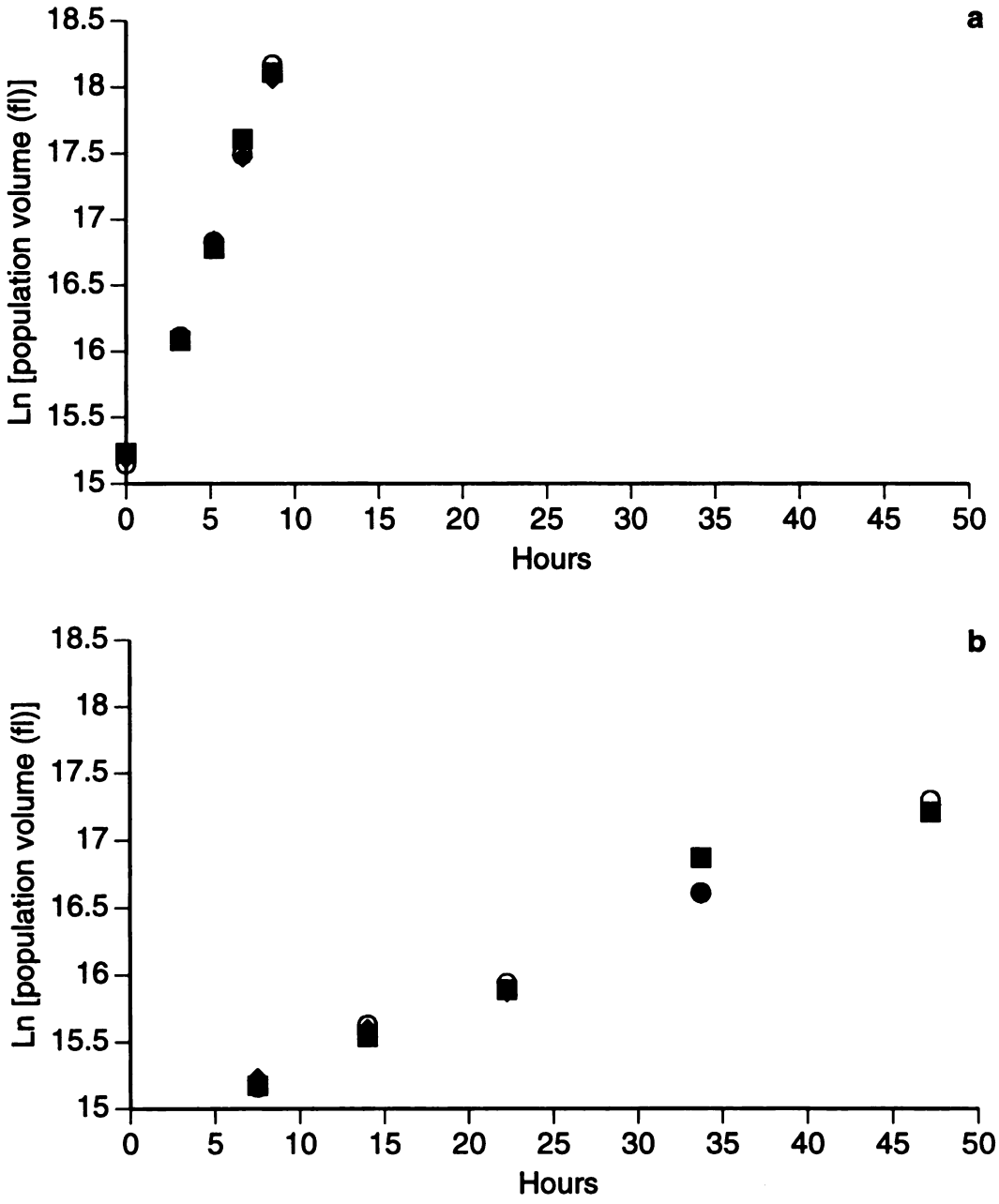

Figure 1. Exponential growth of strains TFD2 and TFD20. Log-transformed population biovolume data (in femtoliters) are shown for (a) a relatively fast growing strain (TFD2), and (b) a slow growing strain (TFD20) during growth on 500 µg/ml succinate. Population biovolume equals the average cell size multiplied by the number of cells. Open circles, closed diamonds and closed squares represent three independent replicate populations, respectively, for both TFD2 and TFD20.

16S ribosomal DNA sequencing

For six of the strains used in this study, 380 basepairs (bp) of 16S ribosomal DNA sequence were available from an earlier study (McGowan 1995). Using the Macromolecular Sequencing Facility at Michigan State University, I also sequenced the seventh strain, TFD13, using the same PCR and sequencing primers that were used previously for the other strains. All of the sequences are downstream of the primer that begins at *Escherichia coli* position 519 in the 16S ribosomal DNA gene. The 16S rDNA gene was first amplified by PCR primers fD1 (5'ccgaattcgtcgacaacAGAGTTTGATCCTGGCTCAG 3') and rD1 (5' cccgggatccaagcttAAGGAGGTGATCCAGCC 3') (Weisburg *et al*. 1991). A sample (1 ml) of stationary phase TFD13 culture (grown in Luria broth) was centrifuged at 14,000 rpm for 10 minutes and resuspended in 1 ml of sterile double-distilled H_2O. PCR was conducted in multiple reaction vials, with each containing 5 µl of resuspended bacterial culture, 0.5 µl of each primer (10 pmol/ml), 10 µl of 10X reaction buffer with $MgCl_2$, 2 µl of mixed dNTP, 1 µl of Taq DNA polymerase, and 81 µl of distilled H_2O. Reaction solutions were overlaid with mineral oil. Amplifications were performed in a Perkin-Elmer DNA Thermal Cycler 480 using a sequence of 6.5 minutes at 95°C; 35 cycles of 60 seconds at 94°C, 60 seconds at 40°C, and 60 seconds at 72°C; 10 minutes at 72°C; and maintenance at 4°C. PCR products were purified using a Qiagen Qiaquick-spin PCR kit, eluted into 50 ml of TE

buffer, and stored at -20°C. Then, 9 µl of the purified PCR product solution was added to 9 µl of H_2O and 2 µl of 10 pmol/ml primer 519R (5' GTATTACCGCGGCTGCTGG 3').

Construction and scaling of the phylogeny

The PAUP (Phylogenetic Analysis Using Parsimony) computer program (Swofford 1991) was used to infer the phylogeny of the bacterial strains included in this study, using all 380 bp of 16S ribosomal DNA sequence (Figure 2). A bootstrap analysis ("branch and bound" method, 100 iterations) was performed to ascertain confidence in the resulting phylogenetic tree, and the percentage of bootstrap samples supporting each grouping is so indicated (Swofford 1991). The estimated branch lengths between ancestral nodes were lengthened slightly using a method that seeks to correct systematic biases in estimating ancestral character states (see Table 1, Felsenstein 1985). Growth rates corresponding to each inferred ancestor were calculated as the average of the growth rates for the extant strains derived from the ancestor, weighted by the estimated branch lengths between ancestors and derived strains (Felsenstein 1985).

RESULTS

Traditional comparative method: application

The tradeoff hypothesis predicts a negative correlation between growth rates at high and low substrate concentrations. I first tested this hypothesis using the

Figure 2. Phylogenetic relationships. The phylogenetic relationships of the seven strains used in this study were inferred from 380 bp of their 16S ribosomal DNA sequences using the PAUP program (Swofford 1991). The letters at each node indicate ancestral nodes. A "branch and bound" bootstrap analysis (Swofford 1991) was performed to ascertain confidence in the inferred relationships. The percentage of bootstrap samples supporting each grouping are indicated on the branch below each node. See Table 1 for branch lengths.

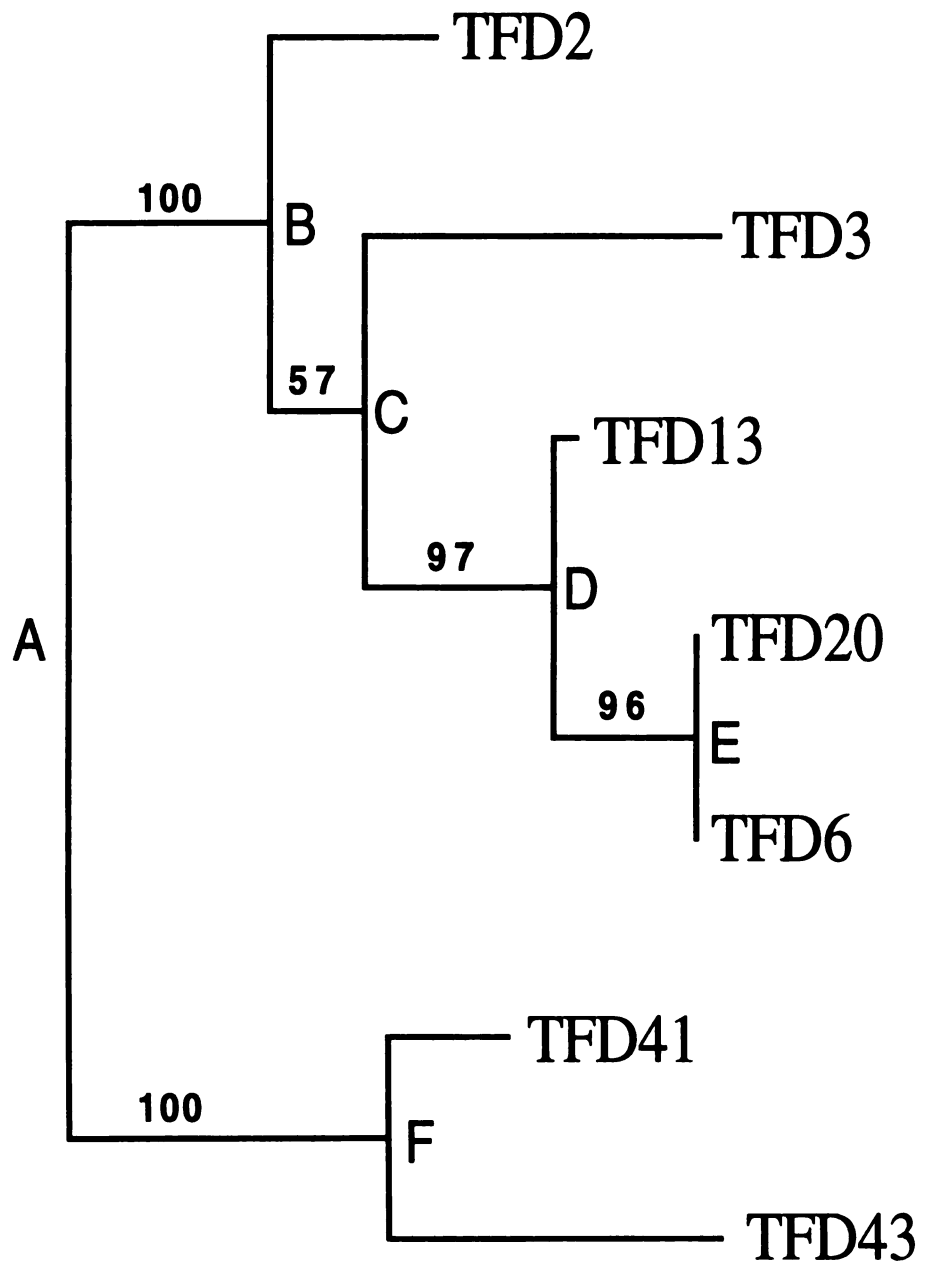

Figure 2. Phylogenetic relationships.

Table 1. Phylogenetic tree branch lengths. Scaled and unscaled branch lengths for the phylogenetic tree of seven strains of 2,4-D degrading bacteria shown in Figure 2. Unscaled branch lengths reflect the number of base pair (bp) differences between strains over 380 bp of 16S rDNA sequence. The estimated branch lengths between ancestral nodes were lengthened slightly by a method that seeks to correct systematic biases in estimating ancestral character states (Felsenstein 1985).

Table 1. Phylogenetic tree branch lengths.

Branch Segment	**Scaled Length**	**Unscaled Length**
A to B	14.55	11
B to 2	5	5
B to C	12.27	7
C to 3	13	13
C to D	8.87	8
D to 13	1	1
D to E	6.5	6
E to 6	1	1
E to 20	1	1
A to F	19.33	16
F to 41	5	5
F to 43	10	10

traditional comparative method, which treats each strain as an independent observation. Both succinate (Figure 3a) and 2,4-D (Figure 3b) gave positive correlations between growth rates measured at 5 and 500 µg/ml. For succinate, this correlation was significant (r = 0.7747, p = 0.0408), whereas for 2,4-D the correlation was not quite significant (r = 0.7431, p = 0.0556).

This traditional analysis clearly does not indicate any tradeoff between performance at high and low substrate concentrations. Rather, it suggests the opposite trend, whereby some strains are superior at both low and high substrate concentrations. However, there are two important considerations that may render this conclusion invalid. First, it is inappropriate to treat each strain as an independent observation, unless one uses phylogenetic information to scale the data in order to meet the assumptions of standard statistical tests. Failure to do so may dramatically alter the apparent correlation between traits. For example, growth rates at high and low substrate concentrations may be positively correlated for one pair of distantly related taxonomic groups, even though several pairs of more closely related strains within each group show the predicted negative correlation (Figure 4). In the next section, I will reanalyze this data using comparative methods that incorporate information on phylogenetic relationships. Second, some strains may be fortuitously preadapted to the laboratory environment for reasons that have nothing to do

Figure 3. Correlations using the older comparative method. Correlations between growth rates at low and high substrate concentrations for (**a**) succinate and (**b**) 2,4-D are shown. The traditional method uses all strains as independent observations, regardless of their phylogenetic relationships to one another. The correlation is significant for succinate (r = 0.7747, p = 0.0408). The correlation is not quite significant for 2,4-D (r = 0.7431, p = 0.0556).

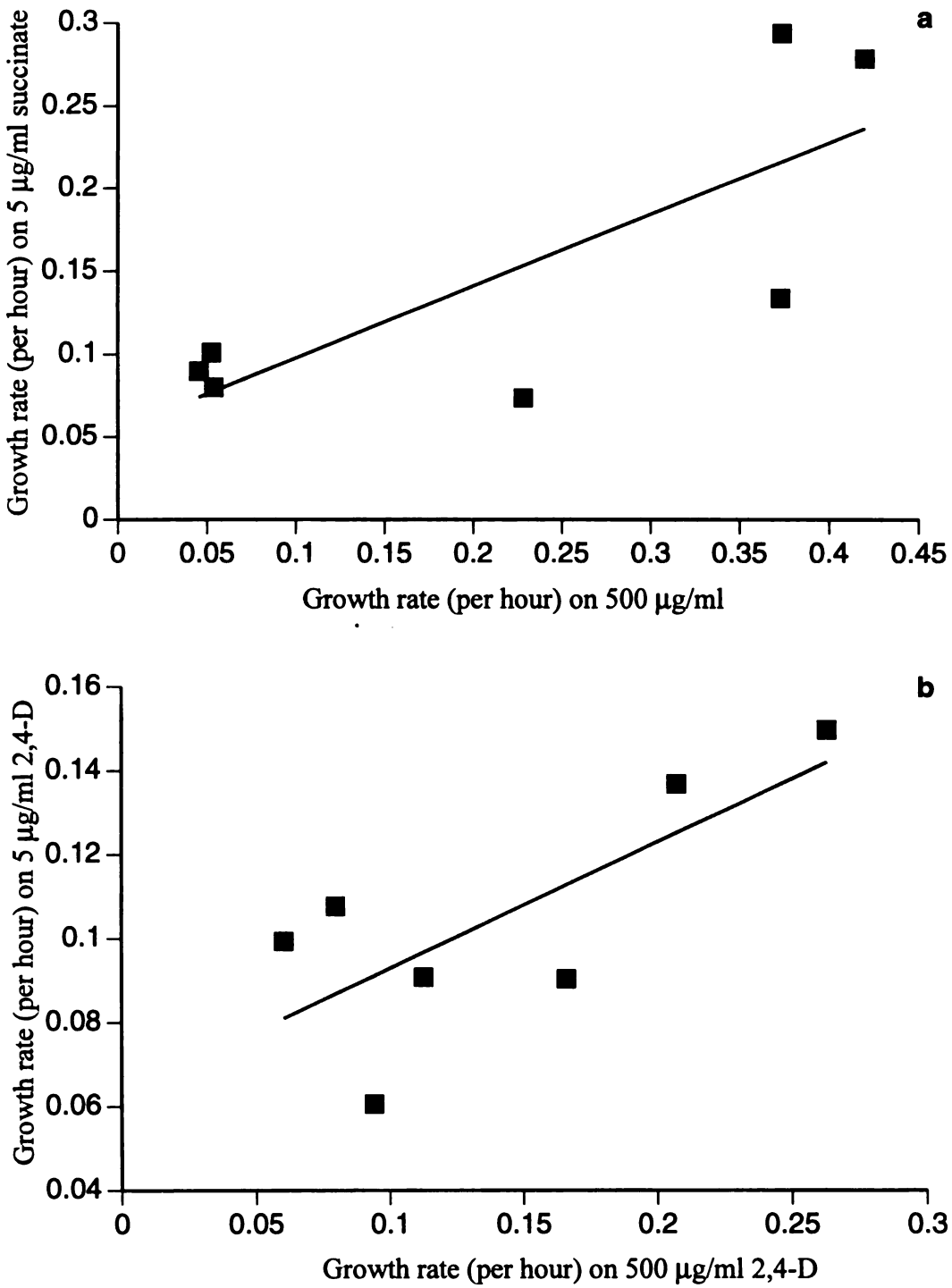

Figure 3. Correlations using the older comparative method.

Figure 4. Phylogenetic effects on correlation analyses. Growth rates schematically illustrated at low and high substrate concentrations are positively correlated across two distantly related taxonomic groups (circles and squares), even though several pairs of more closely related strains within each group exhibit the negative correlation predicted by the tradeoff hypothesis. Lines connect the most closely related pairs. Circles correspond to species A - F in Figure 5b, while squares represent species G - L. The overall correlation would appear to be positive using the traditional comparative method that treats each species as an independent observation. Newer comparative methods take into account phylogenetic relationships among strains and could reveal the preponderance of negative associations between the two traits.

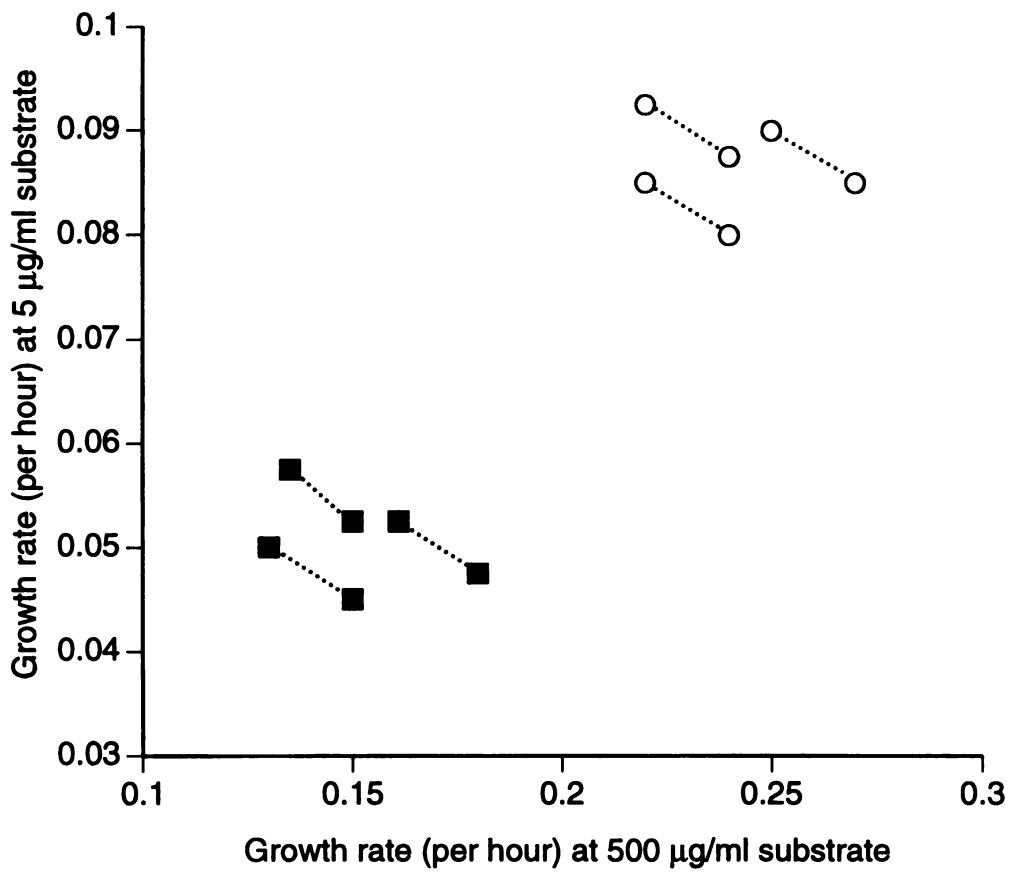

Figure 4. Phylogenetic effects on correlation analyses.

with the competitive relationships between strains in the soil or other natural environments. For example, one strain may grow much faster than another – at both low and high substrate concentrations – simply because the first strain is better suited to the particular combination of base mineral medium, temperature, and other variables used in the laboratory experiments. If one could analyze performance in an environment more similar to that in which the strains had evolved, then one might see the predicted tradeoff. In a later section, I examine evidence that certain strains are fortuitously better adapted to the laboratory environment than are other strains.

Phylogenetically independent contrasts: theory

The purpose of using phylogenetic information in comparative studies is to obtain the truly independent data that are required for robust statistical analysis, data that reflect independent evolutionary events. If all of the species in a study shared a common ancestor from which they had evolved for the same length of time, then these species could be appropriately treated as statistically independent observations (Figure 5a). But if the species are hierarchically related (as in most phylogenies), then statistical problems arise because of the relative lack of independence between the states of more recently diverged lineages (Figure 5b). Statistical problems may also arise if

Figure 5. Equidistant and hierarchical strain phylogenies. Strains (or species) are properly treated as independent observations only if they shared a common ancestor from which they had all evolved for the same length of time. This would require a "star" phylogeny, in which all species pairs are equidistant (**a**). More generally, pairs of strains (or species) are hierarchically related (**b**), and newer comparative methods have been developed that take into account these relationships.

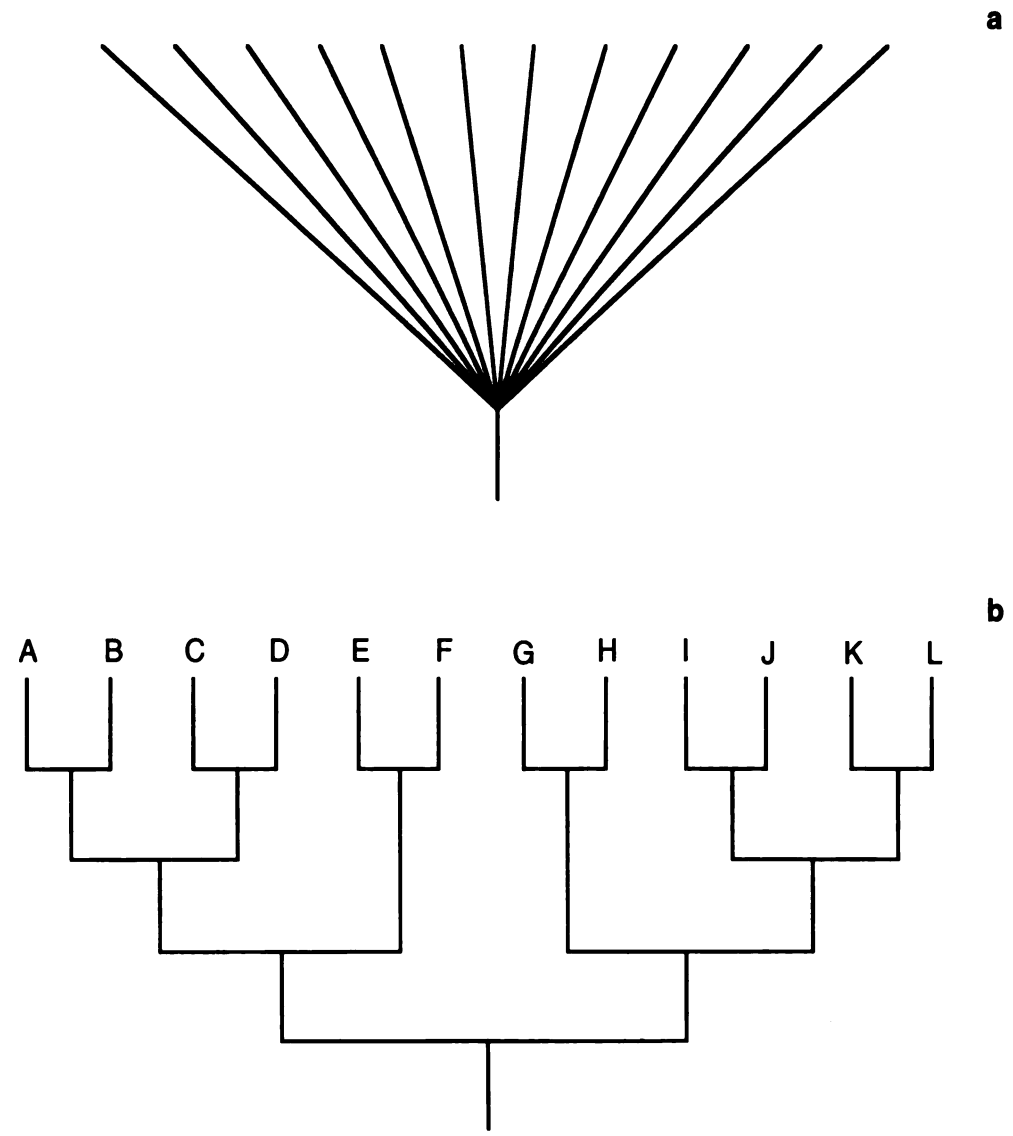

Figure 5. Equidistant and hierarchical strain phylogenies.

evolutionary rates vary among the lineages (Harvey and Pagel 1991).

Several different methods have been developed in recent years to incorporate phylogenetic information into comparative analyses. Some of the earliest methods relied on making contrasts only between independent pairs of extant species (Clutton-Brock and Harvey 1977; Stearns 1983; Cheverud 1985; Lynch 1991), but these methods lacked statistical power because they discarded potentially useful information. More recent methods are preferable because they make use of all the relevant information that is available in the data (Felsenstein 1985; Grafen 1989; Harvey and Pagel 1991).

Felsenstein's (1985) method of independent comparisons makes use of the fact that any two extant species (say, 1 and 2) at adjacent tips on a phylogenetic tree share a unique common ancestor. Consequently, any evolutionary change in the traits of interest (say, X and Y) since 1 and 2 split from their most recent common ancestor is independent of changes in X and Y at all other locations in the phylogenetic tree. Hence, the differences in traits X and Y between species 1 and 2 are statistically independent from the differences in X and Y between other such pairs of adjacent species in the tree. For example, (X1 - X2) and (Y1 - Y2) are independent of (X3 - X4) and (Y3 - Y4). Moreover, this independence holds for adjacent pairs of nodes at higher levels (i.e., earlier in time) in the tree. By using this

method, one can extract n - 1 independent contrasts from n extant species, when all of the differences between adjacent tips and nodes are calculated. Statistical tests of the correlation between traits of interest are then made using these n - 1 independent contrasts (weighted for expected variance based on branch lengths). This method assumes that the true branching pattern and branch lengths of the phylogeny are known, or at least that they can be estimated with some confidence.

Of course, one cannot know the actual values of the traits at the ancestral nodes, but these can be estimated by assuming a particular model of evolutionary change. Felsenstein's method assumes a model of evolutionary change that is equivalent to random Brownian motion. In such a process, each trait has an equal likelihood of a positive or negative change of equal magnitude over any given period of time. Hence, the expected mean change is zero, and the expected variance around this mean is directly proportional to the elapsed time (estimated from the branch lengths). The value of any trait at each ancestral node is therefore estimated simply as the midpoint of its descendant lineages scaled by the distances of those lineages from the node.

Various modifications of these methods have been developed that assume different models of evolutionary change (Grafen 1989; Harvey and Pagel 1991). Martins and Garland (1991) ran computer simulations to examine the effect of these different assumptions on the statistical validity of

the resulting evolutionary inference. Although they found that these assumptions had some effect on statistical validity, fortunately their effects were usually minor. More importantly, all of the methods that incorporate phylogenetic relatedness (but which assume different models of evolutionary change) performed much better than the traditional approach (which ignores phylogeny and treats each species as an independent observation), even when the assumed model of evolution is incorrect. Therefore, in practice, methods that use phylogenetically independent contrasts will yield more reliable inferences than traditional comparative analyses, even when the assumptions that underlie the phylogenetic method are violated.

Phylogenetically independent contrasts: application

I used Felsenstein's method of independent contrasts (see above) to re-examine the correlation between bacterial growth rates at high and low substrate concentrations, using the phylogenetic relationships among the seven strains depicted in Figure 2. Table 2 gives the growth rates that were measured for each strain as well as the growth rates inferred for all of the ancestral nodes. The branch lengths of the phylogenetic tree, both scaled and unscaled, are given in Table 1.

In succinate, the significant positive correlation between growth rates at high and low concentrations, based on the traditional comparative method, is not supported by the

Table 2. Growth rates of strains and anestral nodes. Growth rates were measured for each strain, and growth rates for each ancestral node in the strain phylogeny were also inferred (see Figure 2). The values shown for each strain are averages based on three replicate determinations. The values inferred for each ancestral node used the method of Felsenstein (1985). The growth rates have units of hr^{-1}.

Table 2. Growth rates of strains and ancestral nodes.

Strain or Ancestral Node	Succinate		2,4-D	
	5 μg/ml	500 μg/ml	5 μg/ml	500 μg/ml
TFD2	0.1341	0.3729	0.0905	0.1661
TFD3	0.2790	0.4199	0.1359	0.2073
TFD6	0.0800	0.0539	0.0606	0.0941
TFD13	0.0899	0.0454	0.1078	0.0799
TFD20	0.1011	0.0524	0.0994	0.0605
TFD41	0.0737	0.2283	0.0909	0.1128
TFD43	0.2942	0.3737	0.1498	0.2630
Node A	0.1451	0.3027	0.1035	0.1590
Node B	0.1435	0.3222	0.0982	0.1560
Node C	0.1666	0.1979	0.1170	0.1314
Node D	0.0900	0.0464	0.1041	0.0796
Node E	0.0906	0.0532	0.0800	0.0773
Node F	0.1472	0.2768	0.1105	0.1629

method of phylogenetically independent contrasts (Figure 6a: r = 0.5401; p = 0.2108). In 2,4-D, the phylogenetic method again gives no indication of a significant correlation (Figure 6b: r = 0.5373; p = 0.2136), whereas the traditional method suggested a positive correlation. Thus, by employing phylogenetically independent contrasts, comparative data that seemed to provide compelling evidence that flatly contradicted the tradeoff hypothesis now appears to be merely inconclusive. These changed results, although inconclusive with respect to the tradeoff hypothesis, suffice to show that phylogenetic considerations are important for comparative studies in microbial ecology.

An indirect test of the preadaptation hypothesis

In short, the comparative approach provides no support for the hypothesized tradeoff between relative growth rates at high and low substrate concentrations. A possible explanation for the failure of this hypothesis is that certain strains may be fortuitously preadapted to growth in the laboratory. Thus, one strain may grow faster than another simply because the first strain prefers the particular combination of mineral medium, temperature, and other variables used in laboratory experiments at both high and low substrate concentrations. A direct test of this hypothesis is beyond the scope of this study and would require the ability to perform competition experiments

Figure 6. Correlations using new comparative methods. Correlations between growth rates at low and high substrate concentrations for (**a**) succinate and (**b**) 2,4-D are shown. These new methods take into account phylogenetic relationships among strains. The correlations are insignificant on both succinate (r = 0.5401, p = 0.2108) and 2,4-D (r = 0.5373, p = 0.2136), whereas the traditional comparative method indicated stronger positive correlations (Figure 3).

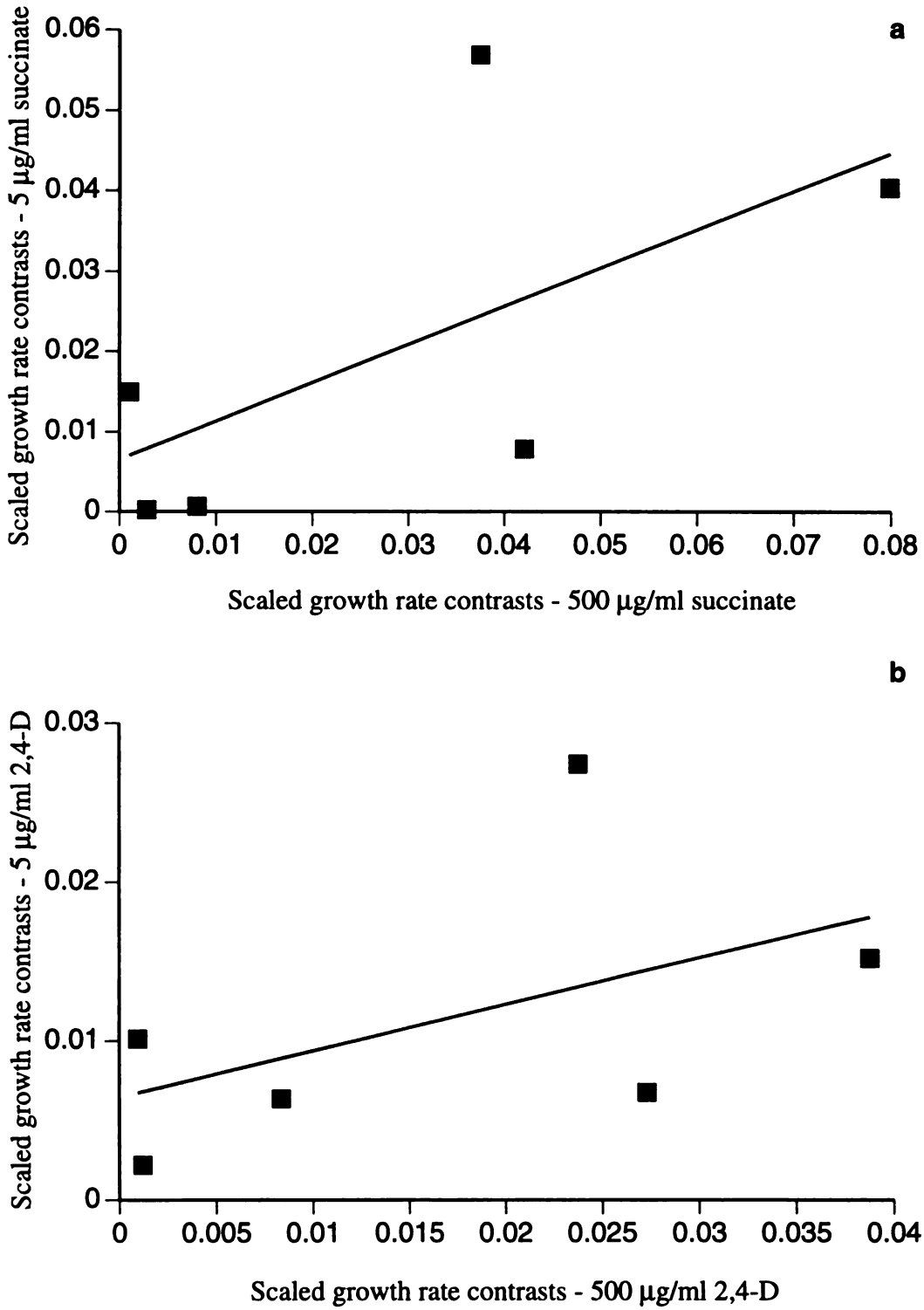

Figure 6. Correlations using new comparative methods.

between strains at high and low substrate concentrations *in situ* in the environments from which the strains were isolated. However, an indirect test can be performed by asking whether growth rates on succinate and 2,4-D are positively correlated. That is, all other aspects of the growth conditions (medium composition, temperature, etc.) are held constant, so that those strains that are fortuitously preadapted to these conditions should benefit regardless of the substrate that is provided.

To that end, I used the method of phylogenetically independent contrasts to calculate the correlation between growth rates on succinate and 2,4-D. At low substrate concentrations (5 µg/ml), there is a marginally nonsignificant positive correlation between growth rates on succinate and 2,4-D (r = 0.7487; p = 0.0528). At high substrate concentrations (500 µg/ml), the correlation between growth rates on the two substrates is even weaker (r = 0.4932; p = 0.2607). The positive correlations across substrates are consistent with the preadaptation hypothesis, but the test is an indirect one and the statistics are again inconclusive.

DISCUSSION

This study had two overlapping goals. The first of these goals was to examine whether bacterial strains that are good competitors at high substrate concentrations tend to be inferior competitors at low substrate concentrations, and

vice versa. This tradeoff hypothesis, if valid, has important implications for understanding the structure of microbial communities as well as for identifying bacterial strains that are most likely to be useful in applications such as bioremediation. Previous tests of this hypothesis have yielded mixed results (Luckinbill 1978, 1979, 1984; Greer et al. 1992; Lenski et al. 1997; Zhou and Tiedje ms), and the results of my study must be added to those that do not support this hypothesis. However, there are important caveats to this interpretation that will be considered below.

The second goal of our study was to illustrate the application of recent advances in the comparative method to microbial ecology (Felsenstein 1985; Harvey and Pagel 1989; Harvey and Pagel 1991). The traditional comparative approach has been to view each species or strain as an independent observation when one calculates correlations between two traits, such as growth rates at high and low substrate concentrations. This approach implicitly ignores the phylogenetic relationships among species or strains. However, there is a general lack of statistical independence of observations with respect to evolutionary hypotheses because of these phylogenetic relationships. As a consequence, the traditional comparative method may often generate spurious correlations (Martins and Garland 1991). A newer comparative method explicitly takes into account these phylogenetic relationships and thereby avoids this problem. In this study, I found that correlations between growth rates

at high and low substrate concentrations were weaker when the phylogenetic relationships were taken into account than when they were ignored (contrast Figures 3 and 6).

These recent advances solve certain statistical problems with the comparative approach as it was formerly applied. Nonetheless, there remain vexing problems in determining the validity of inferences based on the comparative method. I will now discuss two such problems.

It is typically assumed that phylogenetic relationships among organisms can be determined using essentially any set of molecular or other data capable of resolving evolutionary relatedness. The presumption is that any one gene has the same genealogy (at the level of species and higher taxa) as do all other genes. But with bacteria, this assumption may be violated, especially for those traits that are encoded by plasmids or other mobile genetic elements. Phylogenetic relationships among the strains used in this study were inferred from chromosomal sequences encoding the 16S ribosomal subunit. However, *tfdA* and other genes that encode the ability to catabolize 2,4-D are often plasmid-borne (Don and Pemberton 1981), so that the phylogenetic relationships used for independent contrasts may have been inappropriate for testing the correlation between growth rates at high and low concentrations of 2,4-D.

Indeed, McGowan (1995) recently showed that the phylogeny of bacteria that degrade 2,4-D obtained using the 16S ribosomal gene is different from the phylogeny of the

tfdA genes themselves, which implies horizontal gene transfer (see also Dykhuizen and Green 1991). When evaluating the potential tradeoff between growth rates at high and low concentrations of 2,4-D, it is unclear whether the *tfdA* or 16S ribosomal gene phylogeny is more appropriate, because it is unclear *a priori* which genes mediate this hypothetical tradeoff. Therefore, in addition to scaling data based on the 16S ribosomal gene phylogeny, I did the same scaling based on the phylogeny of gene *tfdA* for five of the seven strains used in the ribosomal gene analysis for growth on 2,4-D (McGowan 1995). The qualitative patterns from the *tfdA* based analysis are similar to those from the ribosomal based analysis. Using unscaled growth rate estimates for the five strains, there is a significant positive correlation between growth rates at 5 and 500 μg/ml 2,4-D (r = 0.9466, 2-tailed P = 0.0147). Scaling based on the *tfdA* phylogeny for these five lines reduced the correlation coeffecient of this relationship and made it statistically insignificant (r = 0.8104, 2-tailed P = 0.0962). Because of their qualitative similarity to the results based on the 16S ribosomal gene, these results using the *tfdA* gene suggest that the failure of the tradeoff hypothesis is most likely not due to effects of horizontal gene transfer.

Also, horizontal gene transfer is probably irrelevant to the catabolism of succinate, which is an intermediate compound in central metabolism. The fact that I failed to find any evidence of a tradeoff between growth rates at high

and low succinate concentrations also indicates that possible complications due to horizontal gene transfer were not generally responsible for the failure of the tradeoff hypothesis.

The second caveat arises because the hypothesized tradeoff between performances at high and low substrate concentrations may be masked by fortuitous differences among strains in the extent of their preadaptation to laboratory growth conditions. Imagine, for example, that some strains are adapted to habitats that are similar in temperature, mineral composition, and so on to the conditions used in my laboratory experiments, whereas other strains prefer dissimilar conditions. Certain strains would have a consistent advantage over other strains regardless of substrate concentration, and these differences would therefore promote a positive correlation between growth rates measured at low and high substrate concentrations. This effect might therefore obscure or even override any negative correlation due to the hypothesized tradeoff.

Several evolutionary studies with higher organisms have shown this systematic bias against detecting tradeoffs. These studies have relied on contrasting the results of comparative studies in which the performance capabilities of natural isolates are measured under artificial conditions dissimilar to those under which they evolved with the results of experimental evolutionary studies. In these experimental studies, populations are allowed to evolve in the laboratory,

so that the organisms are adapted evolutionarily to the same artificial conditions in which their performance capabilities will be measured. For example, to test the predictions of the theory of *r*- and *K*-selection, Mueller and Ayala (1981) allowed replicated experimental populations of *Drosophila melanogaster* to evolve for eight generations in the laboratory at either high or low population density. They then estimated the per capita growth rates of the derived populations at different population densities, and they compared these responses across the selected populations. They observed the predicted tradeoff, such that the populations selected at low density grew more slowly at high densities than did those selected at high density, and vice versa. However, this same tradeoff was *not* detected when Mueller and Ayala (1981) performed a comparative study using 25 strains of *D. melanogaster* isolated from nature, presumably because of fortuitous differences among these strains in their preadaptation to the laboratory environment. A direct demonstration of the confounding effect of fortuitous preadaptation to environments that are novel (from the standpoint of an organism's evolutionary history) was made by Service and Rose (1985), also using *D. melanogaster*. By means of experimental evolution, the authors demonstrated a life-history tradeoff in the flies between early fecundity and longevity, when the assays of fecundity and longevity were performed in the same culture medium that had been used during the experimental evolution. However, when these same

traits were measured in a novel culture medium, the tradeoff was largely obscured.

It is clear from these studies that the power of the comparative method suffers when performance traits are measured under conditions that differ substantially from those that prevailed during an organism's evolutionary history. At the same time, it is also clear that evolution experiments performed in the laboratory allow one to circumvent this problem not by eliminating the differences between nature and the laboratory, but rather by using populations that have a defined history of adaptation to the laboratory environment in which the performance assays are conducted. Thus, I have also performed evolution experiments (Chapter 2) to re-examine the tradeoff between growth rates at low and high substrate concentrations. My experiments proceeded in two stages. The first stage allowed bacterial strains isolated from nature to become adapted for many generations to one of two laboratory growth conditions: chemostat culture (wherein cells perpetually experience resource limitation) and serial batch culture (wherein cells are periodically given a surfeit of resources). Then, in a second stage, populations selected under one regime were propagated for many more generations under the alternative regime, and vice versa, with control populations continuing under their former regimes. I then measured the competitive performance of the derived strains at both low and high substrate concentrations to ascertain whether or not

tradeoffs have occurred during evolution under the two regimes.

These evolution experiments are similar in outlook to those performed by Luckinbill (1978, 1984) to test the predictions of *r*- and *K*-selection theory. In Luckinbill's experiment, replicated populations of *E. coli* were propagated by serial batch culture under either *r*- or *K*-selection regimes, with the only difference being that under the *r*-selection regime the bacteria were transferred to fresh medium before they reached their stationary-phase population density. This experiment failed to show any tradeoff in adaptation to these two environments. However, Vasi *et al.* (1994) have suggested that Luckinbill's putative *K*-selection regime may have selected for essentially the same growth parameters as his *r*-selection regime. Their suggestion was based on theoretical and empirical analyses of changes in growth parameters in populations of *E. coli* evolving under a serial batch regime; these analyses indicated that maximum growth rate was the primary target of selection, just as it would be under perpetual exponential growth. Therefore, in my experiment a chemostat culture regime is employed to generate more extreme selection for faster growth rate under conditions of limiting substrate concentrations.

In conclusion, the hypothesis of a tradeoff in bacterial growth rates between low and high substrate concentrations is difficult to test because both comparative and experimental approaches are fraught with subtle inferential problems. The

hypothesis is not supported by the results of this study. Nonetheless, the tradeoff hypothesis is intuitively appealing and may have considerable importance for the field of microbial ecology. Therefore, efforts should continue to develop new ways to test this hypothesis that avoid these problems.

Chapter 2

EXPERIMENTAL TESTS FOR A TRADEOFF IN BACTERIAL FITNESS AT *LOW VERSUS* HIGH SUBSTRATE CONCENTRATION

INTRODUCTION

Pleiotropic tradeoffs involving organismal fitness in different environments have long been recognized as an important dynamic of adaptive evolution (Reznick 1985; Bell and Koufopanou 1986). Such tradeoffs arise from genetic, structural, and physiological constraints on the simultaneous optimization of different traits. Hypothesized tradeoffs include *r*- *vs.* *K*- traits (MacArthur and Wilson 1967; Pianka 1970), adaptation to high *vs.* low temperatures (Mongold et al. 1996), longevity *vs.* fecundity (Service and Rose 1985), and growth *vs.* reproduction (Warner 1984).

r- *vs.* *K*- selection is among the most studied tradeoff theories in ecology. *r*- selection occurs when the primary component of fitness is the intrinsic rate of population increase under low density conditions, while *K*- selection optimizes population carrying capacity under high density conditions (Roughgarden 1971). Characteristic *r*- traits include high growth and death rates, short life spans, and success in low density and fluctuating conditions. Reciprocal *K*- traits include slow population growth and lower mortality rates, increased longevity, and success in stable

and high density environments. Numerous investigations have shown a tradeoff pattern between *r*- and *K*- type traits (Cody 1966; Mueller and Ayala 1981; Andrews and Rouse 1982), while others have failed to reveal predicted patterns (Luckinbill 1978, 1979, 1984). One specific prediction of *r*- and *K*-selection theory is that organisms which compete well in abundant resource environments should perform relatively poorly under resource-limiting conditions (Andrews and Harris 1986).

Microbial ecologists commonly assume that tradeoffs between competitive abilities under abundance *vs.* scarcity are pervasive throughout the microbial world (Matin and Veldkamp 1978; Konings and Veldkamp 1980; Kuenen and Harder 1982; Veldkamp et al. 1984; Greer et al. 1992). The rapid growth of bacteria, their large population sizes, and their ease of manipulation make them excellent organisms for studying tradeoffs such as this one and for the study of resource-based competition theory in general (Levin et al. 1977; Hansen and Hubbell 1980). The hypothesized tradeoff between competitive ability at high *vs.* low resource levels is not only important for ecological life-history theory, but also has applied significance for the selection of microbes useful for bioremediation (National Research Council 1993).

This study experimentally tests the substrate concentration tradeoff hypothesis, as it shall be referred to here. This hypothesis is most commonly expressed in terms of kinetic parameters that are important components of fitness

at high and low substrate concentrations. At high substrate concentrations, the maximum exponential growth rate (μ_{max}) is the major fitness component, whereas under conditions of scarcity, μ_{max}/K_S becomes important, where K_S is the concentration of substrate that allows a growth rate of 1/2 μ_{max}. (μ_{max}/K_S is a better indicator of competitive ability than is K_S alone because an increase in K_S does not necessarily reflect a decrease in fitness at low substrate concentrations if accompanied by a parallel increase in μ_{max}). There is some evidence in favor of the substrate concentration tradeoff hypothesis from studies that compare the kinetic parameters of small numbers of bacterial species (Matin and Veldkamp 1978; Kuenen and Harder 1982; Veldkamp et al. 1984). These studies, however, are not statistically powerful and other more systematic studies have yielded results that do not support the hypothesis (Greer et al. 1992, Lenski et al. 1997, Chapter 1).

The hypothesized tradeoff between the improvement of μ_{max} and μ_{max}/K_S assumes a more general tradeoff involving gross competitive fitness in abundant *vs.* scarce resource conditions. Populations that evolve under abundant resource conditions should simultaneously improve their performance at high substrate levels and lose competitive ability under scarce resource conditions, and vice versa. Therefore, I have tested for a tradeoff between overall competitive ability at high and low resource concentrations. Bacterial populations were allowed to evolve under both types of

conditions and evolutionary changes in the competitive ability of derived strains were measured in both the environment in which they evolved and the alternative environment. My results show that a tradeoff is not compulsory because bacteria were capable of simultaneously improving competitive ability at both high and low resource concentrations. The substrate concentration tradeoff hypothesis is not accurate as a general rule for bacteria, but a significantly modified form of the hypothesis may still be true.

Experimental evolution overview

Evolution experiments were conducted in two temporally distinct stages and in two different environments (Figure 7). In the first environment - serial batch culture - bacterial populations are transferred daily from stationary phase cultures into fresh medium and allowed to grow back to high density; hence populations experience high concentrations of carbon source at regular intervals. Natural selection favors mutants with improved competitive ability under high resource conditions. In the second selective regime - chemostat culture - evolving populations experience high density and low substrate concentrations continuously as fresh media flows into the cultures at a slow, constant rate and the high equilibrium population size holds resource concentrations very low. Here natural selection favors those mutants that are best able to scavenge for scarce resources.

Figure 7. Derivation of bacterial strains. Horizontal lines indicate periods of evolution with the selective regime and sole carbon source indicated above and below, respectively. Each horizontal line represents two independently evolved replicate populations. See MATERIALS AND METHODS for a detailed description of strains and nomenclature as well as the number of evolutionary generations for each line.

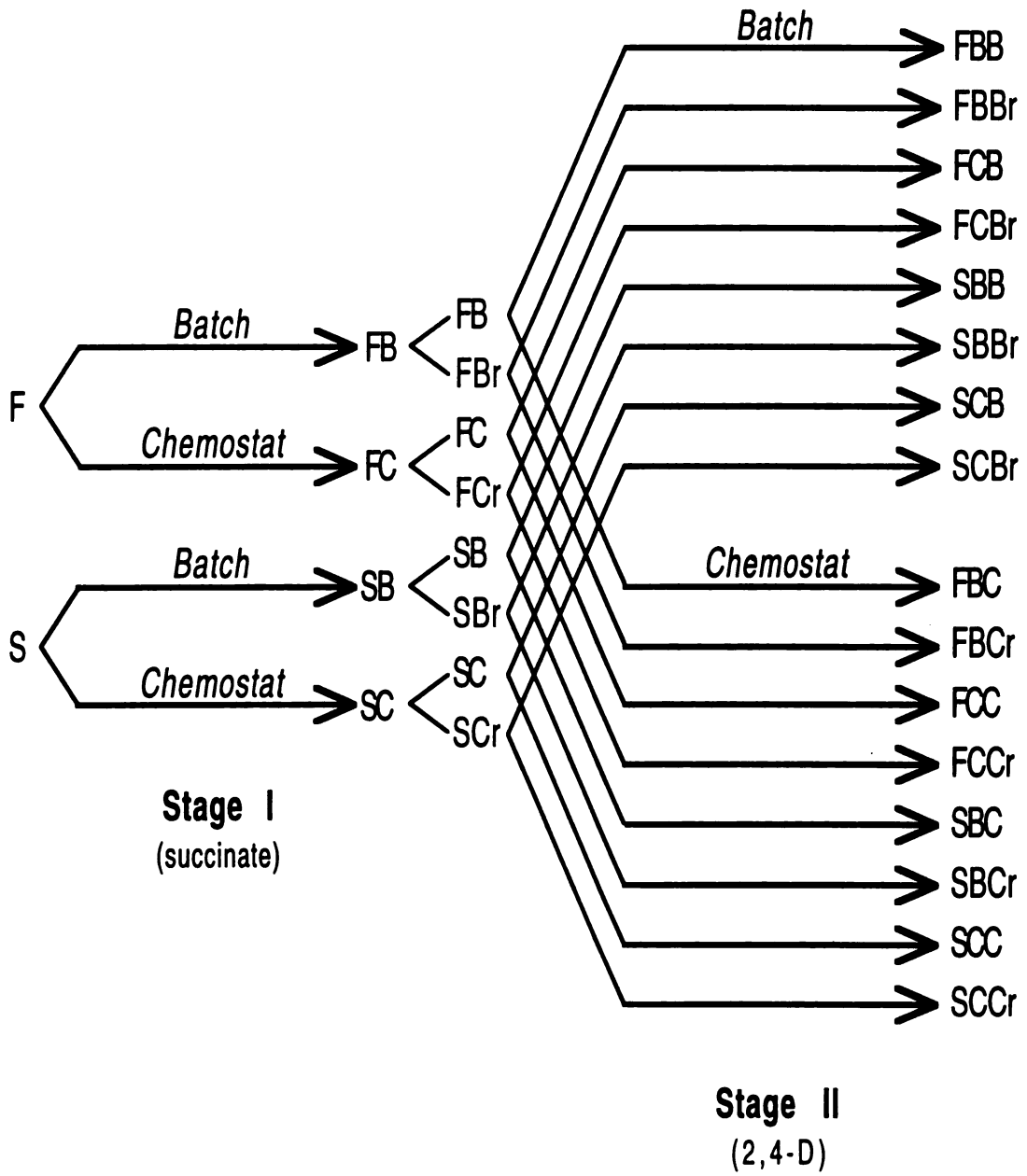

Figure 7. Derivation of bacterial strains.

A problem of testing tradeoff hypotheses by comparing the traits of extant species is the different degrees of "preadaptation" among natural isolates to the environment in which traits are measured. For example, one bacterial strain may perform relatively better than other natural strains at both high and low resource concentrations not because it has optimized performance under both conditions, but rather because it happens to be better fit than are other strains to lab variables other than substrate concentration. Such pre-adaptation has been shown to obscure a tradeoff between early fecundity and longevity when *Drosophila melanogaster* traits are measured in an environment other than that in which they evolved (Service and Rose 1985).

To reduce the effect of any such differences between the natural isolates used here on the rate and extent of adaptation in my experiments, I conducted two stages of evolution. The first stage was designed to allow adaptation to general laboratory culture conditions so that subsequent adaptation on a relatively uncommon carbon source would be largely specific to that substrate. In Stage I of the evolution experiments, two bacterial strains capable of degrading the herbicide 2,4-dichlorophenoxyacetic acid (2,4-D) were used to found populations that underwent 75 days of evolution in either the batch or chemostat regime (Figure 7). I was interested in observing whether bacterial strains that had apparently been shaped by different selective forces in nature responded to the laboratory selection regimes in

similar or different manners. Therefore, I selected one strain that grew fast under laboratory conditions (on both succinate and 2,4-D) and one that grew relatively slowly (on both substrates) to start the evolving lines.

Separate lines were established from each natural isolate in both the batch and chemostat environments for the first stage (Stage I) of evolution, in which succinate (a central substrate in bacterial metabolism) was the sole carbon source. In the second stage (Stage II), populations founded from Stage I lines were propagated in both batch and chemostat environments in a complete factorial design with 2,4-D, which is catabolized into succinate, as the sole carbon source. This design allows examination of the specificity of adaptation not only with respect to culture regime (this chapter), but also substrate type (Chapter 3).

Materials and Methods

Bacterial strains and culture media

The two ancestral bacterial strains used in this study, TFD3 and TFD13, are both capable of catabolizing 2,4-D and were isolated from sludge in Oregon and soil in Michigan, respectively (Tonso et al. 1995). These two strains, which both belong to the genus *Burkholderia* in the β subgroup of Proteobacteria, were selected because one of them (TFD3) grows relatively fast in 2,4-D medium (μ_{max} = 0.21 hr^{-1}), while the other (TFD13) grows relatively slowly (μ_{max} = 0.08 hr^{-1}) (Chapter 1). Hereafter, TFD3 shall be designated "F"

(fast), and TFD13 will be referred to as strain "S" (slow). All laboratory evolution and competition experiments were conducted in the same base mineral salts base medium (AN medium, Chapter 1), with either succinate or 2,4-D added to the base medium at a concentration of 500 µg/ml.

Stage I batch evolution

Two replicate lines of both strain F and strain S were established by inoculating 10 ml flasks of succinate medium from frozen clonal cultures. Upon reaching stationary phase, the lines were transferred into fresh medium. The new cultures were then grown to stationary phase and the cycle repeated daily. The transfer ratios were 1/100 (0.1 ml transferred to 9.9 ml) for the F lines and 18/100 (1.8 ml transferred to 8.2 ml) for the S lines. These ratios allowed approximately 6.6 and 2.4 generations of growth per day, respectively. Lines derived from F and S underwent 75 daily transfer cycles, allowing about 500 generations of evolution for F descendants and 180 generations for S lines. Upon completion of Stage I evolution, each line was stored as a whole population in 10% glycerol at -80° C. Single colonies of Stage I lines evolved from F and S were isolated on agar plates, cultures from which were also stored frozen. These clonal cultures, hereafter designated FB1, FB2, SB1 and SB2 (B = batch), were used for the competition assays comparing ancestral with Stage I evolved strains. Two of these clones,

FB1 and SB1, also were used as ancestors to found lines for the second stage of evolution.

Stage I chemostat evolution

Two evolutionary lines each of strains F and S were established by inoculating succinate medium into chemostat vessels from the frozen, clonal ancestral cultures. Strain F vessels were maintained at 18 ml and strain S vessels at 50 ml. Chemostat flow was maintained at 2.5 ml/hr, setting growth rates at 0.139 hr^{-1} for the F lines and 0.050 hr^{-1} for the S lines. These rates are one third and slightly over one half (0.56) the maximum growth rates of F and S, respectively, on 500 µg/ml succinate as estimated from cell density data for replicate cultures during exponential phase growth. (Chemostat dilution rates relative to ancestral maximum growth rates are shown in Table 3.) Strain F cultures underwent 75 days of chemostat evolution (about 360 generations), while strain S cultures were stored after only 73 days (about 130 generations) due to clogging of the nutrient input tube from bacterial accumulation. Wall growth was allowed to proliferate undisturbed throughout the experiment. This had the effect of increasing cell density and reducing substrate concentrations beyond theoretical expectations and thereby amplifying the selective difference between the batch and chemostat regimes. Chemostat vessels were vortexed prior to withdrawing a sample for freezer

Table 3. Chemostat dilution rates relative to ancestral maximum growth rates. The dilution rates for both stages of chemostat evolution, maximum growth rates (μ_{max}) of proximate ancestors, and the ratios of dilution rate to μ_{max} are shown for each type of evolutionary line. Dilution rate is the flow rate of media divided by chemostat volume.

Line Type	Dilution Rate	μmax of proximate ancestor	Dilution Rate/ μmax
FC	0.139	0.420	0.331
SC	0.050	0.090	0.556
FBC	0.111	0.205	0.542
FBCr	0.111	0.217	0.511
FCC	0.111	0.204	0.545
FCCr	0.056	0.130	0.429
SBC	0.042	0.100	0.419
SBCr	0.042	0.104	0.402
SCCr	0.042	*	*

* A reliable growth rate estimate for strain SCr (proximate ancestor of SCCr1) was not obtained due to extensive clumping of cells during exponential growth.

storage to allow a random mixture of cells from both the liquid and wall populations of the chemostat.

Samples of each evolved culture were stored frozen in 10% glycerol. Single colonies of evolved chemostat cultures were isolated on agar plates, cultures from which were stored frozen. These clonal cultures, hereafter designated FC1, FC2, SC1 and SC2 (C = chemostat), were used for the competition assays comparing ancestral with evolved strains. FC1 and SC1 also became ancestors for the next stage of evolution. All Stage I (and Stage II) evolved clones were confirmed to be true descendants of strains F and S by comparing electrophoretic gel patterns of REP PCR amplification products of the ancestors and descendants (Versalovic et al. 1991). Also, single streptomycin-resistant colonies of FB1, FC1, SB1, and SC1 were selected and stored frozen as clonal cultures. These cultures were designated FBr, FCr, SBr, and SCr, and were used to found one half of the Stage II evolution lines.

Stage II batch evolution

Two evolution lines each from FB1, FBr, FC1, FCr, SB1, SBr, SC1 and SCr were established by inoculating flasks of 10 ml AN medium containing 500 µg/ml 2,4-D with single colonies isolated from the frozen clonal cultures. Transfer cycles were carried out on 2,4-D medium as during Stage I evolution for 75 days. FB1, FBr, and FC1 descendant lines were transferred daily at 100-fold dilutions into fresh medium

(6.6 generations/day, 500 total generations), while FCr descendants were transferred with 13-fold dilutions due to a significantly slower growth rate (3.7 generations/day, 280 total generations). SB1 and SBr descendants were transferred at 5-fold dilutions (2.25 generations/day, 170 total generations), while SC1 and SCr descendants were transferred at 3-fold dilutions (1.5 generations/day, 115 total generations). Mixed population samples were stored frozen at 25 day intervals and after 75 days. Single colonies from each evolved culture were selected, grown up and stored frozen. These clonal samples were used for competition assays and were designated FBB1, FBB2, FBBr1, FBBr2, FCB1, FCB2, FCBr1, FCBr2, SBB1, SBB2, SBBr1, SBBr2, SCB1, SCB2, SCBr1 and SCBr2. In this nomenclature, the first letter reflects the original ancestor, the second and third letters represent the environments in which the strain evolved during Stages I and II, respectively, the letter 'r' indicates that the line is derived from a streptomycin-resistant clone at the start of Stage II, and the numerals distinguish replicate lineages.

Stage II chemostat evolution

Two replicate chemostat cultures each of FB1, FBr, FC1, FCr, SB1, SBr, SC1 and SCr were established by inoculating vessels of AN medium containing 500 µg/ml 2,4-D with single colonies isolated from the frozen clonal cultures. These cultures were grown to turbidity before initiating chemostat

medium flow. A flow rate of 2.0 ml/hr was maintained for 75 days, after which both mixed population and clonal cultures were stored frozen. Vessels containing descendants of strains FB1, FBr, and FC1 contained 18 ml of culture allowing growth at 0.111 per hr (3.8 generations/day, 290 total generations), while vessels containing descendants of FCr held 36 ml, allowing 145 generations of evolution. (Chemostat dilution rates relative to the maximum growth rates of Stage II proximate ancestors are shown in Table 3.)

All vessels with descendants of strain S held 48 ml of culture allowing growth at 0.042 per hour (about 1.4 generations per day, 110 total generations). (Stage II chemostat growth rates were lower than Stage I rates to keep chemostat lines near or below 1/2 of their μ_{max} in 500 µg/ml 2,4-D.) Evolved clonal cultures were designated as FBC1, FBC2, FBCr1, FBCr2, FCC1, FCC2, FCCr1, FCCr2, SBC1, SBC2, SBCr1, SCCr1 and SCCr2. Three of the chemostat lines, SBCr2, SCC1 and SCC2 were lost due to contamination of chemostat vessels. All Stage II derived lines were confirmed to have maintained their ancestral marker state, either resistant or sensitive to streptomycin, and their ancestral REP PCR gel pattern.

Batch competition experiments

All competition experiments were performed with clonal samples of evolved lines in the same culture conditions used to propagate the evolving populations. Two competing

genotypes were mixed together in the same flask and allowed to compete for a common pool of limiting nutrient. In Stage I competitions, the ancestors and descendants all competed against a streptomycin-resistant clone isolated from FB1 or SB1 for the F and S lines, respectively. (These resistant clones, FBr and SBr, were also used to establish 8 of the 32 Stage II lines). For Stage II descendants, one competitor was one of the 28 derived genotypes (four were lost to contamination) and the other was the proximate ancestral genotype carrying the reciprocal streptomycin marker, allowing the competitors to be easily distinguished. (Proximate ancestors of Stage II genotypes are the Stage I derived clones used to begin the Stage II evolution lines). For example, streptomycin-sensitive lines FBB1 and FBB2 competed against streptomycin-resistant FBr, while FBBr1 and FBBr2 competed against FB1.

Both strains in a competition were grown separately for 24 hr (one batch transfer growth cycle) to allow acclimation to competition conditions. Batch competitions lasted either 1 or 2 days for F-derived lines and either 4 or 6 days for S-derived lines, depending on the expected relative fitnesses of the competitors. Competitors were initially mixed in fresh medium (mixing ratios are given below) and shaken thoroughly to allow even dispersion of the two competitors. Densities of the evolved and ancestral strains at the beginning and end of a competition were obtained by diluting the culture onto selective and non-selective agar. The

density of the resistant competitor could be estimated directly from the colony count and dilution factor, while the density of the sensitive competitor was estimated as the difference between the non-selective plate count and the selective plate count adjusted for the dilution factor.

The initial mixing ratios, daily transfer dilutions, and length of duration for each set of batch competition experiments are listed below. These were varied to obtain good resolution of each strain at both initial and final time points. Dilution rates for batch competitions are listed in Table 4.

F lines- 0.05 ml samples of Stage I F-derived lines (F, FB1, FB2, FC1, and FC2) were mixed with equal volumes of the FBr competitor in 9.9 ml of medium. The FC1 and FC2 cultures were plated at 0 and 24 hours, whereas the F, FB1 and FB2 cultures were diluted 100-fold into 9.9 ml of fresh medium at 24 hours and plated at 48 hours. All Stage II lines descended from strain F (sets FBB, FBC, FCB, and FCC; Stage II sets consist of lines that share Stage I and Stage II selective regimes in common) were mixed in equal 0.05 ml volumes with their proximate reciprocal ancestor (FB, FBr, FC, or FCr) and plated at 0 and either 24 or 48 hours, with 48 hour competitions being diluted 100-fold at 24 hours.

S lines- 1 ml samples of S, SB1, and SB2 were mixed with an equal volume the SBr competitor in 8 ml of medium, whereas 1.67 ml samples of SC1 and SC2 were mixed with equal volume of SBr in 6.67 ml of medium. S, SB1 and SB2

Table 4. Dilution rates of competition experiments. Dilution rates of both batch [= ln(daily dilution factor)] and chemostat (= flow rate/chemostat volume) competition experiments are shown for each set of replicate lines.

Line Set	Batch Dilution Rate (per day)	Chemostat Dilution Rate (per day)
FB	4.605	2.667
FC	4.605	2.667
SB	1.609	1.000
SC	1.099	1.000
FBB	4.605	2.667
FCB	4.605	1.333
FCC	4.605	1.333
FBC	4.605	2.667
SBB	1.609	1.000
SCB	1.099	1.000
SCC	1.099	1.000
SBC	1.609	1.000

competition cultures were diluted 5-fold daily and plated at 0 and 96 hours (4 days), while SC1 and SC2 flasks were diluted 3-fold daily and plated at 0 and 144 hours (6 days). Stage II lines from sets SBB and SBC (and the control SB1) were competed with SB1 or SBr in the same manner as the S set competitions described above. The lines of sets SCB and SCC (and the control SC1) competed against SC1 or SCr as per the Stage I SC1 and SC2 competitions described above.

Measurement of selection rate constant for batch competitions

Relative performance between two competitors is expressed as the selection rate constant (SRC), r_{ij}, and is given by

$$r_{ij} = (\ln[N_i(t)/N_i(0)] - \ln[N_j(t)/N_j(0)])/t$$

where $N_i(0)$ and $N_j(0)$ are the starting densities of the two competing genotypes, and $N_i(t)$ and $N_j(t)$ are their corresponding densities after t days of competition (Travisano and Lenski 1996). The selection rate constant equals the difference between the genotypes' realized Malthusian parameters during direct competition for the same pool of limiting resource. (If there is a difference in the plating efficiencies of the genotypes, this does not affect the selection rate constant, granted that plating efficiencies remain the same between initial and final samples.) The selection rate constant, a difference between

Malthusian parameters, was chosen to represent performance rather than the more commonly used *ratio* of Malthusian parameters (Lenski et al. 1991), because the former is less sensitive to sampling error when competitors have very different Malthusian parameters, which was sometimes the case in this study. Marker effects of streptomycin-resistant lines have been factored out of the SRC values presented in Tables 5 and 6, so that a positive SRC reflects superior performance than the relevant ancestor, and a negative value shows decreased performance. This was done to standardize SRC values relative to an ancestral value of zero. For example, to obtain the SRC for strain FBB1 relative to its proximate ancestor FB1, both strains were independently competed directly against strain FBr. Then the SRC of FB1 relative to FBr was subtracted from that of FBB1 relative to FBr to obtain the SRC of FBB1 relative to FB1.

Experimental design of batch competitions

In both the 2,4-D and succinate media, usually five estimates, and a minimum of three, of the selection rate constant for each competitor were obtained. Competitions were performed in blocks of five pairwise matches, with five replicates per match, for a total of 25 separate cultures per block. For the Stage I lines, one block consisted of F, FB1, FB2, FC1, and FC2 each being competed against FBr, and a second with S, SB1, SB2, SC1 and SC2 each competing against SBr. Each block of competitions was performed in both 500

µg/ml 2,4-D and 500 µg/ml succinate AN medium. For Stage II lines, these blocks consisted of one control competition between the two reciprocal ancestors (e.g., FB1 *vs.* FBr) and a set of four lines against their reciprocal ancestors. For example, one block had pairings of FB1 *vs.* FBr, FBB1 *vs.* FBr, FBB2 *vs.* FBr, FBBr1 *vs.* FB1, and FBBr2 *vs.* FB1. (The only exception to this was for the SBC and SCC sets, which both had fewer than four lines due to contamination.)

Protocol and SRC measurement for chemostat competitions

Chemostat competitions were also performed under the same conditions as the chemostat evolution experiments. The genotype pairings and block designs were the same as for the batch competitions, with the exception that two replicates (rather than five) were obtained for each pair of competing genotypes. For each competition, both competing genotypes were first grown to stationary phase in the competition medium. They were then mixed at a ratio of 100 (sensitive competitor) to 1 (resistant competitor) in the chemostat vessels, at which time media flow through the vessels was initiated. To allow a period of physiological acclimation to competition conditions, cultures were allowed to grow overnight (12-24 hours) before being sampled for the first time. Each culture was sampled and plated several times on selective and non-selective agar over a 6-10 day period to monitor changes in density of the two genotypes. Chemostat competition dilution rates are listed in Table 4.

The selection rate constant for each replicate was calculated as the slope of the value $\ln(R/S)$ over time (in days) where R is the density of the resistant competitor and S is the density of the sensitive competitor. This calculation using several density measurements over time is equivalent to that for the batch competitions, which uses density data from only the start and finish of the competition period.

RESULTS

Adaptation to selective regime

Of the 36 evolved lines (Stages I and II) analyzed in this study, 22 of them show significant adaptation to their selective regime based on an SRC > 0 in competition with the ancestor (Tables 5 and 6). In four sets of lines (FC, SB, FBB, and SCB), all lines in each set adapted significantly to their selection regime, while other sets contain lines (total of 13) that show a non-significant increase in competitive performance. Only one strain, FCBr2, actually indicates a decrease in performance relative to its ancestor, but this decrease is not significant.

Of the Stage I lines derived from F, the fast ancestor, neither batch lineage (FB1 or FB2) shows a significant competitive improvement in their batch selective regime, but both lines selected in the chemostat regime (FC1 and FC2) did improve significantly in chemostat. The opposite trend occurred among the Stage I descendants of the slow ancestor

Table 5. Results of competition experiments for evolved lines from Stage I selection in succinate. Selection rate constants (SRC) of Stage I evolved lines (relative to their appropriate ancestors) for both the regime in which evolution occurred and the alternative regime are presented. Lines with SRC values that are significantly different from the appropriate ancestor are presented in bold (*t*-test, 2-tailed $P < 0.05$).

Table 5. Results of competition experiments for evolved lines from Stage I selection in succinate.

Evolved Line	**Selected in**	**SRC (per day)**	
		Selective Regime	**Alternative Regime**
FB1	Batch	0.0260	0.0472
FB2	Batch	0.0910	-0.0374
FC1	Chemostat	**2.8721**	-0.3093
FC2	Chemostat	**2.0268**	**0.8125**
SB1	Batch	**0.2531**	0.3715
SB2	Batch	**0.3759**	**0.6026**
SC1	Chemostat	0.1729	**0.2379**
SC2	Chemostat	0.2521	0.0897

Table 6. Results of competition experiments for evolved lines from Stage II selection in 2,4-D. Selection rate constants (SRC) of Stage I evolved lines (relative to their appropriate ancestors) for both the regime in which evolution occurred and the alternative regime are presented. Lines with SRC values that are significantly different from the appropriate ancestor are presented in bold (*t*-test, 2-tailed $P < 0.05$).

Table 6. Results of competition experiments for evolved lines from Stage II selection in 2,4-D.

Evolved Line	**Selected in**	**SRC (per day)**	
		Selective Regime	**Alternative Regime**
FBB1	Batch	**0.4026**	**0.1821**
FBB2	Batch	**0.9675**	0.2162
FBBr1	Batch	**0.3116**	**0.2268**
FBBr2	Batch	**0.3439**	**-0.4448**
FCB1	Batch	0.4171	0.3177
FCB2	Batch	**0.6390**	-0.2586
FCBr1	Batch	**0.6788**	0.0150
FCBr2	Batch	-0.6600	-0.3296
FCC1	Chemostat	0.2341	**0.3291**
FCC2	Chemostat	**1.2510**	**0.6807**
FCCr1	Chemostat	**0.9161**	**-0.4631**
FCCr2	Chemostat	**0.7944**	-0.0517
FBC1	Chemostat	0.1432	-0.1468
FBC2	Chemostat	0.2944	-0.2777
FBCr1	Chemostat	**0.3265**	-0.3362
FBCr2	Chemostat	**0.6090**	-0.3798

Table 6 (cont'd).

Evolved Line	**Selected in**	**SRC (per day)**	
		Selective Regime	**Alternative Regime**
SBB1	Batch	0.0379	-0.0154
SBB2	Batch	0.1073	**0.1013**
SBBr1	Batch	**0.2230**	-0.0151
SBBr2	Batch	0.5401	0.0098
SCB1	Batch	**0.6924**	0.2494
SCB2	Batch	**0.7670**	0.0758
SCBr1	Batch	**0.4899**	-0.2048
SCBr2	Batch	**0.3680**	-0.4172
SCCr1	Chemostat	0.0013	0.0925
SBC1	Chemostat	**0.0701**	0.1207
SBC2	Chemostat	0.0026	0.0031
SBCr1	Chemostat	**0.0763**	0.0845

(S). SB1 and SB2 both improved significantly in batch, but neither SC1 nor SC2 improved significantly in chemostat.

Among the eight Stage II F lines that continued evolving in the same regime as that of their Stage I ancestral lineage (sets FBB and FCC), seven show significant adaptation (Table 6). In the two sets that switched selective regimes (FBC and FCB), however, only four out of eight improved significantly in their Stage II regime. Those Stage II strains that share strain S as a common ancestor, however, showed the opposite overall trend of adaptation between those lines that did and did not switch selective regimes. In the SBB and SCC sets, only one of five lines show significant improvement in their selective regime, whereas in the sets that switched regimes (SBC and SCB), six of seven lines adapted significantly.

Performance in alternative regime

Of the 36 total lines, ten show significant changes in the alternative regime to that in which they were selected (Tables 5 and 6). Seven of these ten lines (FC2, SB2, FBB1, FBBr1, FBBr2, FCC2, and FCCr1) are also significantly different than their ancestor in their selective regime. The evolutionary changes of these seven lines, therefore, are of greatest consequence for the substrate concentration tradeoff hypothesis. Only two lines (FBBr2 and FCCr1) show the pattern of evolutionary changes predicted by the tradeoff hypothesis - significantly improved performance in its selective environment (chemostat) associated with

significantly diminished competitiveness the alternative regime (batch) (Figure 8a). The other five lines all improved significantly both in their selective and alternative regimes, contrary to expectations of the tradeoff hypothesis (Figure 8b). Among the 22 lines that adapted significantly to their own selective regime, two lines (FBBr2 and FCCr1) show a significant tradeoff pattern, eight show non-significant tradeoff patterns, seven show non-significant improvements in their alternative regime, and five lines (FC2, SB2, FBB1, FBBr1, and FCC2) improved significantly in both regimes.

Homogeneous and heterogeneous responses to selection

Only one of the eight Stage II sets with multiple lines (FBC set) of lines shows a consistent performance response among all lines in both their selective and alternative environments. The competition results for the four FBC lines (Figure 9, open squares) suggest that they all may have improved in their selective regime while worsening in the alternative batch regime. Most of the changes in FBC lines are not significant when individual lines are compared to the ancestor separately. However, when data from separate lines are pooled, the set as a whole shows a statistically significant performance decrease in the non-selective (batch) regime ($t = 2.512$, 21 degrees of freedom, 2-tailed $P = 0.0202$) and a not quite significant improvement ($t = 2.234$, 8 degrees of freedom, 2-tailed $P = 0.0560$) in the selective

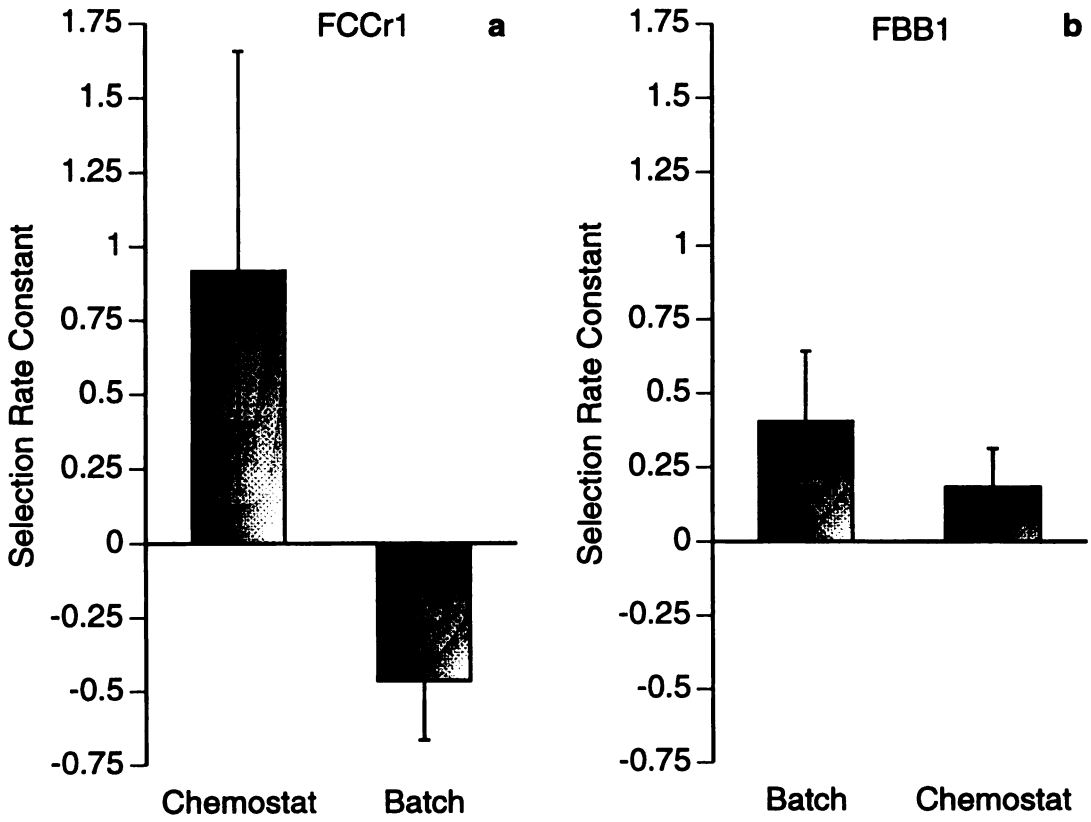

Figure 8. Tradeoff and non-tradeoff patterns of adaptation. (a) Strain FCCr1 shows the pattern of performance in its selective and non-selective regimes that is expected by the substrate concentration tradeoff hypothesis. (b) Strain FBB1 and five other strains (not shown) exhibit the opposite pattern, improving their performance in both batch and chemostat regimes. Error bars indicate 95% confidence intervals.

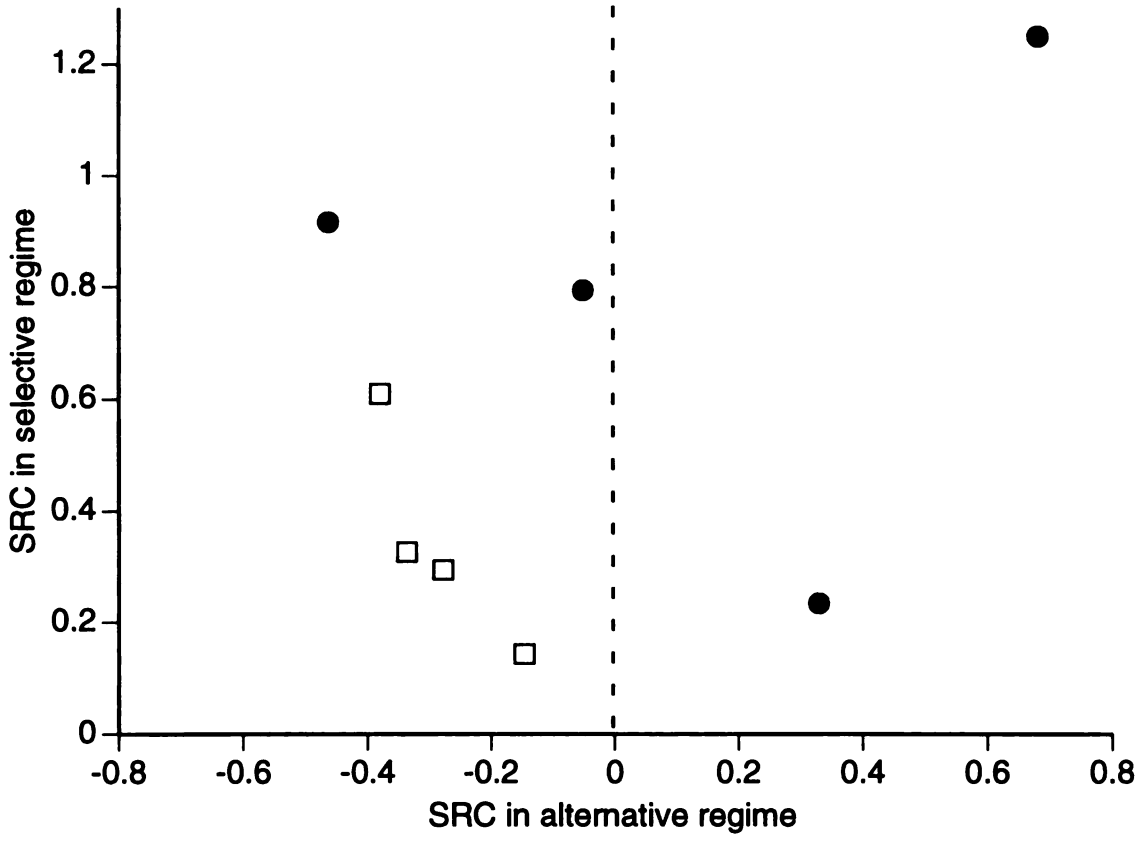

Figure 9. Heterogeneous and homogeneous selection responses. Most Stage II sets show a heterogeneous pattern of performance change in their alternative regime as exemplied by the FCC lines (filled circles), where two lines improved and two became worse. Alternatively, two sets have a homogeneous pattern, as exemplified by the FBC lines (open squares), which all appear to have slightly decreased performance in their alternative (batch) regime.

(chemostat) regime, suggesting an overall tradeoff tendency for the set.

For most Stage II sets, however, there is no consistent improvement or detriment in their non-selective regime. For all six sets showing such variation, one or two lines appear to be worse in the non-selective regime while the other two or three appear to have improved. This is exemplified in Figure 9 by the FCC set (filled circles), in which two lines show improvement in the alternative (batch) regime and two either show or suggest performance decreases. Again, many of the individual changes among all lines in these six sets are not significant, but the overall pattern strongly suggests that improved performance in a selective regime (which occurred or appeared to have occurred in all lines but one) can be associated with either improved or worsened performance in the alternative regime.

Interpretation of chemostat competition data

While analysis of data from the batch competitions is straightforward, there are some features of the chemostat competition data that make its interpretation more challenging and warrant mention. The ideal results of chemostat competitions are log-linear slopes of the ratio of competitors' densities over time (Figure 10a) that are, moreover, very similar for the two independent replicates. This, however, is not always the case. My data can be classified into four basic types, corresponding to the four

Figure 10. Four types of chemostat competition data. The natural log ratios of competing genotypes over time in the chemostat are shown. Circles and diamonds distinguish data points between two independent replicate competition experiments and solid lines represent the two corresponding linear regressions. (a) Both replicates of FCCr1 behave similarly, are linear, and have a mean SRC that is significantly different from the ancestral controls. (b) FBB1 replicates are similar and significantly different from the ancestral controls, but they do not appear to follow a simple linear trajectory. (c) Replicates of SCB2 are similar and linear, but they are not significantly different from the ancestral controls. (d) Replicates of FBC1 are not similar and their mean is not significantly different from the ancestral controls.

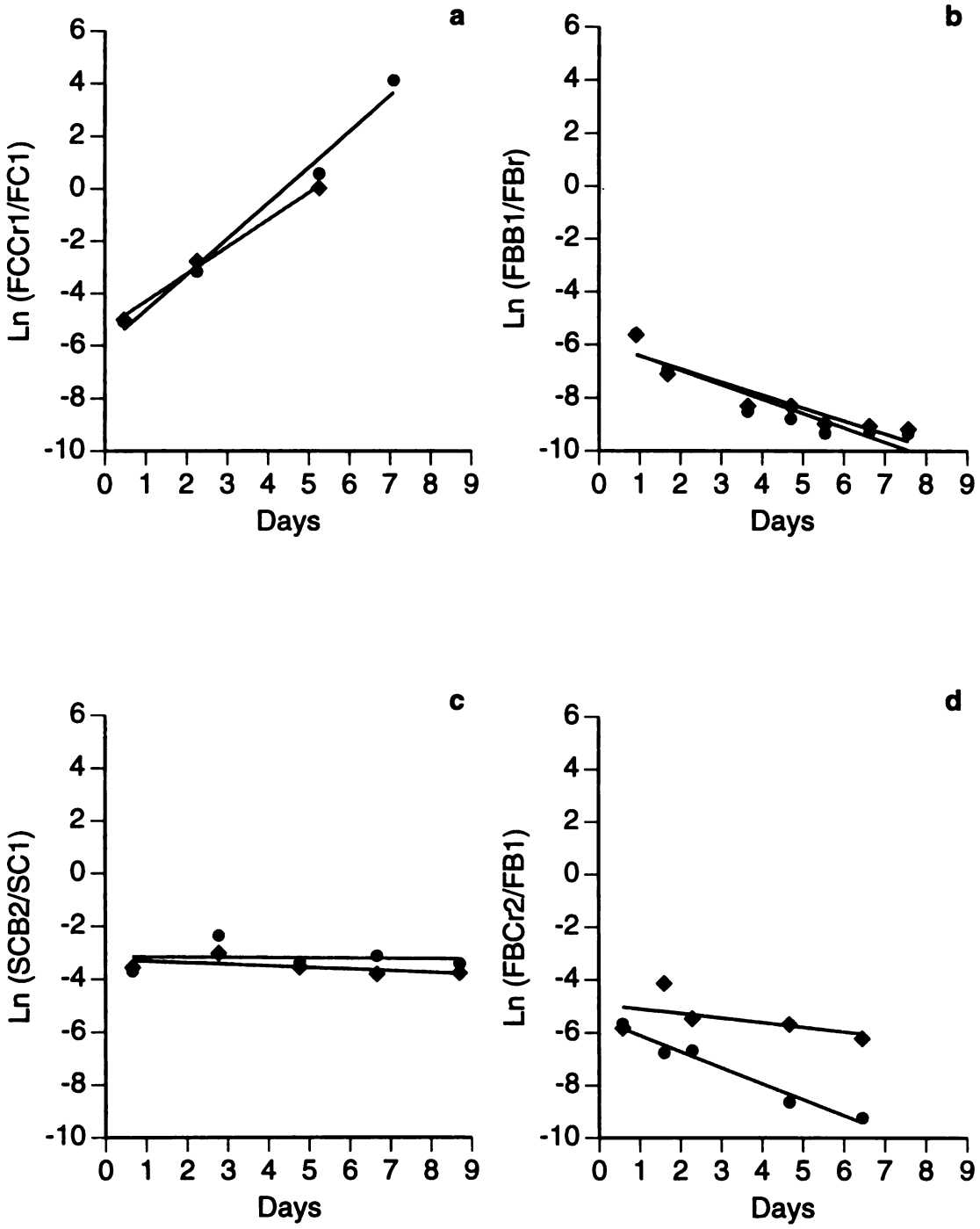

Figure 10. Four types of chemostat competition data.

graphs in Figure 10. In the first case (Figure 10a, strain FCCr1), the data points in each replicate are approximately log linear over time, the two replicate trajectories are very similar, and their mean slope is significantly different from that of the ancestral control competition. A second type of result (Figure 10b, strain FBB1) again exhibits similar replicate patterns and a significantly different slope than the ancestral control, but the data points do not seem to follow a simple linear trajectory over time. Figure 10c (strain SCB2) shows a third case where the replicates are similar and relatively linear, but their mean slope does not differ significantly from the ancestral competition. Finally, there are also instances (Figure 10d, strain FBC1) where the replicate populations seem to show different trajectories and the mean slope is not significantly different from the ancestor.

My results exhibit multiple instances of each of these four types of data, and I will briefly discuss the interpretation of the types that deviate from similarity between replicates (Figure 10d) or from simple linearity (Figure 10b). The causes of these dissimilar or non-linear results are difficult to specify, especially for the former case. Dissimilarity of replicates reduces the statistical significance of any difference in mean slope between derived and ancestral competitions because it increases variance. In all cases where we observe a significant evolutionary change by a derived line relative to its ancestor in the chemostat,

the two replicates of both the derived competition and the ancestral control behave similarly. Because dissimilar replicates tend to mask any actual evolutionary change that took place in a derived line, the effect may be a reduction in the number of statistically significant cases of evolutionary change from which to draw conclusions bearing on the tradeoff hypothesis. Nonetheless, those cases of significant and clear-cut evolutionary change revealed by these competition studies are sufficient to draw robust conclusions about the substrate concentration tradeoff hypothesis.

The other unexpected pattern in chemostat competition data occurs in cases where the slope of the log ratio of competing genotypes changes significantly over time (Figure 10b). Possible causes of this pattern include different rates of chemostat wall accumulation by the two genotypes (Chao and Ramsdell 1985); frequency-dependent selection, where the fitness of one genotype relative to the other changes as the relative frequency of the genotypes changes (Levin 1988); or insufficient time for both competitors to reach equilibrium because one competitor has a longer transition from stationary phase to equilibrium growth than the other (Jannasch 1969; Veldkamp et al. 1984).

Experimentally testing these possibilities was not feasible within this study, given the large number of ancestral and derived strains that were studied. However, there are several considerations which legitimize the use of

the mean slope over the entire time-course of the competitions (rather than selecting only a sub-period of the experiments, in which trajectory appears log-linear, over which to measure performance). First, there may be different causes for this pattern in different competitions. One case may be due to frequency dependent selection and another due to gradual accumulation of wall growth. Hence, it is not clear whether to focus on flatter or steeper parts of the competition trajectory. Second, not all cases of changing slopes follow the same pattern. In some cases, the slope decreases over time, while in others it increases. Third, and most importantly, among those derived strains that show a significant change from their ancestor in chemostat competitiveness, none of them show a change in sign of the slope during the competition, but only a modification in the same direction. All of these considerations suggest that taking a simple log ratio slope over all time points is a feasible, reasonable, and consistent approach to measuring competitiveness for these experiments. However, even if I limited my analysis to those derived strains that are significantly different than their ancestor in chemostat *and* showed constant slopes, this would not alter the fundamental conclusions of this investigation.

DISCUSSION

Implications for the tradeoff hypothesis

Systematic comparative tests of the substrate concentration tradeoff hypothesis (Greer et al. 1992, Lenski et al. 1997, Chapter 1) have failed to support the hypothesis. Greer et al. (1992) and Lenski et al. (1997) both measured the maximum growth rate (μ_{max}) and substrate affinity (μ_{max}/K_S) values for several strains of 2,4-D degrading bacteria. These parameters determine growth rates at high and low substrate levels respectively. Neither study found a significant negative correlation between μ_{max} and μ_{max}/K_S as was expected from the tradeoff hypothesis. I also measured growth rates at high and low substrate concentrations on two different substrates for seven strains of 2,4-D degrading bacteria isolated from a variety of locations and conditions (Chapter 1). While the tradeoff hypothesis predicts a negative correlation between high and low substrate concentration growth rates, the opposite trend was observed. When each data point was treated as an independent observation, there was a significant positive correlation between growth rates at 5 and 500 µg/ml of succinate, and an almost significant positive correlation between growth rates at the same concentrations of 2,4-D. These correlations remained positive but decreased to insignificance after data was scaled to account for phylogenetic relationships among the strains.

A related comparative study was conducted with sixteen strains of benzene degrading bacteria (Zhou and Tiedje, ms). The maximum rates (V_{max}) and half-velocity coefficients (K_m) of benzene utilization were measured for each strain. The strains were then categorized into three basic kinetic types: high V_{max} with high K_m, low V_{max} with low K_m, and high V_{max} with low K_m. The first two categories are expected by the traditional substrate concentration tradeoff hypothesis, but the third category (which included two of the sixteen strains) is not. These two unexpected strains offer additional comparative evidence that the tradeoff hypothesis is in need of modification, even though most strains conformed to the predicted pattern. In summary, systematic comparative studies have either not strongly supported or have contradicted the tradeoff hypothesis.

Experimental evolution has been previously used by Luckinbill (1984) to test predictions of *r*- and *K*- selection theory that are similar to predictions of the substrate concentration hypothesis tested here. In Luckinbill's study, replicate evolving lines of *Escherichia coli* were maintained by serial batch culture using transfer protocols designed to impose either *r*- or *K*- type selection on evolving populations. Cultures under the *r*- selection regime were transferred prior to reaching stationary phase, whereas to impose *K*- selection, populations were allowed to reach and experience a prolonged stationary phase before being transferred to fresh medium. Although no tradeoff was shown

by this experiment, the selective regime intended to impose significant *K*- selection may not have done so. Vasi et al. (1994) conducted theoretical and empirical studies of growth parameter changes during evolution of *E. coli* populations in a batch transfer regime and concluded that even in Luckinbill's putatively *K*- selection regime, maximum exponential growth rate was probably the primary target of selection. This evaluation renders the absence of tradeoff in Luckinbill's experiment inconclusive regarding the relationship of competitive abilities at high *vs.* low nutrient concentrations.

Vasi et al. (1994) measured μ_{max} and K_S values for twelve independently derived lines of *E. coli* before and after 2000 generations of evolution in a serial batch transfer selective regime. Both μ_{max} and K_S increased significantly overall among the twelve lines, but the ratio μ_{max}/K_S did not change significantly. Their study, therefore, also failed to support the tradeoff hypothesis.

In this study, I tested directly for changes in competitive ability at high and low resource concentrations following evolution in regimes that clearly favored adaptation to one condition or the other. The results bear upon the tradeoff hypothesis more clearly and strongly than do those of previous studies. My overall results not only fail to support the hypothesis, but several specific results directly contradict it. Only two strains (FBBr2 and FCCr1) show significant tradeoff patterns, having lost

competitiveness at high 2,4-D concentrations after having evolved and improved competitively under low 2,4-D conditions. Evolutionary changes in the FBC set as a whole are also suggestive of the tradeoff pattern (Figure 9). However, five lines (FC2, SB2, FBB1, FBBr1, and FCC2) show results that flatly contradict the tradeoff hypothesis, improving significantly both in their selective regime and in the alternative regime.

Overall, 14 (12 non-significant) lines show the tradeoff pattern, and 21 (16 non-significant) show the opposite pattern (Table 7). These bi-directional responses to selection indicate no propensity for tradeoffs in competitive performance under the two selective regimes (Table 7). It is likely that at least some evolution has been overlooked among lines not showing significant changes by my competition assays. This could be due to noisy data, insufficient replication, and/or insufficiently long periods of competition. If this is the case, then one expects that more precise data would tend to move cases of masked evolution from insignificance to significance in the direction suggested by my actual data. The overall pattern of evolutionary changes among these lines strongly suggests that there are multiple evolutionary pathways by which bacteria can adapt to high or low substrate conditions and, moreover, that these pathways can be either beneficial or detrimental to fitness under the alternative conditions. These data also

Table 7. Frequency of tradeoff and non-tradeoff patterns. The number of lines that show a statistically significant tradeoff (ST), non-significant tradeoff (NST), non-significant correlated improvements in both batch and chemostat (NSCI), and significant correlated improvements (SCI) are shown. These categories are shown for the 22 lines that adapted significantly to their selective regime and then for the 35 total lines that either show or suggest such adaptation.

	ST	NST	NSCI	SCI
Significantly Adapted	2	8	7	5
Total	2	12	16	5

suggest that such changes in the alternative regime may occur in positive and negative directions at a similar frequency.

Strain history and evolutionary change

Also of interest in the design of these experiments were the effects of strains' selective history on subsequent patterns of adaptation (cf. Travisano et al. 1995). Two patterns in the data are suggestive of such effects. First, the selective histories of F and S in the wild may have influenced the rate and extent of their adaptation to batch and chemostat environments during Stage I evolution. Among the four F-derived lines, neither batch-selected line improved significantly in batch, but both chemostat-selected lines did improve significantly in chemostat. In contrast, S-derived lines show the opposite pattern, with both batch-selected lines becoming significantly better in batch, but neither chemostat-selected line having significantly improved in chemostat. Prior selection in nature had rendered strain F able to grow fast when resources were plentiful, and strain F therefore had much more room for improvement under a condition of resource scarcity (chemostat) than one of abundance (batch culture). The opposite appears to be true for strain S, which prior natural selection left with relatively slow growth in substrate rich conditions and thus with considerable room for improvement in the batch culture regime.

In the second pattern, F-derived Stage II lines show the opposite adaptive trend between lines that did and did not switch regimes from Stage I to II than do S-derived Stage II lines. Seven of the eight F-derived lines that shared a common selective regime for both stages of evolution (FBB and FCC sets) adapted significantly to their Stage II regime, while only four of the eight lines that switched regimes (FBC and FCB sets) are significantly better in their Stage II environment. Among those S-derived lines that switched regimes, however, six of seven improved significantly during Stage II, whereas only one of five among those that did not switch regimes is significantly improved. These results suggest that the selective history of strain S in the wild may have left it with more potential for reversible evolution in alternating environments than did the natural history of F. Because this explanation is *ad hoc*, confirmation would require further experimentation.

Potential mechanisms of adaptation

Here I first discuss the population genetic processes that caused phenotypic changes in these evolving lines and then I speculate on potential demographic mechanisms underlying their adaptation. Seven lines underwent significant fitness changes in both their selective regime and the alternative regime. Were the fitness changes in the alternative regime due to pleiotropic effects of beneficial mutations in the adaptive regime? Or were they due to the

random fixation of mutations that were effectively neutral in the selective regime (Kimura 1983)? The vast majority of mutations that affect fitness in a given environment are expected to be harmful rather than beneficial, because it is generally much easier to disrupt than to improve the performance of a well-functioning entity. Therefore, if one considers mutations that are selectively neutral in one regime (say, batch culture), but affect fitness in an alternative environment (say, chemostat), then one would expect most of them to reduce, rather than improve, fitness in the alternative regime. In this study, however, six of the eight lines mentioned above show significant improvements in both types of regime. These data imply that improvements in the alternative regimes were not due to substitution of random mutations by drift, but instead were caused by the same mutation that were responsible for improvements in the selective regimes. In other words, selected mutations were generally beneficial in both regimes, indicating positive pleiotropy (and contrary to the antagonistic pleiotropy assumed by the tradeoff hypothesis).

The parameters μ_{max} and μ_{max}/K_S are central components of fitness in the batch and chemostat regimes, respectively. They are also the parameters in which the substrate concentration tradeoff hypothesis is most often expressed. The dual improvement of performance by several evolved lines in both regimes suggests that adaptive mutations can occur that simultaneously increase both μ_{max} and μ_{max}/K_S. For

lines evolved in batch culture, the lag and stationary phases of growth are potential objects of selection as well as exponential growth rate. However, a simple increase in μ_{max} would also increase the value μ_{max}/K_S, improving fitness at both high and low resource concentrations. This one-step path to improving both kinetic parameters seems more likely than an increase in μ_{max}/K_S being correlated with an improvement in lag or stationary phase fitness.

The strengths and limitations of this study

The greatest strength of this investigation relative to previous tests of the substrate concentration tradeoff hypothesis is that the evolution relevant to the conclusions of the study occurred under well-defined, experimental laboratory conditions, rather than in unknown conditions in the wild. Instead of basing conclusions on correlative patterns of data for traits with unknown selective histories in the wild, I have been able to compare the competitive performance of derived lines with that of their clonal ancestors after evolution in known selective conditions. This means that differences among evolved lines relative to each other and to the ancestor are due to the selective forces inherent in the lab evolution regimes rather than to phylogenetic relationships, differential preadaptation to laboratory conditions, or unknown selective forces in nature.

Also strengthening this study is the experimental design, which includes multiple starting lines, two stages of

evolution with different resources, independently evolving replicate populations, and two distinct selective regimes. Multiple starting lines allow observation of the influence of strain history, if any, on subsequent evolutionary patterns. Two evolutionary stages allowed adaptation to lab conditions during Stage I and allowed Stage II lines to either continue in the same selective regime or alternate to the other. Most importantly, the two selective regimes used, batch and chemostat culture, are clearly distinct in the selective forces they impose on their evolving populations.

In this test of the substrate concentration tradeoff hypothesis, evolutionary changes in competitive fitness in both selective and alternative regimes were estimated by direct competition experiments between evolved and ancestral strains. This approach tests the hypothesis more directly than would comparison of the kinetic parameters (between evolved and ancestral strains) that are thought to underlie fitness in abundant and scarce resource conditions. While measurement of these parameters would prove useful in clarifying which components of fitness changed most during evolution, evolutionary dynamics (whether in the lab or in the wild) are ultimately a function of overall competitive ability.

Additionally, these experiments were performed with naturally isolated strains of bacteria capable of degrading a common herbicide rather than by using strains with long laboratory histories and no particular applied significance.

The substrates used are potential resources for the strains in nature, increasing the relevance of this study for applied bioremediation issues. The components of this study have resulted in conclusions that almost unambiguously contradict the hypothesis in question.

These experiments provide little or no evidence for a tradeoff in competitive performance under conditions of resource abundance *versus* scarcity. However, the maximum number of evolutionary generations undergone by any of my derived lines was 1000 (FBB set, 500 in batch-succinate, 500 in batch-2,4-D). Although I have shown short-term simultaneous fitness increases in alternative environments to be possible and even probable, the long-term potential for this simultaneous optimization of traits may be limited. For example, a particular strain evolving in the batch regime for 2000 generations may increase its SRC in batch relative to its ancestor from 0 to 1.0 while simultaneously increasing its SRC in the chemostat environment from 0 to 0.5. Further evolution (say, another 2000 generations) in batch, however, may increase its batch SRC from 1.0 to 1.5 while simultaneously reducing its chemostat SRC from 0.5 to 0.25 (Figure 11). Hence, the putative tradeoff may in fact exist, but only beyond a certain threshold level of adaptation to a particular environment. Below this threshold, improvement of fitness in the non-selective environment is possible. The only way to conclusively test whether such a tradeoff threshold exists would be to conduct experimental evolution

Figure 11. Hypothetical adaptive threshold for tradeoff. The competitive ability of an evolving population is shown to increase (with a decreasing *rate* of increase) over 4000 generations of evolution in its selective regime (say, abundant resource conditions; solid curve). The correlated performance of that strain in its alternative regime (scarce resource conditions; dashed curve) also increases, but only up to about 2000 generations, after which time it begins to decrease. In this case, the substrate concentration tradeoff hypothesis would be false when applied to the first 2000 generations of adaptation, but accurate for subsequent adaptation to the selective regime.

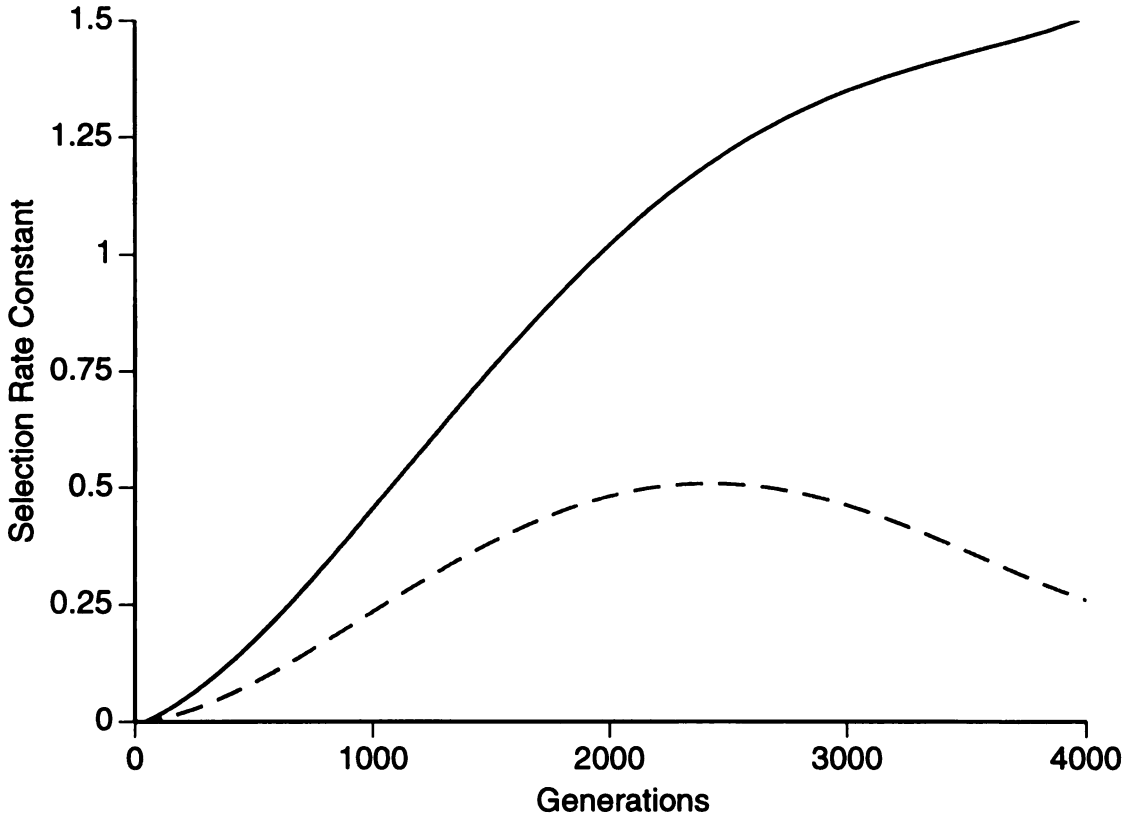

Figure 11. Hypothetical adaptive threshold for tradeoff.

for a long enough period to allow the slope of fitness increase over time to approach zero as the potential for further adaptation to a given environment decreases over time (Lenski and Travisano 1994). Despite this limitation, my results clearly show that a tradeoff between competitive ability at high *vs.* low resource conditions does not exist over the entire trajectory of adaptation to abundant and scarce resource regimes.

Chapter 3

PLEIOTROPIC EFFECTS OF ADAPTATION TO A SINGLE CARBON SOURCE FOR GROWTH ON ALTERNATIVE SUBSTRATES

INTRODUCTION

Biological traits that are not important components of fitness in a particular environment may nonetheless evolve by a variety of mechanisms. Such non-selective mechanisms of evolution include pleiotropic side-effects of adaptive mutations on unselected traits (Wright 1977) and the random fixation of effectively neutral alleles by genetic drift (Kimura 1983). Along with adaptation by natural selection, these more indirect mechanisms of evolution are relevant to such issues as the genetic divergence of populations within a species (Cohan 1984; Travisano et al. 1995) and the fate of genetically modified organisms in natural environments (Tiedje et al. 1989; Lenski 1993; Regal 1993).

Several studies have shown that isolated populations of the same species may evolve different adaptations even to the same selective conditions (Gould and Lewontin 1979; Cohan 1984). Some of clearest evidence for this phenomenon comes from laboratory experiments using fruitflies (Cohan and Graf 1985; Hoffman and Cohan 1987) and bacteria (Travisano et al. 1995; Travisano and Lenski 1996). These results appear to conform with Sewall Wright's concept of an "adaptive

landscape" that has several fitness peaks, each representing a distinct genetic solution to a particular selective environment.

Alternative pathways of adaptation to a particular selective environment may lead to heterogeneous changes in traits that are not important for fitness in that environment. For example, several populations of organisms that are under selective pressure to utilize a particular substrate more efficiently may each find a different physiological mechanism to do so. The effects of these different adaptations on the ability of organisms to utilize some alternative substrate may be positive in some cases, neutral in others, and negative in yet other populations. As a consequence of these different correlated responses, some populations may be fortuitously well adapted, and others poorly adapted to environments that contain this alternative substrate.

In this study, I have used evolving populations of bacteria to investigate the effect of adaptation to one carbon source on competitive performance on an alternative substrate. The bacterial strains employed are two natural isolates of the β-proteobacteria, each capable of degrading the herbicide 2,4-dichlorophenoxyacetic acid (2,4-D). Experimental evolution occurred in two separate stages, one in which succinate was the sole carbon source, and a second in which 2,4-D was the limiting substrate (Figure 12).

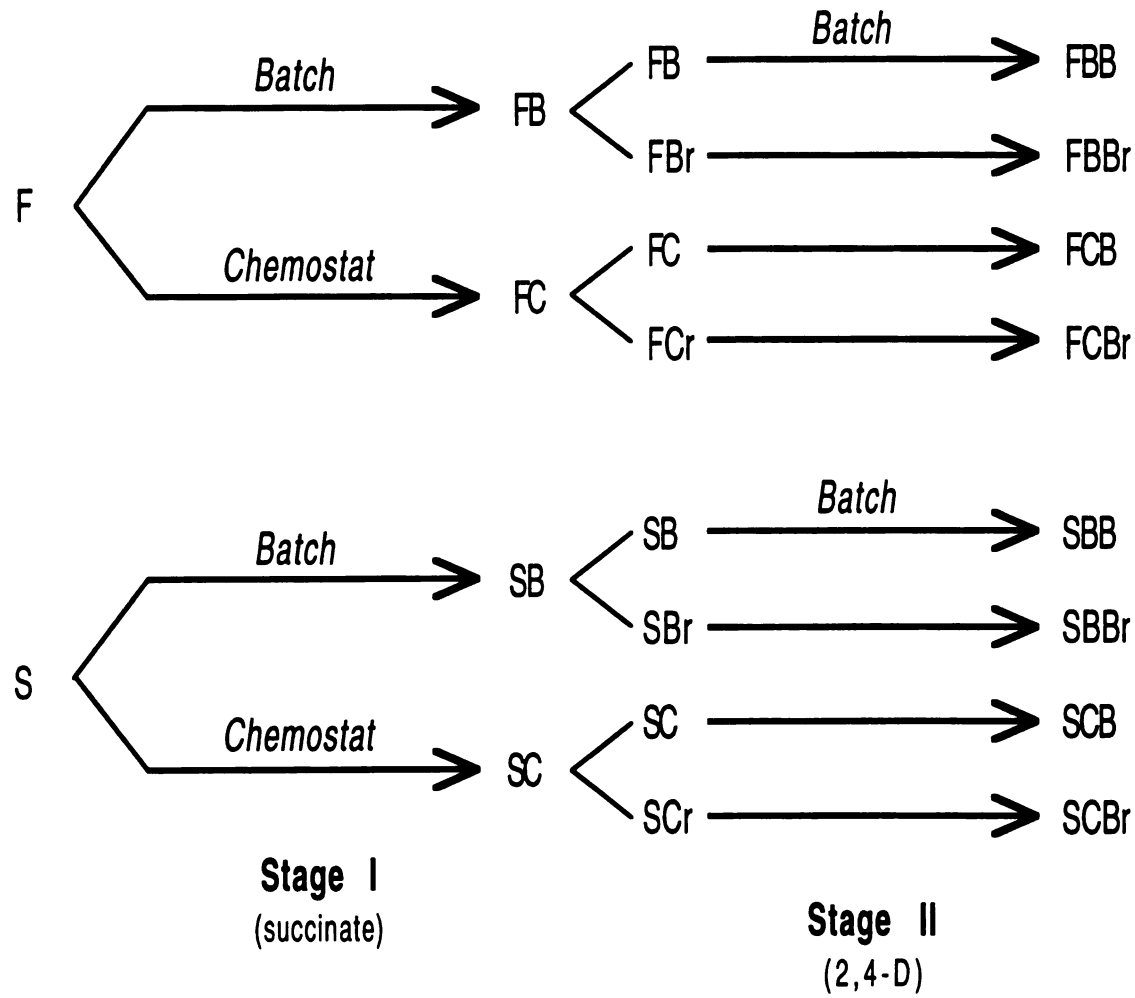

Figure 12. Derivation of bacterial strains. Horizontal lines indicate periods of evolution with the selective regime and sole carbon source indicated above and below, respectively. Each horizontal line represents two independently evolved replicate populations. See MATERIALS AND METHODS (Chapters 2 and 3) for detailed strain and nomenclature descriptions as well as the number of evolutionary generations for each line.

The use of environmentally important substrates increases the relevance of my results to concerns about the evolutionary adaptation of genetically modified organisms subsequent to their release in the environment. One concern is that certain environmental conditions may cause bacteria that are degrading a toxic substrate to produce an even more toxic metabolite in the process. For example, bacteria may produce highly toxic vinyl chloride during the biodegradation of tetrachloroethylene under methanogenic conditions (Vogel and McCarty 1985). In a similar vein, genetically modified bacteria that are used to degrade some toxic compound may *evolve* novel degradative pathways that yield a more toxic metabolite even as they continue to degrade the intended substrate. Concerns over these and other potential adverse effects are magnified if there is a significant likelihood that the released organisms may persist indefinitely in the environment (Tiedje et al. 1989; Lenski 1993; Regal 1993). And as the organisms adapt to the natural environment in which they were released, the likelihood that they can persist will increase.

Of particular relevance to these concerns is the effect that evolutionary adaptation by bioremediative organisms to their intended anthropogenic substrate has on their abilities to grow on, and compete for, naturally occurring substrates. If adaptation to an anthropogenic substrate always leads to a reduction in fitness on natural substrates, then this provides a measure of safety for the release of organisms

modified for bioremediation. If, however, such adaptation may indirectly enhance fitness on natural substrates, then this increases the likelihood that the released organisms (and any adverse effects they may cause) will persist in the environment.

In Stage I of the experimental evolution, two naturally occurring strains of 2,4-D degrading bacteria were used to found replicate populations that were propagated in batch culture with succinate (a naturally occurring substrate) as the sole carbon source (see Figure 12). One of these strains (F) grows relatively fast under laboratory conditions, whereas the other (S) grows more slowly. In Stage II of the evolution experiment, clonal isolates derived from the Stage I batch culture lines were used to found replicate populations that were further propagated in batch culture, except that 2,4-D was provided as the sole carbon source (Figure 12). Additional Stage II batch lines were founded using clones from Stage I lines that had undergone evolution in a different selective regime - chemostat culture. I then measured the competitive performance of all of these derived lines relative to their immediate ancestors on both succinate and 2,4-D. The results of these experiments demonstrate that adaptation to either substrate can be associated with both improvements and losses in competitive performance on the alternative substrate.

MATERIALS AND METHODS

All strains, evolution experiments, and batch competition protocols are the same as described in the MATERIALS AND METHODS section of Chapter 2, except that batch competition assays were conducted in each strain's alternative substrate (succinate or 2,4-D) as well its selective substrate. Dilution factors and the duration of competition experiments were always the same for both substrates. This study focuses on a subset of the strains described in Chapter 2 (Figure 12). Batch competition experiments were performed on both selective and alternative substrates for strains that had been propagated in the batch regime during their most recent stage of experimental evolution. These strains are FB1, FB2, SB1, and SB2, all of which grew on succinate in batch culture during Stage I evolution; and sixteen strains that grew on 2,4-D in batch culture during Stage II evolution. Among the latter group, the FBB and SBB sets had been previously grown on succinate in batch culture during Stage I; whereas sets FCB and SCB had been previously grown on succinate in chemostat culture during Stage I. Competition dilution rates are listed in Table 8.

Table 8. Dilution factors and rates for batch competition experiments. Daily dilution factors and corresponding dilution rates (ln(daily dilution factor) per day), are shown for each set of replicate lines.

Line Set	Daily Dilution Factor	Dilution Rate (per day)
FB	1/100	4.605
SB	1/5	1.609
FBB	1/100	4.605
FCB	1/100	4.605
SBB	1/5	1.609
SCB	1/3	1.099

RESULTS

Adaptation to selective substrate

In this section, I report the extent of adaptation by the various lines to the substrate in which they were most recently selected. In the next section, I describe the effect of this adaptation to one substrate on the performance of each line on the alternative substrate.

Of the Stage I lines propagated on succinate in batch culture, both SB1 and SB2 significantly improved their competitive performance in that environment, whereas neither FB1 nor FB2 showed significant improvement (Table 9 and Figure 13). The greater extent of adaptation by S-derived lines (which underwent fewer than half the number of generations during Stage I as did the F-derived lines) suggests that the slower growing strain S had more room for improvement in the batch regime than did strain F (see Chapter 2).

Six of the eight Stage II F-derived lines significantly improved their competitiveness on 2,4-D, which was the substrate supplied during Stage II. All four lines in the FBB set demonstrably improved, as did two of the four FCB lines (Table 9, Figure 14). Similarly, five of the eight S-derived Stage II lines show significant fitness increases, but only one of these five belongs to the SBB set of lines (Table 9, Figure 15). All four SCB lines, which were subjected to changes in both culture regime and growth

Table 9. Results of competition experiments for evolved lines from Stage I selection in succinate and Stage II selection in 2,4-D. Selection rate constants (SRC) of evolved lines (relative to their appropriate ancestors) for both the substrate in which evolution occurred and the alternative substrate are presented. Lines with SRC values that are significantly different from the appropriate ancestor are presented in bold (*t*-test, 2-tailed $P < 0.05$).

Table 9. Results of competition experiments for evolved lines from Stage I selection in succinate and Stage II selection in 2,4-D.

Line	Selected in	SRC (per day)	
		Selective Substrate	**Alternative Substrate**
FB1	Succinate	0.0260	0.0236
FB2	Succinate	0.0910	**-4.6052***
SB1	Succinate	**0.2531**	0.0366
SB2	Succinate	**0.3759**	**0.1582**
FBB1	2,4-D	**0.4026**	-0.0929
FBB2	2,4-D	**0.9675**	0.0147
FBBr1	2,4-D	**0.3116**	**0.3932**
FBBr2	2,4-D	**0.3439**	**-0.8023**
FCB1	2,4-D	0.4171	**-2.7843**
FCB2	2,4-D	**0.6390**	**-1.1000**
FCBr1	2,4-D	**0.6788**	**1.9561**
FCBr2	2,4-D	-0.6600	-0.5012

* The asterisk marking strain FB2 for performance on 2,4-D indicates that the maximum SRC for FB2 on 2,4-D was inferred, rather than measured directly. The value is based on its competitor's (FBr) ability to grow at least 100-fold per day on 2,4-D, while FB2 is unable to grow on 2,4-D.

Table 9 (cont'd).

SBB1	2,4-D	0.0379	-0.0287
SBB2	2,4-D	0.1073	**0.1892**
SBBr1	2,4-D	**0.2230**	-0.0880
SBBr2	2,4-D	0.4092	-0.0762
SCB1	2,4-D	**0.6924**	**0.2455**
SCB2	2,4-D	**0.7670**	0.0291
SCBr1	2,4-D	**0.4899**	**0.4913**
SCBr2	2,4-D	**0.3680**	**-1.0028**

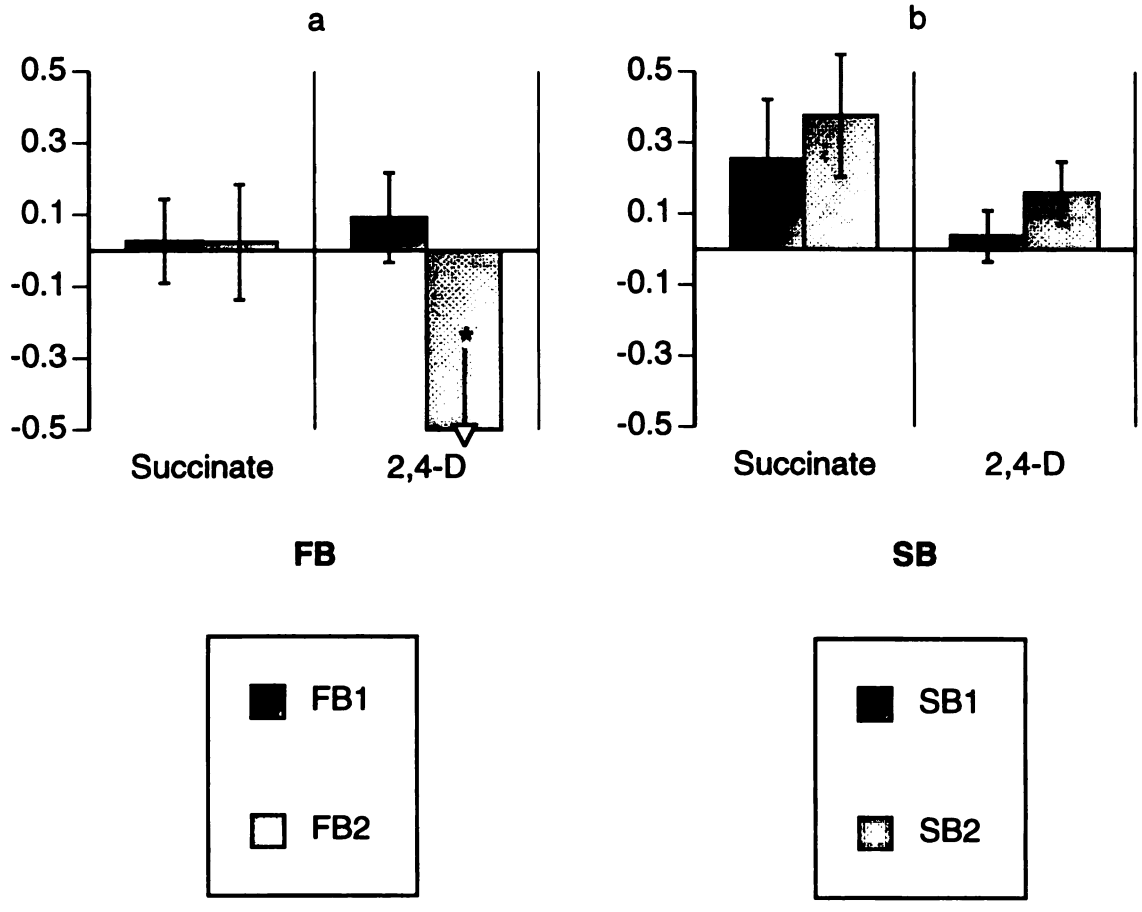

Figure 13. Performance changes of Stage I lines. (a) FB lines; (b) SB lines. Mean SRC values are shown, along with 95% confidence intervals, for both the selective substrate (succinate, left column) and the alternative substrate (2,4-D, right column). * See Table 9.

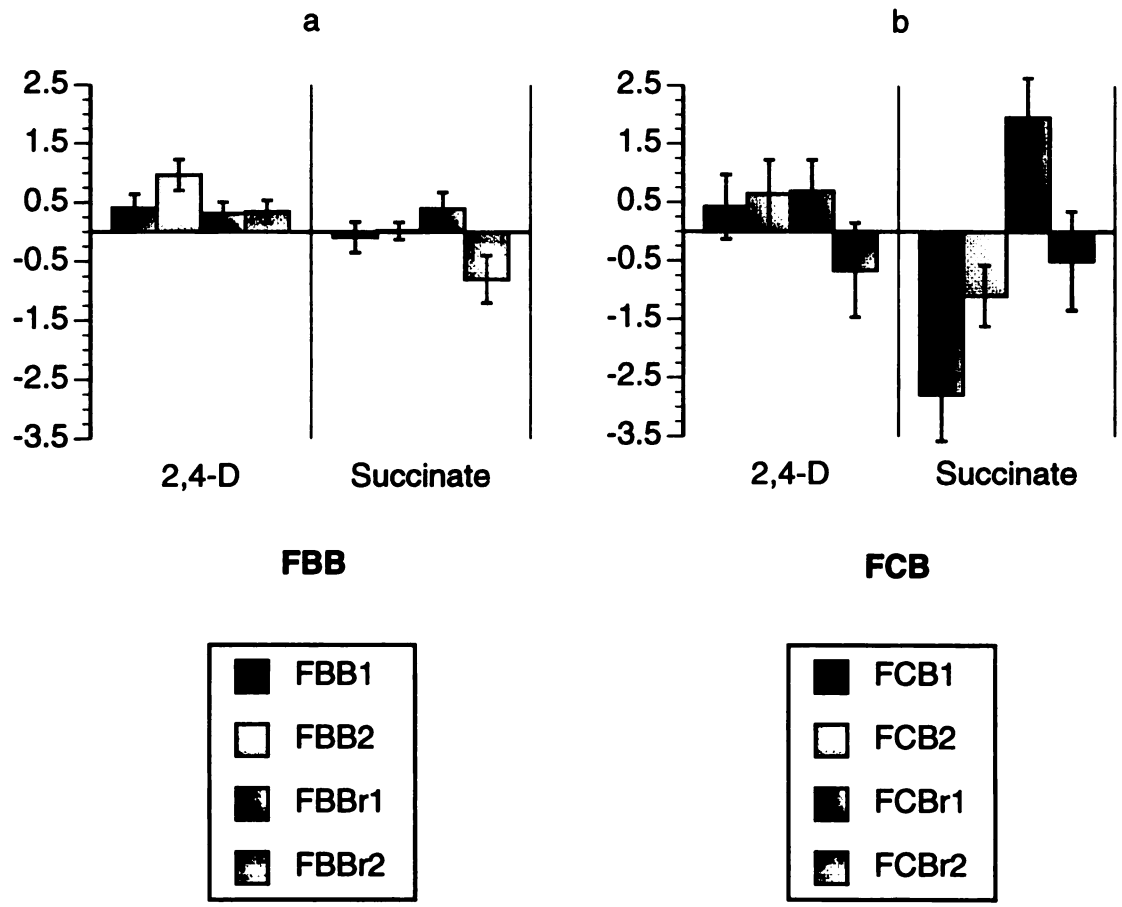

Figure 14. Performance changes of Stage II F-derived lines. (a) FBB lines; (b) FCB lines. Mean SRC values are shown, along with 95% confidence intervals, for both the selective substrate (2,4-D, left column) and the alternative substrate (succinate, right column).

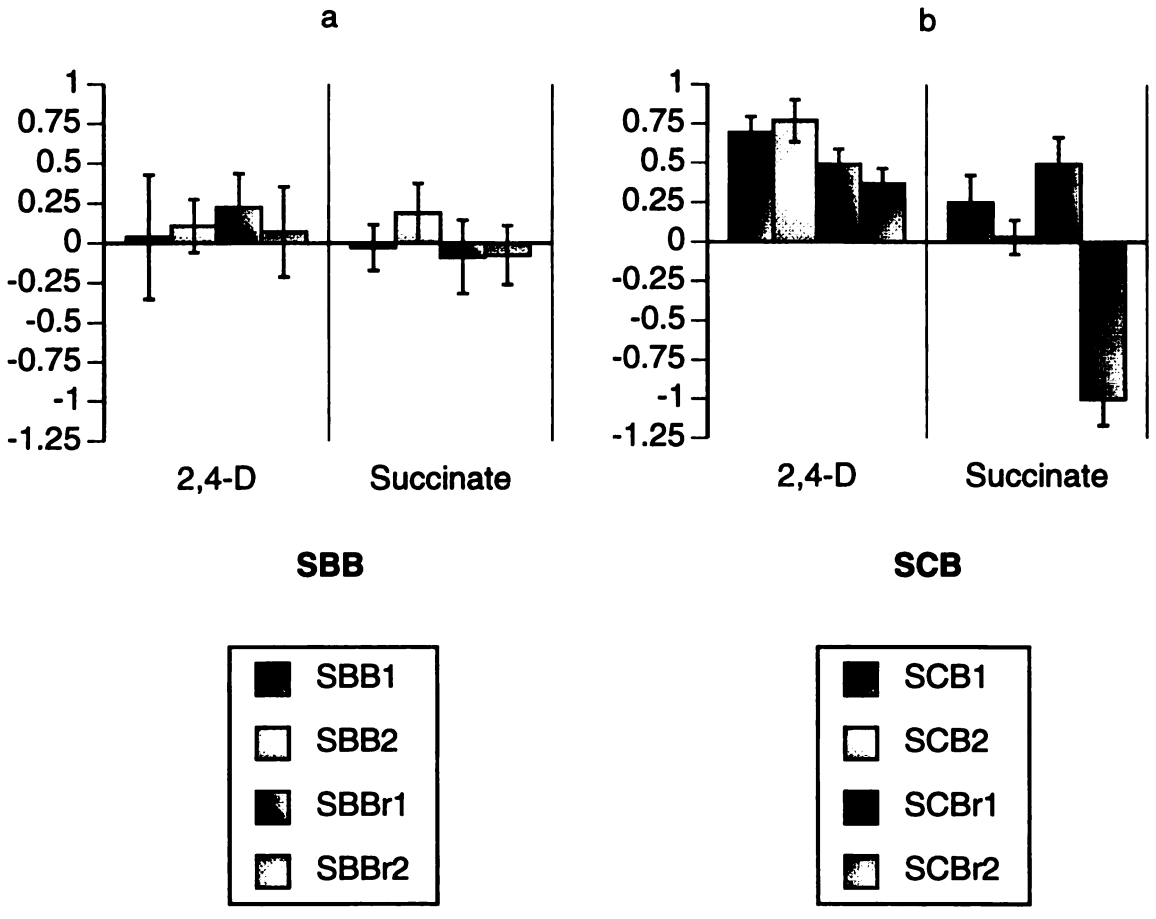

Figure 15. Performance changes of Stage II S-derived lines. (a) SBB lines; (b) SCB lines. Mean SRC values are shown, along with 95% confidence intervals, for both the selective substrate (2,4-D, left column) and the alternative substrate (succinate, right column).

substrate between Stages I and II, improved significantly during Stage II.

Performance on alternative substrate

Among the thirteen lines that showed significant improvement on their selective substrate, five of them (SB2, FBBr1, FCBr1, SCB1 and SCBr1) also improved significantly (Table 10) on the alternative substrate (2,4-D for Stage I and succinate for Stage II). Three additional lines (SB1, FBB1, and SCB2) had non-significant performance increases on the alternative substrate. In contrast, three of the thirteen significantly adapted lines (FBBr2, FCB2, and SCBr2) experienced significant losses of competitive ability on the alternative substrate, and two others (FBB1 and SBBr1) were suggestive of such losses (Table 10). Overall, ten lines either show or suggest correlated improvements in both substrates, while nine show or suggest fitness losses in the alternative substrate (Table 10). One line (FCBr2) suggests unexpected losses of performance on both the selective and alternative substrates, although neither change was statistically significant. Among the six sets of parallel lines, only the SB set exhibited a homogeneous pattern of performance for all its replicate lines (in this case only two) on both selective and alternative substrates. The other five sets showed (or suggested) both performance losses and gains among their lines on the alternative substrate.

Table 10. Frequency of four types of correlated response to selection. The number of lines that show statistically significant correlated improvements (SCI) for both 2,4-D and succinate, non-significant correlated improvements (NSCI) for both substrates, non-significant tradeoffs between substrates (NST), and significant tradeoffs between substrates (ST) are shown. These categories are shown for the thirteen lines that adapted significantly to their selective substrate and then for the 19 total lines that either showed or suggested such adaptation.

	SCI	NSCI	NST	ST
Significantly Adapted	5	3	2	3
Total	5	5	6	3

Three lines where improvements on the selective substrate were not significant nonetheless have significantly changed performance on the alternative substrate. For example, the data for SBB2 only suggest a small performance increase on 2,4-D (SRC = 0.1073), its selective substrate, yet this line shows a significant improvement in succinate (SRC = 0.1892). FB2 and FCB1 data also merely suggest adaptation to their selective substrates of succinate and 2,4-D, respectively. These two lines, however, show significant and very large performance losses on their respective alternative substrates. Indeed, line FB2 appears to have entirely lost its ability to grow on 2,4-D; pure inocula of FB2 into 2,4-D medium never become turbid. Strain FB2 was shown to be a true descendent of strain F by comparison of their REP PCR "fingerprints" (Chapter 2). This genetic test confirms that the inability of FB2 to grow on 2,4-D represents an evolutionary loss of that function (rather than the accidental introduction of a non-2,4-D degrading contaminant strain). Similarly, strain FCB1 shows the second largest fitness loss (SRC = -2.7843) on its alternative substrate (succinate) of the twenty experimental lines.

DISCUSSION

Population dynamics of adaptation

The evolutionary changes in competitive performance on non-selective substrates could be caused either by

pleiotropic effects of adaptive mutations (Wright 1977) or by the random drift of effectively neutral alleles (Kimura 1983). The latter possibility can be readily dismissed with respect to my experiments. Only a very small minority of mutations that affect fitness in a given environment are expected to be beneficial rather than harmful, because it is usually much easier to disrupt than to improve the performance of a complex entity. That is, if one considers mutations that are selectively neutral in one environment (say, 2,4-D medium), but that affect fitness in an alternative environment (say, succinate medium), then one would expect most of them to reduce, rather than improve, fitness in the alternative environment.

In this study, however, five of the eight evolved lines that showed significant performance changes in both 2,4-D and succinate media underwent improvement on both substrates. These data therefore imply that improvements in the alternative substrates were not due to substitution of random mutations by drift, but instead were caused by the same mutations responsible for improvements in the selective regimes. In other words, the selected mutations were often beneficial in both regimes, indicating positive pleiotropy. This being the case, it seems likely that the three cases of significant performance loss on the alternative substrate (associated with significant performance improvement on the selective substrate) are also due to pleiotropy, in this case negative pleiotropy.

It is also likely that each line's improvement in its selective environment was due to only one or a few adaptive mutations. This small number is because the duration of experimental evolution in this study was relatively short (maximum of 500 generations during either stage of evolution). Mathematical models indicate that many generations (often several hundred) are required for each beneficial mutation to sweep sequentially through a large, clonal population (Lenski et al. 1991). The results of previous evolution experiments with *E. coli* support the predictions of these models (Lenski and Travisano 1994; Elena et al. 1996).

Multiple adaptive pathways

My results clearly show that replicate evolving populations often achieved adaptation to their selective substrate by different mechanisms. This inference follows from the fact that in three of the four Stage II sets (each containing four independent replicate lines), at least one line shows significant correlated improvements on both selective and alternative substrates, whereas at least one other line shows a significant loss of performance on its alternative substrate after adaptation to its selective substrate (Table 9, Figures 14 and 15). Another way to describe these patterns is that the variance in competitive fitness among replicate populations on alternative substrates is much greater than the corresponding variance on selective

substrates (Travisano et al. 1995). Thus, among the strains that showed significant improvement on their selective substrate, the corresponding SRC values ranged from 0.25 to 0.97; whereas among the strains that changed significantly in their performance on the alternative substrate, the SRC values ranged from -4.61 to 1.96 (Table 9).

Even for replicate lines within a set that showed the same pattern of performance changes on both selective and alternative substrates, there is sometimes additional evidence that these changes were caused by different underlying physiological mechanisms. For example, lines SCB1 and SCBr1 both adapted significantly to their selective substrate (2,4-D), and they both showed significant correlated improvements on their alternative substrate (succinate). However, data presented in Chapter 2 of this dissertation suggest that one of these lines, SCB1, had improved performance in the chemostat culture regime during adaptation to the batch regime, whereas the other strain, SCBr1, appears to have reduced performance in chemostat culture. [The difference in chemostat performance between each strain and its proximate ancestor was not significant, but the resulting difference between the two evolved strains was highly significant (t = 8.298, 8 degrees of freedom, 2-tailed $P < 0.0001$).] Assuming that these changes in chemostat performance reflect the pleiotropic effects of mutations that were adaptative in the batch regime, then

these data would imply that even dual improvements of SCB1 and SCBr1 had occurred by different underlying mechanisms.

The replicate lines that indicate different mechanisms of adaptation to 2,4-D in batch culture were founded from base populations that were initially isogenic. This corroborates the results of Travisano et al. (1995), which showed that natural selection can cause significant divergence of populations within a species even when the populations are founded from the same progenitor and experience identical environments. Such divergence is presumably due to the fact that the replicate populations incur different sequences of random mutations during evolution, thus providing distinct patterns of genetic variation across populations upon which natural selection may act.

Potential physiological mechanisms of adaptation

This study focuses on the evolution of competitive performance of bacterial strains at the population level, and the results demonstrate a variety of correlated responses for competitive ability on alternative substrates. For these reasons, detailed analyses of the specific mechanisms of genetic adaptation for the experimental lines was not feasible within the scope of this study. Nonetheless, I will briefly review physiological functions that are involved in growth on succinate and 2,4-D, and then discuss a few alternative physiological mechanisms that might account for

the performance changes observed in evolved lines. It will become evident, however, that the performance changes alone are insufficient to distinguish between alternative scenarios.

Succinate is a key metabolite in the TCA cycle, which has presumably been fine-tuned by natural selection over many millions of years. It is conceivable that some regulatory changes to the TCA cycle might be advantageous if the organism is given a steady diet of nothing but succinate. It seems more likely, however, that transport of succinate into the cell would be the physiological step with the greatest room for improvement. By contrast, growth on 2,4-D requires several more specialized enzymes to take it through the various steps by which it is degraded into succinate and enters the central metabolic circuitry. Hence, improvements in growth on 2,4-D might be expected to involve not only transport but also the regulation and expression of any of these enzymes.

The genes responsible for 2,4-D degradation are often plasmid-borne but in some strains they are located on the chromosome (Don and Pemberton 1981, Amy et al. 1985). The most extensively studied plasmid that encodes 2,4-D degradative functions is pJP4 from *Alcaligenes eutrophus* strain JMP134 (Don et al. 1985). This plasmid carries seven genes that are responsible for the breakdown of 2,4-D into succinate, and possibly one or more genes that code for the proteins responsible for 2,4-D transport. The first three

steps in this pathway (encoded by genes *tfdA*, *tfdB*, and *tfdC*), result in cleavage of the aromatic ring. The rate-limiting step for 2,4-D catabolism is widely thought to be one of these first three steps (L. Forney, personal communication). However, circumstantial evidence suggests that permeability of the cell to 2,4-D may also sometimes limit a strain's overall growth rate on 2,4-D (T. Sassanella, personal communication).

Stage I selection on succinate. Line SB2 adapted significantly to growth on succinate, and it simultaneously showed significant improvement in its performance on 2,4-D. Plausible physiological foci of adaptation that could explain correlated improvement on these two substrates include succinate transport system, the TCA cycle, or some other function that is not directly involved in succinate metabolism. For example, an acceleration of a rate-limiting step in the TCA cycle could benefit growth on both substrates, as could an adaptation to some variable other than the type of substrate, such as pH or temperature. And while it seems likely that succinate and 2,4-D are transported into the cell by different mechanisms (given their very different biophysical characteristics), one can nonetheless imagine that a change in some general feature of the cell surface, such as loss of a capsule, could benefit growth on both substrates if transport is limiting (T. Marsh, personal communication).

Another Stage I line, FB2, completely lost its ability to grow on 2,4-D. The 2,4-D degradative pathway of strain F is chromosomally encoded (Amy et al. 1985; strain F is named 'RASC' in this reference), and so the loss of the ability to grow on 2,4-D was not simply due to plasmid segregation, as might have occurred in a strain with a plasmid-borne 2,4-D degradative pathway. Instead, the loss must be due to a mutation in a chromosomal gene that is critical for 2,4-D degradation, but it is not clear whether that mutation was somehow beneficial in succinate or whether it might be an instance of random genetic drift.

Stage II selection on 2,4-D. Among the thirteen Stage II lines that adapted to 2,4-D, some became better competitors for succinate whereas others became worse. Their improved performance on 2,4-D could be due to modifications in 2,4-D transport, 2,4-D specific catabolism, central metabolism, or some function not directly involved in nutrient acquisition, such as pH regulation. Improvements in the last two categories would seem most likely to benefit growth on both 2,4-D and succinate. However, as noted above, any change in the cell envelope that increased its overall permeability could also have this effect, even if the specific transport systems for 2,4-D and succinate were completely different. Hence, it seems safe to exclude only changes that are specific to 2,4-D catabolic functions in the case of those lines that showed correlated improvements on succinate.

The physiological bases of adaptation to 2,4-D for those strains that became worse on succinate is similarly unclear. Improvements in 2,4-D transport or catabolism could prove detrimental for growth on succinate. For example, the duplication of one or more genes specific for growth on 2,4-D may enhance growth rate on 2,4-D. That same duplication, however, may impose a significant metabolic cost that reduces the rate of growth on other substrates such as succinate. Alternatively, a mutation that repressed a membrane protein involved in succinate transport might benefit growth on 2,4-D either by allowing a higher level of expression of an alternative 2,4-D transport protein or by reducing the cost associated with an unused function.

There are undoubtedly many more scenarios that could be put forward to explain any given pattern of evolutionary change with respect to the selective and alternative substrates. I have presented these scenarios to illustrate the difficulties of invoking overly specific relationships between selective factors in the environment and an organism's evolutionary response to those factors. At the same time, the diversity of plausible mechanisms suggests that the physiological heterogeneity among replicate populations may be far greater than even their divergent ecological performances would indicate.

Possible relevance to the adaptation of genetically modified organisms in natural habitats

In the discussion of relative benefits and risks of releasing genetically modified organisms into nature, it has often been argued that genetically altered microorganisms will not persist because they are inherently less fit than indigenous competitors (Brill 1985; Davis 1987; Davies 1988). Lenski (1993) reviewed various arguments for why genetically modified organisms might theoretically be less fit than their natural counterparts, and he summarized some experiments with bacteria that bear on those arguments.

Most of the studies reviewed by Lenski (1993) support the view that modified microorganisms are competitively inferior to the strains from which they are derived. Mutations that alter basic metabolic functions (Lenski 1988), the expression of additional functions (Andrews and Hegeman 1976; Dykhuizen 1978; Koch 1983), the carriage of accessory genetic elements (Lenski and Bouma 1987), and the "domestication" of bacteria as they adapt to laboratory conditions (Davis 1987; Regal 1988) all tend to decrease the fitness of modified organisms relative to their progenitors, although important exceptions to this generality exist (Biel and Hartl, 1983; Edlin et al. 1984; Hartl et al. 1983; Blot et al. 1994). Beyond the immediate fitness effects of genetic manipulations that occur prior to release, there is the further possibility that genetically modified organisms may adapt evolutionarily to the local ecosystem in which they

are released. Such "post-release" adaptation seems more likely than fortuitous preadaptation prior to release, and it could lead to the indefinite persistence of modified organisms in the environment (along with any adverse effects they might cause).

Several studies, including three with bacteria in the laboratory (Bouma and Lenski 1988; Lenski 1988; Modi and Adams 1991) and one with insects in the field (McKenzie et al. 1982), indicate that organisms may evolve so as to mitigate the fitness costs associated with other genetic changes. In one case, a population of *E. coli* carried a plasmid, pACYC184, that encoded resistance to two antibiotics, but which reduced the bacteria's fitness in the absence of antibiotic (Bouma and Lenski 1988). These plasmid-bearing cells were then propagated in medium that contained antibiotic, in order to prevent spontaneous plasmid-free segregants from out-competing the plasmid-bearing cells and taking over the population. After only 500 generations, the plasmid-bearing cells were, suprisingly, more fit than both their plasmid-free progenitor and their isogenic plasmid-free derivatives even in the absence of antibiotic (R. Lenski, personal communication; Bouma and Lenski 1988). In other words, evolution of a genetically modified organism in one environment (with antibiotic) led to a correlated improvement in fitness in an alternative environment (without antibiotic).

The above results point towards a second concern over the evolution of microorganisms that have been genetically modified for bioremediation in the environment. What is the effect of adaptation to an anthropogenic substrate on their competitive fitness for naturally occurring substrates? If such adaptation may have pleiotropic benefits for growth on natural substrates, then this increases the likelihood that these genetically modified organisms will persist in the environment. The results of this study clearly show that evolutionary adaptation of bacteria to growth on a common toxic herbicide (2,4-D) sometimes improved their competitive fitness on a natural substrate (succinate). The strains employed in this investigation were already capable of degrading 2,4-D, without genetic modification in the laboratory. However, there is no obvious reason that qualitatively similar results might be obtained for strains that are genetically modified to degrade chlorinated aromatic compounds (or perform some other bioremediation function) more efficiently than natural strains (Ramos et al. 1987; Chaudhry and Chapalamadugu 1991). In fact, such modified organisms may have even more opportunities for general improvements in their overall vigor, owing to the fitness costs associated with their modification (see above). Therefore, when considering the fate of genetically modified organisms released into the environment for bioremediation of anthropogenic substrates, it seems unrealistic to assume that these organisms will simply do their job and then fade away.

Reference List

REFERENCE LIST

Amy, P. S., J. W. Schulke, L. M. Frazier, and R. J. Seidler. 1985. Characterization of aquatic bacteria and cloning of genes specifiying partial degradation of 2,4-dichlorophenoxyacetic acid. *Applied and Environmental Microbiology* **49**:1237-1245.

Andrews, J. H., and R. F. Harris. 1986. r- and K- selection in microbial ecology. *Advances in Microbial Ecology* **9**:99-147.

Andrews, J. H., and D. I. Rouse. 1982. Plant pathogens and the theory of *r*- and *K*- selection. *American Naturalist* **120**:283-296.

Andrews, K. J., and G. D. Hegeman. 1976. Selective disadvantage of nonfunctional protein synthesis in *Escherichia coli*. *Journal of Molecular Evolution* **8**:317-328.

Bell, G., and V. Koufopanou. 1986. The cost of reproduction. *Oxford Surveys in Evolutionary Biology* **3**:83-131.

Bennett, A. F., and R. E. Lenski. 1993. Evolutionary adaptation to temperature. II. Thermal niches of experimental lines of *Escherichia coli*. *Evolution* **47**:1-12.

Biel, S. W., and D. L. Hartl. 1983. Evolution of transposons: natural selection for Tn*5* in *Escherichia coli* K12. *Genetics* **103**:581-592.

Bouma, J. E., and R. E. Lenski. 1988. Evolution of a bacteria/plasmid association. *Nature* **335**:351-352.

Brill, W. J. Safety concerns and genetic engineering in agriculture. *Science* **227**:381-384.

Brueggeman, J. 1993. Element G. Chemical methods only. Human health effects of 2,4-D. Ebasco Environmental, Bellevue, Washington.

Calabrese, V. G. M. 1994. Ph.D. dissertation. Michigan State University, East Lansing, MI.

Calabrese, V. G. M., N. L. Tonso, A. J. Sexstone, and W. E. Holben. 1992. Characterization and classification of diverse 2,4-dichlorophenoxyacetic acid (2,4-D) degrading populations using whole plasmid probes in colony hybridization analyses. *Abstracts of the 92nd General Meeting of the American Society*

for Microbiology. American Society for Microbiology, Washington, D. C.

Chao, L., and E. C. Cox. 1983. Competition between high and low mutating strains of *Escherichia coli*. *Evolution* **37**:125-134.

Chao, L., and B. R. Levin. 1981. Structured habitats and the evolution of anticompetitor toxins in bacteria. *Proceedings of the National Academy of Sciences USA* **78**:6324-6328.

Chao, L., and G. Ramsdell. 1985. The effects of wall populations on coexistence of bacteria in the liquid phase of chemostat cultures. *Journal of General Microbiology* **131**:1229-1236.

Chaudhry, G. R. and S. Chapalamadugu. 1991. Biodegradation of halogenated organic compounds. *Microbiological Reviews* **55**:59-79.

Chaudhry, G. R., and G. H. Huang. 1988. Isolation and characterization of a new plasmid from a *Flavobacterium* sp. which carries the genes for degradation of 2,4-dichlorophenoxyacetic acid. *Journal of Bacteriology* **170**:3897-3902.

Cheverud, J. M., M. M. Dow, and W. Leutenegger. 1985. The quantitative assessment of phylogenetic constraints in comparative analyses: sexual dimorphism in body weight among primates. *Evolution* **39**:1335-1351.

Clutton-Brock, T. H. and P. H. Harvey. 1977. Primate ecology and social organization. *Journal of Zoology* **183**: 1-33.

Cody, M. L. 1966. A general theory of clutch size. *Evolution* **20**:174-184.

Cohan, F. M. 1984. Can uniform selection retard random genetic divergence between isolated conspecific populations? *Evolution* **38**:495-504.

Cohan, F. M., and J.-D.Graf. 1985. Latitudinal cline in *Drosophila melanogaster* for knockdown resistance to ethanol fumes and for rates of response to selection for further resistance. *Evolution* **39**:278-293.

Davies, J. 1988. Genetic engineering: processes and products. *Trends in Ecology and Evolution* **3**:S7-S11.

Davis, B. D. Bacterial domestication: underlying assumptions. *Science* **235**:1329-1335.

Don, R. H., and J. M. Pemberton. 1981. Properties of six pesticide degradation plasmids isolated from *Alcaligenes paradoxus* and *Alcaligenes eutrophus*. *Journal of Bacteriology* **145**:681-686.

Don, R. H., A. J. Weightman, H. J. Knackmuss, and K. N. Timmis. 1985. Transposon mutagenesis and cloning analysis of the pathyways for degradation of 2,4-dichlorophenoxyacetic acid and 3-chlorobenzoate in *Alcaligenes eutrophus* JMP134 (pJP4). *Journal of Bacteriology* **161**:85-90.

Dykhuizen, D. E., and A. M. Dean. 1990. Enzyme activity and fitness: evolution in solution. *Trends in Ecology and Evolution* **5**:257-262.

Dykhuizen, D. E., A. M. Dean, and D. L. Hartl. 1987. Metabolic flux and fitness. *Genetics* **115**:25-31.

Dykhuizen, D. E., and L. Green. 1991. Recombination in *Escherichia coli* and the definition of biological species. *Journal of Bacteriology* **173**:7257-7268.

Ebasco and Herra. 1993. Element E. Chemical methods only. Environmental effects of glyphosates and 2,4-D. Ebasco Environmental, Bellevue, Washington and Herra Environmental Consultants, Inc., Seattle, Washington.

Edlin, G., R. C. Tait, and R. L. Rodriguez. 1984. A bacteriophage Lambda cohesive ends (*cos*) DNA fragment enhances the fitness of plasmid-containing bacteria growing in energy-limited chemostats. *Bio/Technology* **2**:251-254.

Elena, S. F., V. S. Cooper, and R. E. Lenski. 1996. Punctuated evolution caused by selection of rare beneficial mutations. *Science* **272**:1802-1804.

Felsenstein, J. 1985. Phylogenies and the comparative method. *American Naturalist* **125**:1-15.

Geller, A. 1983. Growth of bacteria in inorganic medium at different levels of airborne organic substances. *Applied and Environmental Microbiology* **46**:1258-1262.

Gerson, U. and I. Chet. 1981. Are allochthonous and autochthonous soil microorganisms r- and K- selected? *Rev. Ecol. Biol. Sol.* **18**:285-289.

Gould, S. J., and R. C. Lewontin. 1979. The spandrels of San Marco and the panglossian paradigm: a critique of the adaptationist program. *Proceedings of the Royal Society of London B* **205**:581-598.

Grafen, A. 1989. The phylogenetic regression. *Philosophical Transactions of the Royal Society* **326**:119-156.

Greer, L. E., J. A. Robinson, and D. R. Shelton. 1992. Kinetic comparison of seven strains of 2,4-dichlorophenoxy-acetic acid-degrading bacteria. *Applied and Environmental Microbiology* **38**:1027-1030.

Hansen, S. R., and S. P. Hubbell. 1980. Single-nutrient microbial competition: qualitative agreement between experimental and theoretically forecast outcomes. *Science* **207**:1491-1493.

Hartl, D. L., D. E. Dykhuizen, R. D. Miller, L. Green, and J. deFramond. 1983. Transposable element IS*50* improves growth rate of *E. coli* cells without transposition. *Cell* **35**:503-510.

Harvey, P. H. and M. D. Pagel. 1989. Comparative studies in evolutionary ecology: using the database. In *Toward a more exact ecology* (ed. P. J. Grubb and J. Whittaker), pp. 209-30, Blackwell, Oxford.

Harvey, P. H. and M. D. Pagel. 1991. *The Comparative Method in Evolutionary Biology.* Oxford University Press, Oxford.

Healey, F. P. 1980. Slope of the Monod equation as an indicator of advantage in nutrient competition. *Microbial Ecology* **5**:281-286.

Hoffman, A. A., and F. M. Cohan. 1987. Genetic divergence under uniform selection. III. Selection for knockdown resistance to ethanol in *Drosophila pseudoobscura* populations and their replicate lines. *Heredity* **58**:425-433.

Jannasch, H. W. 1969. Estimations of bacterial growth rates in natural waters. *Journal of Bacteriology* **99**:156-160.

Kim, J., L. R. Ginzburg, and D. E. Dykhuizen. 1991. Quantifying the risks of invasion by genetically engineered organisms. In L. R. Ginzburg (ed.). *Assessing Ecological Risks of Biotechnology.* Butterworth-Heinemann, Boston. pp. 193-214.

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

Koch, A. L. 1983. The protein burden of *lac* operon products. *Journal of Molecular Evolution* **19**:455-462.

Konings, W. N. and H. Veldkamp. 1980. Phenotypic responses to environmental change. In D. C. Ellwood, J. N. Hedger, M. J. Lathan, J. M. Lynch, and J. H. Slater (eds.). *Contemporary Microbial Ecology.* Academic Press, New York. pp. 161-191.

Korona, R., C. H. Nakatsu, L. J. Forney, and R. E. Lenski. 1994. Evidence for multiple adaptive peaks from populations of bacteria evolving in a structured habitat. *Proceedings of the National Academy of Sciences USA* **91**:9037-9041.

Kuenen, J. G., and Harder, W. 1982. Microbial competition in continuous culture. In R. G. Burns and J. H. Slater (eds.). *Experimental Microbial Ecology*. Blackwell Scientific Publications, Oxford, England. pp. 342-367.

Lenski, R. E. 1988. Experimental studies of pleiotropy and epistasis in *Escherichia coli*. I. Variation in competitive fitness among mutants resistant to virus T4. *Evolution* **42**:425-432.

Lenski, R. E. 1993. Evaluating the fate of genetically modified microorganisms in the environment: Are they inherently less fit? *Experientia* **49**:201-209.

Lenski, R. E., and B. R. Levin. 1985. Constraints on the coevolution of bacteria and virulent phage: a model, some experiments, and predictions for natural communities. *American Naturalist* **125**:585-602.

Lenski, R. E., M. R. Rose, S. C. Simpson and S. C. Tadler. 1991. Long-term experimental evolution in *Escherichia coli*. I. Adaptation and divergence during 2,000 generations. *American Naturalist* **138**:1315-1341.

Lenski, R. E., and M. Travisano. 1994. Dynamics of adaptation and diversification: a 10,000 generation experiment with bacterial populations. *Proceedings of the National Academy of Sciences USA* **91**:6808-6814.

Lenski, R. E., G. J. Velicer and R. Korona. 1997. Experimental studies on the evolution of bacteria that degrade 2,4-dichlorophenoxyacetic acid. In Horikoshi, K., M. Fukuda, and T. Kudo (eds.). *Microbial Diversity and Genetics of Biodegradation*. Japan Scientific Societies Press, Tokyo. In press.

Leroi, A. M., A. K. Chippindale, and M. R. Rose. 1994. Long-term laboratory evolution of a genetic life-history trade-off in *Drosophila melanogaster*. 1. The role of genotype-by-environment interaction. *Evolution* **48**:1244-1257.

Levin, B. R. 1988. Frequency-dependent selection in bacterial populations. *Philosophical Transactions of the Royal Society London B* **319**:459-472.

Levin, B. R., F. M. Stewart, and L. Chao. 1977. Resource-limited growth, competition, and predation: a model and experimental studies with bacteria and bacteriophage. *American Naturalist* **111**:3-24.

Luckinbill, L. S. 1978. r- and K- selection in experimental populations of *Escherichia coli*. *Science* **202**:1201-1203.

Luckinbill, L. S. 1979. Selection and the r/K continuum in experimental populations of protozoa. *American Naturalist* **113**:427-437.

Luckinbill, L. S. 1984. An experimental analysis of a life history theory. *Ecology* **65**:1170-1184.

Lynch, M. 1991. Methods for the analysis of comparative data in evolutionary biology. *Evolution* **45**:1065-1080.

MacArthur, R. H., and E. O. Wilson. 1967. *The Theory of Island Biogeography*. Princeton University Press, Princeton, N.J.

Martins, E. P. and T. H. Garland. 1991. Phylogenetic analyses of the correlated evolution of continuous characters: A simulation study. *Evolution* **45:**534-557.

Matin, A., and H. Veldkamp. 1978. Physiological basis of the selective advantage of a *Spirillum* species in a carbon-limited environment. *Journal of General Microbiology* **105**:187-197.

McGowan, C. 1995. Ph.D. dissertation. Michigan State University, East Lansing, MI.

McKenzie, J. A., M. J. Whitten, and M. A. Adena. 1982. The effect of genetic background on the fitness of diazinon resistance genotypes of the Australian sheep blowfly, *Lucilia cuprina*. *Heredity* **49**:1-9.

Modi, R. I., and Adams, J. 1991. Coevolution in bacteria-plasmid populations. *Evolution* **45**:656-667.

Mongold, J. A., A. F. Bennett, and R. E. Lenski. 1996. Evolutionary adaptation to temperature. IV. Adaptation of *Escherichia coli* at a niche boundary. *Evolution* **50**:35-43.

Mueller, L. D. and F. J. Ayala. 1981. Trade-off between r-selection and K-selection in *Drosophila* populations. *Proceedings of the National Academy of Sciences USA*, **78**:1303-1305.

National Research Council. 1993. In situ *bioremediation: When does it work?* National Academy Press, Washington, D. C.

Pianka, E. R. 1970. On r- and K-selection. *American Naturalist* **104**:592-596.

Ramos, J. L., A. Wasserfallen, K. Rose, and K. N. Timmis. 1987. Redesigning metabolic routes: manipulation of TOL plasmid pathway for catabolism of alkylbenzoates. *Science* **235**:593-596.

Regal, P. J. 1988. The adaptive potential of genetically engineered organisms. *Trends in Ecology and Evolution* **3**:S36-S38.

Regal, P. J. 1993. The true meaning of 'exotic species' as a model for genetically engineered organisms. *Experientia* **49**:225-234.

Reznick, D. 1985. Costs of reproduction: an evaluation of the empirical evidence. *Oikos* **44**:257-267.

Roughgarden, J. 1971. Density-dependent natural selection. *Ecology* **52**:453-468.

Service, P. M. and M. R. Rose. 1985. Genetic covariation among life history components: the effect of novel environments. *Evolution* **36**:1276-1282.

Stearns, S. 1983. The influence of size and phylogeny of patterns of covariation among life-history traits in mammals. *Oikos* **41:**173-187.

Swofford, D. L. 1991. *PAUP: Phylogenetic Analysis Using Parsimony, Version 3.0s.* Computer program distributed by the Illinois Natural History Survey, Champaign, Illinois.

Tiedje, J. M., R. K. Colwell, Y. L. Grossman, R. Hodson, R. E. Lenski, R. N. Mack and P. J. Regal. 1989. The planned introduction of genetically engineered organisms: ecological considerations and recommendations. *Ecology* **70**:298-315.

Tonso, N. L., V. G. Matheson, and W. E. Holben. 1995. Polyphasic characterization of a suite of bacterial isolates capable of degrading 2,4-D. *Microbial Ecology* **30**:3-24.

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 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., F. Vasi, and R. E. Lenski. 1995. Long-term experimental evolution in *Escherichia coli*. III. Variation among replicate populations in correlated responses to novel environments. *Evolution* **49**:189-200.

Vasi, F., M. Travisano, and R. E. Lenski. 1994. Long-term experimental evolution in *Escherichia coli*. II. Changes in life-history traits during adaptation to a seasonal environment. *American Naturalist* **144**:432-456.

Veldkamp, H., H. van Gemerden, W. Harder, and H. J. Laanbroek. 1984. Competition among bacteria: An overview. In M. J. Klug and C. A. Reddy (eds.). *Current Perspectives in Microbial Ecology*. American Society for Microbiology, Washington, D. C. pp. 279-290.

Versalovic, J., T. Koeuth, and J. R. Lupski. 1991. Distribution of repetitive DNA sequences in eubacteria and application to fingerprinting of bacterial genomes. *Nucleic Acids Research* **19**:6823-6831.

Vogel, T. M. and P. L. McCarty. 1985. Biotransformation of tetrachloroethylene to trichloroethylene, dichloroethylene, vinyl chloride, and carbon dioxide under methanogenic conditions. *Applied and Environmental Microbiology* **49**:1080-1083.

Warner, R. R. 1984. Deferred reproduction as a response to sexual selection in a coral reef fish: a test of the life historical consequences. *Evolution* **38**:148-162.

Weisburg, W. G., S. M. Barns, D. A. Pelletier, and D. J. Lane. 1991. 16S ribosomal DNA amplification for phylogenetic study. *Journal of Bacteriology* **173**:697-703.

Wright, S. 1970. "Surfaces" of selective value. *Proceedings of the National Academy of Sciences USA* **58**:165-172.

Zhou, J., and J. M. Tiedje. Manuscript. Kinetic specialization of benzene-degrading bacteria for parts-per-billion concentrations.