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Richard E. Lenski
Major professor

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CONSEQUENCES OF ECOLOGICAL SPECIALIZATION IN LONG-TERM EVOLVING POPULATIONS OF *ESCHERICHIA COLI*

By

Vaughn Scott Cooper

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ABSTRACT

CONSEQUENCES OF EVOLUTIONARY SPECIALIZATION IN LONG-TERM EVOLVING POPULATIONS OF *ESCHERICHIA COLI*

By

VAUGHN SCOTT COOPER

This dissertation asks two major questions: 1) Does adaptation to a specific environment necessarily cause losses in performance or functionality in other environments? and 2) What are the population genetic mechanisms responsible for any such losses? These questions are addressed using 12 populations of *Escherichia coli* that evolved in a simple, glucose-limited laboratory environment for 20,000 generations. During this time, all of the populations became progressively better adapted to growth in this environment. In Chapter I, I examine the consequences of this evolution for the bacteria's capacity to utilize 93 substrates, and thereby quantify the extent to which specialization led to losses of function. On average, diet breadth of the evolved populations declined significantly relative to the ancestors, but complete loss of function on any given substrate was rare. I also measured the relative fitness of the evolved populations in four foreign environments, in which improved fitness was frequently found. In Chapter II, I quantify diet breadth after multiple evolutionary time points to determine whether antagonistic pleiotropy (AP) or mutation accumulation (MA) explains a greater proportion of the observed functional decay. The evolution of increased mutation rates in three populations allowed a critical test of the MA model.

Mutator lines are expected to lose functions much more rapidly than the wildtype lines under MA, but not under AP, as the rate of adaptive substitution was similar across lines. These classes of lines did not significantly differ in diet breadth, contrary to the predictions of MA. Most losses of function occurred early in the experiment when beneficial mutations were being substituted most rapidly, a pattern that is consistent with AP. Thus, AP appears more important than MA for the tendency toward decreased diet breadth in these evolving populations. In Chapter III, I examine the effects of adaptation to a constant 37° environment on thermal niche. The mean maximum growth rate of all evolved populations increased at moderate temperatures, but decreased at extreme high and low temperatures. Most of these changes in thermal niche occurred over the first 2,000 generations, when selection was most intense. Together these results suggest that AP was responsible for improved performance at moderate temperatures but reduced growth at extreme temperatures. Chapter IV is a case study of functional decay, and focuses on the loss of D-ribose catabolic function that occurred in all populations by generation 2,000. The mutation rate for this loss of function was found to be extremely high and caused by deletions associated with an IS*150* element inserted upstream of the operon. Mutants bearing only this mutation also conferred a small benefit in the selective environment. Given these findings, population genetic models suggest that the loss of ribose catabolic function was caused by (i) an unusually high mutation rate, such that these mutants appeared repeatedly in all populations (ii) a selective advantage in the experimental medium that drove these mutants to fixation, and (iii) linkage of these mutations to other beneficial mutations of larger effect.

For my parents, James and Vicki Cooper

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INTRODUCTION

As reflected in the epigraph, evolutionists generally assume that genetic adaptation to any particular environment is associated with the loss of fitness in dissimilar environments. Indeed, nearly all mathematical models of niche breadth – diet, physiological tolerance, life history features, and so on – assume the existence of tradeoffs which fulfill this assumption (e.g. MacArthur and Levins 1964, Levins 1968, Charlesworth 1980, Lynch and Gabriel 1987). Yet despite the central importance of this assumption for theories in evolutionary ecology, it remains largely untested (Futuyma and Moreno 1988). Part of the problem lies in defining specialization in a manner that would allow this assumption to be rigorously tested. Some authors define specialization simply as adaptation to any given subset of an organism's niche (Smiley 1978, Fox and Morrow 1981). For others, some reduction in realized niche (sensu Hutchinson 1958) associated with adaptation must occur, whether it be fewer host plants visited, resources utilized, environments tolerated, etc. (Rausher 1983, Lynch and Gabriel 1987, Jaenike 1990, Fong et al. 1995). Finally, some authors point out the importance of the population genetic processes responsible for functional decay when considering specialization (Futuyma and Moreno 1988). Hence, Futuyma and Moreno (1988, p. 208) suggest that "often specialization must lie in the eye of the beholder."

Throughout this dissertation, I define specialization to be a decline in fundamental niche breadth associated with adaptation to any particular environment. However, a rigorous definition only solves part of the problem. Further challenges are posed by defining alternative environments and accurately measuring relative fitness. If these

problems are surmounted, the effects of adaptation upon general functionality must then be quantified. Only if adaptation is correlated with reductions in the niche of the organism is the use of the term "specialization," as opposed to "specific adaptation," appropriate.

Yet another aspect of the problem of specialization is the widespread failure to distinguish between two distinct population-genetic mechanisms that can produce an association between adaptation in one environment and functional decay in other environments. These two processes are antagonistic pleiotropy and mutation accumulation. The former requires that the same beneficial alleles (mutations) that enhance adaptation cause decay outside the domain of specialization. By contrast, the latter presumes that adaptive decay occurs by the drift-fixation of distinct alleles (mutations) that are effectively neutral in the selective environment but which negatively affect functions necessary for optimal performance elsewhere.

REVIEW OF LITERATURE

Some well-known empirical investigations of specialization include the natural history of cave organisms, numerous experiments using phytophagous insects, and laboratory selection experiments using microbes and parasites. These disparate studies of specialization have three basic components: 1) quantifying adaptation to a specific environment, 2) quantifying functional decay associated with the adaptation, and 3) determining the population genetic mechanism responsible for the functional decay. Most studies have addressed one or two of these components, but few have considered

all three. I now provide a few illustrative examples of this broad field of study.

Several artificial selection experiments in the laboratory have examined the origin of specialization by selecting for one trait of an organism while quantifying other traits. In a series of classic experiments, Spiegelman and coworkers selected for high replication rate in Qβ bacteriophage while providing the Qβ replicase in the experimental medium. Because replication rate was placed at a premium, the phages became little more than replicators and lost not only their infectivity but also 83 percent of their genome (Mills et al. 1967, Saffhill et al. 1970). This drastic genome reduction is an obvious instance of specialization, and the loss of genes was caused by direct selection that had antagonistic effects on other functions. However, selection that results in such dramatic streamlining may be quite unusual in nature.

Another series of experiments by Bennett, Lenski and co-workers demonstrated temperature-specific adaptation in evolving laboratory populations of *Escherichia coli* (Bennett et al. 1992, Bennett and Lenski 1993, Leroi et al. 1994, Mongold et al. 1996). One notable conclusion from these studies is that tradeoffs associated with thermal adaptation were asymmetrical: populations adapted to low temperature often suffered fitness losses at high temperature, but the reverse was not usually the case (Bennett and Lenski 1993). Further, correlated improvements in fitness at foreign (nonselected) temperatures were also observed whereas losses of fitness were infrequent. These experiments raise doubt about whether organisms necessarily lose performance at nonselected temperatures when selected for optimal performance at a different temperature (Bennett and Lenski 1993, Lenski and Bennett 1993, Mongold et al. 1996). Such findings are echoed in a broader literature review by Huey and Hertz (1984), who

found that individuals within different species that were successful at one temperature tended to do well at all temperatures tested.

An alternative form of selection experiment serendipitously occurs in nature when parasitic species are observed to have recently switched hosts. This host shift should in theory bring about host-specific adaptation that, sooner or later, may be costly to performance on the previous host. Bush and coworkers (Bierbaum and Bush 1990, Prokopy and Bush 1993) have documented several shifts between host plants for *Rhagoletis* fruit flies, which are typically monophagous. Recently Filchak et al. (1999) tested whether host-plant dependent fitness tradeoffs had arisen in races of *Rhagoletis* found on either apples or hawthorn. They found it necessary to integrate selection pressures over the entire life cycle of the fly to find plant-specific selection and fitness tradeoffs. Had they not studied selection at different stages of the fly, they would have failed to document these tradeoffs, which emphasizes the importance of an accurate measure for fitness. The coordination between adaptation to one host and loss of adaptation to another host over a relatively short period of time suggests that antagonistic pleiotropy was responsible, but definite evidence has yet to be collected.

The study of specialization is not as novel an enterprise as the previous examples suggest. Natural historians including Darwin (1872) have pondered the character reductions so obvious among cave organisms (see Fong et al. 1995 and Culver et al. 1995 for reviews). These organisms are known to be locally adapted because their fitness tends to suffer when removed from the cave environment, and this decline in performance is usually a direct result of the lost traits (Culver et al. 1995). The first two requirements to characterize specialization, specific adaptation and associated decay,

have therefore been met. Yet the question of which population genetic mechanism led to this trait reduction remains largely unanswered. Take, for example, the loss of vision. The loss of vision may be explained by two different kinds of antagonistic pleiotropy: (i) direct selection against having eyes, because they may be costly in the cave environment due to increased risk of injury or infection, or (ii) selection for enhancements in other systems (Jones and Culver 1989) which may lead to reduced allocation to eye development. The loss of vision may also be explained by mutation accumulation, in which genes responsible for eye development and maintenance accumulated deleterious mutations once the selection for the preservation of vision was eliminated. However, in the absence of a population of organisms currently adapting to a cave environment, we lack evidence for the relationship between the adaptation and the loss of function (Fong et al. 1995). As a result, conclusively testing which mechanism led to specialization remains a daunting task.

A better understanding of specialization requires that specific adaptation and any associated changes in niche breadth be accurately quantified and followed over time. When a loss of function is found, additional studies are necessary to determine whether a genetic tradeoff or drift is the cause. For example, one might follow a series of populations as they adapt to a particular novel environment and determine if specialists emerge. Several conditions are thought to favor specialization, including environmental constancy (Lynch and Gabriel 1987), correlations between habitat choice and function (Cavener 1979, Holt 1987, Prokopy and Bush 1993), predation or parasitism (Ehrlich and Raven 1964, Gould 1979, Futuyma 1983) and more specifically, reduced variance in available resources (Levins and MacArthur 1969, Jaenike 1978, James et al. 1988).

For this dissertation I chose to examine the evolution of both functional diet breadth and thermal niche. The bacterium *Escherichia coli* is well suited for such a study. Its rapid replication rate allows many generations to be followed, its genetics and metabolism are well characterized, and robust techniques for measuring relative fitness exist. Further, a wide variety of characters can be readily sampled to identify any losses of function that do occur and to determine which population genetic process was responsible.

THE EXPERIMENTAL SYSTEM: PREVIOUS FINDINGS AND CURRENT OBJECTIVES

The primary goal of this dissertation is to quantify changes in function associated with adaptation to a particular environment. I chose to use a series of long-term evolving populations of *E. coli* B because their adaptation to the laboratory environment and certain correlated responses to this adaptation have been well studied (Lenski et al. 1991, Lenski and Travisano 1994, Vasi et al. 1994, Travisano et al. 1995, Travisano and Lenski 1996, Elena et al. 1996, Elena and Lenski 1997, Travisano 1997, Sniegowski et al. 1997). Several observations relevant to this study have been made to date. First, each of these lines has undergone significant adaptation to growth in a serially diluted, or seasonal, environment of glucose-limited minimal media (Lenski et al. 1991, Lenski and Travisano 1994, Vasi et al. 1994, Travisano et al. 1995). The dynamics of adaptation are well known: after a period of rapid adaptation during the first 1,000 generations, the rate of improvement has subsequently slowed to almost one-thirtieth of the initial rate (Lenski

and Travisano 1994). Moreover, relatively few mutations generated this early, rapid adaptation (Lenski et al. 1991, Lenski and Travisano 1994, Elena et al. 1996). Thus, these populations have experienced two types of dynamics: one of rapid adaptation followed by one of much slower improvement.

Second, Travisano and Lenski (1996; see also Travisano et al. 1995) demonstrated that adaptations specific to growth on glucose were partly responsible for the observed fitness increases. They found correlated improvements in fitness on substrates that share membrane transport pathways with glucose, but not for substrates that did not. Third, adaptation to glucose led to reductions in performance on some of the substrates that enter the cell by a different mechanism (Travisano and Lenski 1996). In summary, two requirements for studying specialization have been met in this system: adaptation to a particular environment and some associated functional decay. Using a much broader set of assays for functionality over a longer period of quantified adaptation, this study further attempts to describe the association between loss of function and adaptation.

The second objective was to identify which population genetic mechanism was primarily responsible for any observed declines in performance or functionality. The fact that three populations evolved genomic mutation rates approximately two orders of magnitude higher than the ancestor and the other derived lines (Sniegowski et al. 1997) allows one to address the relative importance of mutation accumulation. These "mutator" populations should accumulate 100-fold more mutations at loci no longer under selection. If mutation accumulation is important in causing specialization, mutator populations should have lost a greater number of functions than populations that retained the ancestral

mutation rate. Antagonistic pleiotropy should leave its own distinct fingerprint. Losses of function common to most or all of the 12 independent lineages indicate a strong association with that loss and adaptation to the new environment, supporting antagonistic pleiotropy. This laboratory system is unique for the distinct predictions it allows for each population genetic mechanism.

I pursue these two objectives by measuring the evolution of diet breadth and thermal niche in these 12 laboratory populations of *E. coli*. In Chapter I, I examine the consequences of this evolution for the bacteria's capacity to utilize 93 substrates, and thereby quantify the extent to which specialization led to reduced diet breadth by generation 10,000. I also measured the competitive fitness of the evolved populations in four environments that differ from the evolution conditions. In Chapter II, I extend observations of diet breadth and the dynamic of adaptation to multiple evolutionary timepoints to determine whether antagonistic pleiotropy (AP) or mutation accumulation (MA) explains a greater proportion of the observed functional decay. In Chapter III, I examine the effects of adaptation to a constant 37° C environment on thermal niche. I measured maximum growth rates for population isolates from five evolutionary timepoints at eight foreign temperatures, and compared these data with the ancestor's performance at the particular temperature. In Chapter IV, I investigate the details of the loss of D-ribose catabolic function, which occurred in all populations by generation 2,000. The mutation rate for this loss of function was estimated and the genetic event responsible for the loss was characterized. Further, I quantify the effect of this mutation on fitness in the experimental environment.

Chapter I

EFFECTS OF ADAPTATION TO A SINGLE RESOURCE ON DIET BREADTH IN LONG-TERM EVOLVING POPULATIONS OF *E. COLI*

Adaptation is a process that is surprisingly difficult to quantify, despite being the primary focus of evolutionary biology. As a result, some consequences of adaptation are even less well understood. For example, do these consequences differ for organisms adapting to specific environments versus those adapting to broad or fluctuating environments (Bennett et al. 1992, van Tienderen 1997, Kassen and Bell 1998)? It has long been assumed that adaptation to specific environments will, sooner or later, adversely affect performance in alternative environments (Darwin 1872, Levins 1968, Jaenike 1978, Futuyma and Moreno 1988, Holt 1995). However, evidence in favor of this assumption is scarce partly because opportunities to simultaneously follow adaptation and its consequences are rare.

In this chapter, we present results from one such opportunity. We describe a series of experiments that quantified the catabolic diet breadth of 12 populations of *Escherichia coli* that adapted to a simple environment in which glucose is the sole carbon source. All populations were founded by the same clone and later compared with this clone to identify any evolved losses of function. Such a direct comparison is not possible in many experimental systems, but it is in this one because of the frozen, but revivable, "fossil record" we have of each lineage. These frozen stocks allow genotypes separated by years of laboratory evolution to be compared simultaneously. This first set of

experiments compares the diet breadth of isolates from each population with the ancestral phenotypes after 10,000 generations of evolution. In addition, the competitive fitness of these isolates relative to the ancestor was quantified in four environments that differed from the one in which the populations evolved.

Diet breadth was estimated by the ability of each genotype to catabolize 71 different carbon sources contained on a single microtiter plate. Relative fitness, on the other hand, was determined in the four different environments by a head-to-head competition with the ancestor. Whereas the absolute ability to catabolize various carbon sources is analogous to the fundamental niche of the genotype, competitive ability in different environments is more akin to the realized niche of the genotype. These contrasting interpretations of the niche should provide complementary perspectives on specialization. In each assay three clones from each of 12 populations were used to determine whether observed changes were systematic, restricted to a particular population, or indicative of variation within populations.

Secondly, we sought to identify which population genetic mechanism is more responsible for any observed declines in performance or functionality. Antagonistic pleiotropy occurs by the selection of mutations that are beneficial in the selective environment but deleterious in other environments. In contrast, mutation accumulation occurs by the drift-fixation of mutations that do not affect performance in the selective environment, but which negatively impact performance in other environments. The two mechanisms, antagonistic pleiotropy and mutation accumulation, offer distinct predictions. Three populations evolved increased mutation rates (Sniegowski et al. 1997), and these "mutator" populations should lose more functions than populations that

retain the ancestral mutation rate if mutation accumulation is important in causing specialization. However, if mutator populations have not lost any more functions than the other evolved populations, mutation accumulation is likely unable to explain most loss of performance. Meanwhile, if the same losses of function are found in replicate populations, suggesting parallelism, these losses may be adaptive and caused by the pleiotropic effects of selected mutations.

METHODS

Experimental system. The design of the evolution experiment has been described elsewhere (Lenski et al. 1991). In short, 12 lines were derived from a single, strictly asexual clone of *E. coli* B that has been in the laboratory for several decades (see: http://myxo.css.msu.edu/ecoli/strainsource.html for more information). The strain is prototrophic (it can synthesize all components of a cell from an energy source, such as glucose, and inorganic salts, nitrogen, etc.) and has undoubtedly undergone some general adaptation to the laboratory environment, but has not been selected under any specific conditions like the evolution protocol. A spontaneous mutant of the ancestor capable of using arabinose (Ara+) was used to found six of the replicates, whereas the other six were founded with the Ara- ancestor. This trait can be used to distinguish between populations on indicator plates and is neutral in the selective environment (Lenski et al. 1991). The populations are maintained by the daily transfer of 0.1 ml of culture into 9.9 ml of fresh Davis minimal media supplemented with 25 μg/ml of glucose (DM25). These conditions allow roughly 5 x 10^7 cells/ml at stationary phase. Every 500 generations (75 days),

samples of each population were stored in a glycerol suspension at -80°C.

These experiments focus only on isolates from the populations at 10,000 generations and their common ancestors. Three clones from each population were picked randomly from platings of these cultures and frozen separately; each experiment therefore compares 36 clones (12 populations x 3 clones) with the two ancestors (Ara^- and Ara^+). We studied multiple clones from each population because there are within-population polymorphisms (Elena and Lenski 1997, Papadopolous et al. 1999, Rozen and Lenski 2000). We also focused our investigation in part on traits related to membrane transport (bile salt tolerance and novobiocin resistance, see below), because previous experiments indicated that the outer membrane may have been under selection and hypothetically subject to tradeoffs (Travisano et al. 1995, Travisano and Lenski 1996).

Measurement of diet breadth. We used Biolog® ES (designed for *E. coli* and *Salmonella* spp.) microtiter plates to obtain estimates of catabolic diet breadth. These plates have 95 different carbon sources and a tetrazolium indicator dye whose intensity is proportional to the amount of growth on that substrate. Based on estimates of the proportion of genes in *E. coli* that are involved in membrane transport, catabolism, and electron transport, Biolog plates have been estimated to resolve mutations in about 5-20% of the genome (B. Bochner, personal communication). Of the 95 substrates in these plates, glucose and arabinose were excluded *a priori* because glucose was the target of adaptation and arabinose utilization was a marker in the evolution experiment.

Cultures were preconditioned for two days in LB broth (see below for specifics), and then on the day of the experiment each culture was diluted 1:100 into fresh LB and

incubated for 6 hours. Because glucose is the preferred resource for *E. coli*, it is specifically excluded from preconditioning to avoid catabolite repression, which suppresses other catabolic functions and could result in an underestimate of diet breadth (data not shown; *Biolog ES Microplate Instructions for Use*, 1993). Cells were centrifuged at 10,000 g for 10 minutes and then resuspended in saline to remove any substrate from the previous medium. Biolog wells were then inoculated at a constant density for all clones, after which initial readings were taken immediately with an automatic plate reader (t = 0 hr). A second and final measurement was taken at 24 h; all data discussed in this chapter are the difference in optical density (at wavelength 590 nm, specific to the tetrazolium dye) between 0 and 24 h. Experiments were conducted in two complete blocks of 36 evolved 10,000-generation clones (12 populations x 3 clones) and 6 ancestral genotypes (2 ancestors x 3 replicates).

Competition in Foreign Environments. The Biolog experiments focus more on absolute functionality rather than competitive fitness in foreign environments. It is possible that mutations that cause absolute losses of function are rare but those that cause competitive inferiority are more common. For this reason we sought to measure the relative fitness of the same 10,000 generation isolates in four different environments of increasing complexity and novelty.

The environments are described as follows. First, we both increased and decreased the concentration of glucose (25 μg/ml of glucose is standard) in the evolutionary medium in order to test the specificity of adaptation to the environment. We hypothesized that adaptation to a particular concentration of glucose in the medium might

reduce competitive ability at either lower (2.5 μg/ml) or higher (250 μg/ml) concentrations, perhaps because of tradeoffs between resource affinity and maximum growth rate. Next, we chose to add bile salts, which are large organic detergents that are lethal to most non-enteric bacteria, to the DM25 experimental medium to determine if the evolved populations had compromised their tolerance to bile salts in order to improve glucose transport. Tolerance to bile salts is a membrane characteristic of enteric bacteria shared by the ancestor in our experiment. The bile salt concentration was selected to mimic the concentration of bile salts in Maconkey Agar (1.5 g/L of Sigma Bile Salts, consisting of 50% sodium cholate, 50% sodium deoxycholate), which is typically used to identify enteric bacteria.

The LB environment was chosen because it is so different from the selection medium but it keeps constant many other conditions (e.g. culture vessel, temperature, serial transfer regime). LB media consists of tryptone, yeast extract (which is the water-soluble portion of autolyzed yeast) and salt. In short, it is a complex of nutrients largely devoid of simple carbohydrates like glucose. The LB environment was diluted with distilled water to generate comparable cell density with the DM25 selective environment.

For each competition in a foreign environment, fitness was also simultaneously measured in DM25 to allow direct comparisons of performance. All experiments were conducted in 50 ml Erlenmeyer flasks in a 37° shaking incubator, conditions identical to the long-term evolutionary environment. Cultures were founded from –80° freezer isolates and grown up in DM +1000 μg/ml of glucose. Cultures were then acclimated to the test environment via a 1/4,000 dilution into fresh medium and incubated for 24 h. Each evolved genotype and the ancestor of the opposite Ara marker were each diluted

1/200 into a common flask containing 9.9 ml of the test media, and then incubated 1 day. [Certain clones generated unusually low yields in the LB environment, so competitions were founded with a 2:1 starting ratio of evolved to ancestral genotypes, rather than the typical 1:1. All other details of the competitions for these clones were unchanged.] Initial and final densities of each competition culture were determined by plating aliquots onto TA indicator plates, which allow Ara^- and Ara^+ competitors to be distinguished by their colony color. Relative fitness was quantified by calculating the ratio of the number of doublings for the derived and ancestral competitors (see Lenski et al. 1991 for details). Competition experiments versus the ancestor were conducted in two complete blocks of 36 evolved 10,000-generation clones (12 populations X 3 clones) in each foreign environment and the paired control DM25 environment.

Assay for novobiocin sensitivity. Novobiocin resistance is a character possessed by the ancestor which is sometimes dependent upon mutations in the cell envelope, including the structural component lipopolysaccarides as well as outer membrane porins (Nikaido, 1996). Assays were conducted by distributing approximately 250 cells on LB plates containing 400 μg/ml novobiocin. Replicates of the ancestors produced an average of 40 colonies after 48 hours. If ≤ 2 colonies emerged after 72 hours, the evolved clone was deemed to be sensitive. Three clones for each of the twelve populations were assayed in this manner and replicated twice.

Statistical analyses. The wealth of data collected presented certain analytical challenges. For this investigation we measured performance on 95 carbon sources for 42

clones, twice each, for a total of nearly 8000 individual data points at both 0 and 24 h. We wrote a series of algorithms designed to manage the data and perform more powerful statistical analyses that are appropriately conservative given the large number of tests required. These programs are written in Visual Basic and interface with Microsoft Excel and Access for data management, but not for their statistics. Statistical test I compares the mean of the ancestors with the mean of the evolved populations with Welch's approximate t-test for groups with unequal variances (derived populations are presumed to be more variable than a clonal ancestor; Sokal and Rohlf 1981). This test determines which substrates differentiate the evolved populations, as a group, from the ancestors. Test II is a nested ANOVA that analyzes variation among and within evolved populations, with population and clone as random factors, respectively. This test examines whether populations, or clones within populations, significantly vary on any given substrate.

First, a measure of total catabolic activity, or "diet breadth," was calculated for each genotype by summing the optical density scores of all substrates, to which we applied tests I and II. We excluded glucose and arabinose from these analyses because glucose is the target of adaptation and arabinose is a defined marker state for the system. We further excluded 22 substrates because no growth (population mean optical density < 0.10) was observed for either the ancestors or any evolved populations. Next, each of the 71 remaining individual substrates was similarly analyzed. Because catabolism of each resource is not necessarily an independent physiological activity, we employed a stringent criterion of significance ($p_{crit} < .0005$).

To more generally address how evolved populations perform on each substrate

relative to the ancestors, we calculated the slope of evolved mean performance versus ancestral mean performance using linear regression. A slope derived from the model that is significantly less than 1 (i.e. the 95% confidence limits are less than 1) suggests that evolved populations tend to perform worse on each substrate and have generally lost functionality. If no trend is observed or the slope is greater than 1, then diet breadth has probably not become narrower.

For each competition experiment we conducted one or more of the following statistical tests using SPSS: 1) a nested ANOVA on the fitness data in the novel environment designed to identify significant population- and clone-level variance (population and clone are random factors); 2) a mixed-model nested ANOVA designed to identify the effect of environment (fixed factor) on population and clone-level variance (both random factors); and 3) a paired t-test to differentiate fitness in each experimental environment relative to control. In addition, we inspected the data for potential outlier clones within populations that caused significant effects at the level of clone. We identified one clone from one population (Ara + 6, clone A) that was an outlier in nearly all environments (novel and control). Given that it was inferior even in the control environment, this clone was omitted and analyses 1-3 were recalculated.

RESULTS

Diet Breadth Narrows. We predicted that bacterial populations grown only on glucose for 10,000 generations would lose unused catabolic functions. Only one nearly absolute loss of function, growth on D-ribose, occurred in all twelve populations (Table 1).

Table 1. Significant changes in catabolic function after 10,000 generations of evolution. The first data column is the ancestral mean absorbance for the substrate listed, the second the evolved mean. Data columns 3, 4, and 5 indicate whether there were 3) significant overall declines (t-test), 4) significant variation among populations (ANOVA) and 5) significant variation within populations (ANOVA). P-values were adjusted for multiple non-independent contrasts (p_{crit}<.0005), t-tests were 2-tailed and assumed unequal variances. Substrates grouped by a common font indicate similarities among these carbon sources because of shared cellular transport or catabolism. Substrates for which no significant tests were found are not shown; sum is for all 93 substrates. L denotes functional decline, * denotes p < .0005.

CARBON SOURCES	Ancestral Mean	Evolved Mean	t-test vs. Anc	ANOVA Among	ANOVA Within
D-GALACTOSE	1.083	0.856	L*		*
D-MELIBIOSE	0.954	0.772	L*		
D-LACTOSE	0.990	0.770	L*		*
D-galactonic acid	1.077	0.918			*
methylgalactoside	0.909	0.780			*
D-galacturonic acid	1.023	0.915			*
glycyl-l-aspartic	0.910	0.595	L*		
glycyl-l-glutamic	0.714	0.464	L*		*
glycyl-l-proline	0.643	0.459		*	
D-mannose	1.263	1.047	L*		
L-glutamic acid	0.797	0.538	L*	*	
glucose-6-phosphate	1.160	0.962	L*		
D-ribose	0.968	0.146	L*	*	
L-glutamine	0.697	0.442	L*		
adenosine	0.919	0.766	L*		
D-psicose	0.520	0.433	L*		
pyruvic acid	0.940	0.810	L*		
lactulose	0.589	0.550			*
deoxyadenosine	0.955	0.780			*
L-proline	0.664	0.508		*	
D-alanine	0.354	0.404		*	
tween 20	0.081	0.144		*	
maltose	0.900	0.655		*	
glycolic acid	0.000	0.085		*	
succinic acid	0.303	0.141		*	
SUM	46.450	39.807	L, p=.029		p = .004

However, when we summed the scores for all 93 carbon sources for each genotype, we found a significant decline in diet breadth of an average of 15% for all evolved populations (Figure 1, one-tailed t-test, $t = 4.183$, $df = 1.87$, $p = .029$). This decline in diet breadth proved to be general and not limited to a few substrates (Figure 2, slope $\pm$ 95% CI = $0.80 \pm .044$); on average, evolved populations tended to perform worse than the ancestor.

Significant variance in diet breadth evolved among and within populations. There was no significant variation among populations in the sum of all catabolic functions, but there was significant variation among clones within populations (Table 2). A single "specialist" clone in population A-5 that grew no more than 50% as well as the ancestor on 47 of 95 substrates caused much of this effect, but this clone was otherwise normal on glucose. Another "specialist" clone that was abnormally deficient on fewer substrates was also found in population A+4. These clones were not better competitors than other clones in the evolution environment because of their reduced functionality, however (data not shown). If these two clones are omitted from the nested ANOVA of diet breadth, then significant variation exists among populations ($F_{11,22} = 2.34$, $p = .043$) but no longer among clones within populations ($F_{22,34} = .957$, $p = .534$).

Significant variation among the populations was found for nine individual carbon sources (Table 1). This heterogeneity was the result of scattered losses on six substrates, scattered gains on two substrates, and both gains and losses on one substrate, d-alanine. In general, the observed gains were minor in their effects on the total catabolic function of each population, while the declines tended to have larger effects on the sum. This

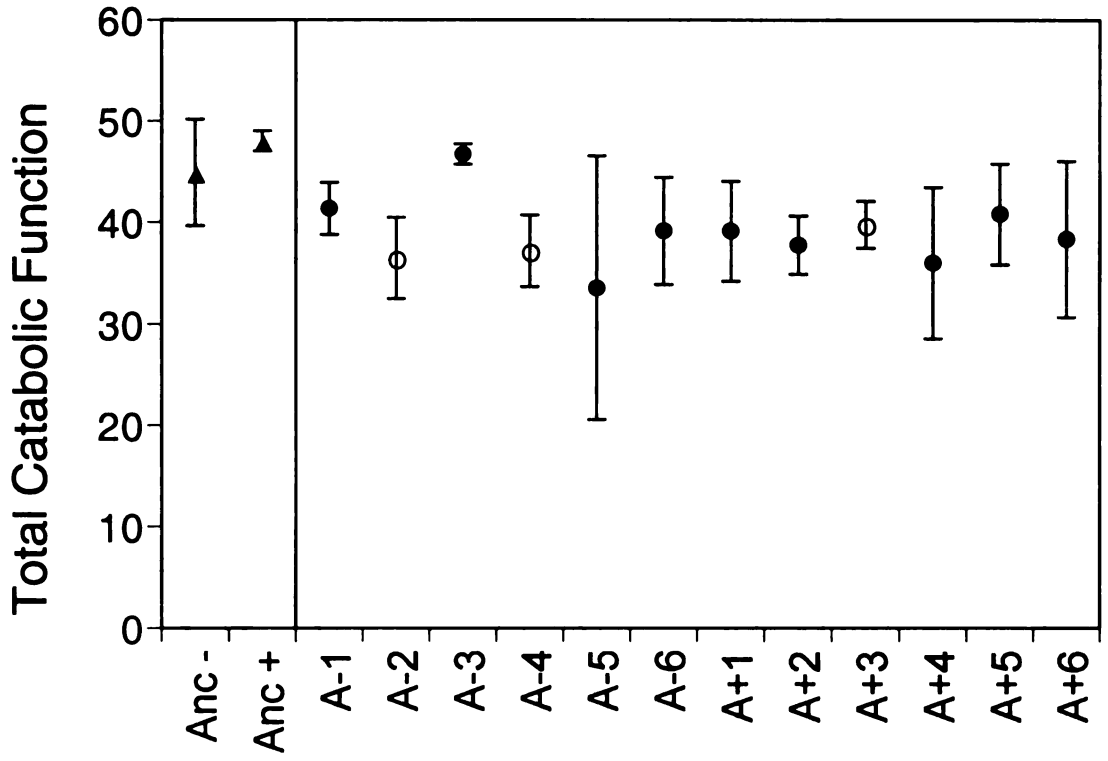

Figure 1. Effect of 10,000 generations of evolution on catabolic diet breadth. Each point is the mean total catabolic activity of 93 substrates. The evolved populations (circles) had significantly lower diet breadth ($t = 4.183$, df = 1.87, $p = 0.029$, one-tailed) than the common ancestors (triangles). The populations with elevated mutation rates (open circles) were not significantly different from the remaining evolved populations (closed circles, $t = .618$, df = 10, $p = 0.275$, one-tailed, ns). Error bars are standard errors.

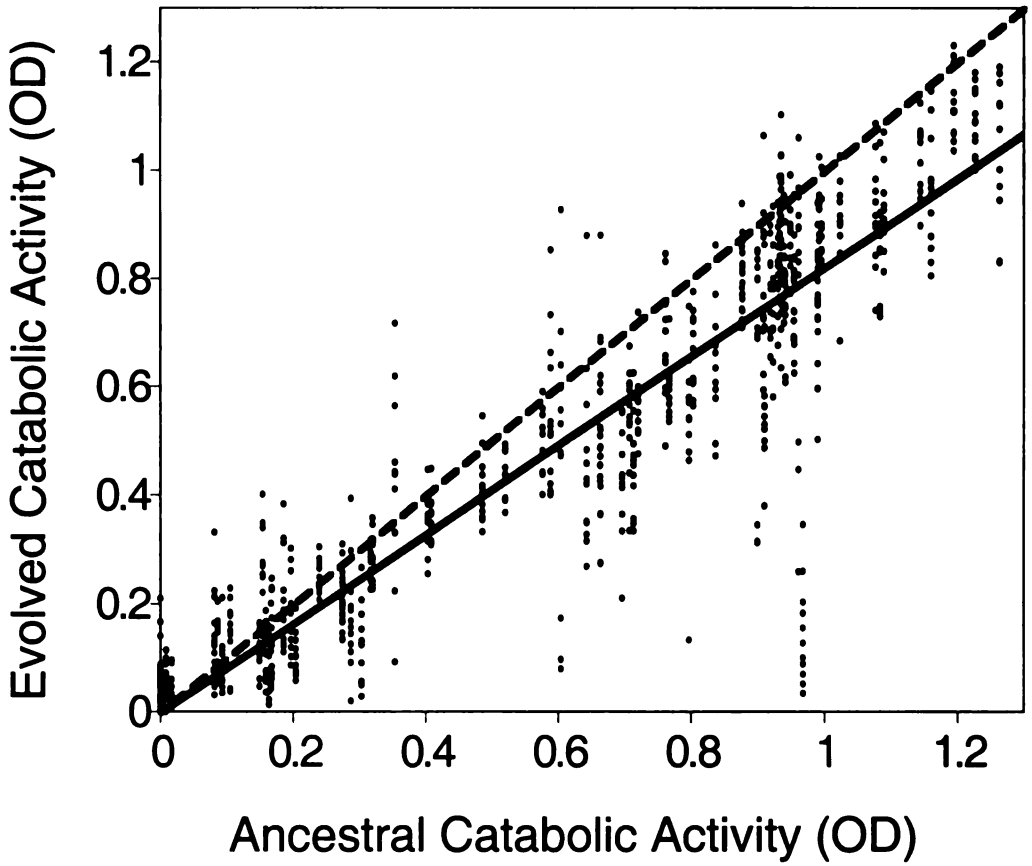

Figure 2. Relationship between evolved and ancestral catabolic function for each substrate. Each point is the mean of 3 clones from each population replicated twice each, for 93 substrates. The dashed line is the isocline where slope = 1; the solid line is the linear regression through the data (R^2 = .935, slope = .800, y-intercept = .018, overall model: $p < .001$).

Table 2. Nested ANOVA for total catabolic activity of the 12 evolved populations. Total catabolic activity is the sum of absorbance scores for 93 different substrates. Population and clone are random effects.

Source	df	MS	F	P
Population	11	61.66	1.203	.339
Clone (Pop)	23	51.26	3.125	.001
Error	35	16.40		

trend can be seen in Figure 2 by the greater number of points below the dashed line (which represent losses) relative to those above it (which represent evolved gains). We also found significant variation within populations for eight carbon sources; however, six of these may be condensed into two separate characters. Four of these substrates share the sugar galactose as a backbone (d-galactose, d-galactonic acid, methylgalactoside, and d-galacturonic acid), while two are lactose sugars (d-lactose and lactulose). Two or three clones that performed extremely poorly explained variance in both substrate classes. We next considered whether the decay in performance on these individual resources translated into decreased competitive ability in foreign environments.

Fitness improved in foreign environments. We evaluated diet breadth in a second, contrasting manner by competing clones from evolved populations directly against the ancestor in four foreign environments. In the first experiment we explored competitive fitness versus the ancestor over a 100-fold range of glucose concentrations in the otherwise standard experimental environment. Clones were competed in two "test" environments (DM2.5 and DM250) as well as in the control environment (DM25). We found a significant effect of glucose concentration on population mean fitness (Table 3). Populations (and clones within populations) also varied significantly in their performances at different glucose concentrations, as evidenced by the significant interaction terms in the ANOVA in Table 3. Not surprisingly, populations were most fit in the DM25 environment in which they evolved (Figure 3; t-tests for paired comparisons: DM2.5 vs. DM25, $t_s = 5.48$, df = 11, $p < .001$, DM25 vs. DM250, $t_s = 14.08$, df = 11, $p < .001$). However, all populations were more fit than the ancestor in

Table 3. Nested ANOVA for relative fitness obtained for the 12 evolved populations in three different glucose concentrations. Population and clone are random effects.

Source	df	MS	F	P
Concentration	2	1.187	35.12	<.001
Population	11	.0678	1.967	.098
Clone (Pop)	23	.015	1.042	.439
Concentration * Pop	22	.0339	2.345	.007
Concentration * Clone (Pop)	46	.0144	1.684	.015
Error	105	.00858		

both DM2.5 and DM250; that is, adaptation to the DM25 environment brought about correlated improvements in fitness, rather than tradeoffs, at other glucose concentrations (Figure 3).

In the second experiment we evaluated the effect of adding bile salts to the experimental medium (Figure 4). We found no overall treatment effect: a paired t-test between the fitness data of the evolved populations in DM25 with and without bile salts showed no significant difference (1-tailed $t_s = 1.10$, df = 11, $p = .138$). A nested ANOVA on the fitness data in DM25 + bile salts did reveal marginally significant variation among and within populations (Table 4). A single clone from population A-5 caused nearly all of this variation within populations; this same clone also was deficient on numerous carbon sources on Biolog plates. When we omitted this clone from the nested ANOVA, we found more significant variation among populations for fitness in bile salts ($F_{11,22} = 2.615$, p = .026) but not among clones within populations ($F_{22,34} = .718$, p =.790)

In the final experiment, we altered the competitive environment in a more pronounced manner by using a much more complex medium, LB, instead of minimal salts supplemented with glucose. We anticipated the greatest difference in fitness between this environment and the control. Indeed, mean fitness in LB was significantly lower than that in the control environment ($t_s = 12.278$, df = 11, p <.001). However, we were surprised that nine of 12 populations had nevertheless increased in fitness in LB relative to the ancestor, and that the fitness of one population (A-1) was statistically indistinguishable between LB and DM25 (Figure 5). On the other hand, three populations were statistically equivalent to the ancestor in LB fitness (i.e. the 95%

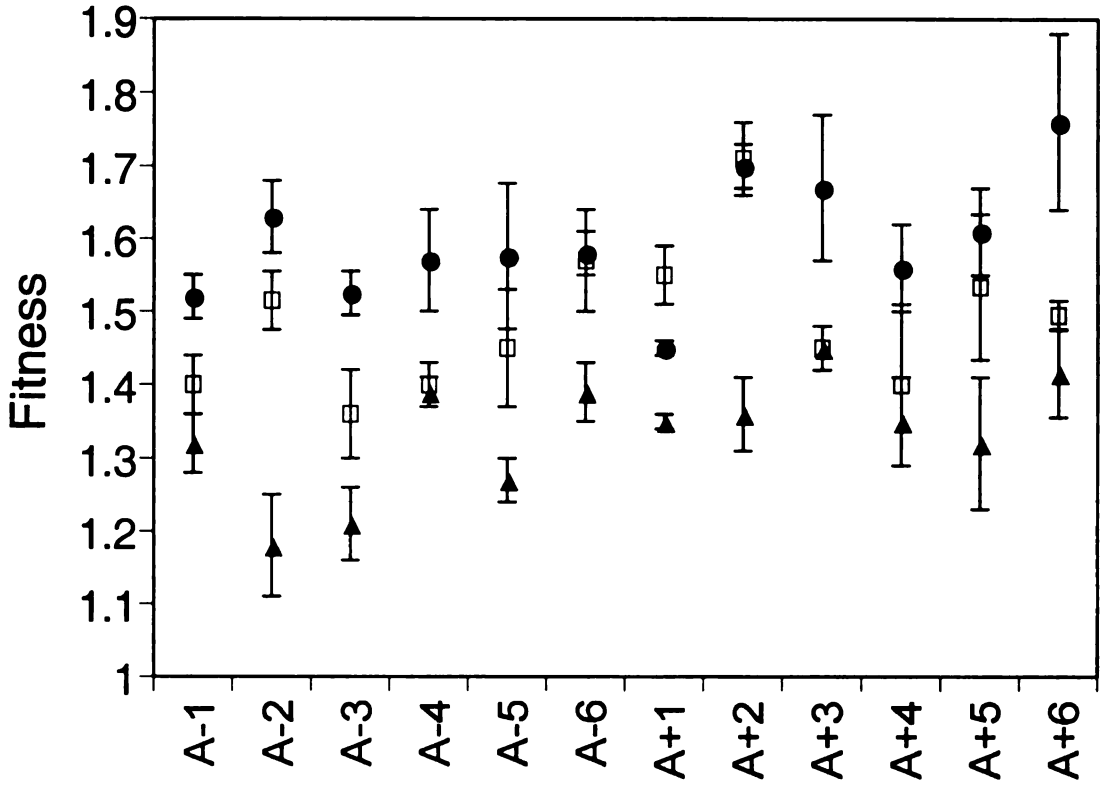

Figure 3. Mean fitness (±SE) relative to the ancestor of evolved populations when measured in the experimental environment at varying concentrations of glucose. Each point is the mean of 3 random clones, each replicated twice. Open squares: 2.5 μg/ml glucose; closed circles: 25 μg/ml glucose; closed triangles: 250 μg/ml glucose.

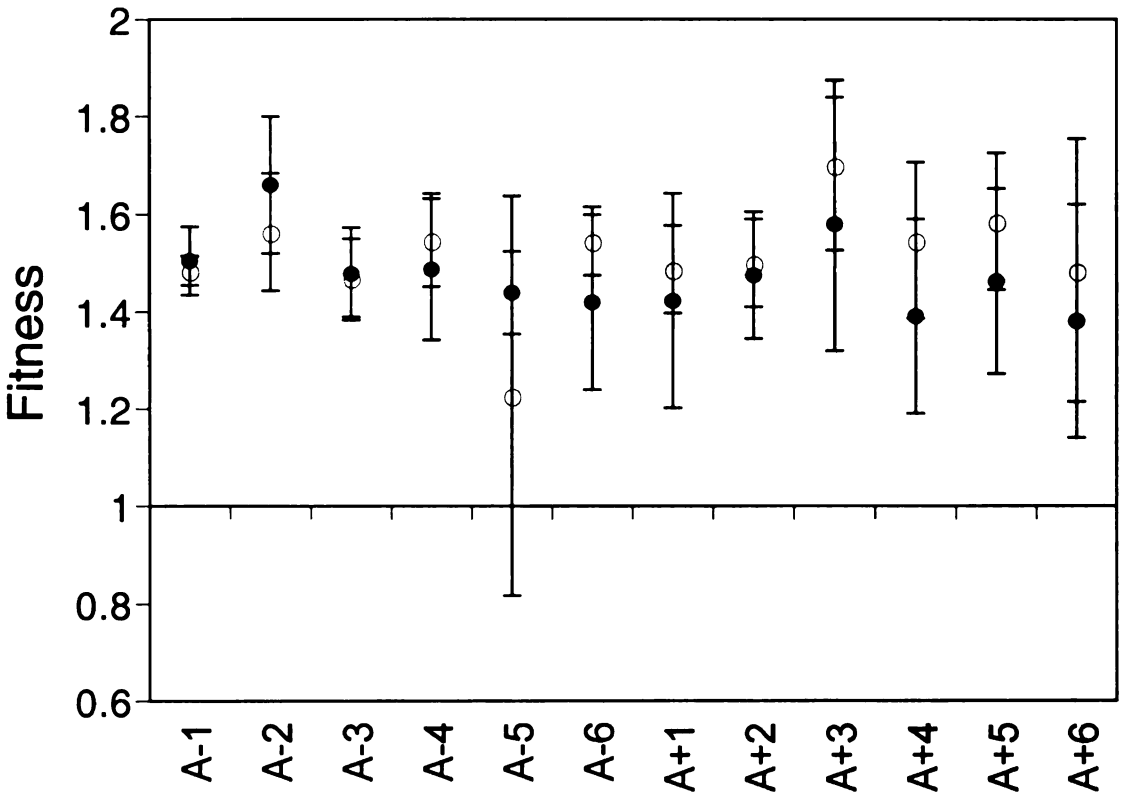

Figure 4. Mean fitness (±SE) relative to the ancestor of evolved populations when measured in the experimental environment with (closed circles) and without the addition of 1.5 g/L bile salts (open circles). Each point is the mean of 3 random clones, each replicated twice.

Table 4. Nested ANOVA for relative fitness obtained for the 12 evolved populations in the experimental environment plus bile salts. Population and clone are random effects.

Source	df	MS	F	P
Population	11	.0716	2.118	.062
Clone (Pop)	23	.0338	1.817	.054
Error	35	.0186		

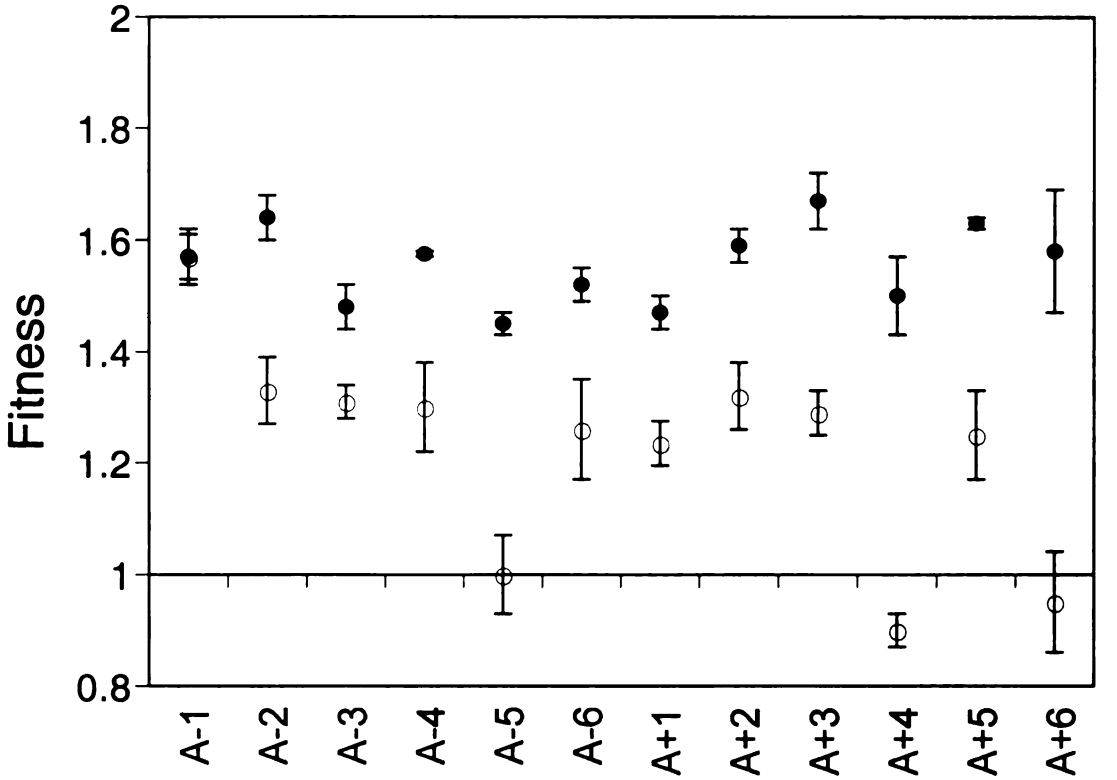

Figure 5. Mean fitness (±SE) relative to the ancestor of evolved populations when measured in the minimal experimental environment (DM25, closed circles) and a novel, complex environment (LB, open circles). Each point is the mean of 3 random clones, each replicated twice.

confidence intervals of these means (not shown) did not exclude the ancestral fitness, which equals 1.0). In addition, a nested ANOVA using fitness in LB revealed highly significant variation among populations but not within populations (Table 5).

Most populations become sensitive to novobiocin. Assays that examined the ability of each population to grow on agar plates containing novobiocin demonstrated that most populations became sensitive to this antibiotic. Nine of 12 populations were completely sensitive to the antibiotic; no colonies were observed for any of the three clones. Only one of the 12 populations retained a level of resistance comparable to that of the ancestor, and two other populations were intermediately sensitive.

Mutator populations were not significantly different from populations that retained the ancestral mutation rate in all assay environments. We compared the performance of the mutator populations relative to the other evolved populations in each assay condition. One-tailed t-tests between these groups resolved no significantly greater loss among the three mutator populations in (a) the sum of Biolog catabolic function ($t = .618$, $df = 10$, $p = .275$); (b) 2.5 μg/ml glucose medium ($t = .796$, $df = 10$, $p = .223$), (c) 250 μg/ml glucose medium ($t = .296$, $df = 10$, $p = .387$); (d) LB medium ($t = .941$, $df = 10$, $p = .210$). Contrary to the direction of the prediction, the mutator populations performed significantly better in bile salts medium ($t = 1.932$, $df = 10$, $p = .041$). In sum, these tests indicate that mutator populations have not lost more functionality in any of these environments than populations retaining the ancestral mutation rate.

Table 5. Nested ANOVA for relative fitness obtained for the 12 evolved populations in the foreign environment LB. Population and clone are random effects.

Source	df	MS	F	P
Population	11	.196	13.072	<.001
Clone (Pop)	23	.015	.896	.693
Error	35	.168		

DISCUSSION

Spiegelman and collaborators (Mills et al. 1967, Saffhill et al. 1970) asked bacteriophage Qβ only to "go forth and replicate" in the medium containing Qβ replicase, and numerous functions were lost. We have issued a similar injunction to these *E. coli* populations, except in a glucose and minimal salts medium. Following this precedent, these long-term evolving *E. coli* populations should have become streamlined scavengers of glucose by eliminating extraneous functions. We found this not to be the case, at least not nearly to the extent observed in Qβ. Only one absolute catabolic loss (D-ribose) was observed in all twelve lineages, and one additional loss (resistance to novobiocin) occurred in most populations. For most functions assayed, no significant reduction was found among evolved populations. Moreover, the significant quantitative reductions in catabolic function that were found were usually small in magnitude.

Nonetheless, the evolved mean diet breadth declined an average of 15%, and no overall gains in function occurred that might have compensated for other losses of function. In these populations, specialization did not involve numerous wholesale losses of function, but rather reduced performance on several substrates not used during the evolution experiment. The degree of decay varied both among and within populations for growth on different substrates (Table 1), and a substantial amount of functional decay occurred in two of the 36 clones sampled. Thus, the fundamental catabolic niche of these populations became narrower while adapting to this simple laboratory environment.

However, we arrived at a strikingly different conclusion when we measured the fitness (which is perhaps more analogous to the realized niche) of the evolved

populations in four different foreign environments. In these assays, performance was quantified by directly competing the evolved genotypes with the ancestors, and in nearly every case the evolved genotype was the superior competitor. These correlated improvements in fitness in novel environments indicate that adaptation to the laboratory environment has been frequently general, and raise questions about the specificity of adaptation.

Each of these novel environments may not be entirely foreign to the evolved populations, however. The two novel glucose concentrations in which we measured fitness are not equally unfamiliar, for example. Because the glucose concentration in the experimental medium drops below 25 μg/ml as the population grows, competitive ability at lower concentrations was likely favored, even if only briefly (Vasi et al. 1994). Thus, it is perhaps to be expected that the populations are considerably more fit than the ancestor in 2.5 μg/ml glucose than in the 250 μg/ml concentration which they never experience, which is supported by a paired t-test ($t_s = 6.37$, df = 11, $p < .001$). The mechanism behind the greater fitness gains in 2.5 μg/ml than in 250 μg/ml glucose need not be the glucose concentration itself, however. Vasi et al. (1994) argued that selection in these lines for improved resource affinity at low concentrations was weak, which perhaps implicates some other factor in the medium (oxygen concentration, different concentrations of metabolic by-products, etc) as the cause of the lesser fitness gains in 250 μg/ml glucose.

Bile salts are an important component of the natural environment of *E. coli*, so much so that resistance to these compounds is often used to define enteric bacteria in microbiological assays. Bile salts are like novobiocin in that both are antibiotics that

attack the cell membrane, but their effects on these populations were completely different. Most populations became completely unable to grow in the presence of novobiocin; however, no significant differences in fitness were found when bile salts were added to the selective medium. If changes in membrane permeability were responsible for the loss of novobiocin resistance, they apparently had specific effects that did not affect other membrane traits like tolerance to bile salts.

We found no systematic cost of adaptation to the simple laboratory environment when we modified the glucose concentration or added bile salts, but rather found correlated improvements in fitness which were occasionally of the magnitude observed in the evolution medium. However, the complex LB environment is certainly different enough from minimal glucose medium that the correlated improvements in the LB environment are surprising. A simple explanation for the correlated improvements in novel environments is that these environments did not sufficiently vary from the selective conditions. Whereas we altered the carbon source and media composition, we did not change other conditions like the speed of shaking (aeration), temperature, the batch culture protocol, or the absence of biotic interactions with other species, including competing bacteria and predatory viruses. It is likely that the 12 populations have adapted in part to several of these environmental components, and that changing only one of them does not affect the complex of other adaptations to the experimental environment. Thus, it is possible that a measure of fitness in an environment that differs by only one factor may sometimes be insufficient to reveal "adaptive decay."

Evidently, these evolving populations acquired mutations that were beneficial under several environmental conditions. For example, mutations that improve fitness in

25 μg/ml glucose may also improve fitness in 250 μg/ml glucose (because of the common resource) as well as in dilute LB (because of the equivalent cell density allowed by the medium). The same genotypes examined here were also tested in undiluted LB, which is a medium that is roughly 100-fold more concentrated. More losses of fitness were found, which suggests some specialization towards resource concentration, but replicates of the same genotype were too inconsistent to be considered reliable (V.S. Cooper, unpublished data). In general, these competitions in novel environments suggest that mutations that were beneficial in the selective environment may sometimes have positive pleiotropic effects. To the contrary, the Biolog results suggest frequent antagonistic effects of beneficial mutations. We now turn to the question of which population genetic mechanism better accounts for the observed reductions in diet breadth.

Is specialization the result of antagonistic pleiotropy or mutation accumulation?

It has been widely assumed that specialization results from tradeoffs that are caused by antagonistic pleiotropy (Futuyma and Moreno 1988). However, a tradeoff may have been generated by either of two processes: antagonistic pleiotropy, or mutation accumulation in loci no longer under selection. If these two processes occur in tandem (as they often do), a third mechanism of functional decay may occur, which is the genetic hitchhiking of conditionally deleterious alleles along with beneficial alleles. Hitchhiking is possible in this system more than in others because these populations are strictly asexual, which completely links conditionally deleterious alleles with beneficial ones.

We may infer the relative importance of these mechanisms even when we lack

direct genetic evidence. Losses of function that consistently occur in replicate populations are best explained by antagonistic pleiotropy because of the strong association between the loss and adaptation. Losses that occur in individual populations may be also due to antagonistic pleiotropy, but independent evidence, such as knowledge of how selection affected the particular catabolic pathway, is required. In the absence of these links between functional decay and adaptation, the loss may more likely be due to mutation accumulation.

A unique feature of this experiment allows us to examine the overall contribution of mutation accumulation to losses of function. Three of the twelve populations evolved genomic mutation rates roughly 100-fold higher than the ancestor and the remaining nine evolved populations. These three populations ought to have fixed 100-fold more conditionally neutral mutations during the time when they were mutators. The margin of difference between these two classes of populations provides an estimate of the effect of mutation accumulation. We tested for an effect of mutation accumulation by comparing the performance of mutator populations with those populations that retained the ancestral mutation rate (non-mutators). Mutator populations should have lost significantly more functionality than non-mutators, but did not: mutators were not significantly worse than non-mutators in any foreign environment in which we measured performance.

This negative result must be explained in the context of the number of mutations fixed by drift in these populations. While the number of individual mutations that have occurred in each population over the course of 10,000 generations of evolution with an effective population size of $\sim 10^7$ (Lenski and Travisano 1994) is extremely large, the number fixed by drift is likely quite low. Given Drake's (1991) estimate of the genomic

mutation rate of *E. coli*, 2.5 x 10^{-3} per generation, which applies to the ancestor and the non-mutator populations, we estimate that an average of only 25 mutations have been fixed in the roughly 5 million bp genome over 10,000 generations.

Biolog plates have the potential to resolve mutations in about 5-20% of the genome (Bochner, personal communication), which reduces the number of mutations we may potentially detect to between 1 and 5. Further, only roughly 1/3 of these mutations should actually modify the phenotype, based upon a survey of mutations in *E. coli* genes (Drake 1991). Realistically, each population can be expected to have an average of about one mutation that affects Biolog catabolic function after 10,000 generations. Thus, mutation accumulation should have had a negligible effect in non-mutator populations during this period. However, the number of detectable mutations fixed in mutator populations should be 100-fold higher after the fixation of the mutator phenotype. The three populations have been mutators for ~7600 (population A-2), ~7,000 (A+3), and ~1500 (A-4) generations, respectively (Sniegowski et al. 1997). If non-mutator populations have roughly one discernible catabolic mutation over 10,000 generations, then the mutator populations have on the order of 76, 70, and 15 mutations fixed by drift, respectively. Given these estimates, our finding that mutator populations have lost no more functions than non-mutators is surprising.

There are two possible explanations for the lack of a mutator effect that are not mutually exclusive. First, mutations that knock out function in alternative environments might also frequently affect fitness in the selective environment. These mutations would never become fixed in these populations unless they were linked to another strongly beneficial mutation, or were themselves beneficial. The large population sizes also

enhance purifying selection and eliminate all but the most neutral mutations. If genuinely neutral mutations are indeed rare, mutation accumulation may require more time and more frequent sampling to be detectable. Second, the Biolog assay may have been insensitive to mutations of small effect and may have only reflected mutations that greatly reduced function. We did not use the Biolog assay to measure growth kinetics on the individual substrates in this experiment, but future experiments will include kinetic information and may be more sensitive to mutations of smaller effect.

Given the lack of any mutator effect, antagonistic pleiotropy is a better alternative explanation for some of the observed losses. Parallel reductions in catabolic function were found on 13 of the 71 substrates upon which the ancestor could grow (Table 1). Such parallelism is itself suggestive that each loss is linked to adaptation, and previous work also supports this hypothesis. Several of these 13 substrates have been previously identified by Travisano and Lenski (1996) as sharing at least part of the same transport mechanism for glucose across the cell membrane, and therefore performance on these substrates may have been constrained by selection for glucose efficiency. Moreover, the loss of D-ribose catabolic function was found to be beneficial in minimal glucose medium (discussed further in Chapter IV). These reductions in function are likely the result of pleiotropic effects of adaptive mutations as opposed to the genetic drift of conditionally neutral mutations.

We can speculate about the types of mutations that led to the parallel reductions in function by considering the transport and catabolism of the relevant substrates. For example, three substrates that reflect losses in Table 1 (glycyl-l-aspartic acid, glycyl-l-glutamic acid, and glycyl-l-proline) are all dipeptides which share glycine and are

transported by the same operon (Oliver 1996). The most parsimonious explanation for these results is that each population acquired a single beneficial mutation for glucose that affected the transport of all three of these substrates. Another three substrates (galactose, melibiose, and lactose) each may be transported by the same permeases, any of which may have been antagonistically affected by selection in the glucose environment (Lin 1996). If these speculations are correct, the number of mutations responsible for the 13 parallel reductions in function may be far less than 13. Thus, much of the observed decay appears to be linked to adaptation and the result of relatively few mutations in each population. The possibility that one mutation may affect multiple traits is precisely why we employed such a conservative statistical criterion ($p < .0005$) to identify changes.

The lack of significant contribution of mutation accumulation to losses of diet breadth does not rule out the effect of genetic hitchhiking. Reductions in function on seven carbon sources presented in Table 1 were limited to certain individual populations, and four additional carbon sources also illustrated variation within populations. This variability in correlated responses to selection could either result from heterogeneity in the effects of mutations favored in the evolution environment (Travisano and Lenski 1996), or different conditionally deleterious mutations hitchhiking to fixation across populations. Some of the reductions in function observed in single populations may have resulted from hitchhiking, and others from antagonistic pleiotropy, but the design of this experiment prevents us from distinguishing these scattered effects. However, the lack of any evidence for mutation accumulation does suggests that the frequency of conditionally deleterious mutations that may hitchhike is probably quite small. This argument, combined with the evidence for antagonistic pleiotropy causing parallel losses of

function, suggests that these losses in certain clones and populations may also be explained by antagonistic pleiotropy.

Finding losses of function in alternative environments is a prerequisite to identifying "adaptive decay." We found surprisingly few losses despite the number of traits we measured. The scarcity of functional decay supports Futuyma and Moreno's (1988) argument that tradeoffs cannot be assumed and must be measured empirically. Further attention to the population genetic process responsible for the losses is also mandated in recent literature (Futuyma and Moreno 1998, Holt 1995). We have begun to address which process better explains specialization in this system; however, it is notable that even in such a simple system it is difficult to identify the target of adaptation and its consequences. The following chapters will continue to disentangle the population genetic processes responsible for specialization in greater detail, and will also focus on different aspects of the niche of the bacterium.

Chapter II

POPULATION GENETIC PROCESSES LEADING TO THE ECOLOGICAL SPECIALIZATION OF EVOLVING *E. COLI* POPULATIONS

When organisms adapt genetically to one environment, they may lose fitness in other environments (Mills et al. 1967, Futuyma and Moreno 1988, Fry 1990, Bennett and Lenski 1993). Two distinct population genetic processes can produce ecological specialization, mutation accumulation (MA) and antagonistic pleiotropy (AP; Rose and Charlesworth 1980, Rose 1991, Holt 1995, Sgrò and Partridge 1999). In MA, mutations occur in genes which are no longer maintained by selection; adaptation to one environment and loss of adaptation to another are therefore caused by different mutations. AP arises from tradeoffs, such that the same mutations that are beneficial in one environment are detrimental in another. In general, it has proven difficult to distinguish these processes (Rose and Charlesworth 1980, Rose 1991, Holt 1995, Sgrò and Partridge 1999). We analyzed the decay of unused catabolic functions in 12 lines of *Escherichia coli* propagated on glucose for 20,000 generations (Lenski et al. 1991, Lenski and Travisano 1994). During that time, several lines evolved elevated mutation rates (Sniegowski et al. 1997), and they were expected under MA to experience more decay than the other lines, but no significant difference was observed. Moreover, most catabolic losses occurred early in the experiment when beneficial mutations were being

substituted most rapidly, a pattern predicted by AP. Thus, AP appears more important than MA for the decay of unused catabolic functions in these evolving populations.

Twelve populations were founded from a strain of *E. coli*, and they all adapted to a minimal medium supplemented with glucose (Lenski et al. 1991, Lenski and Travisano 1994). Competitive fitness increased rapidly in the first few thousand generations and has continued to improve, but the average rate of improvement decelerated sharply over time and was only about one-tenth as fast between generations 15,000 and 20,000 as it was in the first 5,000 generations (Figure 6). While adapting to the glucose medium, the evolving populations also underwent some decay of unused catabolic functions, such that their diet breadth tended to become narrower and more specialized (Chapter I).

We sought to identify the population genetic process primarily responsible for the association between increased fitness in glucose and reduced diet breadth by following both characters through time. If most losses of function happened early in the evolution of the populations, when adaptation to glucose was most rapid, then AP would be supported (Figure 7, curve AP). Moreover, if the same functional losses occurred in most populations, then this parallelism would suggest that the losses were adaptive and caused by AP. We do not expect an association between the dynamics of adaptation and functional decay under a strict MA model. Rather, MA predicts that losses of unused functions should accumulate stochastically at a roughly constant rate (Figure 7, curve MA), and that different functions should decay in the replicate populations. "Bottlenecks" caused by selective sweeps of beneficial alleles should not affect the expected rate of substitution of strictly neutral mutations, which depends on mutation rate

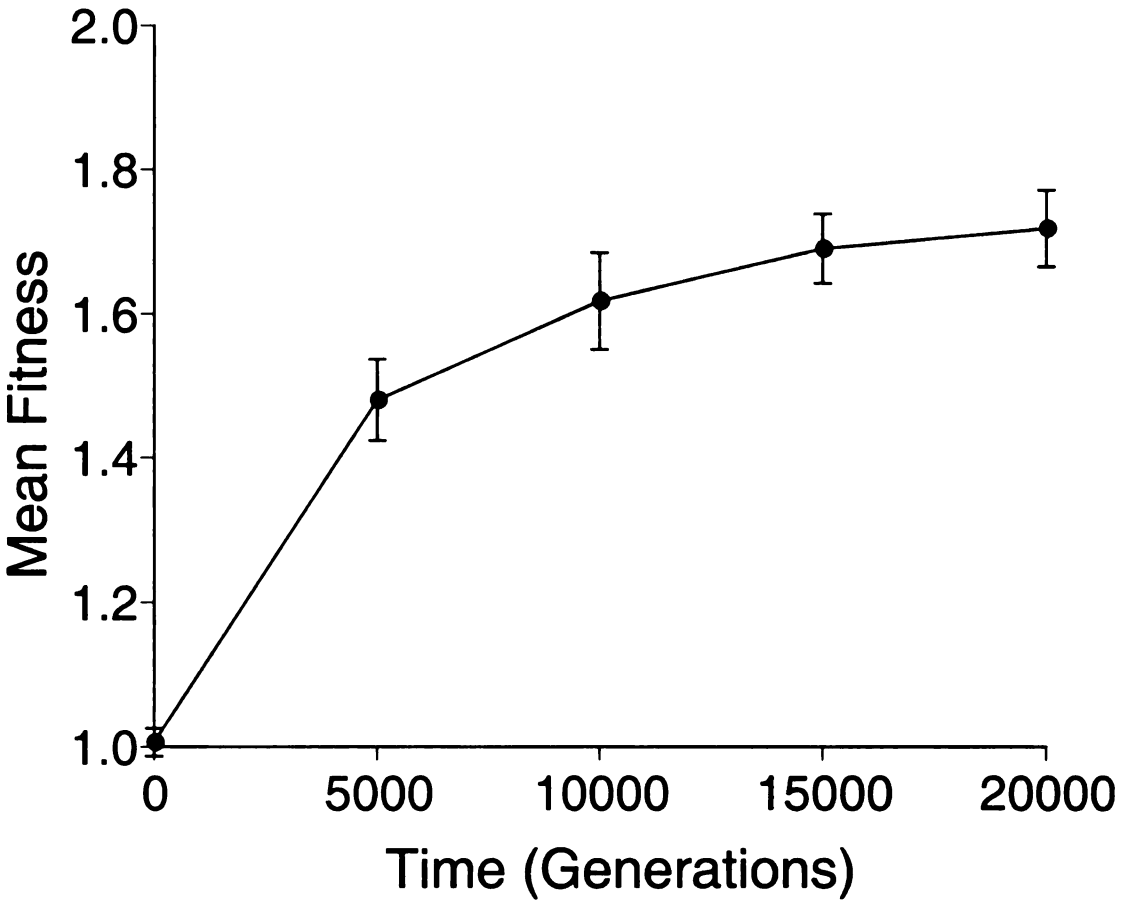

Figure 6. Trajectory for mean fitness of *E. coli* during 20,000 generations in minimal glucose medium. Each point is the mean of all 12 populations, and each population's fitness relative to the ancestor was measured with five-fold replication. Error bars are 95% confidence intervals based on the replicate populations.

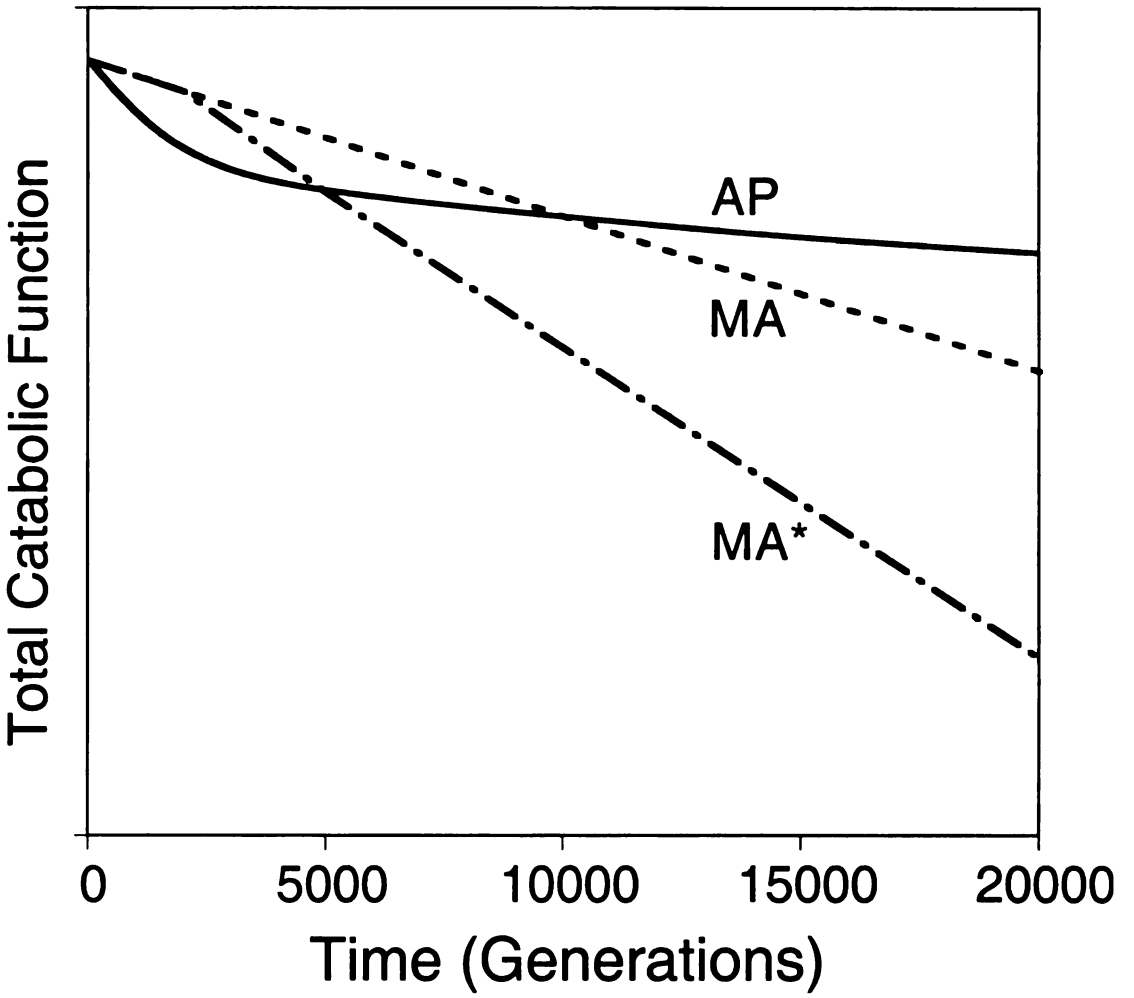

Figure 7. Hypothetical trajectories for the evolution of ecological specialization, as reflected by the decay of unused catabolic functions. Curve AP: antagonistic pleiotropy, in which functional decay is inversely parallel to gains in fitness. Curve MA: mutation accumulation (ancestral mutation rate), in which functional decay occurs at a constant rate and is independent of the pace of adaptation. Curve MA*: mutation accumulation at an accelerated rate that occurs when a population becomes a mutator.

but not effective population size (Kimura 1983). However, three of the 12 lines evolved defects in DNA mismatch repair, which led to genome-wide mutation rates about 100-fold higher than the rates experienced by the ancestor and other lines (Sniegowski et al. 1997). These three lines became mutators around 2,500, 3,000, and 8,500 generations (Sniegowski et al. 1997), respectively, and they retained high mutation rates through generation 20,000 (P. D. Sniegowski, unpublished data); therefore, all three experienced elevated mutation rates for most of the experiment. [A fourth line became a mutator between 16,500 and 18,000 generations (P. D. Sniegowski, unpublished data). Because most of this line's history was at the ancestral mutation rate, it was excluded from statistical comparisons between mutator and non-mutator populations at generation 20,000.] If MA were the primary cause of ecological specialization, then the mutator lines should have evolved much narrower diet breadth than the lines that retained the ancestral mutation rate (Figure 7, curve MA*).

We used Biolog ES plates to measure the catabolic function of the ancestor and three clones isolated from each population at generations 2,000, 10,000 and 20,000. The wells in these plates contain 95 different carbon sources and an indicator dye that reflects the amount of growth on the substrate; 64 substrates were informative for this study. Based on the proportion of genes in *E. coli* that encode catabolic functions (including transport), these plates can resolve mutations in several hundred genes (B. Bochner, personal communication). The weighted average of these 64 phenotypes provides an estimate of total catabolic function, or diet breadth.

Two predictions of AP are that replicate lines should often exhibit parallel decay of the same catabolic functions (those that trade-off with performance on glucose), and

that most losses should occur early (when adaptation to glucose was fastest). To test these predictions, we compared for each substrate the ancestral strain with the 12 evolved populations, as a group, at generations 2,000, 10,000 and 20,000. Because this approach entails multiple comparisons (Miller, 1981), we were very conservative in our statistical criteria; we employed two-tailed *t*-tests, assumed unequal variances for the ancestor and evolved lines, and used 0.0005 as the critical *p*-value for hypothesis testing. A significant test indicates a parallel change in catabolic function that is common to most, if not all, of the lines. Sixteen substrates showed parallel decay in the first 10,000 generations, and there was no further increase in this number over the next 10,000 generations (Table 6). In fact, nine substrates showed parallel decay after only 2,000 generations, at which time none of the populations had yet evolved a mutator phenotype. The findings that there were many parallel reductions of catabolic function, and that these were concentrated early in the evolution experiment, indicate that AP contributed to ecological specialization.

The evidence that AP was important in causing functional decay does not exclude the possibility that MA was also important. To that end, we tested whether the mutator populations accumulated more losses of catabolic function than did the populations that retained the ancestral mutation rate (Figure 8). However, there was no significant difference in the catabolic diet breadth of the two groups after 20,000 generations (t = 0.887, 9 df, 1-tailed p = 0.199). Any difference between these groups in the extent of catabolic decay is small and subtle in comparison with the roughly 100-fold difference in mutation rates that they experienced for most of that time.

Table 6. Statistical summary of parallel changes in catabolic functions, based on comparisons between the evolved populations and common ancestor at three time points. Very stringent significance ($p < 0.0005$) was demanded to account for multiple tests (Miller 1981, 64 substrates tested in each of three generations). Grey fill denotes catabolic functions that consistently decayed; black fill indicates significant gains in function. Each cell also shows the number of replicate populations (out of 12) that grew worse, on average, than the ancestor.

	Time (Generations)		
Carbon source	2,000	10,000	20,000
bromosuccinic acid	7	11	12
D-alanine	1	3	6
D-malic acid	5	12	12
D-ribose	12	12	12
D-saccharic acid	9	11	11
D-serine	12	11	10
D-sorbitol	12	11	11
fructose-6-phosphate	11	10	9
fumaric acid	9	12	12
glucose-1-phosphate	12	11	10
glucose-6-phosphate	11	12	8
glucuronamide	0	4	8
L-asparagine	8	12	12
L-aspartic acid	9	12	12
L-glutamine	12	12	12
L-lactic acid	11	12	10
L-malic acid	7	12	12
malic acid	9	12	12
mono-methylsuccinate	2	12	12
mucic acid	12	8	9
P-hydroxyphenylacetic acid	5	12	11
succinic acid	9	12	12
uridine	12	12	10
Sum of parallel losses	9	16	16

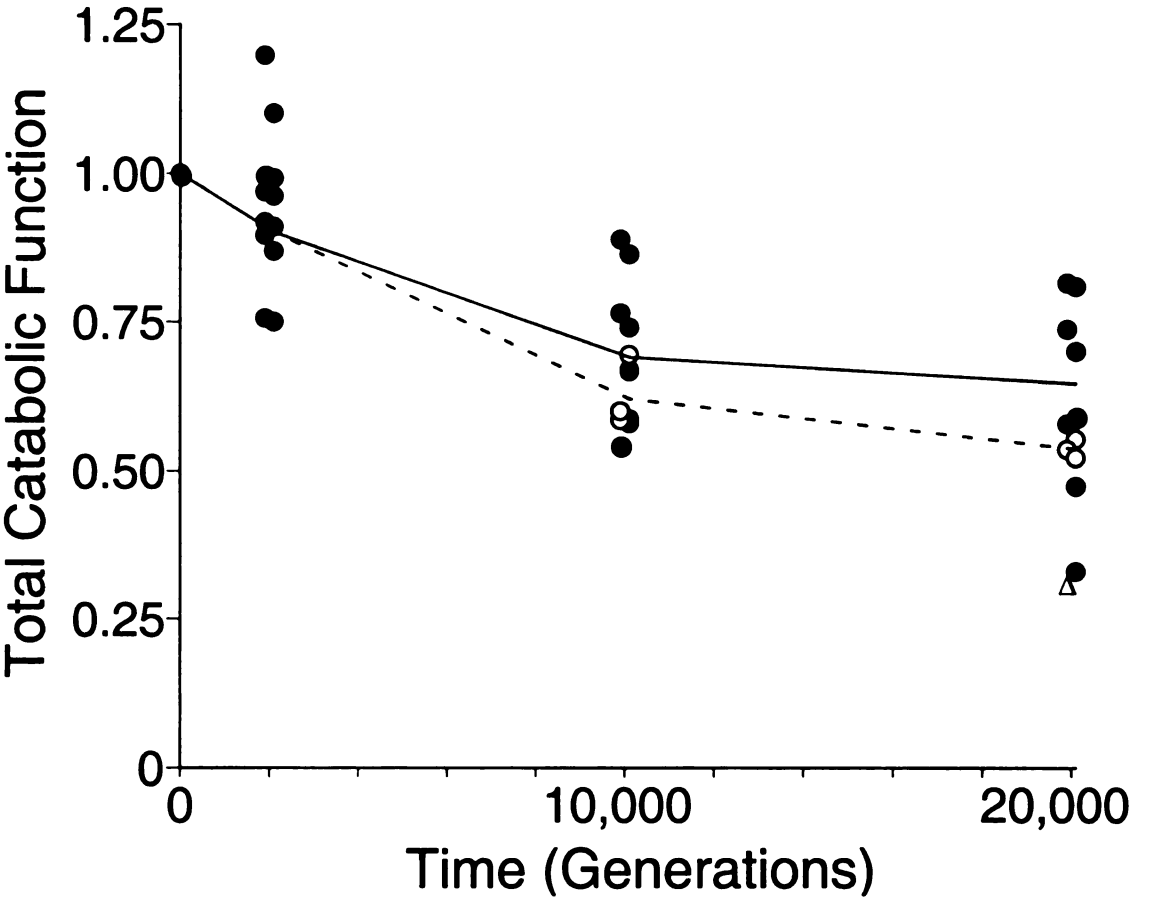

Figure 8. Evolution of total catabolic function, or diet breadth, during 20,000 generations in minimal glucose medium. Total catabolic function is a weighted average across all 64 informative substrates, such that a value of 1.0 is the ancestral level while values less than 1.0 indicate an overall decay of catabolic function. Each point is the mean of three randomly chosen clones from each population. Solid line: mean value of the evolved populations that retained the ancestral mutation rate at that generation; dashed line: corresponding mean of the three mutator populations. Closed circles: populations with the ancestral mutation rate; open circles: mutator populations; open triangle: a late appearing mutator population (excluded from both classes).

On balance, our data indicate that AP was the main contributor to resource specialization that occurred during evolution of *E. coli* lines in minimal glucose medium. That is, the mutations responsible for catabolic decay did not simply accumulate under mutation pressure but were themselves adaptive in the glucose environment. Two recent studies further support this inference. First, we have shown elsewhere that loss of the capacity to use D-ribose, which occurred in all 12 populations during the initial 2,000 generations, resulted from deletion mutations that confer a selective advantage in glucose medium (Chapter IV). Second, Funchain et al. (2000) studied *E. coli* mutator lines that evolved under a regime with severe population bottlenecks, which allowed deleterious mutations to accumulate. In contrast to our study, they found that (i) fitness declined over time, (ii) losses of catabolic function were not concentrated early in the experiment, and (iii) these defects were scattered across catabolic pathways, with parallel losses reflecting hypermutability of certain genes (as opposed to selection for specific losses of function). The large population sizes and intense competition in our experiment would have prevented most deleterious mutations from being fixed as they would be under a severe bottleneck regime.

We note two possible caveats concerning the hypothesized effects of MA and AP. First, we assumed that the amount of AP was equivalent in all lines, which might be invalid if mutator populations had undergone faster adaptive evolution than their less mutable counterparts. Consistent with our assumption, the three mutator lines did not attain significantly higher final fitness than the other eight lines ($t = 1.322$, 9 df, two-tailed $p = 0.219$); nor does the mutation supply limit the rate of adaptive evolution under these circumstances (de Visser et al. 1999). Moreover, most parallel reductions in

catabolic function occurred in the first 2,000 generations (Table 6), before any populations became mutators (Sniegowski et al. 1997). Second, the prediction that unused functions should decay at a constant rate under MA holds true for neutral mutations (Kimura 1983). But some of the decay might reflect deleterious mutations that hitchhiked to fixation with beneficial mutations; during the early period of rapid adaptive evolution, there would have been more opportunities for deleterious mutations to hitchhike, possibly generating a false indication of AP. However, this explanation cannot readily account for the parallel decay of catabolic functions across replicate populations (Table 6). Also, as noted before, we have demonstrated elsewhere that the mutations responsible for the decay of ribose catabolic function were indeed beneficial in the glucose medium (Chapter IV).

In summary, a long-running evolution experiment with bacteria allowed us to distinguish between the effects of two processes that can contribute to ecological specialization, mutation accumulation and antagonistic pleiotropy. Our findings indicate that antagonistic pleiotropy played a greater role in specialization than did mutation accumulation. This conclusion does not contradict theory (Muller 1964, Kondrashov 1988) and experiments (Funchain et al. 2000, Houle et al. 1992, Kibota and Lynch 1996) on the importance of mutation accumulation in populations that become less fit because of high mutation rates, small population sizes, or both. Rather, our work emphasizes the distinction between adaptive evolution, in which unused functions may be lost as a consequence of natural selection for other traits, and nonadaptive evolution, in which deleterious mutations accumulate precisely because selection has become ineffective.

METHODS

Evolution experiment. The design of the long-term evolution experiment has been described in detail elsewhere (Lenski and Travisano 1994). In brief, 12 lines were founded from two variants of *E. coli* B and propagated daily by 1:100 dilution into Davis minimal medium supplemented with glucose at 25 μg/ml. The populations were propagated in this manner for 20,000 cell generations (3,000 days); every 500 generations, samples from each population were stored at –80°C. Six of the evolving populations were founded from a strain that is unable to grow on arabinose (Ara^-), and the other six were founded with a spontaneous Ara^+ mutant; the two ancestors were otherwise isogenic, and the Ara marker itself is neutral in the glucose medium (Lenski et al. 1991).

Fitness assays. The protocol for estimating the competitive fitness of evolved lines relative to the ancestor has been previously described (Lenski et al. 1991). In brief, samples of the evolved lines (containing whatever genetic diversity was present when they were sampled) and ancestral strains were removed from the freezer and separately acclimated to the same medium and culture conditions as used in the evolution experiment. Each evolved line was mixed with an equal volume of the reciprocally marked ancestor, and the two types then grew and competed under the same conditions as prevailed during the evolution experiment. Initial and final densities of the two competitors were enumerated by plating cells on a tetrazolium-arabinose indicator agar that allowed them to be distinguished by the Ara marker. (A different plating procedure was used for competitions with one evolved line that no longer produced distinct colonies

on the indicator agar.) The net growth rate of each competitor was calculated from the data, and the relative fitness of an evolved line is then expressed simply as the ratio of its growth rate to that of the ancestor. Assays were run with five-fold replication (in complete blocks) for all 12 lines.

Biolog assays. Catabolic diet breadth was assayed using Biolog (Hayward, California) ES plates for the two ancestral variants and three clones randomly chosen from each evolved line at generations 2,000, 10,000 and 20,000. Assays were run in three sets, each set comprising all of the clones for four lines plus three replicates of each ancestral variant. The bacteria were grown for two days in LB broth; on the next day, each culture was diluted 1:100 into fresh LB and incubated for 6 h. (LB was used instead of minimal glucose medium to avoid catabolite repression, which depresses other catabolic functions and yields fewer positive readings; Biolog ES Microplate Instructions for Use, 1993.) The cultures were centrifuged at 10,000 rpm for 10 min and resuspended in saline to remove residual medium; this suspension was then used to inoculate each well at a constant density. At 0, 4, 12, 24 and 48 h, optical densities were measured at 590 nm using an automated plate reader, and all measurements were adjusted by subtracting out the reading from the blank well. A trapezoidal area approximation (Guckert et al. 1996) was used to integrate the five measurements for each well into one value, which reflects the area beneath the curve of optical density versus time; this area value is sensitive to both the rate and final level of catabolic function. Of the 95 substrates, glucose and arabinose were excluded *a priori* because glucose was the target of adaptation and arabinose utilization was a marker in the evolution experiment. Another 29 substrates

were excluded because repeated measurements on the ancestor were statistically unreliable (coefficient of variation > 1), leaving 64 informative substrates. To test the evolutionary change of each individual catabolic function, the values for the three clones from each line at a given generation were averaged, and the 12 evolved lines as a group were compared with the two ancestral variants using a two-tailed *t*-test for unequal variances (given divergence among the replicate lines) and a very stringent *p*-value of 0.0005 (to adjust for multiple tests, Miller 1981). Also, for each informative substrate the catabolic function of an evolved clone was standardized to the ancestral value, thus giving equal weight to each substrate, and then log-transformed to give equal weight to proportionally equivalent gains and losses of function. The anti-log of the average of these transformed values then provides a measure of total catabolic function; the ancestral total equals 1.0 (by definition), whereas values less than 1.0 indicate an overall loss of function.

Chapter III

EVOLUTION OF THERMAL PERFORMANCE OF *E. COLI* POPULATIONS DURING 20,000 GENERATIONS IN A CONSTANT ENVIRONMENT

Temperature is one of the most important physical factors that defines a species' fundamental niche. Temperature limits some species' distributions (Stevens & Fox 1991, Hoffmann & Watson 1993, Clarke 1996) because both extreme heat and cold adversely affect metabolic and life-history traits (Hochachka & Somero 1984, Cossins & Bowler 1987, Prosser 1991, Ingraham & Marr 1996). Such effects should lead to optimization of metabolic and demographic traits over the range of temperatures most often experienced by a population; if this range is narrow, then thermal specialists may evolve. This hypothesis presumes an evolutionary tradeoff between capacity to tolerate a broad range of temperatures and peak performance over a narrow range.

Huey & Hertz (1984) posed the question directly: "Is a jack-of-all-temperatures a master of none?" They addressed this issue by examining data on thermal performance of running speed of individuals of two lizard species, and maximum growth rate of several clones of the protozoan *Amoeba*. In both cases, they found that the rank order for performance of individuals within species was nearly constant across temperatures — that is, individuals that excelled at one temperature tended to excel at all temperatures. Huey & Hertz (1984) concluded that there was no tradeoff between peak performance at any given temperature and broad performance across a range of temperatures. However, there are possible limitations to this study, including the fact that the positive correlations

are phenotypic and may not reflect underlying genetic variation. Moreover, lizard performance was quantified using a trait, sprint speed, whose contribution to fitness is unknown; in the case of *Amoeba*, growth rates were measured under conditions that may have been quite different from those which the organisms had previously experienced.

The effects of temperature on bacterial growth have been well studied. *Escherichia coli* can grow over a range of about 40°C; however, important physiological changes (e.g., the heat-shock response) occur over this range that involve differential expression of various proteins (Herendeen et al. 1979, Gross 1996, Ingraham & Marr 1996). Therefore, if plastic responses are hindered by selection for optimum performance at any one temperature, then performance at extreme temperatures may decline. Neidhardt et al. (1990, p. 233) offered a possible mechanism for thermal specialization when they suggested that "a microorganism evolving in the absence of the challenge of elevated temperature would contain very few thermostable proteins."

Laboratory populations of *E. coli* have been employed to study the evolution of thermal performance because of the feasibility of performing long-term selection experiments (Bennett et al. 1992, Bennett & Lenski 1993, Mongold et al. 1996, Mongold et al. 1999). These studies have examined genetic adaptation to various temperatures as well as the correlated effects of this adaptation on performance at other temperatures. In general, experimental populations became better adapted to the temperatures at which they evolved. However, their correlated responses to other temperatures varied depending on their selective temperature and even among the replicate populations maintained at the same selective temperature. For example, populations that evolved at 20°C systematically became inferior competitors at temperatures above about 40°C

(Mongold et al. 1996), whereas most populations that adapted to 42°C did not become inferior competitors at low temperatures but one of them did (Bennett & Lenski 1993). These evolution experiments therefore provide ambiguous support for the existence of tradeoffs in performance across the thermal niche. However, because thermal tolerance varies greatly among taxa, the idea that selection at one temperature will impact performance at other temperatures remains popular.

In this paper, we examine the performance of 12 experimental populations of *E. coli* that have evolved at 37°C for 20,000 generations (Lenski et al. 1991, Lenski & Travisano 1994, Chapter II) in order to test whether they became temperature specialists by losing the capacity to grow as well as their ancestor across a range of temperatures. Our study parallels other research on these same populations that examines whether they became resource specialists during their evolution in a medium in which glucose was their sole source of carbon and energy (Chapter II). By examining performance breadth across a range of temperatures and resources, we test the general theory that evolutionary adaptation to one particular environment leads to loss of performance in alternative environments (Futuyma & Moreno 1988, Holt 1995), and we address whether adaptation to a constant environment similarly affects these two different dimensions of the niche.

The physiological functions that govern thermal performance and resource usage are, of course, quite distinct. Both functions, however, should be subject to the same fundamental population-genetic processes that can lead to specialization: antagonistic pleiotropy (AP) and mutation accumulation (MA). Under AP, adaptation to the selective environment and loss of function in other environments are caused by the exact same mutations, which mediate a tradeoff between performance across environments. Under

MA, adaptation and loss of function are caused by different mutations. Adaptive mutations are fixed by selection but do not themselves cause any loss of function in the other environments; such losses are caused instead by mutations that spread by random drift in genes no longer under positive selection.

These two distinct processes have long been recognized, but their relative contributions have only rarely been examined because they are difficult to disentangle (Rose & Charlesworth 1980, Futuyma & Moreno 1988, Sgrò & Partridge 1999). However, the two processes should lead to different dynamics of functional decay. AP predicts a temporal association between improvement in the selective environment and decay in alternative environments. Thus, if adaptation to a selective environment is initially fast but decelerates over time, then so too should the rate of functional decay be initially more rapid. By contrast, functional decay arising from MA occurs by stochastic drift of neutral alleles; it should therefore occur at a constant rate that depends only on the mutation rate in relevant genes (Kimura 1983). In an asexual population, selection at other loci will cause periodic bottlenecks that influence the dynamic of any particular neutral mutation but not their overall rate of substitution, which is independent of effective population size. If the mutation rate itself should increase (see below), then the rate of MA will increase correspondingly, even if the rate of adaptation is unaffected.

Experimental Overview. The 12 populations studied here are all derived from a single clone of *E. coli* B (Lenski et al. 1991). The populations evolved in and adapted to a constant 37°C environment for 20,000 generations (3,000 days). We isolated three clones at random from each population at five time points (2,000, 5,000, 10,000, 15,000, and 20,000 generations), giving a set of 180 clones (12 populations x 5 time points x 3

clones) plus two ancestral clones that differ only by a neutral marker. We measured the exponential growth rate, V_{max}, of each clone at nine temperatures, ranging from 20°C to 42°C; growth rates were measured in microtiter plates to handle all the clones simultaneously. Exponential growth rate is not the sole component of fitness in the serial transfer regime in which the bacteria evolved, but both theoretical and empirical analyses indicate it is the single most important component (Vasi et al. 1994).

The 12 populations experienced different mutation rates for part of their history, which as noted above has implications for the rate of functional decay expected under MA. Sniegowski et al. (1997) demonstrated that three populations evolved defects in DNA mismatch repair and, as a consequence, had roughly 100-fold higher genomic mutation rates than the other populations or their common ancestor. These populations became "mutators" around 2,500, 3,000 and 8,500 generations (Sniegowski et al. 1997), and all three remained mutators at generation 20,000 (P. D. Sniegowski, unpublished data). Therefore, they experienced substantially elevated mutation rates for the majority of the experiment. A fourth population also became a mutator, but only much later; this mutator was first seen at generation 16,500 and did not become fixed for another 1,500 generations (P. D. Sniegowski, unpublished data). Thus, this line spent most of its history at the ancestral mutation rate.

METHODS

Bacteria. Genetically heterogeneous samples from each experimental population were obtained every 500 generations. From the samples obtained at generations 2,000,

5,000, 10,000, 15,000 and 20,000 generations, aliquots of cells were spread on nutrient-rich agar plates and three clones were randomly chosen from each. The two ancestral variants differ only by a spontaneous mutation that affects arabinose utilization. This mutation was used as a marker in the evolution experiment and in competition assays, where the mutation is selectively neutral (Lenski et al. 1991). Six of the evolving populations were founded from the Ara^- ancestor and six from the Ara^+ variant. The ancestors as well as the genetically heterogeneous samples and clones from the evolving populations were stored frozen at –80°C, where they are available for later analyses.

Assays. Clones were inoculated into 30-ml test tubes containing 10 ml of Davis minimal medium (Lenski et al. 1991) supplemented with 500 μg/ml of glucose (DM500), and the cultures were then incubated for 24 h at 37°C in a shaking incubator. Each culture was then diluted 1:100 into fresh DM500 and incubated for 24 h at the relevant assay temperature, such that all clones were comparably acclimated to the temperature and growth medium in which their exponential growth rates would be measured. The acclimated cultures were diluted 1:100 into fresh DM500, and 250 μl of each new culture was then transferred to one of two 100-well microtiter plates that fit simultaneously into a Bioscreen (Labsystems, Finland). The Bioscreen is a spectrophotometric plate reader that allows precise temperature control and agitates the plate to maintain aeration. A greater nutrient concentration (500 μg glucose/ml) was utilized in these assays than in the selective regime (25 μg glucose/ml) in order to produce concentrations of bacteria that could be easily detected by the growth analyzer. In pilot experiments, the performance of the ancestor was assessed at various well locations to test for site-dependent variation, but none was detected. Six replicates of both ancestral variants were interspersed among the

180 evolved clones at each assay temperature, as were eight blank wells. The optical density (OD) of each well was measured every 10 min for 16 h.

Numerical Analyses. The exponential growth rate, V_{max}, was determined for each clone as follows. The average OD for blank wells was first subtracted from all other values, and each datum was $\log_e$-transformed. The fastest growth generally occurred when $\log_e$-transformed OD values were between –3 and –2 (non-transformed OD between 0.050 and 0.135). V_{max} was then calculated as the slope of the $\log_e$-transformed values over this interval.

Statistical Analyses. We computed the mean V_{max} at a given temperature and generation of the three clones from each population; we also averaged the six replicate measurements for each ancestral variant. A two-tailed t-test was then performed to test whether the 12 evolved populations, on average, grew significantly faster or slower than the two ancestors at a particular temperature. We used Welch's approximate t-test, which assumes unequal variances, because the ancestors are genetically homogeneous whereas the evolved populations have diverged from one another. In principle, one might adjust the significance levels of these tests to reflect the fact that multiple tests were performed across many generations and temperatures. However, we have chosen not to do so because the results across generations are generally consistent with one another, while the results across temperatures indicate that the differences fall consistently into only three distinct thermal ranges: low (20°C), moderate (27-39°C), and high (40-42°C). We also employed Welch's approximate t-test to compare average V_{max} values for the mutator and non-mutator populations at a given temperature and generation. We excluded from the comparisons at generation 20,000 the population that became a mutator late in the

experiment, because of the ambiguity of assigning it to either class with respect to a possible effect of mutation accumulation. The among-population genetic variance was estimated using the VARCOMP procedure in SPSS. All other statistical analyses were also conducted using SPSS.

Note: Images in this chapter are presented in color.

RESULTS

Figure 9 shows the exponential growth rate, V_{max}, of the ancestral *E. coli* as a function of temperature. Figure 10 shows the average exponential growth rate of the evolving populations across generations and temperatures; these averages are all expressed relative to the ancestral value at that temperature.

Growth Rates Improved at Moderate Temperatures. Exponential growth rates tended to increase in the evolving populations at all five assay temperatures from 27 to 39°C (Figure 10). After 20,000 generations, the greatest improvement of the evolved populations relative to the ancestor occurred at 37°C, the temperature that prevailed during the evolution experiment; however, the populations had improved by almost as much at slightly lower and slightly higher temperatures (Figure 10). The vast majority of statistical comparisons between the evolved populations, as a group, with the ancestors were significant at $P < 0.05$ across this range of temperatures and at each generation sampled (Table 7). Most of these correlated improvements in growth rate arose during the first 5,000 generations of evolution, as indicated by the overall flattening of the upper curves in Figure 10.

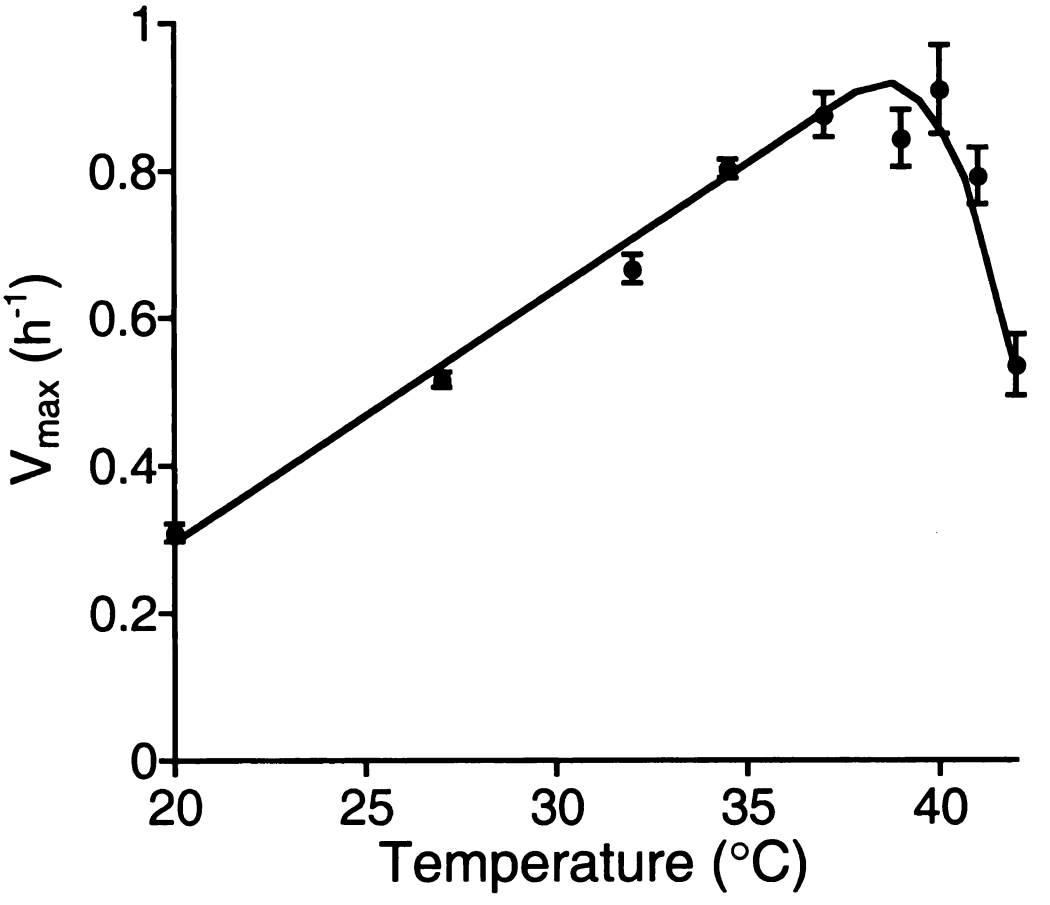

Figure 9. Exponential growth rate, V_{max}, of the ancestral *E. coli* strain as a function of temperature. Error bars represent 95% confidence intervals, based on 12-fold replication (six assays for each of two neutrally marked ancestral variants). The curve was drawn by eye to capture overall shape.

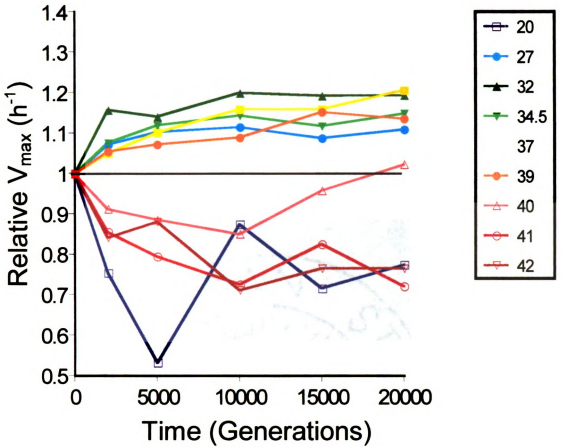

Figure 10. Evolution of exponential growth rate, V_{max}, scaled relative to the ancestor, measured at nine different temperatures (see boxed legend). Each point is the grand mean of 12 replicate populations; each population is the mean of three clones.

Table 7. Statistical summary of the effects of evolution at 37°C on growth rates measured at nine temperatures. For each generation and temperature, the mean of the 12 replicate populations was compared with the ancestral mean using a two-tailed t-test that assumes unequal variances. Grey fill indicates that the evolved populations, as a group, grew significantly more slowly than the ancestor ($P < 0.05$). Black fill indicates the evolved populations, as a group, grew significantly faster than the ancestor ($P < 0.05$). Each cell also shows the number of replicate populations (out of 12) that grew faster, on average, than did the ancestor.

	Generations				
Temperature (°C)	2,000	5,000	10,000	15,000	20,000
20	5	3	3	5	3
27	12	11	11	11	11
32	12	12	12	12	12
34.5	12	12	12	11	12
37	11	12	12	12	12
39	11	12	10	12	11
40	3	4	6	7	8
41	4	4	6	6	5
42	6	6	5	6	5

Growth Rates Decayed at Extreme Temperatures. Exponential growth rates measured at more extreme temperatures, both low (20°C) and high (41 and 42°C), tended to decline in many of the evolving populations (Table 7). As with the gains seen at moderate temperature, most of the reductions in V_{max} at the thermal extremes occurred in the first few thousand generations (Figure 10). The decay at 20°C was significant at $P < 0.05$ in three of the five generations sampled, and the decay at 41°C was significant at two time points (Table 7). At each extreme temperature, three or more of the evolved populations performed better than the ancestor over time (Table 7), but these populations were only marginally better than the ancestor and affected the evolved mean less than the more substantial decay in other populations. Although the proportional decay measured at 42°C was similar in magnitude to that seen at 41°C, the difference at the higher temperature was marginally non-significant in each generation. As shown in the next section, the inconsistency of the decay at extreme temperatures — in contrast to the consistency of the improvements at moderate temperatures — reflects greater genetic variation among populations at the extreme temperatures.

More Genetic Variance among Evolved Populations at Extreme Temperatures. We estimated the among-population genetic variance at each time point and temperature assayed (Figure 11). The U-shaped pattern indicates that the replicate evolved populations generally had more similar growth rates at moderate temperatures, with genetic variation in their performance increasing at extreme temperatures, both low and high.

The greater variance among the evolved populations at extreme temperatures resulted, in large part, from certain clones that failed to grow (i.e., never reached the OD

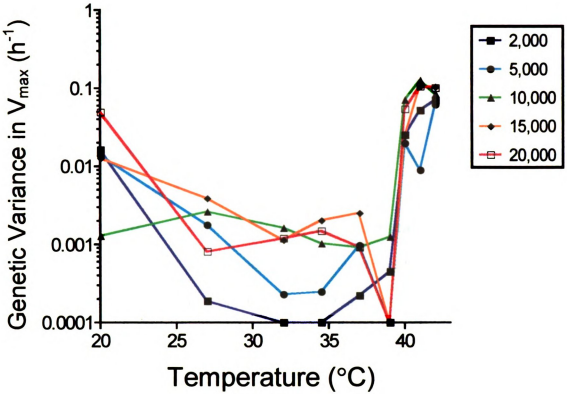

Figure 11. Genetic variance among populations in V_{max} measured across temperatures. The different lines and symbols represent variances estimated in different generations (see boxed legend). Note the logarithmic scale for the vertical axis. Estimated variances ≤0.0001 are shown as 0.0001. The among-population genetic variance component was estimated as the variance among replicate populations in excess of the average clonal variance within each population (Sokal & Rohlf 1981).

range over which V_{max} was measured). In some cases, only one or two of the three clones in a particular sample failed to grow; we cannot say whether this clonal variation reflects within-population genetic variation or simply experimental noise, because we assayed each clone only once per temperature. However, there were 13 cases in which all three clones from an evolved population failed to grow, and all of these cases occurred at extreme temperatures (20, 41, or 42°C).

Spurious Difference between Mutator and Non-mutator Populations at Extreme High Temperatures. We compared growth rates of the mutator and non-mutator populations in order to examine the possible impact of increased mutation rate, and hence MA, on the decay of thermal performance. We used the 20,000-generation samples, because this time point should provide the maximum signal associated with MA in the three populations that became mutators between 2,000 and 10,000 generations. The mutators performed significantly worse than the non-mutators did at 40, 41, and 42°C (Figure 12a). The mutators were not significantly worse than the non-mutators at any other temperature tested (Figure 12), including the low extreme of 20°C (two-tailed P = 0.463). However, the deficiency of the mutator populations at high temperatures cannot be attributed to their mutator status per se, because the growth-rate differences between the three populations that would become mutators and the other nine populations had already arisen by generation 2,000 (Figure 12b), prior to the evolution of the mutator genotype in any of the evolved lines. Therefore, the association between mutator populations and reduced performance at high temperatures appears to be spurious rather than indicating any direct cause and effect.

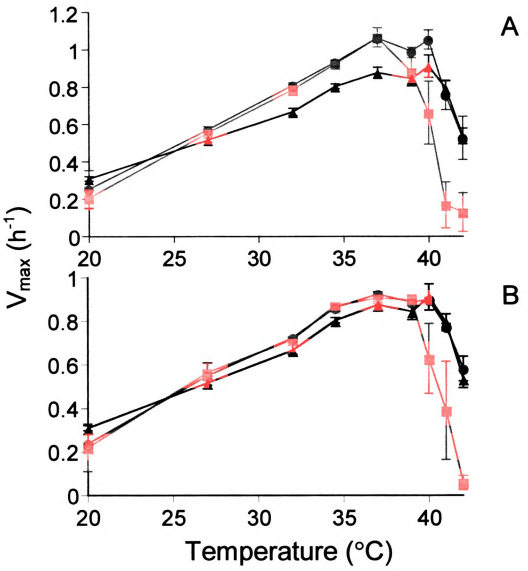

Figure 12. Differences in V_{max} between mutator and non-mutator populations from generation 20,000 across a range of temperatures. Black triangles: ancestral mean. Blue circles: mean of evolved populations that retained the ancestral mutation rate. Red squares: mean of evolved populations that became mutators. Error bars are standard errors. (A) Data in generation 20,000. (B) Data from generation 2,000, which was prior to the evolution of the mutators.

DISCUSSION

We have studied genetic changes in thermal performance, specifically exponential growth rate, of *Escherichia coli* populations during 20,000 generations of experimental evolution in a defined medium at constant 37°C. Our main results can be summarized as follows. (i) Populations showed consistent improvement at moderate temperatures, ranging from 27°C to 39°C. (ii) Most of the improvement at these moderate temperatures occurred during the initial period of rapid adaptation. (iii) Some populations tended to decay in performance at thermal extremes, both low and high. (iv) Most of this decay also occurred during the initial period of rapid adaptation. (v) There was more genetic variation among the replicate populations in their performance at both thermal extremes than at moderate temperatures. In some populations, there was no measurable growth at one of the extreme temperatures. (vi) There appeared to be some association between the loss of performance at high temperature and the evolution of an elevated mutation rate, which occurred in several populations. However, this association is spurious because the loss of high-temperature performance was already manifest in these same populations even before they became mutators.

These results support, in broad outline, the hypothesis that adaptation to a constant thermal environment leads to temperature specialists, with reduced performance at extreme temperatures but enhanced performance at intermediate temperatures. Beyond that, one might be surprised by how little (or much) performance was lost at the thermal extremes, and by the breadth of temperatures over which the correlated gains occurred. These quantitative details presumably depend on the underlying physiology of the organism as well as the evolutionary duration of the environmental constancy. We would

presumably have observed deeper and more frequent losses at the thermal extremes, and perhaps losses over a wider range of temperatures, if our evolution experiment had extended for 20,000,000 generations instead of a mere 20,000 generations.

Let us now consider the population-genetic processes that can best explain the observed pattern of thermal performance in these bacterial populations. A fundamental issue in population genetics — but one that remains poorly resolved — is whether the same beneficial mutations that enhance performance in the selective environment (here, 37°C) are, through their pleiotropic effects, also responsible for the changes in performance in "foreign" environments (here, all other temperatures). Alternatively, other mutations that were neutral in the selective environment may have accumulated by drift, and these other mutations could be responsible for the changes in performance in the foreign environments.

Focusing first on the correlated improvement in performance at moderate temperatures, it seems obvious that these must be synergistic pleiotropic effects of the mutations that enhanced growth at 37°C. In any environment, it is generally accepted that many more mutations are deleterious than are advantageous. If the random accumulation of mutations that are neutral at 37°C were responsible for the performance gains at other moderate temperatures, then we would have to suppose that many more unselected mutations were beneficial than deleterious in these foreign environments. It is probable that many of the mutations that adapt the bacteria to 37°C also improve performance at somewhat higher and lower temperatures as well. Exactly how far in either direction this positive correlation extends may depend, as noted above, on physiological details and evolutionary circumstances. In addition to this intuitive

reasoning, three lines of evidence also support the conclusion that the performance gains at moderate foreign temperatures were pleiotropic effects of the mutations that enhanced fitness at 37°C. First, the magnitude of improvement in moderate foreign environments, especially between 32 and 39°C, was quite similar to the improvement at 37°C itself (Figure 10). Second, the gains at moderate temperatures were temporally correlated with the genetic adaptation; that is, these gains tended to occur early in the evolution experiment, as did the adaptation to 37°C (Figure 10). Third, the low genetic variance among populations in their performance at moderate temperatures (Figure 4) is consistent with parallel selection on the traits that underlie performance in this thermal range.

We now turn to the question of whether antagonistic pleiotropy (AP) or mutation accumulation (MA) better explains the observed losses of performance at the thermal extremes. Recall that AP means that the same mutations that improved performance at 37°C (and other moderate temperatures) caused these losses, whereas MA means that these losses were caused by other mutations that accumulated in the evolving populations by drift. Unlike the improvements at moderate temperatures, it is not easy to dismiss either possibility on intuitive grounds, and we must instead use quantitative reasoning. Under AP, one would expect more losses to occur while adaptive evolution is faster than when it is slower, because more beneficial substitutions should translate into greater losses. In contrast, under MA, losses should occur stochastically at a constant rate that depends only on the underlying mutation rate, and should not be correlated with either selection or population size (Kimura 1983).

One might think that drift substitution of neutral mutations cannot occur in populations containing millions of individuals, because the expected number of

generations for fixation by drift is of the same order of magnitude as the population size (Kimura 1983). However, in asexual populations, such as those studied here, each selective sweep creates a bottleneck of a single individual, and therefore the effective population size with respect to this process is fairly small. In effect, each selective sweep creates an opportunity for a neutral mutation to drift very quickly to fixation by hitchhiking, just as that same sweep purges the vast majority of neutral mutations already in the population. The remarkable feature of neutral theory is that the expected overall rate of neutral substitutions is independent of effective population size. Thus, fluctuations caused by selective sweeps should not influence the overall dynamic of decay, even though they radically affect the trajectory of any particular neutral mutation.

Turning back to the interpretation of our experimental data, we have shown previously, by competing ancestral and derived bacteria, that the rate of fitness increase during the first few thousand generations was an order of magnitude faster than during the later generations of this experiment (Lenski & Travisano 1994; Chapter II). The losses of function at extreme temperatures also generally occurred early in the experiment (Figure 10), a temporal association consistent with AP but not MA. At 20°C, the average growth rate declined by 25% (from 0.310 to 0.234 per h) by generation 2,000 but did not decline further over the next 18,000 generations. At 42°C, the average growth rate declined by 16% (from 0.536 to 0.451 per h) in the first 2,000 generations but declined only 9% more (from 0.451 to 0.410 per h) between 2,000 and 20,000 generations.

AP and MA are not mutually exclusive, and therefore evidence in support of AP does not exclude the possibility that MA might also contribute to the evolution of specialization. If MA were a significant force, then its effect should have been much

greater in those populations that acquired defects in DNA repair and thus became mutators. Three populations evolved into mutators before generation 10,000, leaving more than 10,000 subsequent generations for their higher mutation rates to generate mutations that harmed performance at the thermal extremes (but which were neutral at 37°C). At generation 20,000 the mutator populations grew more slowly at high temperatures (40-42°C) than did the non-mutators (Figure 12a), seemingly consistent with a role for MA. However, on closer examination, the difference between the two sets of populations is spurious, because decreased growth rates were already manifest at generation 2,000 (Figure 12b), before any of the populations had become mutators (Sniegowski et al. 1997). In other words, the three populations that would later become mutators evolved lower growth rates at high temperatures before they evolved into mutators. The statistical association thus appears to be a false positive, although we cannot exclude the possibility that loss of high-temperature performance somehow predisposes a population to lose its DNA repair at a later time. In any case, these early losses of performance at high temperature cannot be explained by additional MA in mutators. Instead, they support AP as the main population-genetic process responsible for specialization, because they reinforce the observation that most of the decay at high temperatures occurred during the period of most rapid adaptation.

The high among-population variance in growth rate at extreme temperatures indicates an absence of selection for maintaining performance in these environments (Figure 11). This finding is consistent with either MA or AP, and thus provides no compelling evidence with respect to their relative influence. According to MA, divergence of replicate populations would indicate random drift. According to AP, this

divergence would imply heterogeneity among beneficial mutations selected at 37°C with respect to their pleiotropic effects on performance at the thermal extremes. Without isolating the particular mutations that produced adaptation to 37°C, we cannot exclude either explanation for the among-population variance in performance at the extreme temperatures. However, as discussed above, the dynamics of decay indicate that AP was primarily responsible for the losses of performance at the thermal extremes.

In conclusion, our findings show that the bacteria tended to become thermal specialists, relative to their ancestor, during 20,000 generations of evolution at a constant temperature. In addition, the data indicate that antagonistic pleiotropy — in which mutations that improved performance at moderate temperatures were deleterious at extreme temperatures — was the main population-genetic force leading to ecological specialization. Both of these conclusions run parallel to, and thus reinforce, findings from a recent study of the evolution of catabolic function in these same *E. coli* populations, which found that unused catabolic functions tended to decline and that antagonistic pleiotropy was primarily responsible for catabolic specialization (Chapter II).

Chapter IV

MECHANISMS CAUSING RAPID AND PARALLEL LOSSES OF RIBOSE CATABOLISM IN EVOLVING POPULATIONS OF *E. COLI* B

We have been studying the dynamics of phenotypic and genomic changes in 12 populations of *Escherichia coli* B while they have evolved in, and adapted to, a minimal glucose medium for more than 10,000 generations (Lenski et al. 1991, Lenski and Travisano 1994, Papadopoulos et al. 1999, Schneider et al. submitted, Sniegowski et al. 1997, Travisano et al. 1995). As part of this research, the performance of the derived lines and the common ancestor was quantified on a large set of diverse substrates, to determine if losses of catabolic function accumulated during evolution in an environment in which glucose was the sole carbon source (Chapter I). We observed a general decline in catabolic function, which was largely attributable to subtle reductions in function on several carbon sources. However, absolute losses of function were very rare, with one conspicuous exception: all 12 populations lost the ancestral ability to catabolize D-ribose. In this paper, we examine the mechanisms responsible for these parallel losses, including both population genetic processes and the underlying molecular genetic events.

Parallel evolution of a trait across multiple lineages is often used as an indicator that the change is adaptive and has been shaped by natural selection (Cunningham et al. 1997, Harvey and Pagel 1991, Huey et al. 2000, Nakatsu et al. 1998, Simpson 1953). Therefore, we hypothesized that the loss of the ability to use ribose may have improved fitness in the glucose-limited environment. The alternative explanation – that mutations

accumulated in genes affecting ribose function, but without those mutations enhancing fitness on glucose – seemed less likely on two grounds. First, other catabolic functions also experienced relaxed selection but did not exhibit such losses. Second, given typical mutation rates and the duration of the evolution experiment (Drake 1991, Travisano et al. 1995), we would expect the accumulation of neutral mutations to cause sporadic but not parallel losses of function in the replicate populations.

In this paper, we report experiments that examine: (i) the phenotypic dynamics of loss of ribose function in each population; (ii) the underlying mutation rate from Rbs^+ to Rbs^-; (iii) the affected loci and mutational mechanism responsible for the losses of ribose function; and (iv) the effects of the losses on competitive fitness in glucose medium. We then use these data to examine quantitatively the contributions of mutation and selection to the evolutionary dynamics that drive this systematic loss of function.

METHODS

Long-term evolution experiment and culture media. The design of the long-term evolution experiment is described in detail elsewhere (Lenski et al. 1991). Briefly, 12 populations evolved from a single clone of *E. coli* B (ref. 16: strain Bc251 *T6-r str-r* rm_{111} ara^-; see also http://myxo.css.msu.edu/~dlenski/strainsource.html). This ancestor is unable to grow on arabinose (Ara^-) and was used to found six lines; the other six lines were founded with a spontaneous Ara^+ mutant of the ancestor. The Ara marker is neutral in the experimental environment (Lenski et al. 1991), which consists of daily 1:100 dilution into fresh Davis minimal broth supplemented with limiting glucose at 25 μg/ml. The 100-fold dilution and re-growth allows ~6.6 (= $\log_2 100$) generations per day. The

12 populations have now been evolving under the same conditions for more than 20,000 generations (3000 days); the experiments described here focus on the first 2,000 generations, during which time all 12 populations lost catabolic function on D-ribose. Every 500 generations, samples from the populations were stored in glycerol at –80°C, where they are available for study at any time.

Two other media used in this study are tetrazolium-ribose (TR) indicator agar and minimal-ribose (MR) selective agar. Rbs^+ and Rbs^- cells produce pink and red colonies, respectively, on TR agar, while only Rbs^+ cells can form colonies on MR agar.

Phenotypic screening. The loss of D-ribose function was initially discovered in experiments with Biolog™ microtiter plates used to detect changes in catabolic functions during the evolution experiment (Chapter I). These plates contain different carbon sources in each of 95 wells plus a tetrazolium indicator dye. Three clones from each of the 12 population samples stored at generation 2,000 were evaluated using these plates; all but one of the 36 clones (the exception being a clone from population Ara+2) showed virtually zero growth after 48 h in the well containing D-ribose (Chapter I).

To characterize the dynamics of the loss of ribose catabolic function, samples of each of the evolving populations from generations 500, 1,000 and 2,000 were spread on TR indicator agar, which allowed the relative frequency of the ancestral Rbs^+ and derived Rbs^- types to be estimated with greater precision. The declining frequency of the Rbs^+ type was confirmed by plating samples onto MR agar.

Direct measurement of the mutation rate from Rbs^+ to Rbs^-. In preliminary experiments, we observed that the ancestral Rbs^+ strain generated Rbs^- mutants at a high rate. We then performed a Luria-Delbrück fluctuation test (Luria and Delbrück 1943) to

obtain the distribution of spontaneous mutations among 56 replicate cultures. Independent cultures were each founded from a small number (~50) of cells of the Rbs^+ ancestor in flasks containing 10 ml of the same glucose-supplemented Davis minimal medium. After 24 h, each culture was diluted, several hundred cells were spread on TR agar, and the numbers of Rbs^+ and Rbs^- colonies counted. These data yielded the total number of cells and the number of Rbs^- mutants in each culture. We then performed iterative numerical calculations to estimate the mutation rate from Rbs^+ to Rbs^- consistent with these data; further details are provided with the Results.

Fitness assays. The protocol used for estimating the relative fitness of two strains during direct competition is described in detail elsewhere (Lenski et al. 1991). In this study, we estimated the fitness of seven independently isolated Rbs^- mutants, each relative to the Rbs^+ ancestor. Equal culture volumes of the mutant and the ancestor were mixed and diluted in a flask containing fresh medium. Initial and final densities of each competitor were enumerated by plating onto indicator agar. Fitness is expressed as the ratio of the realized population growth rates for each type as they compete for the common pool of nutrients.

DNA handling. Genomic DNA was prepared from 3 ml cultures using standard methods (Sambrook et al. 1989). DNA fragments used as probes were cold-labeled, and hybridizations were performed with the DIG-labeling and detection kit sold by Roche. All hybridizations and washings were performed at 68°C under high stringency conditions.

PCR experiments. The sequences adjacent to IS*150* were cloned from *Hinc*II-digested genomic DNA of the ancestor by inverse PCR. Genomic DNA of the ancestor

was digested with *Hin*cII and the resulting fragments were separated on a 0.8% agarose gel with *Pst*I- and *Hin*dIII-digested lambda DNA used as size markers. The gel fraction containing the expected IS element was cut, and DNA was purified using a modification of the Geneclean™ kit of Bio101 (Boyle and Lew 1995). The fragments were self-ligated with T4 DNA ligase (Roche) at 5 to 10 μg/ml, and the ligated mixtures were used as templates in PCR experiments. Primers used for inverse PCR to amplify sequences adjacent to IS*150* were G5: 5' GAT CCT GTA ACC ATC ATC AG 3', and G6: 5' CTG AAG GAT GCT GTT ACG G 3'. Both sequences lie near the IS*150* extremities and are directed outward. All PCR reactions were performed with Expand Taq DNA polymerase (Roche) according to the manufacturer's recommendations. The PCR product containing the IS*150*-adjacent sequences was cloned into the pCRII-Topo vector (Invitrogen). After transformation of *E. coli* TOP10 competent cells (Invitrogen), the plasmid content of white colonies was digested with *Eco*RI and *Hin*cII, giving rise to three fragments. One fragment contained only vector sequence, while each of the others contained one of the two IS*150*-adjacent sequences. The pCRII-Topo vector contains no *Hin*cII and two *Eco*RI restriction sites, which are located on either side of the inserted DNA in the multiple cloning sites. The inverse PCR was performed with *Hin*cII-digested DNA, such that a single *Hin*cII restriction site was present in the PCR product; in this particular case, there were no *Eco*RI restriction sites in the PCR product. The two IS*150*-adjacent sequences were used as probes in subsequent hybridization experiments and they were also sequenced.

PCR reactions to characterize mutations in the ribose operon were also performed using the Expand Taq polymerase (Roche) according to manufacturer's

recommendations. The primers were G76: 5' TGC CGG ATG ATG GAA ACC TC 3', and G77: 5' GAT GGC CTT CTT CAT GCA GG 3'. Sequences were obtained following the method of Sanger *et al.* (1977) with an ABI automated sequencer. Sequences of the different PCR products were obtained using primers G5 and G6. Sequences were compared with the databases using the BLAST program (Altschul et al. 1997).

RESULTS

Differences in ribose utilization in *E. coli* K-12 and B strains. *E. coli* K-12 is able to catabolize D-ribose, and it has a single functional *rbs* operon located at 83 min on the chromosome (Lin 1996). The particular strain of *E. coli* B with we began the long-term evolution experiment is also able to grow on ribose. However, other widely used strains of *E. coli* B are unable to catabolize ribose and have two defective *rbs* operons located at 2 and 83 min (Abou-Sabé et al. 1982, Lin 1996). Possible explanations for the differences between *E. coli* B strains in ribose function will be considered in the Discussion.

Ribose catabolism is rapidly lost from evolving populations. After only 500 generations, Rbs^- mutants had taken over seven of the 12 populations, and by generation 2,000 the Rbs^- phenotype had become fixed, or nearly so, in all 12 of the populations (Figure 13). Previous studies indicated that several beneficial mutations swept through each of these populations in this interval, and these mutations conferred fitness gains of about 10% each (Lenski et al. 1991, Lenski and Travisano 1994). The rapidity and reproducibility with which the losses of ribose catabolic function arose suggested to us

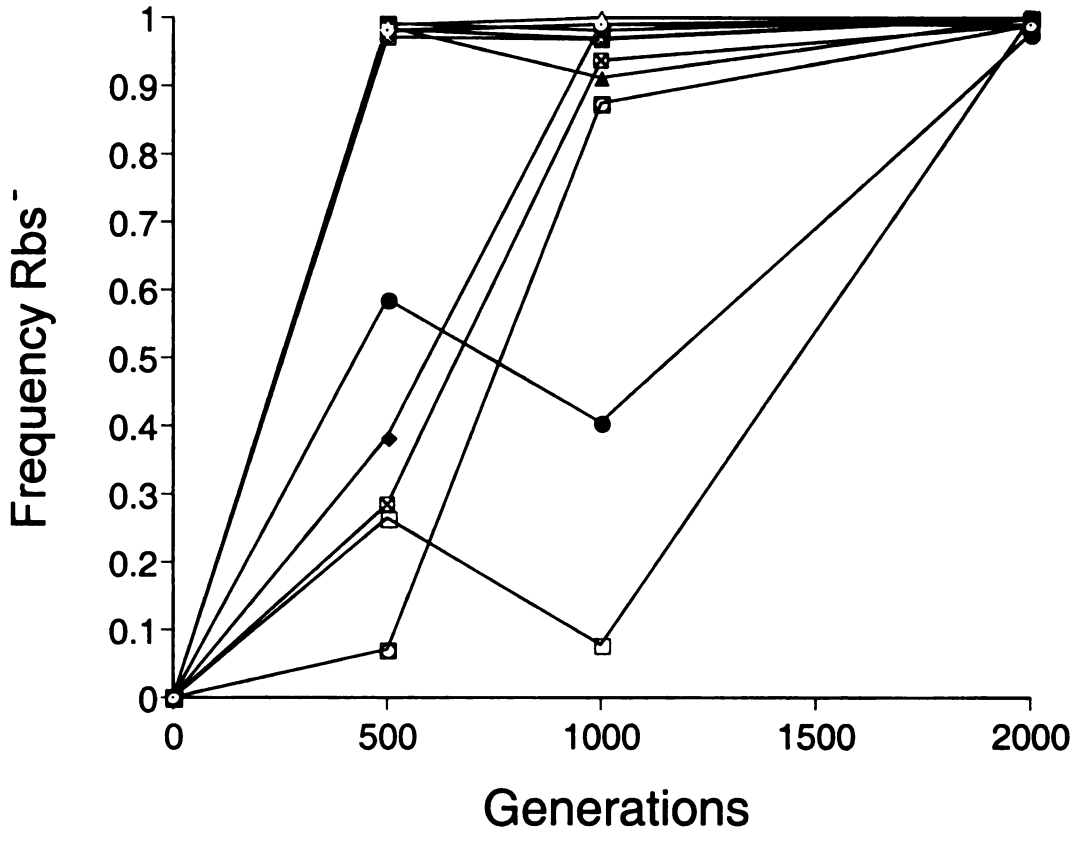

Figure 13. Frequency of Rbs^- cells over time in the 12 evolving populations. At generation 2,000 all the populations contained between 97 and 100% Rbs^- cells.

that the Rbs^- mutants might therefore represent one of these strongly beneficial mutations.

Mutation rate from Rbs^+ to Rbs^- is extremely high. An alternative explanation for the rapid parallel losses of ribose function is that this character is genetically unstable. This alternative was first suggested to us by the unexpected finding that spontaneous Rbs^- mutants were observed when several hundred ancestral cells were plated on TR indicator agar. This observation led us then to perform a fluctuation test to measure the ancestor's rate of mutation from Rbs^+ to Rbs^-. Among 56 independent cultures (each inoculated from ~50 cells), the final average total density was 4.2×10^8 cells and the average frequency of Rbs^- mutants was 0.000512. We then ran numerical simulations of growing and mutating populations to solve for the mutation rate consistent with these data, employing several slight variations in procedures (Appendix A). First, we allowed mutations to accumulate and replicate continuously from the outset, such that even a fractional mutant was able to replicate; this procedure gave an estimated mutation rate of 4.5×10^{-5} per cell per generation. Second, we imposed a constraint such that mutations would accumulate from the outset but could replicate only after the median time to the first appearance of a mutant; this more realistic procedure yielded an estimated mutation rate of 6.0×10^{-5} per cell per generation. Third, we repeated the second procedure but now taking into account the fitness advantage that we measured for the Rbs^- mutants over their Rbs^+ progenitor (see below). This procedure gave a mutation rate from Rbs^+ to Rbs^- of 5.4×10^{-5} per cell per generation, which is our best estimate of the true rate. For a more explicit description of these calculations, see Appendix A.

In *E. coli*, the typical mutation rate per base pair has been estimated to be about 5 $\times 10^{-10}$ (Drake 1991). The entire *rbs* operon is approximately 7000 base pairs; extrapolating this mutation rate to the operon as a whole yields a mutation rate of about 3.5×10^{-6}, and only a small fraction of these point mutations would be expected to knock-out ribose catabolic function. Evidently, the actual mutation rate from Rbs^+ to Rbs^- is unusually high, which implies that an unusual mutational mechanism affects one or more of the loci required for D-ribose catabolism.

Rbs^- mutants are slightly more fit than the ancestor. Each of the experimental populations carries many mutations (Papadopoulos et al. 1999, Schneider et al. submitted), including several that are beneficial (Lenski et al. 1991, Lenski and Travisano 1994). It would therefore be inappropriate to use genotypes from these populations to measure the effect of the Rbs^- mutation on fitness, as these genotypes contain other confounding mutations. Hence, we ran competition experiments, with five-fold replication, between seven spontaneous Rbs^- mutants and the Rbs^+ ancestor to quantify the effects of the Rbs^- mutations on competitive fitness in the glucose minimal medium. All seven Rbs^- mutants were more fit than the Rbs^+ ancestor (Figure 14), with an average advantage of about 1.4% ± 0.4% (95% confidence interval). There was no discernible heterogeneity among the independent mutants (one-way ANOVA: $F = 1.492$, 6 and 28 degrees of freedom, $P = 0.217$), indicating that they all have similar effects on fitness. Loss of D-ribose catabolic function thus confers a small, but consistent, selective advantage in the environment that prevailed during the evolution experiment. Evidently, both selection for loss of function and a high underlying mutation rate contributed to the rapid and parallel evolution of the Rbs^- phenotype in the 12 experimental populations.

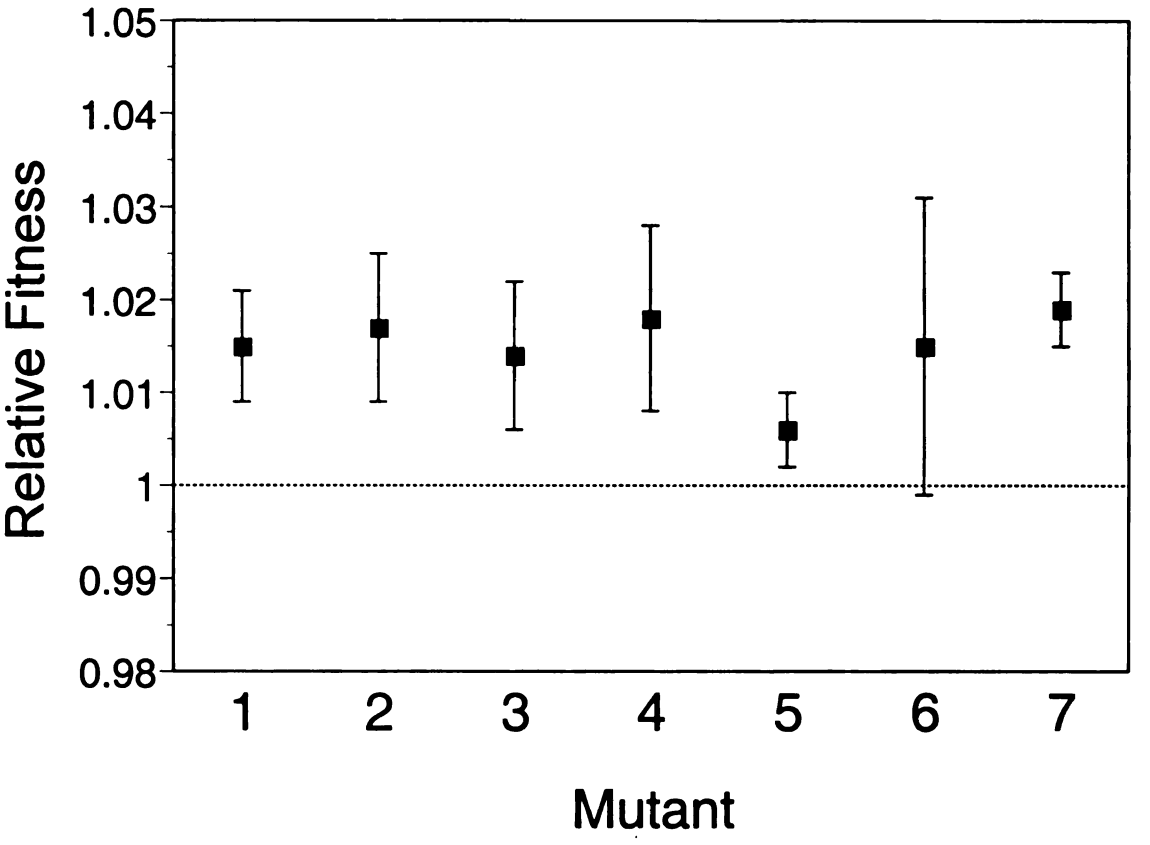

Figure 14. Fitnesses of seven spontaneous Rbs^- mutants relative to their progenitor estimated from competition experiments. Error bars are 95% confidence intervals, based on five-fold replication for each mutant. There was no significant heterogeneity among the mutants (see text for details).

An IS*150* element adjacent to the *rbs* operon mediated deletions during the evolution experiment. Given the unusually high mutation rate from Rbs^+ to Rbs^-, we sought to identify the loci affected and molecular basis of the mutations that were fixed during the experimental evolution. RFLP analysis (Papadopoulos et al. 1999, Schneider et al. submitted) of *Hin*cII-digested genomic DNA from the ancestor and from clones sampled at generation 10,000 using IS*150* as a probe revealed parallel losses in all 12 populations of a 2.7-kb fragment that contained an IS*150* element. The regions adjacent to this element were cloned from *Hin*cII-digested genomic DNA of the ancestor and then sequenced. The IS*150* element was found to be located immediately upstream of the *rbs* operon (Iida et al. 1984) that is involved in ribose utilization (Figure 15). The insertion itself led to a 3-bp duplication of the target site.

The two sequences adjacent to IS*150* were then used as DIG-labeled probes with *Hin*cII-digested genomic DNA isolated from one clone randomly chosen from each of the 12 evolving populations at generation 2,000. Eleven of the 12 evolved clones were Rbs^-, while the clone from population Ara+2 happened to belong to the small Rbs^+ minority (~1.5%) still present in that population. The control hybridization with *Hin*cII-digested genomic DNA of the ancestor revealed, as expected, a 2.7-kb signal using both probes. The same pattern was also observed in hybridizations with genomic DNA from the one evolved Rbs^+ clone. In hybridizations with all 11 Rbs^- evolved clones, the left IS*150*-adjacent sequence hybridized with one *Hin*cII fragment, which ranged in size from 2.0 kb to 4.2 kb; no hybridization signal was detected with these clones using the right IS*150*-adjacent sequence as a probe (Figure 15). These results indicated the presence of deletions affecting at least part of the *rbs* operon in the Rbs^- clones.

To characterize the extent of the deletion in the 11 Rbs$^-$ clones, the endpoints were PCR-amplified using two primers, one located upstream of the IS*150* insertion, and one at the beginning of *yieO*, a gene of unknown function located immediately downstream of the *rbs* operon (Figure 15). PCR products ranging from 2.1 kb to 7.4 kb were obtained depending on the clone, and these were sequenced using primers G5 and G6. In each case, ~700 bp of sequence were obtained with each primer, and comparison with the *E. coli* K-12 genome sequence (Blattner et al. 1997) allowed precise mapping of the deletion endpoints in or near the *rbs* operon (Figure 15). In all cases, the left end of the deletion coincided exactly with the right end of IS*150*, which strongly implicated the involvement of this element in the rearrangement. In seven of the 11 clones, the right deletion endpoints were located within *yieO*, although the precise location of the endpoints within *yieO* varied among the clones. Therefore, these deletion events removed the entire *rbs* operon. In the remaining four cases, the right endpoint of the deletion fell twice within *rbsR* (the last gene of the operon), once within *rbsB*, and once within *rbsA*. Even the smallest deletion therefore included the promoter region, the first gene of the operon (*rbsD*), and part of *rbsA*. In two populations, Ara-1 and Ara+1, we also examined the extent of the deletions in clones that were isolated as early as generation 500 and as late as generation 10,000. In each population, the same hybridization pattern was detected over time, indicating that no further changes in the extent of the deletion occurred after it first arose.

Similar deletions of the *rbs* operon occur in spontaneous Rbs$^-$ mutants. We then performed the same PCR assays used to identify deletion endpoints in evolved clones to characterize the seven spontaneous Rbs$^-$ mutants whose fitnesses we had

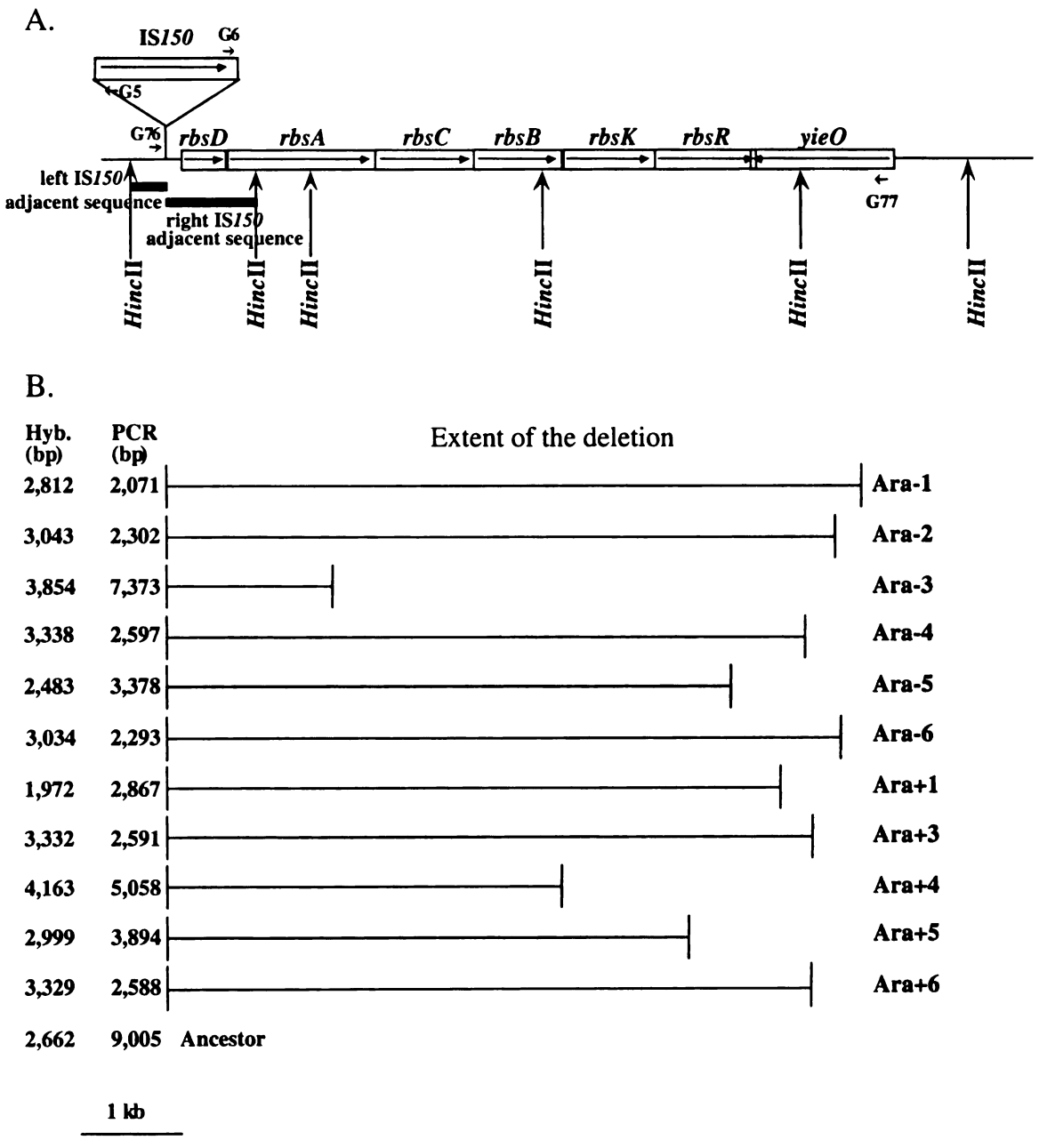
A.
IS150
G6
G5
G76
rbsD
rbsA
rbsC
rbsB
rbsK
rbsR
yieO
left IS150 adjacent sequence
right IS150 adjacent sequence
G77
HincII
HincII
HincII
HincII
HincII
HincII
B.
Hyb. (bp)
PCR (bp)
Extent of the deletion
2,812 2,071 Ara-1
3,043 2,302 Ara-2
3,854 7,373 Ara-3
3,338 2,597 Ara-4
2,483 3,378 Ara-5
3,034 2,293 Ara-6
1,972 2,867 Ara+1
3,332 2,591 Ara+3
4,163 5,058 Ara+4
2,999 3,894 Ara+5
3,329 2,588 Ara+6
2,662 9,005 Ancestor
1 kb

Figure 15.

Figure 15. Losses of D-ribose catabolism in evolving populations caused by deletions in the *rbs* operon. (A) Map of the *rbs* operon in *E. coli* B ancestor, based on the genome sequence for *E. coli* K-12 (Blattner et al. 1997). The directions of gene transcription are shown by arrows. An IS*150* element is inserted upstream of the *rbs* operon. Thick lines indicate the left and right adjacent sequences used as probes. The primers used in PCR experiments (G5, G6, G76, and G77) are also shown, along with the relevant *Hin*cII restriction sites. (B) The deletions in clones isolated from 11 of the 12 populations are shown as horizontal lines. Population Ara+2 is not pictured because a Rbs^+ minority clone was sampled from this population (see text). The right adjacent sequence of IS*150* did not hybridize with any of the Rbs^- clones, whereas the left adjacent sequence hybridized in every case with a *Hin*cII fragment, whose size is shown in the column labeled Hyb. The sizes of the PCR products obtained from these clones using the G76 and G77 primers are indicated in the column labeled PCR. The sizes, in the ancestral strain, of the hybridizing *Hin*cII fragment and PCR product are indicated at the bottom.

measured. In all seven mutants, the loss of D-ribose catabolism was caused by deletions of various sizes within and near the *rbs* operon: three had the right endpoint of their deletion in the *rbsK* gene, two in *yieO*, and one each in *rbsA* and *rbsC*. We reported in a previous section that these seven mutations had equivalent effects on competitive performance, in each case increasing fitness by about 1.4% in glucose minimal medium. Thus, different underlying mutations conferred the same selective benefit, with the commonality being that the *rbs* operon was rendered nonfunctional by a deletion beginning adjacent to the IS*150* element located just upstream of this operon.

DISCUSSION

Twelve populations of *E. coli* B experienced rapid and parallel losses of D-ribose catabolic function during evolution in glucose minimal medium (Figure 13). We showed that these losses were caused by similar deletions of the *rbs* operon; in all cases, one end of the deletion was immediately adjacent to an IS*150* element located just upstream of the operon, whereas the other endpoint varied (Figure 15). We further showed that mutations from Rbs^+ to Rbs^- occurred at an unusually high rate; that these mutations were caused by deletions of the *rbs* operon similar to those during the evolution experiment; and that Rbs^- mutations confer a small but consistent advantage in glucose minimal medium (Figure 14).

It may be instructive to examine the logic that led us to identify the evolutionary forces responsible for the loss of ribose function. The rapid and parallel evolution of the Rbs^- phenotype initially suggested to us that this change was adaptive. Repeatable change across lineages is widely taken as evidence of adaptation by natural selection

(Cunningham et al. 1997, Harvey and Pagel 1991, Huey et al. 2000, Nakatsu et al. 1998, Simpson 1953). Furthermore, the fact that this loss of function occurred when adaptation to the new environment was most rapid (Lenski et al. 1991, Lenski and Travisano 1994) reinforced our view that the loss was beneficial for the bacteria in glucose minimal medium. However, our observation that Rbs^- mutants were readily isolated from the ancestor led us to question our preconceptions and focus on the alternative possibility that the losses of ribose catabolic function might be caused by a hypermutable locus of some sort. Indeed, a fluctuation test confirmed this possibility, and the data provided an estimate for the mutation rate from Rbs^+ to Rbs^- of 5.4×10^{-5} per cell per generation. We then performed competition experiments between spontaneous Rbs^- mutants and their Rbs^+ progenitor, which showed that the Rbs^- mutants were also more fit than their progenitor in the glucose minimal medium. Evidently, selection for the loss of ribose catabolic function and its hypermutable genetic basis both contributed to the rapid and parallel phenotypic evolution we observed.

Molecular basis of the high mutation rate from Rbs^+ to Rbs^-. The same class of molecular events led to the loss of ribose function in the evolved lines and spontaneous Rbs^- mutants. A total of 18 Rbs^- genotypes (11 evolved lines and 7 spontaneous mutants) all showed deletions in which one endpoint was located precisely at the end of an IS*150* element that was inserted upstream of the *rbs* operon. The extent of the deletion varied among the genotypes, but always encompassed the promoter region and first gene (*rbsD*) and in some cases included all six genes in the *rbs* operon and part of an adjacent gene of unknown function (*yieO*). The fact that one endpoint of the deletion was consistently and precisely located at the end of this IS*150* element suggests that the

mechanism of deletion involved first the transposition of an IS*150* element (either the one upstream of *rbs* or any other) into the site corresponding to the other endpoint and in the same orientation as the one upstream of *rbs*. This transposition was presumably then followed by a recombination event between the new IS*150* copy and the one upstream of *rbs*, thereby leading to the deletion of the intervening region. Whether the transposition and recombination events occurred simultaneously or successively is unknown; however, the fact that none of the spontaneous Rbs^- mutants showed a simple transposition that inactivated the *rbs* operon (without the associated deletion) suggests that the two events occurred in the same cell generation. Analysis of the nucleotide sequences at the right endpoints of the different deletions indicated no homology whatsoever with the end of IS*150* corresponding to the left deletion endpoint, which further suggests rearrangements associated with an initial transposition event. Examination of the nucleotide sequence of the presumptive target sites of the IS*150* transpositions in the different genotypes failed to reveal any obvious preference for its insertion.

All IS-associated mutations have been studied (Papadopoulos et al. 1999, Schneider et al. submitted) in two of the 12 evolving populations. In both of these focal populations, *rbs* deletions were present at generation 500 and in all clones sampled in later generations. Thus, the *rbs* deletions are "pivotal" mutations (Papadopoulos et al. 1999) that became fixed in these evolving populations. By performing RFLP analyses using IS elements as probes with *Eco*RV-digested genomic DNA extracted from clones sampled over time, Papadopoulos *et al.* (1999) found several other pivotal mutations. By characterizing these mutations at the sequence level, Schneider *et al.* (submitted) showed that these pivotal mutations involved IS-mediated events, including transpositions,

inversions, and deletions; and they further showed that none of the pivotal mutations in the two focal populations involved the same genes. However, Papadopoulos *et al.* (1999) did not detect the parallel *rbs* deletions that we have described here, for the following reason: *Eco*RV cuts at the right end of IS*150*, whereas the probe they used corresponded to the left end of IS*150*, so that the type of rearrangement involved in the *rbs* operon (deletions at the right end of IS*150*) went undetected. Based on our analysis of *Hin*cII-digested genomic DNA of the evolved clones, the *rbs* deletions are the only pivotal IS-associated mutations in the two focal populations that were not detected by Papadopoulos *et al.* (1999).

As noted in the Results, other studies have reported that certain strains of *E. coli* B are Rbs^- (Abou-Sabé et al. 1982, Lin 1996), whereas our founding strain was clearly Rbs^+ but predisposed genetically to become Rbs^-. One plausible explanation for the reports that other strains of B are Rbs^- is that these strains lost the ribose catabolic function during propagation in the laboratory, much as we have observed in our experimental lines. Under this hypothesis, the common ancestor of all B strains would have had two *rbs* operons, a non-functional one at 2 min and a functional one at 83 min, with deletions independently arising in the sub-lineages at different points in time. However, other sources of genetic instability may also contribute to such differences. For example, the operon at 2 min appears to have been generated by a duplication of the one at 83 min, and a transposable element encoding the *rbs* operon was found in an experiment, suggesting that the operon itself is mobile in *E. coli* B (Abou-Sabé et al. 1982).

IS and other transposable elements generate a substantial fraction of the mutations in bacteria, and they are therefore an important evolutionary factor (Blot 1994). Nonetheless, there are conflicting views about their costs and benefits, and the balance of forces that maintain these elements in populations. One view emphasizes that a much higher proportion of mutations are deleterious than are beneficial; infers that transposable elements impose a burden on adaptation by substantially increasing the overall mutation rate; and concludes that active elements can therefore be maintained only if horizontal gene transfer allows them to exist as genomic parasites. Another view emphasizes that transposable elements may, on balance, be adaptive to an evolving population just as mutator alleles are under certain conditions; and like mutators, transposable elements may spread, even in asexual populations, by hitchhiking along with occasional beneficial mutations that are produced by their activities (Blot 1994, Chao et al. 1983, de Visser et al. 1999, Sniegowski et al. 1997, Taddei et al. 1997). Our findings demonstrate the high mutation rate that can result locally from the presence of just one IS element in a particular gene region. If these deletions occurred near any essential gene, then the load created by the IS element would be equal to the mutation rate, implying a weak but non-trivial selection coefficient of about 0.00005 against that element alone. Our results also demonstrate that some IS-mediated mutations are beneficial and promote the adaptation of an evolving population. Interestingly, and in contrast to an evolution experiment in which point mutations and IS transpositions generated functionally equivalent beneficial mutations (Treves et al. 1998), in our study all of the many beneficial knock-outs of the ribose function were associated with IS activity.

It is clear that deletions of part or all of the *rbs* operon are beneficial to *E. coli* B in glucose minimal medium, and that the IS*150* element located immediately upstream of the operon plays a role in generating those deletions. However, the physiological basis for the benefit that accrues is unclear. In all 18 deletions examined, the promoter region and first gene (*rbsD*) of the operon were eliminated, suggesting that it was silencing of the operon that provided the selective advantage. The fact that comparable benefits accrued whether the deleted region was 2 kb or 7 kb implies that neither energetic savings associated with chromosomal replication nor conformational changes in the chromosome can account for the beneficial phenotypic effect.

Quantitative analysis of the contributions of mutation and selection to the evolution of the Rbs$^-$ phenotype. Both positive selection for loss of the *rbs* operon and its underlying mutability contributed to the evolutionary losses of ribose catabolic function in the 12 experimental populations. Here, we examine mathematically their contributions as well as their interplay with one another and with selection at other loci.

We consider first the expected time course if selection had favored the loss of this function, but without any hypermutable basis. For simplicity, we assume the mutation is just common enough that there is no waiting time for the mutation to appear, and then we calculate the time for the mutant genotype to increase from one cell to 50% of the total population. In our calculation, we use the 1.4% selective advantage, s, that we measured in this study for Rbs$^-$ mutants, and the effective population size $N_e = 3 \times 10^7$ that prevailed during the evolution experiment (Lenski et al. 1991). The ratio, R, of genotypes changes in a log-linear fashion under constant selection (Dykhuisen and Hartl 1983, Lenski et al. 1991). We express bacterial generations using a $\log_2$-transformation

of the daily dilution and re-growth. Thus, the time in generations, g, for a single mutant to increase to 50% (a final ratio of 1) of the population is calculated as:

$$g = \log_2 (R_g / R_0) / s \qquad \text{[Eqn. 1]}$$

$$= \log_2 (3 \times 10^7) / 0.014 = 1{,}774 \text{ generations.}$$

In fact, however, the actual frequency of mutants reached 50% much sooner than this in most of the experimental populations (Figure 13).

Next we calculate the time required for the mutant to achieve 50% assuming that Rbs$^-$ mutants increase by recurring mutation only, without the benefit of any selection. In that case, the frequency, p, of the Rbs$^+$ progenitor should decay in an exponential fashion from an initial frequency of 1. Given an estimated mutation rate, μ, of 5.4×10^{-5}, the time for the progenitor to decline to 50% (and the mutant to reach 50%) is given by:

$$g = -(\log_2 p) / \mu \qquad \text{[Eqn. 2]}$$

$$= -(\log_2 0.5) / 5.4 \times 10^{-5} = 18{,}519 \text{ generations.}$$

The actual spread of Rbs$^-$ mutants was much faster than this in all populations (Figure 1).

Finally, we calculate the approximate time that is required for the mutant to reach 50% given both its observed selective advantage and its high mutation rate. To do so, we note that mutants should be present after the first day of the evolution experiment in the same frequency as observed in the fluctuation test, which was about 0.000512. We then use the fact that $s >> \mu$ to deduce that selection will drive the subsequent increase in the frequency of mutants, so that we can apply Eqn. 1 to calculate the time to reach 50% (a final ratio of 1) given an initial ratio of 0.000512. We obtain the estimated time as follows:

$$g = \log_2 (1 / 0.000512) / 0.014 = 781 \text{ generations.}$$

This last calculation gives better agreement with the observed spread of Rbs^- mutants than does either calculation that ignores the contribution of mutation or selection.

Two features of the dynamics of the Rbs^- mutants that are not explained by these simple models are: (i) the pronounced variability in the frequency of Rbs^- mutants among the replicate populations, especially at generations 500 and 1,000; and (ii) the temporary reversals in the frequency of Rbs^- mutants in a few populations over that interval (Figure 13). Both features are understood by realizing that selection was simultaneously acting on mutations at other loci, including some mutations that were much more beneficial than were the *rbs* deletions. Indeed, each population underwent several sweeps by beneficial mutations during these 2,000 generations, and the underlying mutations conferred fitness advantages, on average, of about 10% (Lenski et al. 1991, Lenski and Travisano 1994). Given the asexual nature of the evolving populations, the short-term fate of Rbs^- mutants in any population would depend on how long it took for one of the highly beneficial mutants to appear in a Rbs^- clone, and whether an even more beneficial mutation appeared in a Rbs^+ clone, which could cause a reversal owing to clonal interference (Gerrish and Lenski 1998). Thus, the variation among populations in the dynamics of the Rbs^- mutants is expected from the stochastic appearance of beneficial mutations at other loci, which leads to divergence in the genetic linkage among beneficial alleles across the replicate populations.

APPENDIX

Appendix A

ESTIMATION OF RBS$^+$ TO RBS$^-$ MUTATION RATE USING NUMERICAL SIMULATIONS.

The estimation of mutation rate requires that the frequency of observed mutations be measured. In 56 independent cultures of the ancestral strain of *E. coli* B, we observed 25 Rbs$^-$ mutants. An average of 872 cells was sampled per culture. Thus, the frequency, f, of Rbs$^-$ mutants was:

(1) f = (25 mutants / 56 cultures) / (872 cells / culture) = 0.000512.

We must also determine the number of generations that occurred in each culture during its population expansion. The initial population size of each culture was approximately 50 cells, and the average final population size was 4.19×10^8 cells. The number of generations, g, during binary fission is given by:

(2) $g = \log_2 (4.19 \times 10^8 \text{ cells} / 50 \text{ cells}) = 22.98 \approx 23$ generations.

If as many mutants arose in each culture in the final generation (# 23) as in the previous generation (# 22), then the mutation rate, μ, is given by:

(3) $\mu = 2f / g = 2 \times .000512 / 23 = 4.45 \times 10^{-5}$.

This estimate allows even "partial mutations" to accumulate, and doubles the estimate because n - 1 doublings occur in n generations, with twice the number of mutants in each successive generation.

Next, we estimated mutation rate if "virtual" mutants are not permitted to replicate until their frequency = 0.5. That is, "virtual" mutants accumulate at the mutation rate in

(3) until their frequency reaches 0.5, and then they double at the same rate as the rest of the dividing population. These two different rates of accumulation are given by equations 4a and 4b, below, given N, population size, m, the frequency of mutants, and μ.

(4a) $m_t = \mu N_t + m_{t-1}$

(4b) $m_t = \mu N_t + 2(m_{t-1})$

By generation 23, the frequency of mutations generated by methods 4a and 4b (.000378) is less than observed in the experiments (.000512). Thus, a correspondingly higher mutation rate is required. By trial and error, we find a rate that generates the observed frequency of mutants: $\mu = \mathbf{6.03 \times 10^{-5}}$.

We found that Rbs$^-$ mutants are competitively more fit in the selective medium than the ancestor. This selective advantage, s (equaling 1.4%, on average), would lead to a greater representation of these mutants in each successive generation, and therefore cause an overestimation of mutation rate. We revised method (4) as follows, allowing mutants to replicate with the 1.4% advantage, s.

(5a) $m_t = \mu N_t + m_{t-1}$

(5b) $m_t = \mu N_t + 2s(m_{t-1})$

We found that the calculated frequency of mutations (.000573) was actually greater than that observed in our experiments (.000512). By trial and error, we find a rate that generates the observed frequency of mutants: $\mu = \mathbf{5.39 \times 10^{-5}}$.

We believe method (5), above, best approximates the population expansion of mutants in these cultures. It therefore provides our best estimate of the true mutation rate in this experiment.

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