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ABSTRACT

ADAPTATION AND DIVERGENCE IN EXPERIMENTAL POPULATONS OF THE BACTERIUM *ESCHERICHIA COLI*: THE ROLES OF ENVIRONMENT, PHYLOGENY AND CHANCE

By

Michael Travisano

This dissertation examines the roles of environment, phylogeny, and chance in adaptation and divergence. Populations of *Escherichia coli* were selected and their competitive fitness measured under defined laboratory conditions. In Chapter I, correlated responses of twelve replicate populations which had undergone 2,000 generations of selection under glucose-limited batch culture were examined. These twelve populations were founded from the same ancestor, so that chance events could be the only factor causing divergence of these populations. The twelve glucose-selected populations exhibited extreme parallelism in fitness improvement, but had diverse correlated responses in novel nutrient environments (such as maltose-limited medium), indicating that the twelve populations had diverged phenotypically during adaptation. In Chapter II, the mechanistic bases for the divergence of the twelve glucose-selected populations were examined. Correlated responses to

selection, as measured by fitness in twelve different nutrient-limited environments, implied that the twelve glucose-selected populations had diverged into at least six distinct groups with different adaptations to improve glucose uptake from the environment. In Chapter III, environmental effects on evolution were examined in twelve replicate populations of *Escherichia coli*, having the same ancestor as the glucose-selected populations, but selected in maltose-limited batch culture. The maltose-selected populations exhibited similar dynamics of adaptation, as measured by fitness in the maltose environment, to those of the glucose-selected populations in the glucose environment. However, the twelve maltose-selected populations did not exhibit diverse correlated responses in the glucose environment (contrasting with the diverse responses of the glucose-selected populations in maltose). In Chapter IV, the relative effects of phylogeny, chance, and adaptation on evolution were examined in thirty-six populations propagated for 1,000 generations in maltose-limited batch culture. The thirty-six populations were founded as three-fold replicates from the twelve glucose-selected populations, which had exhibited diverse correlated fitness responses in the maltose-limited environment. Adaptation to the maltose-selective environment was almost five-fold more important than phylogeny to fitness at 1,000 generations, and phylogeny was three-fold more important than chance.

This dissertation is dedicated to

JoAnne P. Scriffiano

&

Billy Travisano

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INTRODUCTION

There are many examples where sub-divided populations have adapted to similar environmental conditions by different mechanisms (see Cohan, 1984a for a review). For example, populations of the two-spotted spider mite evolved different mechanisms of malathion resistance in Europe and North America (Matsumura and Voss, 1964; Voss and Matsumura, 1964). Similarly, resistance to malaria in humans has occurred via sickle-cell anemia, thalassemia, and other hemoglobin variants (Luzzatto, 1979). These and other studies show that alternative adaptive solutions can occur when different populations of the same species are exposed to similar environmental conditions.

What causes different populations of the same species to exhibit different adaptations to similar environmental conditions? There are at least three possible sources of this divergence. First, divergent adaptations may result from subtle differences in environmental conditions. For example, although malathion was almost certainly the primary selective agent responsible for the resistant genotypes mentioned above, there may well exist ecological differences between Europe and North America that constrain the mites' adaptation to that particular component of the environment. Second, divergent adaptations may result from differences in initial genetic composition. For example, the European and North American mite populations may have been predisposed to different physiological mechanisms of resistance as a consequence of differences in their genetic background, which arose as a consequence of their different evolutionary histories prior to malathion exposure. Third, different adaptations may reflect the stochastic nature of the processes involved in the generation and loss of genetic

variants. For example, a particular spontaneous mutation to malathion resistance may have occurred in the European mite population that did not occur (or that was lost due to genetic drift) in the North American population. These alternative explanations are not mutually exclusive, nor are they easily disentangled without appropriate experimental design.

Cohan and associates (Cohan, 1984b; Cohan and Graf, 1985; Cohan and Hoffmann, 1986; Hoffmann and Cohan, 1987; Cohan et al., 1989) undertook a pioneering experimental study to examine some of the causes of genetic divergence. In their study, populations of *Drosophilia melanogaster* and *D. pseudoobscura* were selected for resistance to ethanol vapor. Their results suggested that both initial genetic composition and genetic drift contributed to the divergence of populations, where divergence was manifest as heterogeneity in such correlated responses as resistance to other chemical agents and/or certain fitness components.

More recently, Lenski et al. (1991) examined the adaptation and divergence of initially identical populations of *Escherichia coli* propagated in the same glucose-limited environment for 2,000 generations. The dynamics of adaptation and divergence were quantified by periodic assays of each population's mean fitness as well as the between-population genetic variance in fitness. Mean fitness increased by ~35% relative to the common ancestor over the course of the 2,000 generations, although the rate of fitness improvement was substantially diminished towards the end of this period. Lenski et al. also detected significant between-population variance for fitness. However, the magnitude of the between-population fitness variance was rather small,

with evolved populations differing in fitness from one another by only 3% or so on average. Moreover, the dynamics of mean fitness and of the between-population variance in fitness were such that they could not reject the hypothesis that the replicate populations would eventually converge towards similar adaptive states. Taken at face value, the results of Lenski et al. (1991) thus suggest a remarkable degree of evolutionary parallelism.

However, at least two issues were unresolved by Lenski et al. (1991). (1) Did the apparent lack of strong evidence for divergence result from having measured only fitness, and not its components, correlated responses, or the physiological and genetic bases of the fitness improvement? It is possible that the bacterial populations studied by Lenski et al. (1991) adapted to the same environment by very different means, despite their similar fitnesses. (2) Would populations that differed in their initial genetic composition been more likely to diverge than those which were initially identical? A particular mutation could be advantageous in one genetic background, even if it is neutral or disadvantageous in a different genetic background. Although Lenski et al. (1991) did not demonstrate sustained divergence in fitness among the replicate populations, their experimental design and divergence criteria were extremely conservative. A less conservative approach, examining additional characters and employing diverse founding genotypes, might yield a greater amount of divergence.

This dissertation addresses both of these questions. Following Lenski et al. (1991) I have used experimental populations of *Escherichia coli* as a model system. Bacterial populations are easy to maintain

under controlled laboratory environmental conditions, they can be propagated for many generations over relatively short periods of time, and they can be stored indefinitely at -80°C. In addition, there is an extensive body of knowledge on the physiology, biochemistry and genetics of *Escherichia coli*, which facilitates the interpretation of the phenotypic bases of adaptation and divergence.

The organization of this dissertation is as follows. Chapter I examines whether the similarity in mean fitness of populations studied by Lenski et al. (1991) masks underlying genetic and phenotypic divergence. The complex biochemical mechanisms of glucose uptake are used to motivate fitness assays in novel nutrient environments. Hypothesizing that the populations may have improved glucose uptake by different mechanisms, the replicate populations' fitnesses are assayed in two unselected nutrient environments, maltose and lactose. In Chapter II, the same populations are further examined in nine additional nutrient environments to identify the specific targets of selection and further characterize the the divergence of the populations. In Chapter III, the experiment by Lenski et al. (1991) is essentially repeated in a different nutrient environment, maltose-limited medium, to examine whether a simple change in the selective regime alters the evolutionary outcomes observed by Lenski et al. (1991) and in Chapter II in glucose. Of particular interest is whether correlated responses during selection in maltose versus glucose are symmetric or asymmetric. Chapter IV examines experimentally the relative effects of adaptation to the current environment, phylogeny, and chance during evolution. Three replicate populations were derived from each of the twelve populations studied by Lenski et al. (1991). These thirty-six new populations were

then propagated for 1,000 generations in maltose-limited medium, a novel environment where they varied considerably in their initial fitnesses.

Chapter I
DIVERGENCE IN CORRELATED RESPONSES UNDER UNIFORM SELECTION

Gould and Lewontin (1979, p. 593) remark that "subpopulations within a species often develop different adaptations as solutions to the same problem." Moreover, it is often the case that different adaptations result by modification of the very same traits but by slightly different means. For example (following Gould, 1989), a West Indian land snail, *Cerion*, has typically adapted to windy rocky coasts by developing squat shells, but has done so by at least two different developmental pathways for modification of shell allometry. Thus, selection may result in similar phenotypic responses in different populations, but these responses do not necessarily occur by the exact same underlying mechanism.

Gould examined different populations of *Cerion* that were indistinguishable by adult size. However, adult size of an individual snail is affected by both whorl size (essentially the size of the spiraling snail tube) and whorl number (the number of spirals) with adult size varying positively with each. Moreover, in populations where an intermediate adult size is a strongly selected trait, whorl size and number vary inversely due to the selective constraint placed upon adult size and the geometric constraint that "if the adult size of a shell lies within a limited range, then an increase in the size of whorls requires that the adult shell contain a smaller number of whorls" (Gould, 1979). Thus, interpopulation variation of the directly selected trait may not be as large as interpopulation variation of the correlated traits, even though the correlated traits are mechanistically responsible for the selected trait. Different populations can

potentially be distinguished by their correlated responses

Although Gould's observations suggest that uniform selection may produce phenotypic differences among populations in their response, they do not indicate what evolutionary mechanisms result in divergence under uniform selective conditions. There are at least three possible mechanisms. First, divergent adaptations may result from subtle differences in environmental conditions. Second, divergent adaptations may result from differences in initial genetic composition. Third, different adaptations may reflect the stochastic nature of the processes involved in the generation and loss of genetic variants.

Lenski et al. (1991), in an experimental study, examined the propensity for divergence during adaptation among replicate populations of bacteria in a uniform environment. Their experimental design allowed them to exclude the first two of the three possible sources of divergence described above. Twelve initially isogenic populations of bacteria were serially propagated in a glucose-limited minimal medium for 2,000 generations. After 2,000 generations, the mean fitness of each population had increased roughly 35%, with significant between population variance in mean fitness. However, the average difference in mean fitness between any two populations was only about 3%. In addition, the rate of fitness improvement decreased during the course of the 2,000 generations, suggesting that each population was approaching a common selective plateau. Moreover, a subsequent study (Vasi et al., submitted) also indicates that the replicate populations exhibited a high degree of parallelism in the demographic parameters that confer higher fitness in the derived populations. Thus, the replicate populations showed little or no tendency to diverge either in mean

fitness or in major components of fitness, and it could therefore be argued that they were approaching a common adaptive solution.

Alternatively, the similar fitness increases among the replicate populations may result from common functional constraints, which limit the potential for improvement even if the replicate populations adapted in functionally distinct ways. I sought to investigate this possibility by comparing the performances of the replicate populations in two nutrient environments other than the glucose-limited environment in which they had been selected. Comparing genotype-by-environment (GxE) interactions is a method others (e.g. Weis and Gorman, 1990) have used to distinguish genotypes and populations. In addition, any differences in GxE interaction might be helpful in identifying the functions which are the targets of selection.

EXPERIMENTAL OVERVIEW

Lenski et al. (1991) founded twelve replicate populations that were propagated for 2,000 generations in a resource-limited medium containing glucose as the sole carbon and energy source usable by the bacteria. All populations were founded from a single ancestor and thus were initially isogenic, except for a neutral marker. The populations were maintained on a 24 hour schedule, where a small sample (0.1 ml) of the previous day's culture was transferred into (9.9 ml) fresh media. At 2,000 generations, a single clone was randomly chosen from each population to serve as a representative genotype derived from each replicate population.

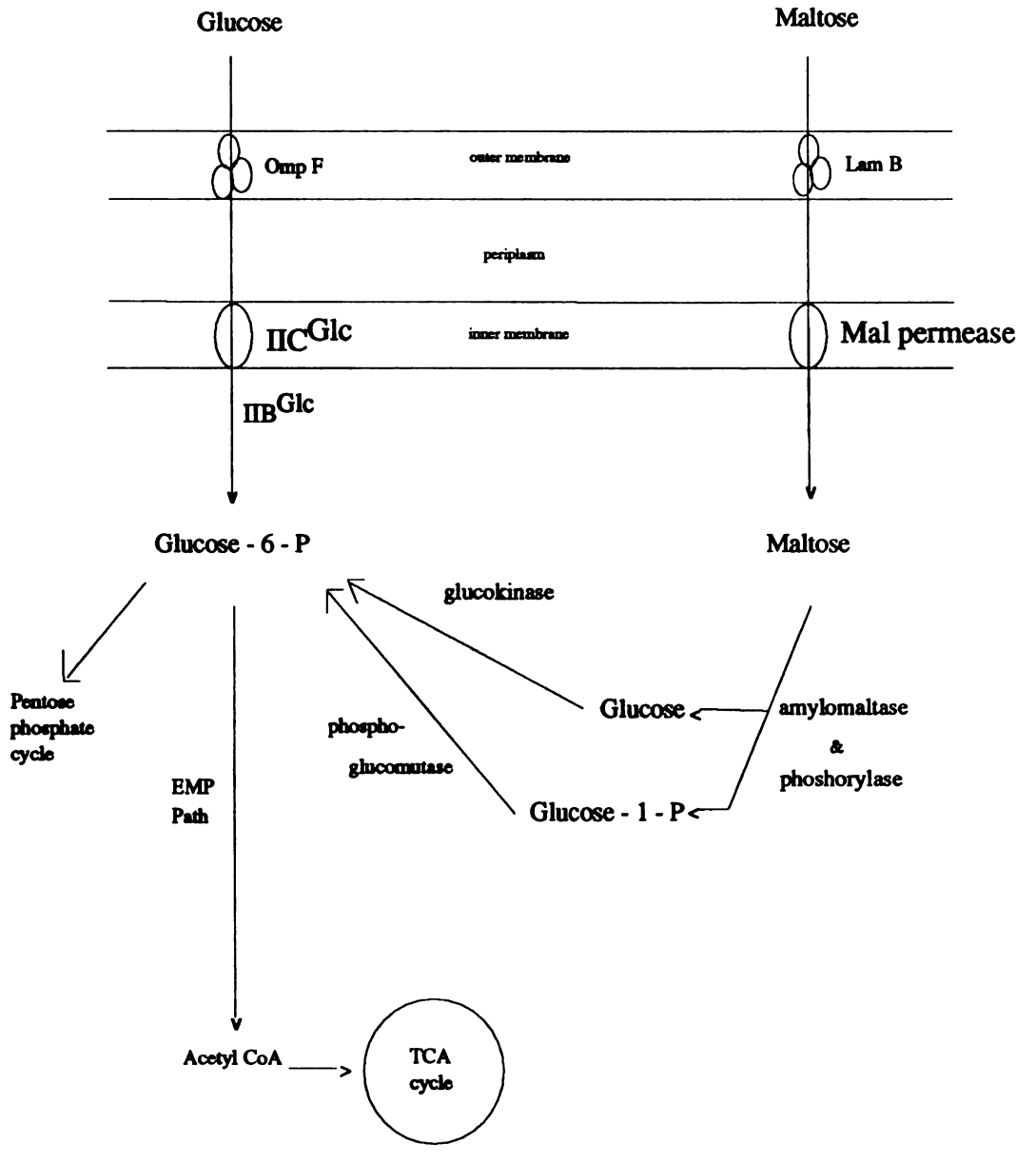
To determine whether the observed similarity in the fitnesses of the derived genotypes of Lenski et al. (1991) might exist despite

underlying genetic variability, I examined these genotypes in three nutrient environments, glucose, maltose and lactose. The representative genotypes were competed against their common ancestor (or, in some cases, against one another), from which estimates of genetic variance for fitness were calculated. If the similarity in fitness of the derived genotypes in the glucose-limited environment masks underlying heterogeneity in the physiological bases of their adaptation, then the genetic variance for fitness should be higher in the maltose- and lactose-limited environments.

The comparison in maltose is especially interesting in that maltose is a disaccharide made up of two glucose molecules; although maltose is catabolized by essentially the same biochemical pathway as glucose, it has a completely different pathway for uptake from the environment (Figure 1). Hence, fitnesses of the replicate derived genotypes are expected to be quite similar in maltose and glucose if the physiological bases of the adaptations responsible for improved fitness in glucose involve any aspect of cell function *except* nutrient uptake. However, if the physiological basis for adaptation to the glucose-limited environment is improved transport of glucose, then such adaptations would not be expected to confer any fitness improvement in a maltose-limited environment.

In addition, an experiment was performed to examine the concordance of fitnesses inferred by competing derived genotypes against one another. If frequency-dependent selection was important, then one would expect statistical interactions such that relative fitnesses would be non-additive (Paquin and Adams, 1983; Heiling et al. 1987).

Figure 1. Uptake and subsequent catabolism of glucose and maltose.



See Schwartz (1987) for a more complete description of these pathways.

MATERIALS AND METHODS

Bacterial strains. -- The isolation of the bacterial strains used in this chapter has been previously described (Lenski et al., 1991). Briefly, all strains were derived from a single clone of a strain of *Escherichia coli* B. The founding strain did not contain any plasmids or functional phage, and therefore it is strictly clonal. The strain is prototrophic, although it is unable to use L(+)arabinose as a nutrient (i.e. Ara^-). Spontaneous arabinose utilizing mutants (Ara^+) are easily selected when large numbers of cells are spread on minimal arabinose medium. The Ara^+ mutants form white colonies, while Ara^- colonies are red, when cells are grown on tetrazolium-arabinose (TA) indicator agar.

Twelve clones, six Ara^- and six Ara^+ (which were derived from the single initial Ara^- clone), were each used to found separate populations that were then propagated for 2,000 generations at 37°C in Davis minimal medium (Carlton and Brown, 1981) containing 25μg/ml glucose as the sole usable carbon and energy source (Lenski et al., 1991). Propagation was performed daily, by transferring 0.1 ml of the previous day's culture into 9.9 ml of fresh medium (a 100-fold dilution). After 2,000 generations, single clones were randomly picked from each population and stored at -80°C in a glycerol based suspension. All competition experiments in this chapter were performed using these single colony isolates and their Ara^- and Ara^+ progenitors.

Measurement of selection rate constants in competition experiments. -- Competition experiments were performed under the same culture conditions originally used to propagate the populations, except that two other carbon/energy sources (maltose and lactose) were substituted for glucose as indicated. The nutrient concentration used

in the competition experiments was the same (25μg/ml) for all three carbon/energy sources. These experiments were done in a 'head to head' fashion, where two genotypes were propagated simultaneously in a single culture so that they competed for the same pool of limiting resource. In order to distinguish the two competitors when estimating fitness, one competitor was always Ara^+ while the other was Ara^-.

The competition protocol was as follows. Both competitors were first preconditioned separately for 24 hours in the particular medium in which they were going to be competed. Then each competitor was diluted 200-fold into a single competition culture which contained fresh medium. Thus, the total initial density of bacteria was approximately 100-fold less than the final density achieved at the end of the standard daily growth cycle of 24 hours. Initial and final population densities of each competitor were determined by plating samples from the competition culture onto TA agar.

The Selection Rate Constant (SRC) is the difference in Malthusian parameters between the two competitors and provides a relative measure of fitness (Nagylaki, 1977; Lenski et al., 1991). The Malthusian parameter of a genotype is determined by taking the natural logarithm of the ratio of the final cell density to the initial cell density. Any difference in plating efficiency between Ara^+ and Ara^- competitors does not affect estimates of the SRC, as long as plating efficiencies remain constant between initial and final samples.

Lenski et al. (1991) used relative fitnesses to compare the evolutionary responses of the populations. Relative fitness was estimated as the ratio of the number of doublings the two competitors undergo during competition, which is equal to the ratio of Malthusian

parameters. For consistency, relative fitness would have been the preferred statistic in this study as well. However, relative fitness, being a ratio, is very sensitive to sampling error if the competitors' Malthusian parameters are very different. Thus, the variance of estimates of relative fitness can be very large if fitness differences are extreme, which was the case for some comparisons in this study.

Experimental designs and statistical analyses. -- To test the selective neutrality of the arabinose-utilization marker, the two marker variants of the ancestral genotype were competed against one another in each type of medium (with replication). These marker controls were performed simultaneously with the competition experiments comparing the evolved genotypes with the ancestors.

Five, six, and four estimates of the selection rate constant for each of the twelve derived genotypes (relative to the reciprocally marked common ancestor) were obtained in media containing glucose, maltose and lactose, respectively. Replicates were performed in sets of complete blocks. The genetic variance in fitness (on a particular sugar) among independently derived genotypes was tested using a two-way analysis of variance, in which derived genotype and block were random effects. The among-genotype genetic variance component, V_G, was estimated as the difference in the genotype and error mean-squares, divided by the number of replicate assays (=blocks) per group. Confidence intervals for V_G were computed using the methods described by Sokal and Rohlf (1981, pp. 217-218). Although the twelve independently derived genotypes are regarded as random rather than fixed entities for most purposes, a Bonferroni-corrected T-test for multiple comparisons (Miller, 1981, SAS Institute, 1988, pp. 593-595) was

performed to ask how many distinct classes of genotypes could be distinguished on the basis of fitness in the experimental sugars.

Variation among the derived genotype in their performance may have been due to interactions other than competition strictly for the exogenously provided resource. Such interactions may be mediated by compounds released into the media by the bacteria (e.g., cross-feeding, allelopathy). These interactions may result in non-additive effects, including even non-transitive relative fitnesses wherein genotype A is competitively superior to B, and B is superior to C, but A is inferior to C. To test for non-additive and even non-transitive interactions, each Ara^- derived genotype was competed against each Ara^+ derived genotype in lactose-limited medium, the media which gave the most extreme fitness. Two blocks of measurements were performed, each containing a single replicate of every pairwise comparison. The data were analyzed in a two-way ANOVA with among-genotype variation in each arabinose-utilization marker class as the main effects and the interaction term reflecting the possible non-additive interactions between particular Ara^+ and Ara^- genotypes.

RESULTS

Selective neutrality of the genetic marker -- The neutrality of the arabinose-utilization marker was examined in the ancestral genotype in all three media types (Table 1). In every medium, the marker is neutral in the ancestral genotype. Thus, the SRC estimates are unbiased with respect to the arabinose marker, within the resolution of these experiments. This result does not preclude the possibility that the

Table 1. Effective neutrality of the arabinose-utilization marker in the ancestral genotype in medium containing three different sugars.

Sugar	Selection Rate Constant (Ara^+ Relative to Ara^-) Mean (± SE)[1]	P[2]
Glucose (n = 5)	0.0184 (± 0.0579)	0.767
Maltose (n = 6)	-0.1380 (± 0.0860)	0.169
Lactose (n = 11)	0.2979 (± 0.2183)	0.202

[1] Mean (and standard error of the mean) selection rate constant based on n assays in medium containing a particular sugar.

[2] Two-tailed probability computed from the *t*-distribution with n - 1 degrees of freedom; the null hypothesis is that the selection rate constant equals zero, indicating equal fitness for the Ara^+ and Ara^- marker states.

ancestral marker state had an effect upon the observed responses to selection. However, the six Ara^+ derived genotypes are not more or less fit (as a group) than the six Ara^- derived genotypes in any of the three sugars (Table 2). These results, coupled with the fact that all experiments are fully balanced with respect to marker, suggest that pooling the data for the Ara^+ and Ara^- genotypes in subsequent analyses is statistically valid.

Parallel fitness improvements in glucose among replicate genotypes. -- In glucose, the grand mean selection rate constant for the twelve independently derived genotypes, relative to their common ancestor, is 1.318 (Table 3, first column). The corresponding 95% confidence interval (based on the *t*-distribution with 11 degrees of freedom) ranges from 1.236 to 1.400. An analysis of variance (based on the repeated measures of the selection rate for each of the derived lines) shows no significant heterogeneity among the twelve genotypes in their selection rate constants (Table 4). The estimated among-genotype variance component, V_G, is 0.0050; the upper limit of the 95% confidence interval for that variance is 0.0366. The corresponding standard deviations ($\sqrt{V_G}$) are 0.071 and 0.191, respectively, which are quite small in comparison with the grand mean. Evidently, the average change in fitness is quite large relative to the variation in fitness among the independently derived genotypes, consistent with the results reported by Lenski et al. (1991).

Genetic adaptation to glucose does not increase average fitness in maltose. -- The selection rate constant for each derived genotype in maltose is given in Table 3. Although the genotypes showed substantial

Table 2. Effective neutrality of the arabinose-utilization marker in the derived genotypes in medium containing three different sugars.

	Mean Selection Rate Constant			
Sugar	Ara^+ Derived Relative to Ara^- Ancestor	Ara^- Derived Relative to Ara^+ Ancestor	Difference (±SED)[1]	P[2]
Glucose	1.300	1.335	-0.035 (± 0.078)	0.663
Maltose	-0.244	0.244	-0.487 (± 0.468)	0.322
Lactose	1.102	2.615	-1.513 (± 1.278)	0.264

[1] Difference (and standard error of the difference) in the selection rate constants based on six independently derived genotypes in each marker class.

[2] Two-tailed probability computed from the *t*-distribution with 6 + 6 - 2 = 10 degrees of freedom; the null hypothesis is that the difference in the mean selection rate constant for the two marker classes of derived genotype equals zero (indicating equal changes on average, in the fitness for the Ara^+ and Ara^- derived genotypes).

Table 3. Differential adaptation to glucose and maltose.

Twelve replicate genotypes selected by Lenski et al. (1991) during 2,000 generations in glucose-limited minimal medium.

Derived Genotype	Mean Selection Rate Constant Relative to the Common Ancestor in[1]		
	Glucose	Maltose	Difference
Ara^-1	1.098	-0.229	1.327
Ara^-2	1.415	-1.637	3.052
Ara^-3	1.430	0.250	1.180
Ara^-4	1.244	0.670	0.574
Ara^-5	1.168	-0.346	1.514
Ara^-6	1.446	-0.169	1.615
Ara^+1	1.475	0.526	0.949
Ara^+2	1.229	0.394	0.835
Ara^+3	1.431	0.637	0.794
Ara^+4	1.398	-1.420	2.818
Ara^+5	1.187	0.386	0.801
Ara^+6	1.292	0.940	0.352
Mean	1.318	0.000	1.318
SE	0.037	0.235	0.243
P[2]	< 0.001	0.998	< 0.001

[1] The selection rate constant for each derived genotype is based on five or six assays for glucose or maltose, respectively.

[2] Two-tailed probability computed from the *t*-distribution with n - 1 = 11 degrees of freedom; the null hypothesis is that the mean selection rate constant (or difference between two means) is zero, indicating equal fitness for the average derived genotype and the common ancestor (or equal fitness in the two sugars).

Table 4. ANOVA for Selection Rate Constants for derived genotypes (Lenski et al., 1991) in glucose.

Analysis of variance for the selection rate constants obtained for the twelve independently derived genotypes in glucose, relative to the common ancestor. Both genotype and block are random effects.

Source	df	MS	F	P
Genotype	11	0.0837	1.421	0.198
Block	4	0.0492	0.835	0.510
Error	44	0.0589		

improvement relative to their ancestor in medium containing glucose (Table 3, first column), they showed no significant improvement, as a group, in maltose (Table 3, second column). A t-test for paired comparisons indicates that the difference in the extent of adaptation to these two sugars is highly significant (Table 3, third column). In fact, the selection rate constant is larger in glucose than it is in maltose for all twelve genotypes.

Derived genotypes vary in their fitnesses in maltose. -- The derived genotypes are homogeneous in their fitnesses in the glucose-limited environment (Table 4). And the derived genotypes, as a group, do not show any significant change in fitness relative to the ancestor in medium containing maltose (Table 3). However, there is genetic heterogeneity among the derived genotypes in their performance on maltose; an analysis of variance (based on the repeated measures of the selection rate constant in maltose for each genotype) indicates highly significant variation in fitness among the genotypes in this sugar (Table 5). The estimated genetic variance component, V_G, is 0.6469; the lower limit of the 95% confidence interval for this variance is 0.3176. The corresponding standard deviations are 0.804 and 0.564, respectively, which are very large in comparison with the average change in maltose (~0: Table 4, second column). In addition, a Kendall's rank test for correlation of the SRC in glucose and maltose was not statistically significant ($\tau = 0.091$, two tailed P-value > 0.1), indicating that the variation in maltose cannot be attributed to any variation in glucose. The genetic variation for fitness in maltose is significantly greater than the corresponding variation in glucose; in fact, the 95% confidence

Table 5. ANOVA for Selection Rate Constant for derived genotypes (Lenski et al., 1991) in maltose.

Analysis of variance for the selection rate constants obtained for the twelve independently derived genotypes in maltose, relative to the common ancestor. Both genotype and block are random effects.

Source	df	MS	F	P
Genotype	11	3.9698	45.050	< 0.001
Block	5	0.0806	0.914	0.479
Error	55	0.0881		

limits for V_G in the two sugars are completely non-overlapping. Among the twelve independently derived lines, a Bonferroni-corrected multiple range test indicates at least three distinct classes in terms of fitness in maltose.

Thus, the relationship between mean change in fitness and divergence in fitness among the independently derived genotypes is the exact opposite for glucose and maltose. In the environment in which the bacteria were selected (glucose), the mean improvement was large and the variation among derived genotypes in the extent of improvement was very small. In the novel environment (maltose), there was no improvement on average but significant variation arose among the independent genotypes.

Additional variability is observed in lactose. -- Based on fitness in medium containing glucose, the twelve independently derived genotypes appeared to be a single homogeneous class. However, significant heterogeneity in fitness among the derived genotypes in medium containing maltose indicates the existence of at least three distinct phenotypic classes. One might reasonably ask whether these three classes, each of which appears to be homogeneous within the statistical limitations of the glucose and maltose data, might not also be shown to be heterogeneous if fitnesses were measured in medium containing yet another sugar. To that end, I estimated the selection rate constants for each of the twelve derived genotypes (relative to the common ancestor) in lactose. The grand mean selection rate constant is 1.858; the corresponding 95% confidence interval (based on the *t*-distribution with 11 degrees of freedom) ranges from 0.426 to 3.290, indicating that the derived genotypes, as a group, show significant improvement relative

to their ancestor when competing for lactose.

An analysis of variance (based on repeated measures of the selection rate constant) also indicates highly significant variation among the genotypes in their fitness in lactose (Table 6). The estimated genetic variance, V_G, is 4.5832, and the lower limit of its 95% confidence interval is 2.7078. The corresponding standard deviations are 2.141 and 1.646, respectively. The genetic variance for fitness on lactose is significantly greater than that on glucose (and even greater than that on maltose). So during the 2,000 generations of experimental evolution, there arose significantly greater variation among the independent genotypes in their fitnesses in both novel environments (maltose and lactose) then in the environment in which the bacteria were selected (glucose).

Among the twelve independently derived genotypes, a Bonferroni-corrected multiple range test indicates at least two distinct classes in terms of fitness in lactose. Although the product-moment correlation of fitness in lactose and maltose is significant ($r = 0.673$, 10 d.f., $P = 0.015$), the multiple range test for lactose nonetheless separates two pairs of derived genotypes that were not separated by the multiple range test for maltose. Each multiple range test has an experiment-wise type I error rate of 0.05. When a further Bonferroni correction is introduced because two separate multiple range tests were performed (thus using an experiment-wise type I error rate of $0.05 / 2 = 0.025$ for each multiple range test), fitness in lactose still distinguishes one pair of genotypes that cannot be discriminated on the basis of fitness in maltose. In other words, the twelve derived genotypes selected for performance on glucose -- and which appear homogeneous in fitness on

Table 6. ANOVA for Selection Rate Constant for derived genotypes (Lenski et al., 1991) in lactose.

Analysis of variance for the selection rate constants obtained for the twelve independently derived genotypes in lactose, relative to the common ancestor. Both genotype and block are random effects.

Source	df	MS	F	P
Genotype	11	20.3189	10.231	< 0.001
Block	3	6.5820	3.314	0.032
Error	33	1.9861		

that sugar -- can be divided into at least four distinct groups based on their fitnesses in maltose and lactose.

Relative fitnesses are additive and transitive. -- In all of the preceding experiments, the fitnesses of the derived genotypes were measured relative to their common ancestor. This design assumes that any difference in fitness resulted from the ability of the derived genotypes to utilize the sugars (glucose, maltose and lactose) that were added to the medium, rather than to utilize (or resist toxic effects of) metabolites that might be produced by the bacteria. If such by-products are important, then genotypes may not simply be competing for a single limiting resource, increasing the likelihood of non-transitive interactions and other forms of frequency-dependent selection. To examine the possible importance of such complex interactions, derived genotypes were competed against one another, as well as against their common ancestor. These additional competition experiments were performed in lactose because the genetic variation in fitness was greatest, therefore providing the largest signal for possible deviations from additivity. In these experiments, all 36 (= 6 x 6) pairs of derived genotypes that differed in their arabinose-utilization marker state were competed.

Significant heterogeneity was observed among the six Ara^+ derived genotypes and among the six Ara^- derived genotypes; but there was no indication whatsoever of any statistical interaction (non-additivity) in the selection rate constants (Table 7). Also, the fitnesses of the derived genotypes relative to their common ancestor and relative to one another were highly correlated ($r = 0.933$, $d.f = 10$, $P < 0.0001$).

Table 7. ANOVA for Selection Rate Constant obtained by competing reciprocally marked derived genotypes against one another in lactose.

Differences among genotypes in both the Ara^+ and Ara^- groups are random effects.

Source	df	MS	F	P
Among Ara^+ genotypes	5	36.657	24.903	< 0.001
Among Ara^- genotypes	5	37.895	25.744	< 0.001
Interaction	25	1.472	1.010	0.479
Error	36	1.456		

Evidently, if genotype A is more fit than genotype B when the fitness of each is measured in competition with genotype C, then A is also more fit than B (and to a similar degree) when their fitnesses are measured in competition against one another. Thus, there is no evidence for any non-additivity, and hence no support for the supposition that competition other than for the resources which were deliberately added to the medium is important.

DISCUSSION

Populations of *Escherichia coli* that were propagated for 2,000 generations in a glucose-limited environment greatly improved their fitness in this environment, relative to their common ancestor, but replicate populations diverged very little from one another in mean fitness (Lenski et al., 1991). Measurements of five underlying life history components of fitness showed significant evolutionary responses for four of the five components, but little variation among populations for any of the five components (Vasi et al., submitted).

However, the similarity of the lines in glucose-limited medium belied large genotypic differences among the lines. The among-genotype variance for fitness on two other sugars (maltose and lactose), neither of which the bacteria had encountered for 2,000 generations, increased by more than 100-fold relative to that on the sugar (glucose) that had been the sole source of carbon and energy during the 2,000 generations. Evidently, the striking similarity in the fitness of the replicate genotypes in the glucose-limited environment masks much greater heterogeneity in their fitness in other environments.

What process(es) could have generated both the extreme parallelism when assayed in glucose and yet divergence when assayed in either maltose or lactose? Two potential causes for the extreme parallelism of the derived genotypes are: (1) the fitness improvement was limited by the rate at which adaptive mutations were generated and fixed, or (2) the fitness improvement was limited by a constraint common to all genotypes. Since the rate of fitness improvement of the populations was significantly faster during the first 1,000 generations than in the second, this strongly suggests that a constraint common to all populations was limiting fitness improvement.

Given a common constraint, there are two possible scenarios, drift and pleiotropy, which can explain the pattern of direct and correlated fitness responses. The scenarios differ in the manner by which the variation in correlated fitness responses is generated. In the drift scenario, the populations have all acquired mechanistically equivalent adaptations. Although exactly the same nucleotide changes resulting in the improved fitness were unlikely to have occurred in each population, each population may have acquired nucleotide changes coding for mechanistically equivalent adaptations. Differences in genotype-by-environment interactions among derived genotypes (e.g. fitness variation in maltose) are presumed to be caused by mutations which had little or no selective effect in glucose medium. Different neutral mutations became fixed in each population, and although they had little effect in the glucose environment, some of the neutral mutations were deleterious in the maltose and/or lactose nutrient environments. Under this scenario, the heterogeneity in correlated fitness responses and the larger variation in fitness in maltose and lactose compared to glucose

are due to the accumulation of mutations that are neutral in glucose but deleterious in maltose and lactose. Overall, the populations evolved in parallel, and any differences among them arose as a result of secondary, *non-adaptive* mutations.

In the pleiotropy scenario, the populations have not adapted in a mechanistically equivalent manner. Instead, the populations exhibit multiple distinct mechanisms of adaptation, but which result in similar fitness improvements in glucose due to a common constraint that limits the improvement that can be achieved by any of the mechanisms. The heterogeneity in correlated responses and the larger fitness variation in maltose and lactose result from the particular details of the different genotypes' adaptations to glucose uptake. Overall, the populations evolved in parallel in terms of all improving uptake, but they may have diverged in the specific mechanisms of improving uptake, so that differences among them arose as heterogeneous pleiotropic effect of the primary, *adaptive* mutations.

The two scenarios are not mutually exclusive, and both may have contributed to the results. The relative contributions of each can be evaluated by considering the process by which each generates divergence. The drift scenario requires many mutations since it implies that the various fitness effects in different nutrient environments are the result of independent mutations in the different nutrient pathways. The pleiotropy scenario requires relatively fewer mutations because each mutation is more likely to affect multiple nutrient pathways.

How many non-adaptive mutations can be expected over this course of the experiment which have deleterious effects in maltose or lactose, but not in glucose? The potential number of neutral substitutions

affecting a particular trait depends on the mutation rate, the number of nucleotide bases possibly involved, and the number of generations during which the population was subject to drift. The known and suspected maltose-specific catabolic genes are *lamB, malE, malF, malG, malI, malK, malM, malP, malQ, malT, malX,* and *malY,* which total about 20,000 nucleotides (Clement and Hofnung, 1981; Cole and Raibaud, 1986; Dassa and Hofnung, 1985; Duplay et al., 1984; Froshauer and Beckwith, 1984; Gilson et al., 1982; Gilson et al., 1986; Palm et al., 1987; Reidl et al., 1989; Reidl and Boos, 1991; Schwartz, 1987). The corresponding number of bases for lactose is even fewer. Assuming a mutation rate per base per generation, u, of ~4 x 10^{-10} (Drake, 1974), then the expected number of mutations in maltose-specific genes over the entire 2,000 generations for each population is given by:

(u) (# generations) (# bases) = 0.016 mutations.

And yet, all twelve independently derived genotypes are less fit in maltose that in glucose. In fact, it would require roughly 1.3 x 10^6 maltose-specific bases, or about 1/4th of the *E. coli* genome, to achieve an expectation of even one maltose-specific mutation in each population. Since both of these estimates include mutations that are neutral as well as deleterious in maltose medium, they overestimate the potential effects of non-adaptive mutations on the heterogeneous correlated responses of the genotypes in maltose. Even so, these values strongly suggest that the correlated responses observed in maltose are very unlikely to have resulted from non-adaptive mutations but must instead be due mostly to mutations that are adaptive in glucose.

Evolutionary implications. -- Although the derived genotypes have evolved in a parallel fashion as measured by fitness in the selected environment and underlying fitness components, the genotypes have diverged substantially in fitness when assayed in maltose- or lactose-limited media. This divergence apparently occurred via differences in pleiotropic effects associated with adaptive mutations. This interpretation suggests that an extreme Fisherian view of adaptation in a clonal organism, whereby the same most fit genotype is fixed in all replicate populations (see Lenski et al., 1991), is an oversimplification of a more complex process. In terms of an adaptive landscape (Wright, 1932), where fitness is viewed as a surface in a gene space, the populations appear to have approached either a common fitness plateau or separate peaks of equal height (i.e. equal fitness). Given the significant decline in the rate of further fitness improvement of the populations during the second 1,000 generations, it is not likely that the adaptive differences manifested by the heterogeneous correlated responses are transient. Thus, genetic diversity for adaptive traits can be generated and maintained simply by the stochastic nature of adaptive evolution acting on isolated populations, without recourse to environmental differences or initial genetic differences.

The existence of multiple adaptive peaks of the same height or a common fitness plateau implies the existence of many mutations which are equivalent in their effects on some primary phenotype that is subject to selection. These adaptive mutations might therefore be expected to interact epistatically, but in a sub-additive manner. More explicitly, two adaptive mutations which each confer 10% fitness improvement when separate, would together confer less than a 20% fitness improvement if

they interact in a sub-additive epistatic manner.

Dykhuizen and Hartl (1980, 1983), Hartl and Dykhuizen (1981, 1985) and Silva and Dykhuizen (1993) observed greater selective differences among several enzyme alleles (allozymic variants) when fitness was assayed in alternative or novel environments rather than the prevailing or selected environment. In this study, substantially more variation in fitness was observed in alternative nutrients than in the selected environment. This "latent potential for selection" may affect subsequent evolution in two ways. First, if there is a change in the external environment, then mutations with equivalent selective value in the original environment, but which have large fitness differences in the new environment, may cause in phenotypic divergence between populations that were phenotypically similar in the old environment, even if the environmental conditions (both before and after the change) are the same for all populations. Thus, a latent selection potential may be the "raw material for adaptive evolution" (Kimura, 1983) and anagenetic evolution may often come about "from capitalizing on preexisting latent selection potentials in the presence of novel ecological opportunity" (Stebbins and Hartl, 1988). Second, epistasis can alter the selective value of a specific mutation depending upon the genetic background in which it occurs. Therefore, selectively equivalent genetic differences may engender different constraints on subsequent evolution because of different genetic interactions (Wright, 1932, 1982, 1988; Lewontin, 1974; Gould, 1989).

The extreme divergence in fitness between genotypes in alternative nutrients suggests that subsequent selection in either maltose or lactose may result in sustained divergence between the genotypes. That

is, the genotypes may have accumulated so many genetic differences that they respond very differently to a change in their selective environment. This possibility is tested experimentally in Chapter IV.

Chapter II
IDENTIFYING TARGETS OF SELECTION ON THE BASIS OF GENETIC SIMILARITIES AND DIFFERENCES AMONG REPLICATE POPULATIONS

Evolutionary models which emphasize the role of natural selection and ecology can often predict some phenotypic outcomes of selection, without incorporating much mechanistic biological detail (Rose 1984; Lenski, 1988b; Mueller, 1988; Bull et al., 1991). However, such models may miss important aspects of evolutionary change; whether models that ignore underlying mechanistic detail are sufficient to predict evolutionary change is a matter of contention (Lewontin, 1974; Rose et al, 1987; Dykhuizen and Dean, 1990; Lenski et al., 1991; Bull and Moulineux, 1992; Via, 1993a, 1993b; Schlichting and Pigliucci, 1993; Scheiner, 1993). A potential limitation of non-mechanistic models stems from the highly integrated nature of organisms (Gould, 1979). Selection acts upon the whole organism, and because organisms are not simply collections of various traits, selective responses may be constrained from behaving as predicted by non-mechanistic models. An in-depth evaluation of the phenotypic bases of evolutionary change is one method by which the benefits of mechanistic models can be evaluated.

This chapter seeks to examine the biochemical bases of adaptation and divergence exhibited by the twelve bacterial populations from Lenski et al. (1991) by carefully analyzing correlated responses to selection and genetic variation in the correlated responses. The fitnesses of populations of *Escherichia coli* that were propagated for 2,000 generations in glucose-limited medium (Lenski et al., 1991) are assayed in nine additional nutrient media to determine their correlated responses to selection. These correlated responses were then "mapped"

to known physiological mechanisms for nutrient uptake (see below) so that: 1) targets of selection during the 2,000 generations of evolution could be determined; 2) the populations could be further characterized in terms of genetic divergence in adapting to the selected environment (see also Chapter I); and 3) the relationship between bacterial physiology and fitness variation in both selected and unselected environments could be explored.

The remainder of this chapter is organized as follows. The next section provides essential background information on the physiological genetics of resource transport in the study organism. There is then an experimental overview that describes the experimental system and includes expectations based on particular hypotheses. The remainder is organized as sections on Materials and Methods, Results, and Discussion.

PHYSIOLOGICAL BACKGROUND INFORMATION

The mechanisms by which bacteria transport carbohydrates are some of the best characterized systems in biology. Carbohydrate transport in gram-negative bacteria requires three steps. The first step is passage from the extracellular media through the outer membrane to the periplasmic space, which is typically achieved by passive diffusion through an outer membrane pore. The second step is passage from the periplasmic space through the inner membrane to the cytoplasm, and this is typically performed by a substrate-specific permease. The third step is either phosphorylation of the monosaccharide carbohydrate or hydrolysis of a disaccharide, both of which prevent the back-flow of the carbohydrate to the periplasm (Cronan et al., 1987).

In *Escherichia coli* B, the experimental organism used in this

study, carbohydrate transport through the outer membrane occurs via the outer membrane protein OmpF, or through LamB which is only produced at high levels during growth on maltose, maltodextrins, or trehalose. Although many carbohydrates can diffuse through both OmpF and LamB, lack of LamB production results in no growth on maltodextrins, severely reduced growth on maltose, and slow growth on trehalose (Klein and Boos, 1993).

Of the mechanisms for carbohydrate transport across the inner membrane, the phosphoenolpyruvate:carbohydrate phosphotransferase system (PTS) may be the most important for bacterial growth. Carbohydrates transported by PTS are preferred growth substrates for *Escherichia coli*, so that during growth on PTS substrates, growth on non-PTS substrates is repressed. The overall chemical reaction of the PTS is:

enolpyruvate-P + carbohydrate -----> pyruvate + carbohydrate-P.

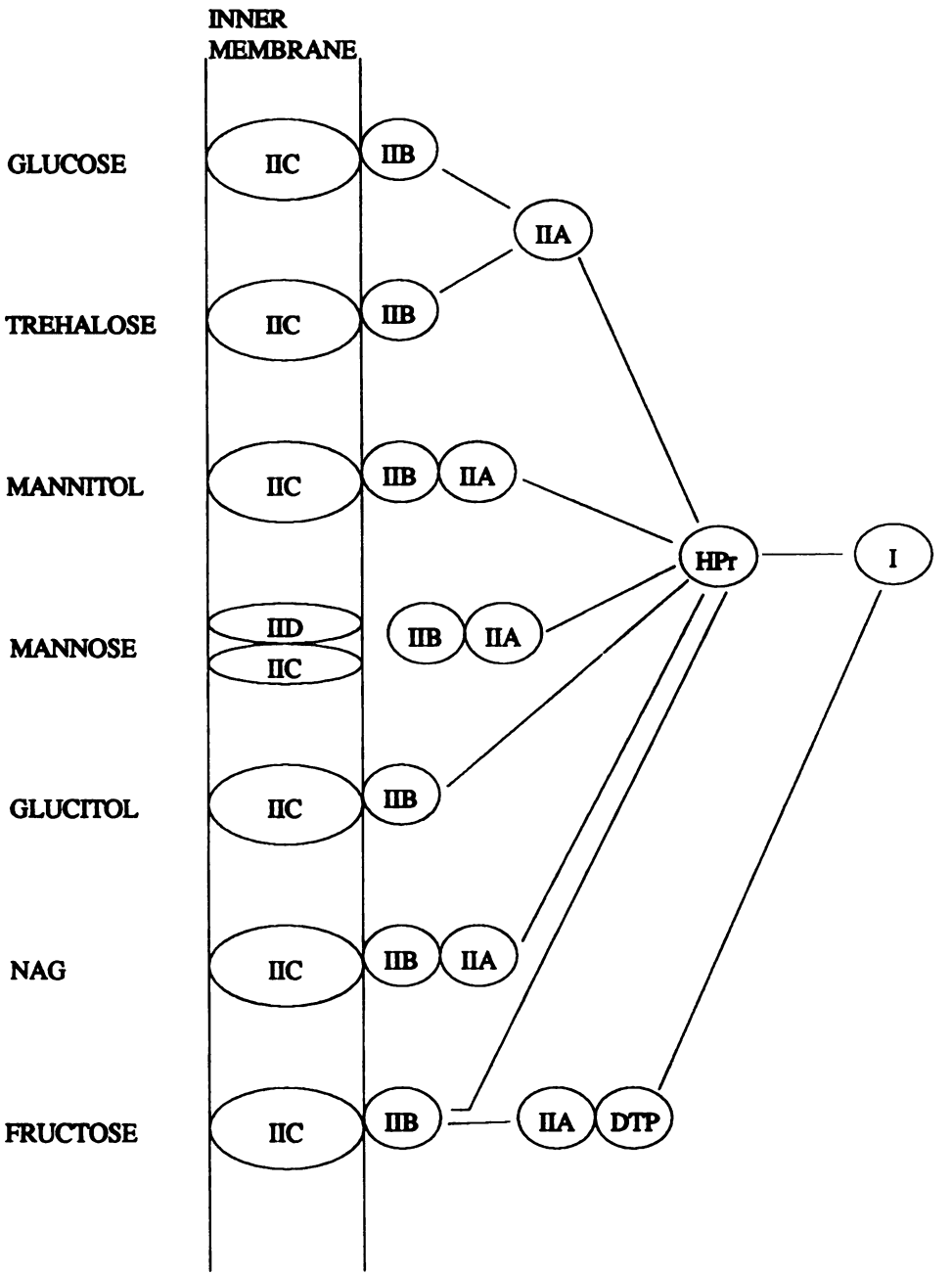
Concomitant with the progress of this reaction is the transport of the carbohydrate from the periplasmic space across the inner membrane into the cytoplasm. Overall, the PTS performs both of the last two functions of carbohydrate transport: active passage through the inner membrane and phosphorylation to prevent backflow.

The PTS can be divided into two parts, general and substrate-specific enzyme pathways. The general part is a two-step pathway which first picks up phosphate from phosphoenolpyruvate (PEP) (performed by enzyme I) and then transfers the phosphate from enzyme I to the substrate-specific enzymes (performed by HPr). The substrate-specific part carries out transport and phosphorylation (Figure 2) (Saier and

Reizer, 1992). For each substrate-specific pathway there are two, three, or four corresponding proteins: IIA, IIB, IIC, and IID. The class IIC and IID proteins form nutrient-specific transmembrane channels. The class IIB enzyme phosphorylates the substrate as it is transported across the inner membrane. The class IIA enzyme transfers the phosphate from HPr to IIB. Although there are three or four substrate-specific enzyme domains for each nutrient, the actual number of enzymes may be two, three, or four, as the domains may occur in the same polypeptide, depending upon the particular nutrient. In addition, there is some overlap among the nutrient-specific enzymes in their nutrient specificity, so that IIA^{Glc} (the class IIA enzyme for glucose) is used during growth in trehalose, and glucose will act as a substrate for the mannose specific enzymes, if they are expressed.

Transport, and growth, on non-PTS substrates is greatly affected by the transport of a PTS substrate. The keystone protein by which the PTS regulates growth on non-PTS substrates is IIA^{Glc}. As alluded to above, IIA^{Glc} exists in either a phosphorylated or unphosphorylated state, and the regulatory effects of IIA^{Glc} depend upon the relative amounts of phosphorylated IIA^{Glc} to unphosphorylated IIA^{Glc} (Roseman and Meadow, 1990). Unphosphorylated IIA^{Glc} greatly reduces growth on non-PTS substrates, while the presence of phosphorylated IIA^{Glc} stimulates non-PTS substrate utilization. The amounts of phosphorylated and unphosphorylated IIA^{Glc} depend upon the phosphorylation state of $IIBC^{Glc}$, to which IIA^{Glc} donates phosphate, and HPr, which donates phosphate to IIA^{Glc}. The extent to which $IIBC^{Glc}$ and HPr are phosphorylated depends in turn upon the presence of a PTS substrate, such that growth on a PTS substrate shifts the balance of IIA^{Glc} to the

Figure 2. PTS carbohydrate uptake. All uptake pathways share proteins I and HPr, but do not share substrate-specific proteins as well: IIA, IIB, IIC, and IID (see text for additional details).



unphosphorylated state and lack of a PTS substrate shifts the balance to the phosphorylated state. When IIA^{Glc} is in the unphosphorylated state, it prevents growth on non-PTS substrates by binding to enzymes essential for the transport of non-PTS substrate (generally to the inner membrane permeases), thereby inhibiting them. When IIA^{Glc} is in the unphosphorylated state, it stimulates the production of cyclic AMP (cAMP), which is required for the expression of many catabolic genes. Thus, the PTS has both a negative and positive regulatory role in growth on non-PTS substrates.

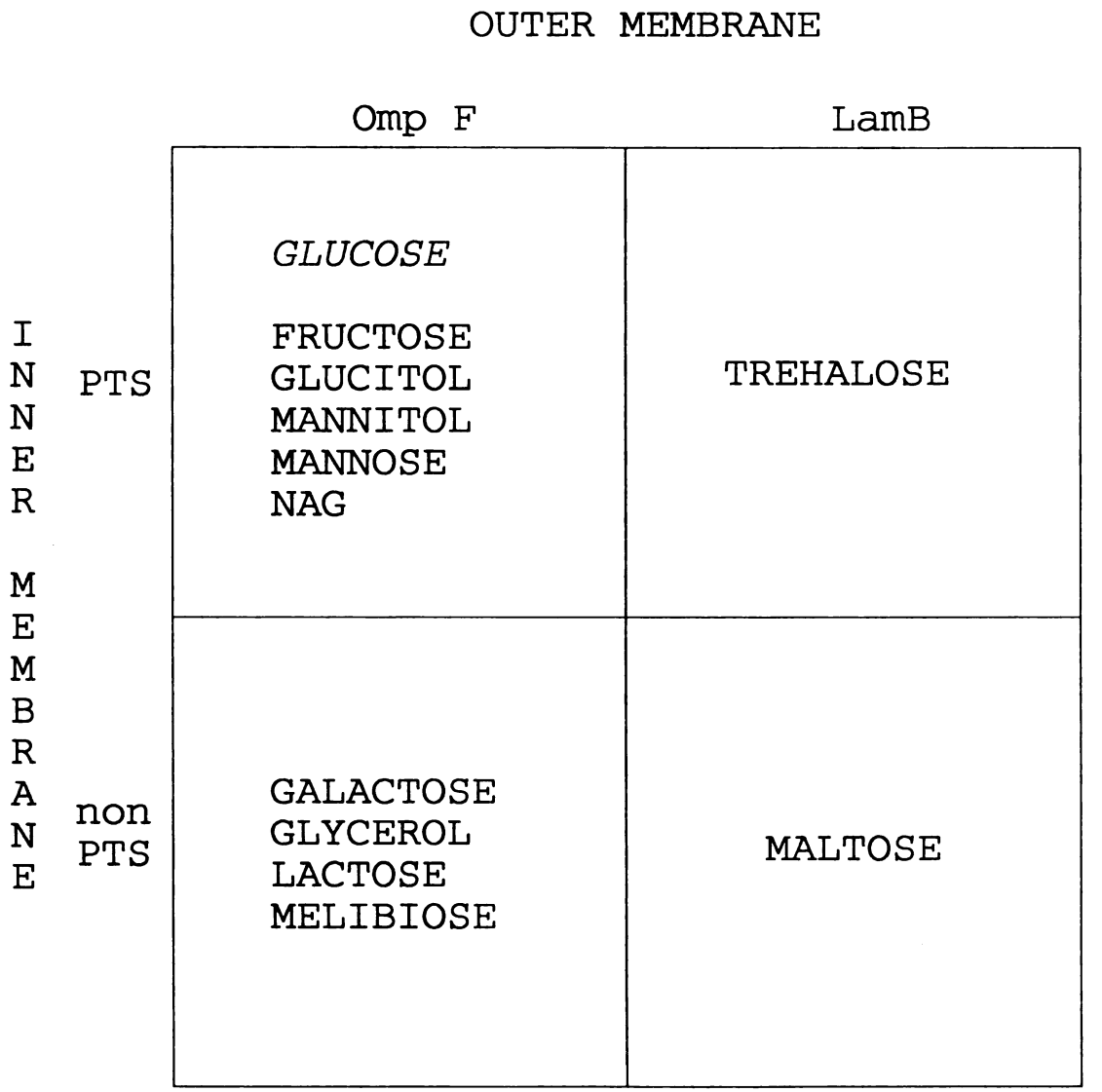
The regulation of PTS protein synthesis mostly follows the division between general and substrate-specific enzyme pathways. All substrate-specific enzymes, excepting IIA^{Glc}, are grouped together in operons by substrate (Postma, 1993). The two general enzymes, HPr and I, as well as the glucose-specific enzyme IIA^{Glc}, are coded for by *ptsH*, *ptsI*, and *crr* genes, respectfully, which in that order together form the *pts* operon. The levels of these enzymes are always fairly high due to constitutive expression of the *pts* operon (Rephaeli and Saier, 1980). However, up to a three-fold increase in expression is possible during growth on PTS substrates. The *pts* operon regulation is fairly complex, with three or possibly four promoters. Two promoters are important for *ptsH* and *ptsI* transcription, P0 and P1, with P0 as the major promoter when cAMP levels are high, and P1 when cAMP levels are lower. The third promoter, P2, is cAMP-independent and is the major promoter for *crr* transcription.

EXPERIMENTAL OVERVIEW

The experiments described in this chapter build on those described in chapter I, with a stronger emphasis on determining the targets of selection. The bacterial genotypes have been previously described in chapter I. Briefly re-iterating, Lenski et al. (1991) propagated twelve replicate populations of *Escherichia coli* for 2,000 generations in a resource-limited media containing glucose as the sole carbon and energy source usable by the bacteria. All populations were initiated from a common clonal ancestor and thus were initially isogenic and homogeneous, except for a neutral marker. The populations were maintained on a 24 hour schedule, where a small sample (0.1 ml) of the previous day's culture was transferred into 9.9 ml of fresh media. At 2,000 generations, a single clone was isolated from each population to serve as a representative genotype of that population.

The experiments reported here consisted of comparing the competitive ability of the twelve genotypes in nine additional nutrient environments (fructose, mannose, mannitol, N-acetylglucosamine [NAG], sorbitol, galactose, glycerol, melibiose, and trehalose), so that (with glucose, maltose, and lactose: see Chapter I) the populations have been examined in twelve nutrient environments overall. The twelve nutrients fall into four groups, in terms of the physiological basis of their transport through the outer and inner membranes (figure 3). In *E. coli* B, transport of carbohydrates through the outer membrane occurs primarily through either of two outer membrane proteins, OmpF or LamB (Nikaido and Vaara, 1987). Transport through the inner membrane occurs by either the phosphotransferase system (PTS), in which case the nutrients are phosphorylated during transport (Lin, 1987), or by

Figure 3. Classification of nutrients by uptake.



nutrient-specific non-PTS transport systems, where phosphorylation occurs after transport. Although transport of the PTS nutrients through the inner membrane does involve some nutrient-specific enzymes, all of the uptake pathways share one or more enzymes involved with phosphorylation (figure 2). Non-PTS transport is regulated by PTS transport as non-PTS substrate uptake is repressed when a PTS substrate is present and is derepressed when a PTS substrate is absent (Saier and Stiles, 1975).

Outer membrane functions. Glucose, fructose, mannose, mannitol, N-acetylglucosamine (NAG), glucitol, galactose, glycerol, lactose, and melibiose have not been found to induce production of any outer membrane transport protein other than OmpF, and are assumed to pass through the outer membrane via OmpF. Maltose transport through the outer membrane occurs primarily via outer membrane transport protein LamB (Szmelcman et. al., 1976), although there is also substantial OmpF production during growth on maltose, perhaps for maintenance of membrane stability (Sato and Yura, 1981). Trehalose also induces LamB production (Klein and Boos, 1993), and there is substantial OmpF production, but it is not known what role OmpF has in the passage of trehalose through the outer membrane.

Inner membrane functions. -- Glucose, fructose, mannitol, mannose, NAG, glucitol, and trehalose are PTS nutrients. For each of these nutrients, there are one or more specific transmembrane proteins that transport the nutrient through the inner membrane (Postma, 1987). These transmembrane proteins are denoted IIC (and IID) proteins (Saier and Reizer, 1992). All of the PTS uptake pathways described in this work

share two enzymes, HPr and I, except for fructose. Fructose uptake at 25μg/ml requires enzyme I, but in addition to HPr it also uses a fructose specific HPr enzyme, DTP (Kornberg, 1990) (figure 2).

Maltose, galactose, glycerol, lactose, and melibiose are non-PTS nutrients and are not phosphorylated during transport across the inner membrane. Each nutrient has specific permeases and kinases, and their expression is regulated by the presence of glucose or other PTS nutrients (Saier, 1989) as well as by the presence of the particular non-PTS nutrient. Galactose utilization is unusual for non-PTS nutrients in that expression of its permease is independent of cAMP, and expression of the catabolite genes is promoted by both a cAMP-dependent promoter (P_{G1}) and a promoter that operates in the absence of cAMP (P_{G2}) (Adhya, 1987). Galactose and lactose uptake through the inner membrane are driven by the co-transport of a proton into the cell. Maltose uptake is active and is thought to be ATP-dependent (Mimack et al., 1989). Glycerol transport occurs by facilitated diffusion, and is not energy dependent. Melibiose transport is active, driven by the co-transport of a sodium atom into the cell (Lin, 1987).

Expectations. -- The outcome of these experiments was judged in terms of four expectations. *Expectation 1* - If OmpF was a target of selection, then correlated fitness responses of the populations in trehalose media (in which LamB expression is required for rapid growth) are expected to be smaller than the correlated fitness responses of the populations in other PTS nutrients. *Expectation 2* - If the PTS was a target of selection, then larger positive correlated fitness responses are expected for the OmpF/PTS nutrients (excludes trehalose) than for the OmpF/non-PTS nutrients (excludes maltose). *Expectation 3* - If the

independently derived genotypes acquired heterogeneous adaptations in the PTS system, then larger genetic variances for the correlated fitness responses are expected for the OmpF/non-PTS nutrients than for the OmpF/PTS nutrients. This expectation relies on the complex regulatory role that PTS transport plays in the transport of non-PTS nutrients.

Expectation 4 - If the correlated responses reflect traits that are important for fitness in glucose, then the correlated responses and their among-genotype variances, taken together, are expected to demonstrate a qualitatively different pattern in OmpF/PTS nutrients than in OmpF/non-PTS nutrients. In particular, correlated responses that are coupled to traits important for fitness in the selected environment should show both (i) a large positive fitness increase, and (ii) very similar responses in the replicated lines.

MATERIALS AND METHODS

Measurement of selection rate constants in competition experiments. -- Competition experiments were performed under the same culture conditions originally used to propagate the populations, except that other carbon/energy sources were substituted for glucose. The nutrient concentration was the same (25μg/ml) for all competition experiments. These experiments were done in a 'head to head' fashion, where two genotypes were grown simultaneously in a single culture, thereby competing for the same pool of limiting substrate. In order to distinguish the two competitors when estimating fitness, one competitor was always Ara^+ while the other was Ara^-; also, one competitor was one of the twelve derived genotypes while the other was the reciprocally marked common ancestor.

The competition protocol was as follows. Both competitors were first preconditioned separately for 24 hours in the same type of media in which they were going to be competed. Then each competitor was diluted 200-fold into a single competition culture which contained fresh medium. Thus, the total initial population size of bacteria was approximately 100-fold less than the final population size achieved at the end of the standard daily growth cycle of 24 hours. Initial and final population densities of each competitor were determined by plating the competition culture onto TA agar.

The Selection Rate Constant (SRC) is the difference in Malthusian parameters between the two competitors and is a relative measure of fitness (Nagylaki, 1977; Lenski et al., 1991). The Malthusian parameter of a genotype is determined by taking the natural logarithm of the ratio obtained as the final cell density divided by the initial cell density. Any difference in plating efficiency between Ara^+ and Ara^- competitors does not affect SRC estimates, as long as plating efficiencies remain constant between initial and final samples.

Experimental designs and statistical analyses. -- To test the selective neutrality of the arabinose-utilization marker, replicated competitions were performed on the two marker states (Ara^+ and Ara^-) of the ancestral genotype against one another in each type of medium. These marker controls were performed simultaneously with the competition experiments used to estimate the fitness of the derived genotypes relative to their ancestors. We also tested for possible interactions between the arabinose marker and the derived versus ancestral backgrounds by comparing the average fitnesses of the two classes (Ara^- and Ara^+) of the derived genotypes in each medium.

A minimum of three estimates of the selection rate constant were obtained for each of the twelve derived genotypes (relative to the reciprocally marked common ancestor) in each nutrient media. Replicates were performed in sets of complete blocks. The genetic variance in fitness (in a particular sugar) among independently derived genotypes was determined by a two-way analysis of variance, where block and genotype were random effects. The genetic variance component, V_G, was estimated as the difference in the genotype and error mean-squares, divided by the number of replicate assays (=blocks) per group. Although the twelve independently derived genotypes are regarded as random rather that fixed entities for most purposes, a Bonferroni-corrected T-test for multiple comparisons (Miller, 1981, SAS Institute, 1988, pp. 593-595) was performed to ask how many distinct classes of genotypes could be distinguished on the basis of fitness in the experimental sugars.

Comparison of correlated fitness responses in trehalose with the five OmpF PTS nutrients, excluding glucose, was performed on the mean SRC estimates of each genotype by one-tailed Dunnett's t-tests with an experimentwise confidence interval of 0.05 (Dunnett, 1964; SAS Institute, 1988).

Comparison of correlated fitness responses in the five OmpF/PTS (excluding glucose) and four OmpF/non-PTS nutrient environments was done by two methods. First, an unbalanced nested three-way analysis of variance was computed for differences among the genotypes in SRC between the PTS and non-PTS nutrients (see Table 8 for design). The null hypothesis was that the OmpF/PTS and OmpF/non-PTS nutrient environments did not differ in mean correlated fitness response and the alternative hypothesis was that the mean correlated fitness response was larger in

Table 8. Expected mean squares for OmpF/PTS vs. OmpF/non-PTS ANOVA.

Source of Variation	Expected Mean Square[1]
TRANSPORT (Fixed Effect)	Var(Error) + 0.004 Var(GENOTYPE) + 14.997 Var(BLOCK) + 39.171 Var(SUGAR) + Q(TRANSPORT)[2]
SUGAR (Random Effect, Nested within Transport)	Var(Error) + 0.0053 Var(GENOTYPE) + 10.204 Var(BLOCK) + 38.253 Var(SUGAR)
BLOCK (Random Effect)	Var(Error) + 0.0064 Var(GENOTYPE) + 14.032 Var(BLOCK)
GENOTYPE (Random Effect)	Var(Error) + 28.819 Var(GENOTYPE)

[1] Expected Mean Squares are calculated for an unbalanced ANOVA as per Sokal and Rohlf (1981, pp. 293 - 308).

2 Component of Mean Square due to a fixed effect, whose values are determined by quadratic form.

the PTS nutrients. Second, the nutrients were ranked by the number of genotypes that had mean SRC values larger than zero in each nutrient and a one-tailed Wilcoxon two-value test (identical to a Mann-Whitney U test) was performed on this ranking. The null hypothesis was that the genotypes were equally likely to have positive fitness responses in OmpF/PTS nutrients as in OmpF/non-PTS nutrients. The alternative hypothesis was that the genotypes were more likely to have positive correlated fitness responses in a PTS nutrient than in a non-PTS nutrient.

Comparison of the relative magnitudes of genetic variation in OmpF/PTS and OmpF/non-PTS nutrients was performed by ranking the nutrients by the genetic variation of the genotypes in each nutrient. A one-tailed Wilcoxon two-value test was then performed on the rankings with the null hypothesis being that the genetic variation among the twelve evolved genotypes was not different in the PTS and non-PTS nutrient environments. The alternative hypothesis is that the genetic variation was larger in non-PTS nutrients than in PTS nutrients (excluding trehalose and maltose).

To simultaneously address both expectations that if a trait is important for fitness it should (i) show a large positive response (large SRC), and (ii) respond similarly in the replicated genotypes yielding a small genetic variance, a composite statistic was calculated. This statistic was the proportion of genotypes (out of twelve) with positive SRC, divided by the genetic variance of the genotypes in that nutrient. A one-tailed Wilcoxon two-value test was then performed on the rankings with the null hypothesis being that the overall correlated

fitness response, as summarized by the composite statistic, of the twelve evolved genotypes was not different in the PTS and non-PTS nutrient environments. The alternative hypothesis was that the overall correlated fitness responses were qualitatively different, such that the composite statistic was larger in OmpF/PTS nutrients than in OmpF/non-PTS nutrients.

RESULTS

Selective neutrality of the genetic marker. -- The neutrality of the arabinose-utilization marker was examined in the ancestral genotype in all nutrients (Table 9). In every medium the marker is neutral in the ancestral genotype at the $P < 0.05$ level. In one nutrient (mannose), the marker had a marginally significant effect ($0.05 < P < 0.1$), but given that nine such tests were performed, this outcome is not even suggestive of a marker effect. Although neutral in the ancestral genetic background, it is possible that this marker might interact epistatically with (and be subject to selection in) the derived genetic backgrounds. However, the six Ara+ derived genotypes (as a group) are no more or less fit then the six Ara- derived genotypes (as a group) in any of these sugars, excepting mannitol (Table 10; also see Table 39, appendix A which gives the average selection rate constants estimated for each of the twelve derived genotypes in each of the twelve nutrients, as well as the number of replicate assays on which each average is based). Given the multiplicity of tests, this outcome is not indicative of a marker effect. These results, coupled with the fact that all experiments were fully balanced with respect to marker, suggest that pooling the data for the Ara^+ and Ara^- genotypes in subsequent analyses

Table 9. Effective neutrality of the arabinose-utilization marker in the ancestral genotype in medium containing the nine different sugars used in this study[1].

Sugar	Repetitions (n)	Selection Rate Constant (Ara^+ Relative to Ara^-) Mean (± SE)[2]	p[3]
Fructose	7	0.0674 (± 0.0928)	0.495
Glucitol	9	-0.0432 (± 0.1146)	0.716
Mannose	9	-0.2969 (± 0.1318)	0.054
Mannitol	9	0.0411 (± 0.0568)	0.490
NAG	12	0.0340 (± 0.0752)	0.660
Trehalose	12	0.0000 (± 0.0429)	0.999
Galactose	9	0.0793 (± 0.0914)	0.411
Glycerol	9	0.0723 (± 0.0752)	0.364
Melibiose	9	-0.1030 (± 0.0832)	0.251

[1] See table 2 in Chapter I for comparable data for glucose, maltose, and lactose.

[2] Mean (and standard error of the mean) selection rate constant based on n assays in the medium containing a particular sugar.

[3] Two-tailed probability computed from the *t*-distribution with n - 1 degrees of freedom; the null hypothesis is that the selection rate constant equals zero, indicating equal fitness for the Ara^+ and Ara^- marker states.

Table 10. Effective neutrality of the arabinose-utilization marker in the derived genotypes in medium containing the nine different sugars used in this study[1].

Sugar	Mean Selection Rate Constant: Ara^+ Derived Relative to Ara^- Ancestor	Mean Selection Rate Constant: Ara^- Derived Relative to Ara^+ Ancestor	Difference (±SED)[1]	P[2]
Fructose	1.338	0.993	0.345 (+ 0.240)	0.182
Glucitol	0.953	1.340	-0.388 (+ 0.348)	0.292
Mannose	0.227	0.629	-0.403 (+ 0.333)	0.255
Mannitol	1.490	1.746	-0.256 (+ 0.123)	0.044
NAG	1.566	1.442	0.123 (+ 0.096)	0.277
Trehalose	0.441	-0.084	0.494 (+ 0.687)	0.489
Galactose	-0.442	-0.633	0.191 (+ 0.229)	0.423
Glycerol	0.905	1.637	-0.732 (+ 0.786)	0.373
Melibiose	-2.751	-1.772	-0.979 (+ 0.535)	0.097

[1] See table 2 in Chapter I for comparable data for glucose, maltose, and lactose.

[2] Difference (and standard error of the difference) in the selection rate constants based on six independently derived genotypes in each marker class.

[3] Two-tailed probability computed from the *t*-distribution with 6 + 6 - 2 = 10 degrees of freedom; the null hypothesis is that the difference in the mean selection rate constants for the two marker classes of derived genotype equals zero (indicating equal changes, on average, in the fitness for the Ara^+ and Ara^- derived genotypes).

is statistically valid.

Fitness in trehalose is lower than in OmpF/PTS nutrients. -- The genotypes have statistically different correlated fitness responses in trehalose versus four of the five OmpF/PTS nutrients (Table 11), even after adjusting for multiple comparisons. This result supports the hypothesis that the outer membrane in general, and OmpF in particular, were targets of selection during the long-term propagation in glucose.

Mean fitness does not distinguish OmpF/PTS and OmpF/non-PTS nutrients. -- The genotypes' correlated responses do not differ, on average, between OmpF/non-PTS nutrients (excluding glucose) and OmpF/PTS nutrients (Table 12). This result suggests that inner membrane transport was not a target of selection. However, this analysis assumes that the underlying variation among genotypes was relatively equal among the two classes of nutrient, which is shown below to be incorrect.

The genotypes are more likely to have improved fitness in OmpF/PTS than OmpF/non-PTS nutrients. -- A one-tailed Wilcoxon two-value test on the ranking based upon the number genotypes (out of 12) with positive correlated fitness responses does differentiate the nutrients ($p = 0.0317$) (Table 13). That the number of genotypes with positive mean SRC differentiates the nutrients by PTS versus non-PTS transport mechanism, whereas grand mean differences do not, results from the much greater variation in correlated fitness responses in non-PTS nutrients than in PTS nutrients (see next section). Each genotype was less likely to have improved in fitness in non-PTS than in PTS nutrients, but genotypes that did have higher fitness in a non-PTS nutrient often had substantially higher fitness, raising the mean response. Therefore, with respect to

Table 11. Paired t-tests for OmpF/PTS nutrients versus trehalose.

Nutrient Comparison	Mean Difference in SRC	Confidence Interval Lower	Upper	Significance
NAG-Trehalose	1.341	0.821	1.861	*
Mannitol-Trehalose	1.454	0.934	1.975	*
Fructose-Trehalose	1.002	0.482	1.522	*
Glucitol-Trehalose	0.983	0.463	1.503	*
Mannose-Trehalose	0.265	−0.256	0.785	

* $P < 0.05$ after adjusting for nonorthogonal multiple comparisons using Dunnetts' t-tests for paired SRC values for 12 derived genotypes.

Table 12. Significance tests for OmpF/PTS vs. OmpF/non-PTS ANOVA.

Source: TRANSPORT
Error: 1.024*MS(SUGAR) + 0.3241*MS(BLOCK) - 0.0001*MS(GENOTYPE)
- 0.348*MS(Error)

Numerator		Denominator			
DF	MS	DF	MS	F Value	P[1]
1	81.397	7.01	64.833	1.255	0.1500

Source: SUGAR
Error: 0.7272*MS(BLOCK) + 205E-7*MS(GENOTYPE) + 0.2728*MS(Error)

Numerator		Denominator			
DF	MS	DF	MS	F Value	P
7	63.263	29.28	1.630	38.821	0.0001

Source: BLOCK
Error: 0.0002*MS(GENOTYPE) + 0.9998*MS(Error)

Numerator		Denominator			
DF	MS	DF	MS	F Value	P
17	1.703	309.46	1.435	1.187	0.2738

Source: GENOTYPE
Error: MS(Error)

Numerator		Denominator			
DF	MS	DF	MS	F Value	P
10	4.824	309	1.434	3.363	0.0002

[1] P is expressed based on corresponding one-tailed t-test.

Table 13. Consistency of positive fitness response in OmpF nutrients.

Nutrient	Repetitions	number of Mean SRC > 0	rank	p[1]
				0.0317
OmpF/PTS				
Fructose	3	11	6.5	
Glucitol	3	11	6.5	
Mannose	3	8	3	
Mannitol	3	12	8.5	
NAG	4	12	8.5	
OmpF/Non-PTS				
Galactose	3	1	2	
Glycerol	3	10	5	
Lactose[2]	4	9	4	
Melibiose	3	0	1	
Selected				
Glucose[2,3]	5	12	(na)	
LamB/PTS				
Trehalose	4	7	(na)	
LamB/non-PTS				
Maltose[2]	6	7	(na)	

[1] One-tailed Wilcoxon two-value test of hypothesis that the number of genotypes (out of twelve) with positive mean SRC is greater in OmpF/PTS than in OmpF/non-PTS nutrients.

[2] See Chapter 1.

[3] Carbohydrate added to the medium in which the derived genotypes were selected (OmpF/PTS).

the likelihood that a genotype would have a positive correlated fitness response:

$$\text{OmpF/PTS} > \text{OmpF/non-PTS}$$

Thus, improvements to transport of nutrients across the inner membrane, and particularly to the PTS, were responsible for some of the higher fitness in glucose.

The genotypes have larger genetic variance in PTS nutrients than in non-PTS nutrients. -- The ANOVAs for among-genotype variation in each media are given in Table 14, and the estimated genetic variance components for each medium are given in Table 15. A Wilcoxon two-value test of the one-tailed hypothesis that genetic variation is greater in OmpF/non-PTS nutrients than in OmpF/PTS nutrients is marginally significant (P = 0.0556), indicating that:

$$V_{G_{\text{OmpF/PTS}}} < V_{G_{\text{OmpF/non-PTS}}}.$$

Thus, the independently derived genotypes may have improved PTS transport by different mechanisms, which have heterogeneous pleiotropic effects on fitness in non-PTS nutrients.

The genotypes have a qualitatively different response in OmpF/PTS nutrients than in OmpF/non-PTS nutrients. -- The genotypes generally are both more fit and less variable in OmpF/PTS nutrients than in OmpF/non-PTS nutrients (Table 16 and Figure 4). Thus, simultaneous analysis of both the correlated fitness response and the genetic variation between populations differentiates the nutrient environments to a greater extent

Table 14. ANOVAs for fitness (SRC relative to the common ancestor) of the twelve independently derived genotypes from Lenski et al. (1991) in twelve different nutrients.

	Block[1]			Genotype Heterogeneity[1]			Error	
Nutrient	df	MS	P	df	MS	P	df	MS
OmpF/PTS								
Fructose	2	0.0264	0.8478	11	0.5703	0.0051	22	0.1585
Glucitol	2	0.2773	0.2753	11	1.1141	0.0003	22	0.2026
Mannose	2	1.7373	0.0415	11	1.0268	0.0570	21[2]	0.4648
Mannitol	2	0.7792	0.0027	11	0.1766	0.1223	22	0.0997
NAG	3	0.1995	0.0377	11	0.1177	0.0819	32[2]	0.0628
OmpF/Non-PTS								
Galactose	2	0.2064	0.0511	11	0.4596	< 0.0001	22	0.0604
Glycerol	2	0.4442	0.6080	11	5.4900	< 0.0001	22	0.8726
Lactose[3]	3	6.5820	0.0318	11	20.3190	< 0.0001	33	1.9861
Melibiose	2	3.7221	0.0634	11	3.1232	0.0257	22	1.1872
Selected								
Glucose[3,4]	4	0.0492	0.5103	11	0.0837	0.1978	44	0.0589
LamB/PTS								
Trehalose	3	4.2868	< 0.0001	11	5.4242	< 0.0001	32[2]	0.2230
LamB/non-PTS								
Maltose[3]	5	0.0806	0.4785	11	3.9698	< 0.0001	55	0.0881

[1] Block and genotype are random effects.

[2] Indicates one missing value.

[3] See Chapter 1.

[4] Carbohydrate added to the medium in which the derived genotypes were selected (OmpF/PTS).

Table 15. Comparison between estimated genetic variance components for derived genotypes in OmpF/PTS and OmpF/non-PTS nutrients.

Nutrient	Genetic Variation (V_G)	rank	p[1]
			0.0556
OmpF/PTS			
Fructose	0.1373	6	
Glucitol	0.3038	4	
Mannose	0.1932	5	
Mannitol	0.0256	8	
NAG	0.0140	9	
OmpF/Non-PTS			
Galactose	0.1331	7	
Glycerol	1.5392	2	
Lactose[2]	4.5832	1	
Melibiose	0.6453	3	
Selected			
Glucose[2,3]	0.0050	(na)	
LamB/PTS			
Trehalose	1.3305	(na)	
LamB/non-PTS			
Maltose[2]	0.6469	(na)	

[1] One-tailed Wilcoxon two-value test of hypothesis that the genetic variance among derived genotypes is greater in OmpF/PTS than in OmpF/non-PTS nutrients.

[2] See Chapter 1.

[3] Carbohydrate added to the medium in which the derived genotypes were selected (OmpF/PTS).

Table 16. Comparison between derived genotypes in OmpF/PTS and OmpF/non-PTS nutrients by a composite statistic.

Nutrient	(Proportion SRC > 0)/(V_G)	rank	P[1]
			0.0079
OmpF/PTS			
Fructose	6.67	3	
Glucitol	3.02	5	
Mannose	3.45	4	
Mannitol	39.06	2	
NAG	71.43	1	
OmpF/Non-PTS			
Galactose	0.63	6	
Glycerol	0.54	7	
Lactose[2]	0.16	8	
Melibiose	0.00	9	
Selected			
Glucose[2,3]	200.00	(na)	
LamB/PTS			
Trehalose	0.44	(na)	
LamB/non-PTS			
Maltose[2]	0.90	(na)	

[1] One-tailed Wilcoxon two-value test of hypothesis that the composite statistic is greater in OmpF/PTS than in OmpF/non-PTS nutrients.

[2] See Chapter 1.

[3] Carbohydrate added to the medium in which the derived genotypes were selected (OmpF/PTS).

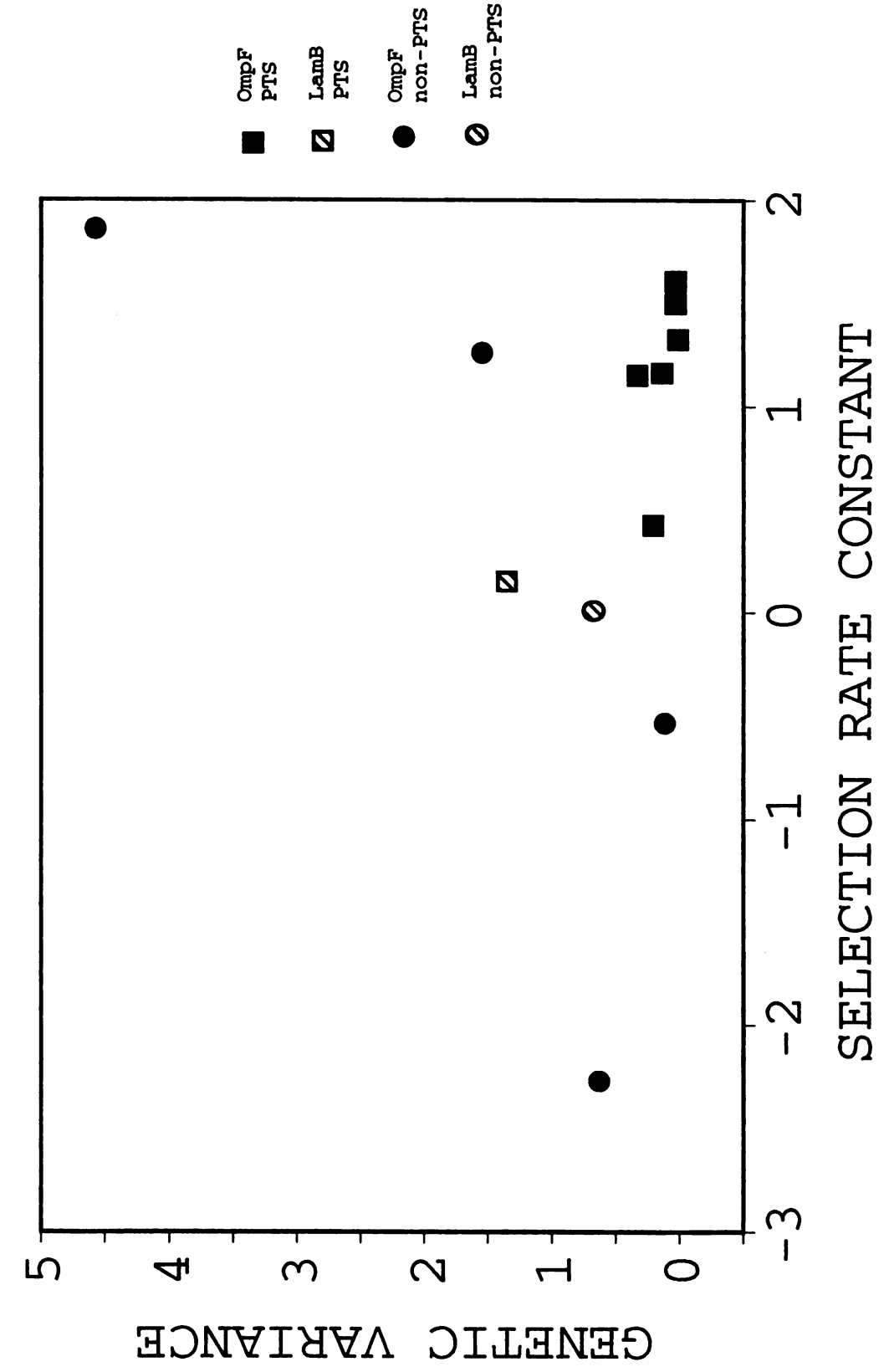


Figure 4. Genetic variance versus mean SRC for the derived strains in twelve nutrients.

than the analysis of either did alone. This finding is consistent with the expectation that traits important to fitness should have *both* a large positive fitness effect and result in similar responses in the replicated populations.

More generally, it is striking that this composite statistic is highest for the selected nutrient (glucose), followed by the five nutrients that share transport mechanisms across both the outer and inner membrane (OmpF/PTS) with the selected nutrient, with the lowest values for the six nutrients that differ in one or both of these mechanisms.

DISCUSSION

Specific targets of selection. -- Several findings indicate that improving glucose uptake was an important target of selection: the reduction of SRC in maltose medium relative to glucose medium (Chapter I, Table 3); the lower SRCs in trehalose (LamB/PTS) relative to the OmpF/PTS nutrients (Table 11); and the qualitatively different fitness response between OmpF/PTS nutrients and OmpF/non-PTS nutrients (Table 16). In addition, the results taken together suggest that adaptations affected transport through both the outer and inner membranes, presumably by improving aspects of both OmpF and PTS. Given that glucose is the preferred carbohydrate source for *Escherichia coli*, it might be surprising that improvements in transport could be readily achieved. However, in nature membrane structures must also protect the cell from a wide variety of environmental stresses while responding to varying nutrient availabilities, so the opportunity for specialized

adaptation of their functions to a fairly benign and constant environment existed in the laboratory.

The limited improvement in trehalose, which requires both LamB and OmpF outer membrane proteins for effective uptake (Klein and Boos, 1993), suggests that alterations in OmpF, or its expression, were responsible for some of the fitness improvement. LamB and OmpF are both transmembrane proteins that allow solutes to pass from the external medium to the periplasmic space located between the two membranes (Hancock, 1987). LamB forms a large maltodextrin-specific channel (Ferenci et al., 1980) which is highly expressed during growth in trehalose or maltodextrin media, but not in other media. Thus changes in OmpF, or its expression are more likely to improve fitness in other OmpF/PTS nutrients than in trehalose. As glucose is not sterically hindered in passing through OmpF, increasing the channel size cannot be expected to greatly increase the rate of diffusion through OmpF (Schindler and Rosenbusch, 1978; Cowan et al., 1992). Rather, it is more likely that there has been an increase in the production of OmpF resulting in a greater number of OmpF channels in the outer membrane.

Adaptations improving transport of glucose across the inner membrane are indicated by the more general improvement and smaller genetic variance of the genotypes in OmpF/PTS than in OmpF/non-PTS nutrients. The phosphotransferase system (PTS) transfers phosphate from phosphoenolpyruvate, a central metabolic intermediate, to a nutrient molecule concomitant with nutrient transport into the cytoplasm. This process is carried out by a combination of general proteins (enzymes I and HPr) and nutrient-specific permease complexes. At the concentration of nutrients used in this study, enzyme I and HPr are both used by all

the PTS nutrients, except for fructose, which in addition to using enzyme HPr, also has a substrate specialized enzyme HPr, DTP (Saier et al., 1970; Kornberg, 1990). The general improvement of all OmpF/PTS nutrients thus suggests that improvements in enzymes I and/or HPr, or their expression, are likely to be responsible for improving glucose uptake, and thus fitness. Circumstantial evidence suggests that enhanced expression of enzymes I and HPr, and not structural alterations, are responsible for the fitness improvement. First, extensive adaptation towards optimal expression is evinced by the codon choice bias towards optimal codon use of the *ptsI* and *ptsH* genes, which code for enzymes I and HPr respectfully (Reizer, et al., 1993). Second, enzymes I and HPr are cotranscribed and are both expressed at high basal levels, but transcription is greatly increased during growth in glucose media (De Reuse and Danchin, 1988). Taken together, optimal codon use and regulated expression suggest that enzymes I and HPr have already been strongly selected for improved catalytic efficiency.

A more hypothetical line of reasoning also suggests that enhanced expression of enzymes I and HPr are responsible for the fitness improvement, and may provide some insight into the regulation of the PTS enzymes. Transcription of I and HPr are positively regulated by glucose permease ($IIBC^{Glc}$) but not by mannose permease (IIC^{Man} and IID^{Man}), although the mechanism by which this regulation is performed is unknown (De Reuse and Danchin, 1991). Of the OmpF/PTS nutrients used in this study, the smallest fitness improvement occurred in mannose, where the genotypes have an improvement less than half that in the other OmpF/PTS nutrients. One might speculate that, excepting mannose, all the permeases of the other OmpF/PTS nutrients used in this study may also

positively regulate the expression of enzymes I and HPr. Phylogenetic relationships of permeases, performed by comparing four homologous regions of each permease, show that mannose and glucitol permeases "appear to be strongly divergent in sequence from the other sequenced PTS permeases" (Saier et al., 1992b). However, the glucitol permease is related to the other PTS permeases for regions 1 and 4, while the mannose permease is related to the other permeases only in region 1. This suggests that region 4 of the PTS permeases may play an especially important role in the regulation of the PTS system.

Among the OmpF/non-PTS nutrients, the lack of much genetic variation in the correlated responses in galactose-limited medium suggests that alterations in the amount of cAMP may have been one mechanism by which transcription of enzymes I and HPr were enhanced. Increased levels of cAMP could potentially increase fitness in glucose-, maltose-, lactose, and glycerol-limited media, and yet have little overall effect in galactose, which has a cAMP-independent permease gene promoter (as well as catabolite genes which have cAMP dependent and independent promoters). The consistently low fitness in melibiose might argue against this, but a possible alternative explanation for the low fitness in melibiose is that the sodium gradient across the inner membrane was smaller in the derived strains than their ancestor, reducing the transport of melibiose. Thus, the lack of variation for fitness in melibiose (relative to other OmpF/non-PTS nutrients) may result from an overall reduction in transport, an fitness, in melibiose-limited medium which obscures the effect of cAMP on fitness in melibiose-limited medium. A specific adaptationist reason for a smaller sodium gradient in the derived genotypes is difficult to devise, but it

may involve a higher proton gradient which can be achieved by reducing the sodium gradient.

Population Divergence

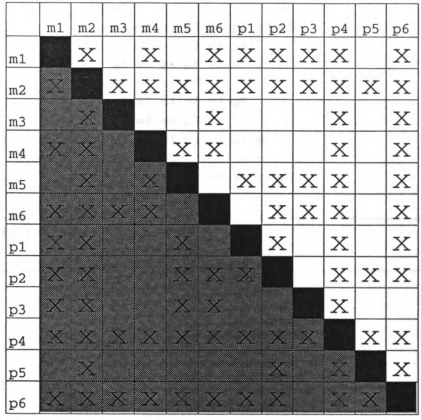
How much metabolic diversity was generated among the genotypes during their independent adaptation to glucose-limited medium? Figure 5 shows the results of the Bonferroni corrected T-tests of the genotypes in the various nutrients. The minimum number of phenotypically distinct groups can be determined by noting that no two groups of genotypes can, in principle, contain the same members, unless the groups are identical. For example, if we have two groups of genotypes, α and β, then:

$$\alpha \cap \beta = \{\varnothing\}, \text{ unless } \alpha = \beta.$$

From Figure 5, the genotype groups shown in Table 18, part A can be read off by moving down the columns left to right, and these can be subsequently reduced to the genotype groups in Table 18, part B, by removing groups that are supersets of smaller groups. Two of the resulting genotype groups are unambiguously determined, β and φ, but the other six groups contain at least one genotype that also belongs to another group. Of these six ambiguous groups, three (α,η, and λ) contain genotypes (m1, p2, and p6, respectfully) that are members *only* of that group. The three unique members therefore must denote three groups, even if the other members of each group cannot be determined unambiguously. The remaining three groups (ε, θ,and γ) do each not contain single unique members, but share two genotypes (m6 and p1) that do not appear in any of the above five groups, so that there must exist a sixth group. Overall the twelve genotypes must form at least five

Figure 5. Bonferroni corrected t-tests for the derived genotypes in twelve nutrient media.

G E N O T Y P E S[1]



Any pair of genotypes indicated by an 'X' are significantly different ($P < 0.05$), even adjusting for *both* the number of such pairs (12 X 11/2) and the number of nutrients (12) using the highly conservative Bonferroni criterion. The lightly shaded region of the figure is the mirror image of the unshaded region of the graph, and does not include additional information. The darkly shaded diagonal is where the column for a single derived genotype meets the row for that same genotype.

[1] Genotypes previously designated $Ara^{-}1$ through $Ara^{+}6$, are identified in this and the following two tables and one figure as m1 through p6.

Table 17. Ambiguous genotype groups.

A. With supersets.

Group	genotypes
α	m1 m3 m5 p5
β	m2
χ	m3 m1 m4 m5 p1 p2 p3 p5
δ	m4 m3 p1 p2 p3 p5
ε	m5 m3 m6 p5
θ	m6 m5 p1 p5
γ	p1 m3 m4 m6 p3 p5
η	p2 m3 m4 p3
ι	p3 m3 m4 p1 p2 p5 p6
φ	p4
κ	p5 m1 m3 m4 m5 m6 p1 p3
λ	p6 p3

B. Supersets removed.

Group	genotypes
α	m1 m3 m5 p5
β	m2
ε	m5 m3 m6 p5
θ	m6 m5 p1 p5
γ	p1 m3 m4 m6 p3 p5
η	p2 m3 m4 p3
φ	p4
λ	p6 p3

genotype groups, although only two of the groups can be unambiguously determined.

The above procedure is highly conservative in terms of determining the number of groups and does not necessarily result in the groupings which best fit the data, but does indicate five genotypes (m1, m2, p2, p4, and p6) and one pair (m6 and p1) which must fall into six separate groups (Figure 6). To distinguish different adaptive solutions of the five unambigously assignable genotypes, the distinguishing phenotypes were associated with each genotype (Table 18). Although a great deal is known about the relevant mechanisms of nutrient utilization, the variety of responses is not easily interpreted in terms of specific mechanisms. This limitation in interpretive ability may be the result of the action of multiple adaptive changes in each genotype, obscuring the effect of any single adaptive change. In addition, limitations in interpretation may arise from the general lack of information on the functioning of *Escherichia coli* as an organism rather than as an atomized grouping of its various traits. Often, bacterial physiology and biochemistry reaserch is performed on cells that lack specific protein, or greatly overexpress a specific protein, which can make understanding of more subtle quantitative differences among genotypes difficult.

Evolutionary Implications

Sequential Adaptation. -- Both the inner and outer membranes were evidently important targets of selection, resulting in improved transport of glucose during the 2,000 generations of propagation of the bacterial populations studied by Lenski et al. (1991). Carbohydrate

Figure 6. Venn diagram of the grouping of derived genotypes.

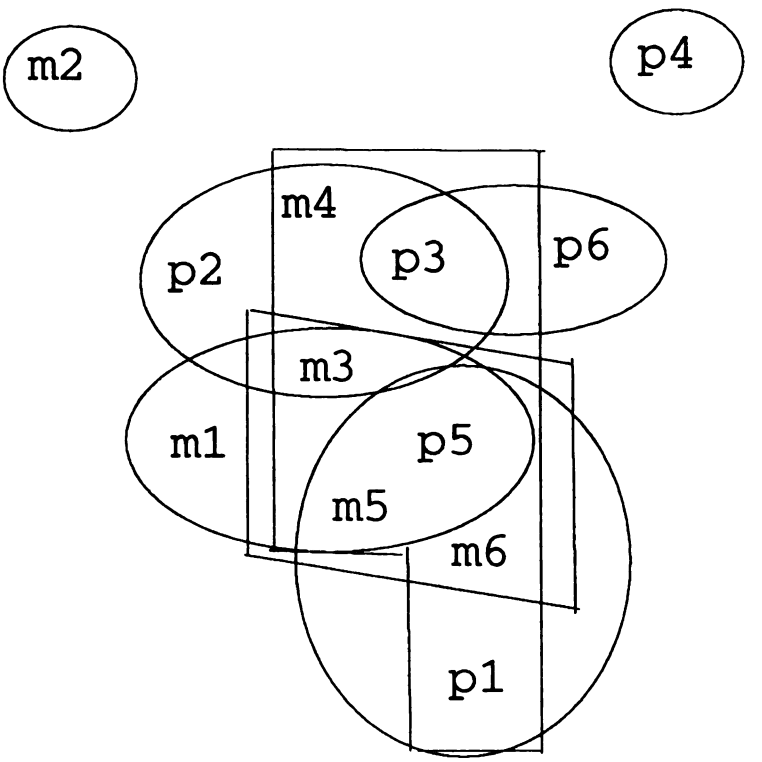


Table 18. Association of genotypes to potential mechanisms of adaptation.

Genotypes	Distinguishing Phenotype[1,2]
m1	low **galactose**, glucose
m2	low **fructose**, **maltose**, **trehalose**, NAG
p2	high **galactose**, **trehalose**, glycerol, NAG
p4	low **maltose**, **trehalose**, **galactose**, **lactose**, **glycerol**
p6	low **glucitol**, melibiose, mannitol, mannose high **maltose**, **lactose**

[1] Fitness on a particular carbohydrate relative to the other derived genotypes, not the common ancestor.

[2] Statistically significant differences are given in bold; unbolded differences indicate that the genotype is either the least or most fit derived genotype in that nutrient, but is not significantly different from other derived genotypes.

transport from the extracellular medium into the cell cytoplasm is necessarily a sequential process and adaptations affecting one step of nutrient uptake can affect the intensity of selection at other steps. Under conditions where there is an excess of nutrient, greatly improved nutrient transport across the inner membrane may result in only a moderate improvement in fitness if transport across the outer membrane is limiting, while at the same time resulting in increased selection on transport through the outer membrane. In general, the selective importance of one step in a linear pathway may change due to adaptive changes in other steps in the pathway (Kacser and Burns, 1979; Dykhuizen and Dean, 1990). The effects of multiple adaptations in such a system are necessarily non-linear, suggesting that adaptation in the populations selected by Lenksi et al. (1991) followed a fairly specific, but as yet unknown, trajectory of improvements in inner and outer membrane transport.

Mechanistic models of selection. -- Bull and Molineux (1992, p. 892) remark that "elementary models of selection predict the outcome of evolution with respect to the phenotype under direct selection, but the models are not successful at predicting either the correlated responses to selection or the multiplicity of genetic states satisfying the selected phenotypic criterion." However, one may ask what amount of genetic information would be required to predict either correlated responses to selection or the existence of multiple genetic states? Initially it might seem that the amount of genetic information required would include all that which is currently associated with fitness in the selected environment. But even this may not be sufficient if there

occur multiple adaptive changes in a single lineage, because of potential epistatic interactions. Thus, if one desires to predict specific mechanistic evolutionary outcomes that involve more than a single adaptive mutation, then one requires not only all the genetic information which is currently associated with fitness, but also all the information on traits that may potentially affect fitness (Lewontin, 1974).

As mentioned in the introduction to this chapter, limitations of non-mechanistic models can arise because of the integrated nature of organisms. Organisms are composed of numerous parts, and the interactions of the parts may complicate responses to selection. Thus a great deal of information about a single trait may not be useful in predicting evolutionary change. However, acquiring information about how organisms work as whole units is difficult. Even using 'simple' systems such as *Escherichia coli* does not completely alleviate this difficulty. Although direct responses to selection for fitness in a very simple experimental regime were understood in terms of known physiology, many correlated responses to selection could not be easily reconciled with that physiology. If correlated responses to selection are important for subsequent adaptive evolution, then even highly detailed mechanistic models of evolutionary change may not necessarily overcome the limitations of non-mechanistic models.

Examination of all the results of the 2,000 generation evolution study (Lenski et al., 1991; Vasi et al., submitted; Chapter I; this chapter) suggests that the populations had quite parallel evolution when examining fitness or traits directly related to fitness. Differences among populations, indicating divergent evolution, became more apparent

as traits less directly associated with fitness in the selected environment were examined. Both the lack of variation of traits tightly associated with fitness and the variation in traits less tightly associated with fitness may have resulted from sub-additive epistatic interactions among loci, since the rate of fitness improvement declined during the 2,000 generation experiment.

Whether these different adaptive solutions are important for subsequent evolutionary potential in a previously unselected environment is considered in Chapter IV.

Chapter III
CHANCE AND ENVIRONMENTAL EFFECTS ON THE OUTCOME OF SELECTION

The power of adaptation to generate biological diversity among isolated populations in similar environments is generally attributed to two factors. The first is that subtle differences among environments result in different selection pressures, and so each population adapts differently in response. The second is that populations may already be genetically distinct, so that each population is predisposed to adapt to the same environment in different ways.

A third factor, often not considered, is that diversity among populations may result from the stochastic (chance) processes that underlie the adaptive process: mutation and genetic drift (Lenski et al., 1991; Johnson et al., submitted). Mutation is the ultimate source for all genetic variation, and because mutants are generated randomly (Luria and Delbruck, 1943; Lenski and Mittler, 1993), different adaptive mutants may occur in different populations and thus result in population divergence. Genetic drift also introduces stochasticity, because even if the same adaptive mutation occurs in different populations, the mutation can be lost due to chance sampling events when present at low frequency within a population (Wright, 1932, 1982, 1988).

Wright illustrated the potential importance of chance events during adaptation via the adaptive landscape. The adaptive landscape is the 'surface' defined by the fitnesses of all potential genotypes within any particular environment, such that different heights on the surface represent higher or lower fitness. If the adaptive surface is 'rugged', having many peaks and valleys, then chance events could move populations into the domain of attraction for different peaks. Populations in the

domain of attraction for different peaks will tend to diverge as natural selection pushes each population up the nearest peak. However, if the adaptive landscape is generally smooth, then chance events are unlikely to result in sustained divergence, since all populations will generally stay in the domain of the same adaptive peak.

In this chapter, the relative influences of adaptation to the current environment and chance in generating diversity are examined. An evolution experiment was performed, where twelve populations were propagated for 1,000 generations in maltose-limited minimal medium. All populations were founded from a single ancestral genotype, and were therefore initially homogeneous and identical to one another, so that chance events in the appearance and fixation of *de novo* mutants could be the only source of divergence. The results of this experiment are compared and contrasted with those of Lenski et al. (1991), where a similar experiment was performed in glucose-limited minimal medium.

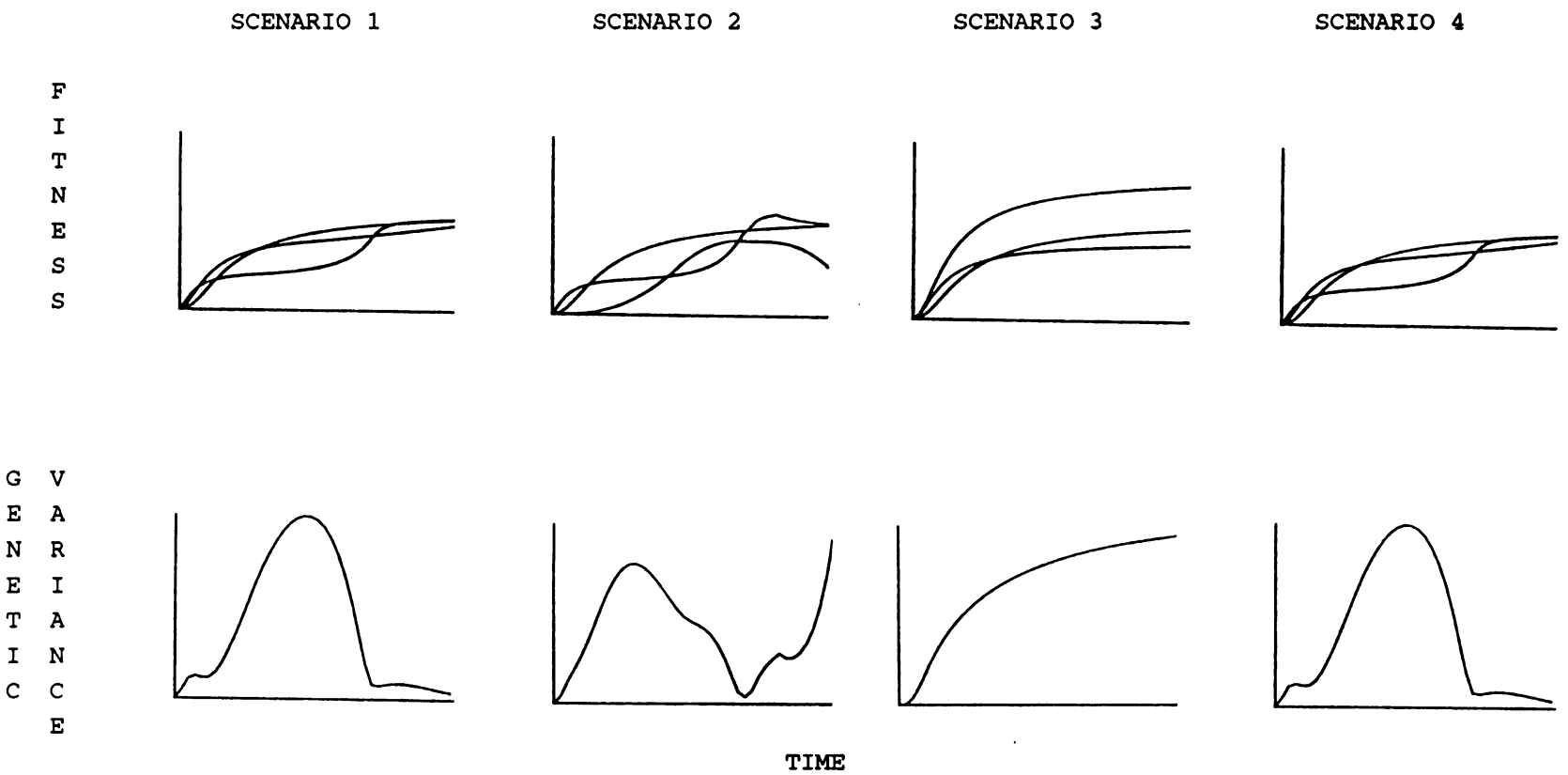
BACKGROUND AND EXPERIMENTAL OVERVIEW

In an experimental study, Lenski and associates (Lenski et al., 1991; Vasi et al., submitted; Travisano et al., submitted, Travisano and Lenski, in prep.) examined the relationship of adaptation and chance in twelve replicate populations (henceforth denoted as G populations) of *Escherichia coli* propagated for 2,000 generations in a glucose-limited medium. All G populations were founded frcm a single ancestral genotype, the same one as was used to found the populations described in this chapter. After 2,000 generations, representative strains isolated from each of the twelve G populations had improved in fitness by approximately 35% relative to their common ancestor, but they differed

from each other by only a few percent (Lenski et al., 1991). In fact, the dynamics of adaptation and divergence in mean fitness were such that it was not possible to exclude the hypothesis that the twelve replicate G populations were converging towards the same adaptive peak. Analyses of demographic components of fitness largely supported the same interpretation, as there were systematic changes in five components of fitness across the twelve representative G genotypes, but relatively little variation among the genotypes for these fitness components (Vasi et al., submitted). However, correlated responses to selection, as measured by fitness in maltose-limited medium, showed that the G populations had diverged into at least three phenotypically distinct groups (Chapter I). Fitness in other nutrient-limited media expanded the number of distinct groups to a least six (Chapter II) This outcome indicated that the adaptive surface consisted either of a broad plateau or of different fitness peaks of similar height.

The experiment described in this chapter essentially replicates the study of Lenski et al. (1991) for twelve populations (henceforth denoted as M) propagated for 1,000 generations in maltose-limited medium. The outcome of this experiment was evaluated in terms of four potential scenarios (see Lenski et al., 1991; Figure 7). According to the first scenario, the adaptive landscape is smooth and has essentially one peak, so that all populations eventually converge to the same adaptive solution, although transient divergence among populations will occur due to the stochastic nature of the appearance and fixation of adaptive mutants. According to the second scenario, the adaptive landscape is unstable because of ecological interactions among genotypes within populations, so that populations "chase" temporally varying

Figure 7. Four scenarios of adaptive evolution in a uniform environment.



Scenarios 1 and 4 cannot be distinguished by mean fitness and its genetic variance alone, but may be distinguished by correlated responses to selection.

adaptive peaks. For example, if non-transitive interactions among genotypes are important (e.g., genotype A is more fit than genotype B, which is more fit than genotype C, but C is more fit than A), then the adaptive landscape itself depends on the genotypic composition of the population. According to the third scenario, the adaptive landscape is very rugged with multiple peaks of different height, so that there is sustained divergence in the populations' mean fitnesses. According to the fourth scenario, there is not so much an adaptive peak as an adaptive ridge or plateau of genotypes with equivalent fitnesses. As in the third scenario, the genotypes may differ in correlated characters; however, unlike the third scenario, with its distinct adaptive peaks separated by valleys, the genotypes comprising an adaptive ridge or plateau are *not* separated by maladapted intermediates.

To characterize the evolutionary responses of the M populations, the populations were assayed for mean fitness and genetic variation for mean fitness in both the selected (maltose) and a novel (glucose) nutrient environment. Mean fitness in the maltose environment, and genetic variation for fitness, were estimated periodically throughout the course of the experiment. Mean fitness and genetic variation for fitness in the glucose nutrient environment were estimated only at 1,000 generations.

Glucose was chosen as the novel nutrient because of the fairly well understood similarities and differences in glucose utilization to that of maltose. Maltose is a dimer of glucose, and once inside the cell maltose is converted into two glucose-6-phosphate molecules so that subsequent utilization is identical to that of glucose (Figure 1,

Chapter I). However, maltose and glucose are taken up from the environment by two completely different mechanisms and sets of gene products (see Schwartz, 1987). In Chapter I, it was argued that the G populations studied by Lenski et al. (1991) had improved in fitness by improving glucose transport, but had done so by somewhat different mechanisms, based on the lack of systematic improvement in maltose and increased genetic variance for fitness in that sugar (~100-fold greater fitness variation in maltose than in glucose). By examining the performance of the M populations in glucose, and comparing these results with the G populations in maltose, the effects of environment-specific adaptation on correlated responses to selection were examined.

A particular issue of interest is whether correlated responses to selection in glucose and maltose are symmetric or asymmetric. During selection in glucose, grand mean fitness improved greatly in glucose and there was very little among-population genetic variance for fitness in glucose; however, the G populations showed no improvement, on average, in maltose and there was very substantial among-population variation in fitness in maltose (Chapter I). If correlated responses are symmetric, then the populations selected in maltose should have improved fitness in maltose, little variation among populations for fitness in maltose, no net improvement in fitness in glucose, and high among-population variation for fitness in glucose.

MATERIALS AND METHODS

Bacterial strains. -- All of the strains described in this study were derived from a single clone of *Escherichia coli* B which has been used in a number of other evolutionary studies (Chao et al. 1977;

Lenski, 1984; Lenski and Levin, 1985; Bouma and Lenski, 1988; Lenski, 1988a, 1988b; Lenski et al., 1991; Bennett et al., 1992; Bennett and Lenski, 1993; Vasi et al., submitted). Briefly, this strain carries no plasmids and harbors no functional bacteriophage, and is strictly asexual. It is prototrophic but unable to use L-arabinose as a nutrient source. From this strain, an Ara^+ mutant was selected by plating $>10^9$ cells on minimal arabinose medium (Lenski, 1988a). The Ara^- and Ara^+ marker versions of the ancestral genotype form red and white colonies, respectively, when spread on tetrazolium arabinose (TA) plates, allowing the genotypes to be easily distinguished (Levin et al., 1977). The arabinose marker has been shown to be effectively neutral in a wide variety of carbohydrate-limited minimal media, including maltose (Table 1, Chapter I). These two ancestral clones, as well as their derived descendants, are stored in glycerol based medium at -80^oC, making them available for simultaneous comparison with one another at any time.

The twelve G populations had been founded from the two ancestral clones (six Ara^- and six Ara^+) (Lenski et al., 1991). They were propagated under conditions identical to those in this study, except that the genotypes were cultured in shaking 50 ml Erlenmeyer flasks, rather than stationary 18 mm tubes as used in this study (see below), and in glucose- rather than maltose-limited minimal medium. Lenski et al. (1991) reported the evolutionary dynamics of the G populations for 2,000 generations. However, I used the G populations stored after 1,000 generations in order to allow a more direct comparison with the M populations that were propagated for 1,000 generations.

Culture conditions. -- The populations were propagated by daily 100-fold serial dilution from 10 ml cultures containing roughly $5x10^7$

cells ml^{-1} into fresh Davis minimal media (Carlton and Brown, 1981) supplemented with 2 x 10^{-6} g thiamine hydrochloride and 25 $\mu g\ ml^{-1}$ maltose. Cultures were maintained in 18 mm tubes at 37°C, and were not shaken. The 100-fold dilution and regrowth results in 6.64 generations ($\log_2 100$) of binary fission per day.

Evolving populations. -- All populations were founded from single colonies, so that there was no initial variation within populations. The twelve (M) populations were founded using the same Ara^+ and Ara^- marker variants of the genotype ancestral to the twelve G populations. Hence, there was no initial genetic variation among the genotypes used to found the M populations, except for the arabinose utilization marker. The M populations were propagated for 150 days, or approximately 1,000 generations of binary fission. Daily transfers of the genotypes into fresh media were performed by alternating Ara^- and Ara^+ populations, so that any cross-contamination from one population to the next would be detectable by changes in marker state. No cross contamination was observed.

Sampling. -- Samples of each population were collected approximately every 100 generations. After performing the daily transfer, glycerol was added to a final concentration of roughly 15%, and a sample (~3-4 ml) was removed to a screwcap vial, which was stored at -80°C.

Measurement of fitness in competition experiments. -- Competition experiments were performed for both G and M populations under culture conditions similar to those in which the derived populations had been propagated. However, the competition experiments sometimes differed from the selective culture conditions in two ways. First, maltose or

glucose was sometimes substituted for the selected resource (as indicated). Second, the G populations were propagated in 50 ml Erlenmeyer flasks, while the M populations were propagated in 18 mm tubes, so additional competitions in flasks and tubes were carried out to control for this difference. The two marker versions of the ancestral genotype, which were directly ancestral to the G and M populations, were used as the common competitors in all of the experiments reported here. The protocol for the competition experiments was as follows. After growing up aliquots of the appropriate genotypes from the freezer for one day, both competitors were separately conditioned for one or two days (one or two cycles of dilution and regrowth) in the competition medium. Each competitor was then diluted 200-fold into a competition culture containing fresh medium (so that the total initial population size was equivalent to the usual 100-fold dilution of the stationary-phase population). This competition culture was then incubated under standard conditions for one day. Initial and final population densities of each competitor were determined by plating aliquots from the competition flask onto TA indicator agar, which allowed Ara^- and Ara^+ competitors to be distinguished by their colony color. Relative fitness (W) was defined as the ratio of the number of doublings for the evolved and ancestral competitors:

$$W = \log_2 (N_f' / N_i') / \log_2 (N_f / N_i)$$

where N_i' and N_i are the initial densities and N_f' and N_f are the final densities of the evolved and ancestral types, respectively.

Experimental design and statistical analysis. -- To test the

selective neutrality of the arabinose marker, replicate competition experiments were performed using the reciprocally marked ancestors in maltose- and glucose-limited minimal media. To test for possible interactions between the arabinose marker and the derived versus ancestral genetic backgrounds, the average fitnesses of the Ara^+ and Ara^- groups were compared for both the G and M populations at 1,000 generations.

A total of twenty-eight fitness estimates were performed prior to propagation of the M populations, and ten estimates were obtained at 1,000 generations for each of the twelve M populations. Also, one, two, or three fitness estimates were obtained per M population at generations 100, 200, 300, 420, 500, and 800. All of the above estimates were performed in maltose-limited minimal media. In addition, I performed two fitness assays for each M population (at 1,000 generations) in glucose-limited minimal medium in flasks; two fitness assays for each M population (at 1,000 generations) in glucose-limited minimal medium in tubes; two for each G population (at 1,000 generations) in glucose-limited minimal medium in flasks; and two for each G population (at 1,000 generations) in maltose-limited minimal medium in tubes.

In general, statistical tests on the above data took two forms, depending upon whether the test was for a mean effect or a variance effect. Tests of mean fitness were performed on the data without regard to possible block effects, whether they were comparisons of initial versus final fitness, or fitness of the G and M populations in the two sugars. In these cases, the number of degrees of freedom was based on the number of replicate populations, without regard to the number of repeated fitness assays per population. Tests of variance, and

determination of variance components, were performed by including block effects in a two-way analysis of variance. Here the number of replicate populations determined the numerator degrees of freedom, while the number of repeated assays per population determined the denominator degrees of freedom.

Genetic variance for relative fitness was estimated as the difference in the population and error mean squares, divided by the number of replicate assays per population. Confidence intervals for genetic variance estimates were computed using the methods described by Sokal and Rohlf (1981, pp. 217-218).

RESULTS

Selective neutrality of the genetic marker. -- There were a number of different conditions that required tests of the neutrality of the arabinose utilization marker: in ancestral and derived backgrounds, in glucose and maltose nutrient media, and in flasks and test tubes. Previous studies have shown that the Ara marker is effectively neutral in the ancestral genotype when assayed in flasks containing either glucose- or maltose-limited media (Lenski et al., 1991; Table 1, Chapter I). In test tubes, the arabinose marker was effectively neutral in the ancestral background in glucose- and maltose-limited minimal media (Table 19).

The effective neutrality of the arabinose marker in the ancestral background does not necessarily indicate that the arabinose marker remained neutral in the derived populations; it is possible that this marker might interact epistatically with the derived genetic backgrounds. However, the six Ara^+ derived G populations (as a group)

Table 19. Neutrality of the arabinose marker for the ancestral genotype in glucose- and maltose-limited minimal media in test tubes.

Nutrient	Mean Fitness (Ara+ Relative to Ara-) Mean (± SE)[1]	n	p[2]
Glucose	1.0124 (± 0.0097)	7	0.2397
Maltose	0.9984 (± 0.0069)	28	0.8210

[1] Mean fitness and standard error of the mean based on n assays.

[2] Two-tailed probability computed from the *t*-distribution with n - 1 degrees of freedom; the null hypothesis is that relative fitness equals one, indicating equal fitness for the Ara+ and Ara- genotypes.

are no more or less fit than the six Ara^- derived G populations (as a group) in either glucose-limited medium in flasks or maltose-limited medium in tubes (Table 20). Likewise, the six Ara^+ derived M populations (as a group) are no more or less fit than the six Ara^- derived M populations in either flasks or tubes containing glucose-limited minimal media, or in tubes containing maltose-limited minimal medium (Table 21). These results, coupled with the fact that all experiments were fully balanced with respect to marker, suggest that pooling the data for Ara^+ and Ara^- populations in the subsequent analyses is statistically valid.

Trajectory of fitness improvement among M populations in maltose-limited minimal medium. -- Figure 8 plots grand mean fitness relative to the common ancestor against time, in generations, averaged over all twelve M populations in maltose-limited minimal medium. At 1,000 generations, the grand mean fitness for the twelve independently derived M populations, relative to their common ancestor, is 1.225. The corresponding 95% confidence interval (based on the *t*-distribution with 11 degrees of freedom) ranges from 1.200 to 1.250.

Mean fitness over all twelve M populations in maltose-limited minimal medium never dropped below that of the ancestor and increased throughout the course of the experiment. This suggests that non-transitive fitness effects, if present, had relatively minor effects on the fitness trajectory. Previous attempts to demonstrate non-transitivity in the G populations have found none (Lenski et al., 1991;Table 7, Chapter I).

Visual inspection of the trajectories of mean fitness over time

Table 20. Neutrality of the arabinose marker for the G populations in glucose in flasks and maltose in test tubes.

	Mean Fitness			
	Ara^+ Derived Relative to Ara^- Ancestor	Ara^- Derived Relative to Ara^+ Ancestor	Difference[1] (± SED)	P[2]
Glucose (flasks)	1.3708	1.3527	0.0180 (± 0.0379)	0.6446
Maltose (tubes)	1.0537	0.9682	0.0041 (± 0.0480)	0.1048

[1] Difference (and standard error of the difference) in fitness based on six independent populations in each derived marker class.

[2] Two-tailed probability computed from the *t*-distribution with 6 + 6 - 2 = 10 degrees of freedom; the null hypothesis is that the difference in relative fitness for the two marker classes of derived populations equals zero, indicating equal changes in mean fitness for the Ara^+ and Ara^- derived populations.

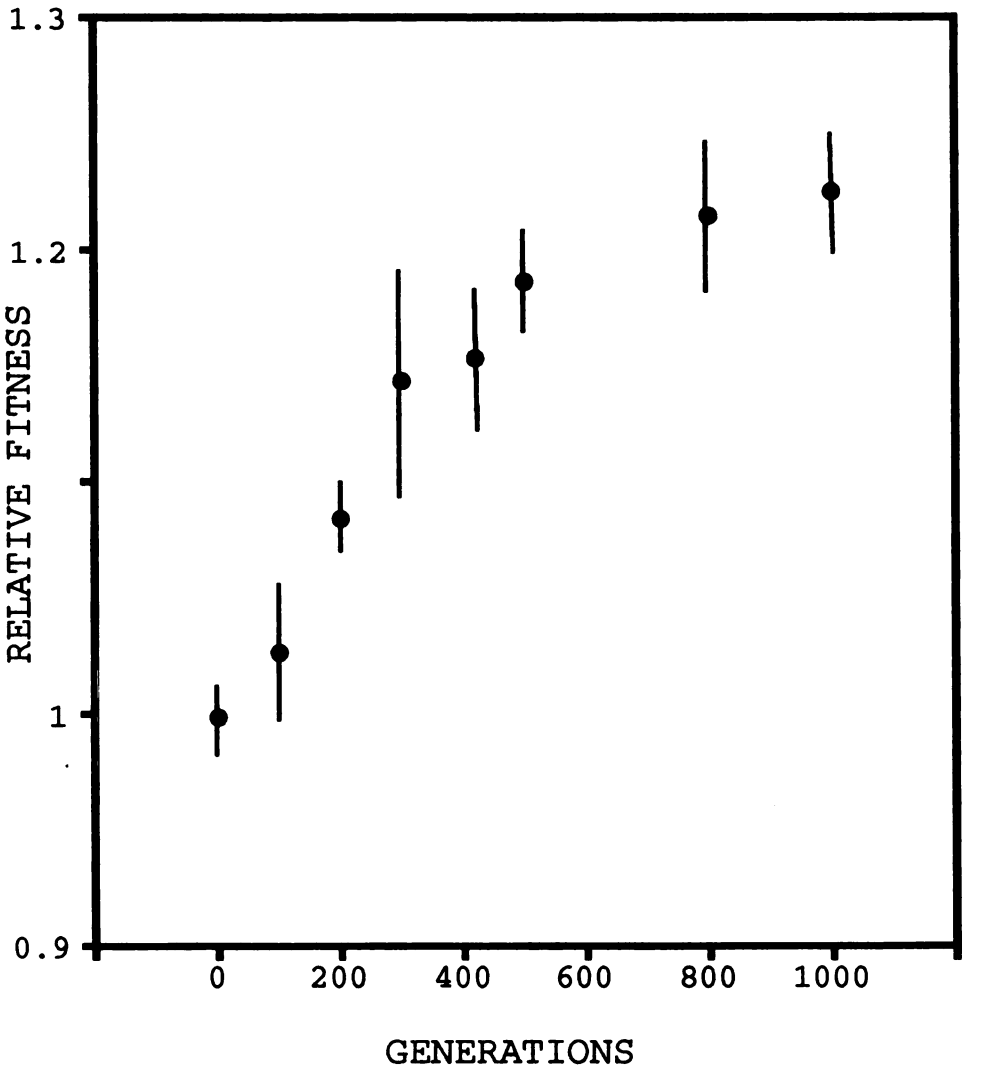
Table 21. Neutrality of the arabinose marker for the M populations in glucose- and maltose-limited minimal media.

	Mean Fitness			
	Ara^+ Derived Relative to Ara^- Ancestor	Ara^- Derived Relative to Ara^+ Ancestor	Difference[1] (± SED)	p[2]
Glucose (flasks)	1.2281	1.1773	0.0508 (± 0.0286)	0.1056
Maltose (tubes)	1.2146	1.2353	−0.0208 (± 0.0226)	0.3802
Glucose (tubes)	1.2413	1.2372	0.0041 (± 0.0145)	0.7850

[1] Difference (and standard error of the difference) in fitness based on six independent populations in each derived marker class.

[2] Two-tailed probability computed from the *t*-distribution with 6 + 6 - 2 = 10 degrees of freedom; the null hypothesis is that the difference in relative fitness for the two marker classes of derived populations equals zero, indicating equal changes in average fitness for the Ara^+ and Ara^- derived populations.

Figure 8. Trajectory of grand mean fitness for M populations during 1,000 generations in maltose-limited minimal medium.



Fitness is expressed relative to the reciprocally marked ancestral genotype. Fitness was estimated for each population, and a grand mean fitness was calculated on the means of all twelve M populations. The error bars show the 95% confidence intervals based on a t-distribution with 11 degrees of freeedom.

(Figure 8) for the M populations suggests that mean fitness improved at a more rapid rate during the first 500 generations, than in the last 500 generations. To test this, the rate of fitness improvement for the first 500 generations was compared to that of the second 500 generations. Linear regressions of the mean fitness were computed for each population over the entire 1,000 generations and for just the first 500 generations. Regressions were performed by fixing the y-intercept to one (see Lenski et al., 1991). The rate of fitness improvement over the second 500 generations was computed by subtracting the rate over the first 500 generations from twice the rate over the entire 1,000 generations (see Table 22). Mean fitness during the first 500 generations improved at a rate which was approximately three times that during the last 500 generations.

Trajectory of genetic variation for fitness among M populations in maltose-limited minimal medium. -- Table 23 shows the results of analyses of variance (based on repeated measures of relative fitness for each of the derived M populations) at generations 200, 300, 420, 500, 800, and 1000. Statistically significant genetic variation among populations was detected at generations 300, 420, and 1,000. As there was no initial genetic variation among the M populations, chance events were the only source of such variation.

Figure 9 plots among-population genetic variation for mean fitness against time, in generations, for the twelve M populations in maltose-limited minimal medium, with 95% confidence intervals. Although genetic variance increased during the experiment, all the confidence intervals overlap, and there is no apparent overall trend for genetic variance.

Table 22. Analysis of trajectories for mean fitness of the M populations during 1,000 generations in maltose-limited minimal medium.

Rate of change in mean fitness (per 1,000 generations)		
	Mean ± SE[1]	ts[2]
A. During all 1,000 gen.	0.249 ± 0.011	23.629***
B. During first 500 gen.	0.387 ± 0.020	18.965***
C. During last 500 gen.[3]	0.112 ± 0.016	6.808***
D. Difference[4]	0.275 ± 0.030	9.052***

[1] Means and standard errors based on twelve M populations.

[2] Two-tailed probability computed from the *t*-distribution with 12 - 1 = 11 degrees of freedom. Null hypothesis is that the slope (or difference in slopes) equal to zero.

[3] Rate of change during the last 500 generations was calculated as twice quantity A minus quantity B.

[4] Difference calculated as quantity B minus quantity C.

*** $p < 0.001$

Table 23. ANOVAs for relative fitness of the twelve independently derived M populations in maltose-limited minimal medium over 1,000 generations.

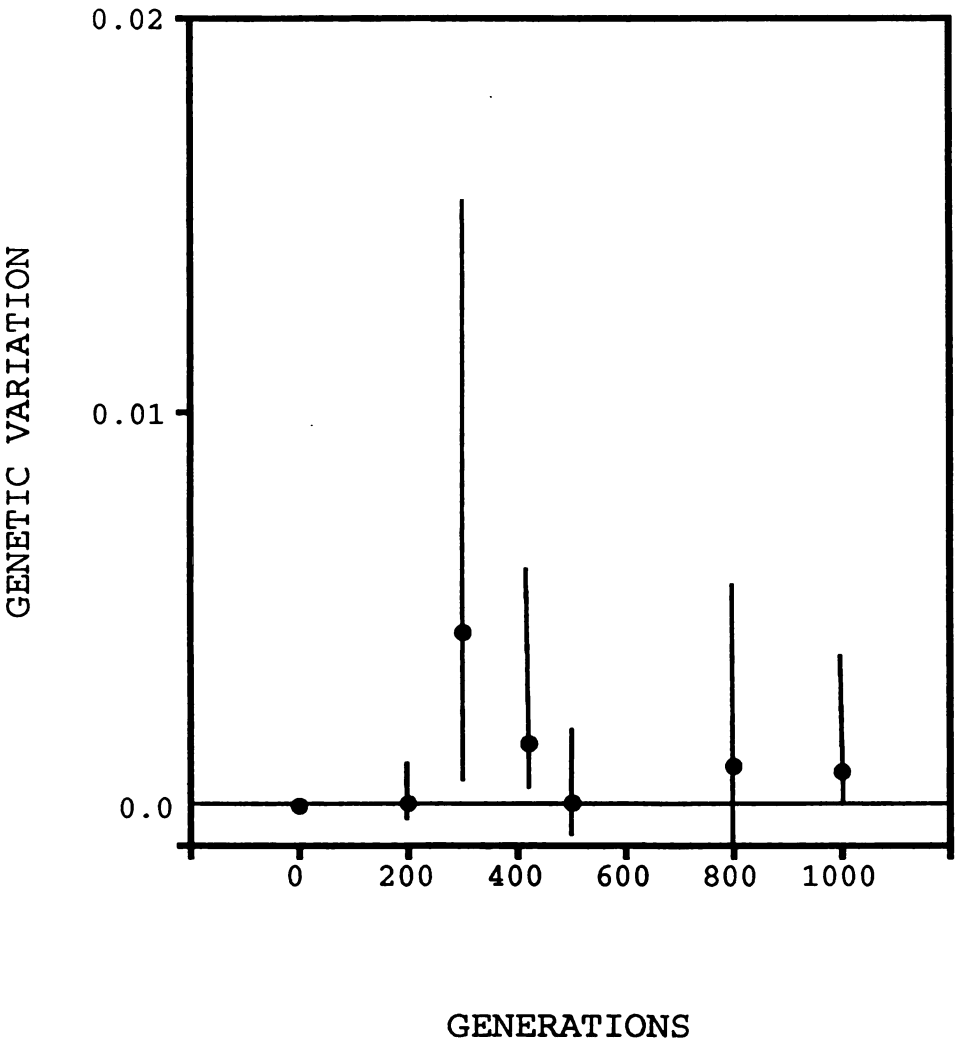
	Block[1]			Genetic Heterogeneity[1]			Error	
Generation	df	MS	P	df	MS	P	df	MS
200	1	0.0190	0.0012	11	0.0016	0.3734	23	0.0014
300	1	0.0523	0.0015	11	0.0117	0.0156	11	0.0030
420	1	0.0031	0.2599	11	0.0662	0.0127	21[2]	0.0021
500	1	0.0247	0.0081	11	0.0032	0.4176	23	0.0029
800	1	0.0068	0.1476	11	0.0048	0.1917	11	0.0028
1000	9	0.0843	< 0.0001	11	0.0151	0.0120[3]	99	0.0064

[1] Block and genetic heterogeneity among populations are random effects.

[2] Indicates two missing values.

[3] Genetic variance among M populations after 1,000 generations in maltose-limited medium is 0.000875, with lower and upper 95% confidence limits of 0.0000834 and 0.00372, respectively.

Figure 9. Trajectory of among-population variation for mean fitness of the M populations during 1,000 generations in maltose-limited minimal medium.



Genetic variation for mean fitness determined by the difference in population and error mean squares divided by the number of replicate measures of fitness per population. Error bars indicate 95% confidence intervals.

This result is similar to that which Lenski et al. (1991) observed, where eventual convergence of the populations to a common fitness could not be excluded.

Genetic adaptation of the M populations to maltose increases average fitness in glucose. -- The fitnesses of the M populations were also measured in minimal medium containing glucose rather than maltose. All twelve populations showed substantial improvement relative to their common ancestor in medium containing either maltose (Table 24, first column) or glucose (Table 24, second column). A t-test for paired comparisons indicates that the difference in the extent of adaptation to these two sugars is not significant (Table 24, third column). Thus the M populations, as a group, adapted as well to glucose as they had to maltose.

Genetic adaptation of the M populations to maltose does not increase genetic variation for fitness in glucose. -- An analysis of variance (based on the repeated measures of fitness in glucose for each M population) shows no significant genetic variation for fitness among the M populations in glucose (Table 25). The estimated genetic variance component, V_G, is 0.0 (actually -0.00014, but a negative variance component is meaningless); the lower and upper limits of the 95% confidence interval for the variance are 0.0 (-0.00272) and 0.000986, respectively. Thus the genetic variance among M populations in the novel environment (glucose-limited medium) is not larger, and is actually smaller (though not significantly so), than in the selected environment (maltose-limited medium). The M populations also show equivalent improvement in fitness during growth in glucose-limited

Table 24. Adaptation of the M populations to maltose- and glucose-limited minimal media in test tubes.

Twelve replicate bacterial populations selected during 1,000 generations in maltose-limited minimal medium.

M Derived Population	Fitness Relative[1] to the Common Ancestor in Maltose[2]	Glucose[2]	Difference[3]
Ara^-1	1.333	1.274	0.059
Ara^-2	1.224	1.257	-0.033
Ara^-3	1.198	1.199	-0.001
Ara^-4	1.196	1.206	-0.009
Ara^-5	1.242	1.236	0.006
Ara^-6	1.218	1.251	-0.033
Ara^+1	1.179	1.255	-0.076
Ara^+2	1.220	1.251	-0.031
Ara^+3	1.239	1.223	0.016
Ara^+4	1.225	1.212	0.013
Ara^+5	1.198	1.245	-0.047
Ara^+6	1.227	1.262	-0.035
Mean	1.225	1.240	-0.014
SE	0.011	0.007	0.010
P	< 0.001	< 0.001	0.1898

[1] Relative fitness for each derived M population is based on ten or two assays for maltose or glucose, respectfully.

[2] Two-tailed probability computed from the *t*-distribution with n - 1 = 11 degrees of freedom; the null hypothesis is that mean fitness is one.

[3] Two-tailed probability computed from the *t*-distribution with n - 1 = 11 degrees of freedom; the null hypothesis is that the difference between the two means is zero, indicating equal fitness for the average derived M population in the two sugars.

Table 25. ANOVA for relative fitness of the M populations in glucose-limited medium, in tubes.

Analysis of variance for fitness obtained for the twelve independently derived M populations in glucose in tubes, relative to the common ancestor. Both population and block are random effects.

Source	df	MS	F	P
Population	11	0.0011	0.807	0.6360[1]
Block	1	0.0493	34.654	< 0.0001
Error	11	0.0014		

[1] Genetic variance among M populations after 1,000 generations in glucose-limited medium in tubes is 0.0 (-0.00014), with lower and upper 95% confidence limits of 0.0 (-0.00272) and 0.000986, respectively.

medium when in flasks (mean fitness = 1.202; the 95% confidence interval, based on a *t*-distribution with n - 1 = 11 degrees of freedom, is 1.168 to 1.237), with little variation among M populations (Table 26). In general, the M populations' adaptation to medium containing maltose resulted in equivalent adaptation to medium containing glucose.

Parallel fitness improvements among the G populations in glucose. -- In glucose, the grand mean fitness in flasks for the twelve independently derived G populations after 1,000 generations in glucose-limited minimal media is 1.362 (Table 27, second column). The corresponding 95% confidence interval (based on the *t*-distribution with 11 degrees of freedom) ranges from 1.322 to 1.402. An analysis of variance (based on repeated measures of fitness for each population) suggests some genetic heterogeneity among the twelve G populations (Table 28). The estimated genetic variance component, V_G, is 0.0023. Although there is a suggestion of genetic variation among the G populations, these results indicate that the evolutionary responses of the G populations were quite similar, consistent with the results reported by Lenski et al. (1991).

Genetic adaptation of the G populations to glucose does not increase average fitness in maltose. -- Previous work (Table 3, Chapter I) has shown that randomly chosen representative genotypes of the G populations, after 2,000 generations in glucose-limited medium, had significantly improved in relative fitness in glucose-limited medium but not in maltose-limited medium. Moreover, the estimated genetic variance for fitness among the representative G genotypes was ~100-fold larger in maltose-limited medium than in glucose-limited medium. To compare the

Table 26. ANOVA for relative fitness of the M populations in glucose-limited minimal medium, in flasks.

Analysis of variance for fitness obtained for the twelve independently derived M populations in glucose in flasks, relative to the common ancestor. Both population and block are random effects.

Source	df	MS	F	P
Population	11	0.0059	1.479	0.2653[1]
Block	1	0.0006	0.143	0.7126
Error	11	0.0040		

[1] Genetic variance among M populations after 1,000 generations in glucose-limited medium in flasks is 0.000948, with lower and upper 95% confidence limits of 0.0 (-0.00400) and 0.00652, respectively.

Table 27. Adaptation of the G populations to maltose- and glucose-minimal media.

Twelve replicate bacterial populations selected during 1,000 generations in glucose-limited minimal medium in flasks.

G Derived Population	Fitness Relative[1] to the Common Ancestor in Maltose tubes[2]	Glucose flasks[2]	Difference[3]
Ara$^-$1	1.113	1.218	-0.105
Ara$^-$2	0.912	1.334	-0.422
Ara$^-$3	0.891	1.350	-0.458
Ara$^-$4	1.066	1.458	-0.391
Ara$^-$5	0.875	1.388	-0.513
Ara$^-$6	0.951	1.369	-0.418
Ara$^+$1	1.015	1.318	-0.303
Ara$^+$2	1.100	1.419	-0.319
Ara$^+$3	1.111	1.324	-0.214
Ara$^+$4	0.943	1.438	-0.495
Ara$^+$5	1.073	1.364	-0.291
Ara$^+$6	1.080	1.361	-0.281
Mean	1.011	1.362	-0.351
SE	0.026	0.018	0.035
P	0.6849	< 0.001	< 0.001

[1] Relative fitness for each derived G population is based on two assays for in each environment.

[2] Two-tailed probability computed from the *t*-distribution with n - 1 = 11 degrees of freedom; the null hypothesis is that mean fitness is one.

[3] Two-tailed probability computed from the *t*-distribution with n - 1 = 11 degrees of freedom; the null hypothesis is that the difference between the two means is zero, indicating equal fitness for the average derived M population in the two sugars.

Table 28. ANOVA for relative fitness of the G populations in glucose-limited medium, in flasks.

Analysis of variance for fitness obtained for the twelve independently derived G populations in glucose in flasks, relative to the common ancestor. Both population and block are random effects.

Source	df	MS	F	P
Population	11	0.0080	2.406	0.0805[1]
Block	1	0.0148	4.446	0.0587
Error	11	0.0033		

[1] Genetic variance among G populations after 1,000 generations in glucose-limited medium is 0.00234, with lower and upper 95% confidence limits of 0.0 (-0.00400) and 0.00652, respectively.

correlated responses to selection of the G and M populations, competition experiments were performed in maltose-limited minimal medium in tubes using the G populations after only 1,000 generations in glucose-limited medium.

The G populations after 1,000 generations, as a group, do not show any significant change in fitness relative to the ancestor in medium containing maltose (Table 27, first column), and a t-test for paired comparisons indicates that the difference in the extent of adaptation to these two sugars is highly significant (Table 27, third column). This observation is consistent with the results in Chapter I (Table 3) for the G populations after 2,000 generations, but contrasts sharply with that of the M populations in glucose (Table 24). Comparing the fitness for the M populations in glucose flasks and the G populations in maltose tubes (Table 29), indicates that the correlated responses to selection between the G and M populations are dissimilar. The populations evolved in maltose (M) improved in fitness, as a group, in both maltose and glucose, while the populations evolved in glucose (G) improved in fitness, as a group, *only* in glucose.

G populations vary in their fitnesses in maltose. -- An analysis of variance (based on the repeated measures of fitness in maltose for each G population) shows significant genetic variation for fitness among the G populations in maltose (Table 30). The estimated genetic variance component, V_G, is 0.00766; the lower and upper limits of the 95% confidence interval for the variance are 0.00343 and 0.0232, respectively. Thus, the genetic variation for fitness among the G populations when assayed for fitness in maltose-limited minimal medium

Table 29. Contrast of correlated responses for the G and M populations in glucose- and maltose-limited minimal media.

Mean Fitness			
Glucose adapted populations (G) in maltose tubes mean (± SE)	Maltose adapted populations (M) in glucose flasks mean (± SE)	Difference[1] mean (± SED)	P[2]
1.011 (± 0.026)	1.202 (± 0.016)	0.192 (+ 0.031)	< 0.0001

[1] Difference (and standard error of the difference) in fitness based on twelve independent populations in each selected group.

[2] Two-tailed probability computed from the *t*-distribution with 12 + 12 - 2 = 22 degrees of freedom; the null hypothesis is that the difference in relative fitness for the two selected group equals zero, indicating equal correlated responses for fitness.

Table 30. ANOVA for relative fitness of the G populations, after 1,000 generations, in maltose-limited minimal medium.

Analysis of variance for fitness obtained for the twelve independently derived G populations in maltose in tubes, relative to the common ancestor. Both population and block are random effects.

Source	df	MS	F	P
Population	11	0.0165	13.522	< 0.0001[1]
Block	1	0.0517	4.230	0.0642
Error	11	0.0012		

[1] Genetic variance among G populations after 1,000 generations in maltose-limited medium in tubes is 0.00766, with lower and upper 95% confidence interval estimates of 0.00343 and 0.0232, respectively.

in tubes, is significantly greater than that for the M populations in glucose, either in tubes (Table 25) or in flasks (Table 26).

DISCUSSION

The major findings of this chapter can be summarized as follows. (1) Mean fitness increased in all twelve evolving M populations, by average of ~22%, relative to their common ancestor. (2) The rate of fitness improvement declined over time, suggesting that the M populations were approaching a fitness peak or plateau. (3) Genetic variation increased during the course of the experiment; however the variation in fitness among populations was small, and potentially transient rather than sustained (see Lenski et al., 1991). (4) The M populations increased in fitness in glucose-limited minimal medium to a similar extent, with no greater genetic variation in glucose-limited minimal medium than in maltose-limited minimal medium. (5) The G populations after 1,000 generations in glucose-limited minimal medium increased in fitness in glucose-limited minimal medium, relative to the common ancestor. (6) However, the G populations, as a group, did *not* increase in fitness in maltose-limited minimal medium. (7) Genetic variance among the G populations for fitness in maltose-limited minimal medium was much greater than that among the M populations for fitness in glucose-limited minimal medium.

Diversity

In the introduction (after Lenski et al., 1991), four different scenarios were presented for the adaptation and divergence of replicate populations propagated in uniform environments. In the first scenario,

all the populations eventually converge to the same adaptive peak, although there may be some transient divergence due to the stochastic nature of the appearance and fixation of mutants. According to scenario two, complex ecological interactions among genotypes within populations result in a temporally varying adaptive landscape. In the third scenario, the populations do not eventually converge to the same adaptive peak, but rather diverge to different adaptive peaks of unequal mean fitness. In the fourth scenario, the populations converge to the same mean fitness; however, they do not converge to the exact same adaptive peak but instead to a ridge or plateau of functionally equivalent genotypes (in the selected environment) that may differ in their correlated responses. The first and fourth scenarios cannot be distinguished solely by measurements of mean fitness and its genetic variance in the selected environment, but require additional phenotypic traits to be scored.

To characterize further the relative effects of chance (which was measured as a variance component for fitness) and adaptation (which was measured as a change in mean fitness), it is useful to put the two quantities into comparable units. This is most easily done by converting the variance component to its corresponding standard deviation.

At the start of the selection experiment, the grand mean fitness of the M populations relative to their common ancestor was one, by definition. After 1,000 generations, the grand mean fitness in maltose had increased to 1.225, with 95% confidence limits of 1.200 and 1.250 (Table 24). Therefore, the mean change in fitness due to adaptation was 0.225, with the confidence interval ranging from 0.200 to 0.250. After

1,000 generations, the genetic variance among populations due to chance was 0.000875, with 95% confidence limits of 0.0000834 and 0.00372 (Table 23). The corresponding standard deviation is 0.0296, with confidence limits ranging from 0.0091 to 0.0610.

Using the point estimates, the effect on fitness due to adaptation is almost 8-fold (0.225/0.0296) larger than the effect due to chance. Even conservatively using the extremes of each confidence interval, the effect of adaptation is more than 3-fold (0.200/0.0610) that of chance. (These relative effects would be approximately squared if the units of comparison were based on mean squares and variances rather than means and standard deviations.) Thus, although there was statistically significant genetic variation in mean fitness among the twelve M populations, each independently derived from the same common ancestor, the *magnitude* of this variation was rather small relative to the overall change in mean fitness.

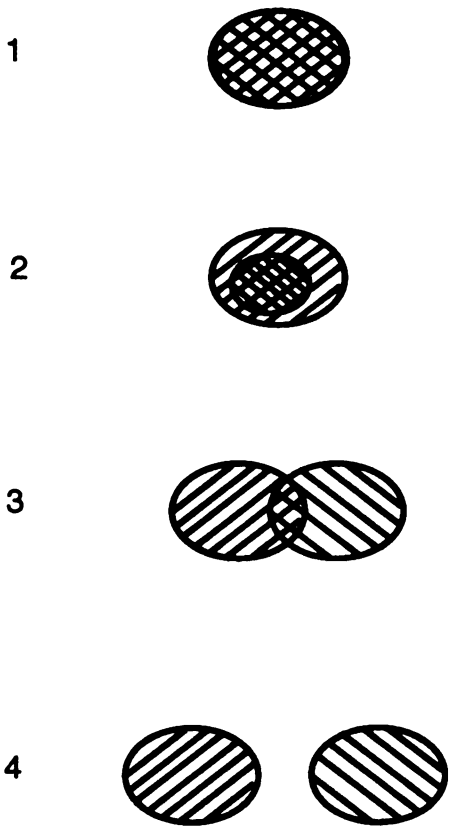
It is unclear whether the M populations would sustain this variation in mean fitness over periods much longer than 1,000 generations (see Lenski et al., 1991). However, the *rate* of adaptation declined significantly between the first 500 generations and the next 500 generations (Table 22), and it likely that further improvements would have become progressively even more difficult to achieve. This deceleration in the rate of adaptation limits the opportunity for further divergence in mean fitness, because any further divergence in fitness requires further adaptation. This dynamical constraint, coupled with the already small effect of chance relative to adaptation, suggests that the effects of chance divergence on fitness would be small relative to the overall increase in mean fitness due to adaptation even over

longer time scales.

Correlated responses and the adaptive landscape. -- Phenotypic differences between populations that have adapted to different environments is expected. In some cases, certain phenotypic traits may be identified for which there are *a priori* expectations concerning such differences (Bull and Mollineux, 1992). However, a complicating factor is that organisms are not simply collections of various "atomized" traits, but are integrated systems. Thus, adaptive differences in phenotype between populations in different environments may not follow simple patterns, even when they might be expected. The degree to which adaptive responses are similar or different results from genotype-by-environment (GxE) interactions.

When two initially identical populations adapt to two different environments, there are four possible relationships between the adaptive mutations that occur in the two populations (Figure 10). These four relationships can be thought of as a transformation scheme from the adaptive landscape associated with one environment to the adaptive landscape associated with a different environment. According to the first possibility, every adaptive mutation in one environment is adaptive in the other, so that every adaptive peak in one environment is also an adaptive peak in the other environment. In this case, there is no GxE interaction and the two populations adapt as if they are in the same environment. According to the second possibility, all of the adaptive mutations in one environment are beneficial in the other, but the converse is not true, so that all adaptive peaks in one environment are adaptive peaks in the other but not vice versa. Essentially, the GxE interaction canalizes adaptation in one environment more so than in

Figure 10. Four possible relationships of adaptive mutations in two environments.



the other, so that a population can be well adapted to both environments, but this is less likely if the population is has evolved the less canalizing environment. According to the third possibility, there are a number of mutations that are adaptive for both populations, but also many which are environment-specific, so that the adaptive landscapes share some peaks but not all. The GxE interaction partially distinguishes the environments, so that populations may or may not be well adapted to both environments, depending upon the amount of overlap. According to the fourth possibility, there is no overlap of adaptive mutations, such that adaptive peaks in one landscape are not peaks in the other adaptive landscape. The GxE interaction completely separates the two groups of adaptive possibilities.

The results of this chapter, in combination with those of Chapter I, strongly support the second possibility. All twelve of the maltose-selected populations (M) improved substantially in their correlated performance in glucose (and to a similar extent as in maltose). But only a few of the twelve glucose-selected populations (G) improved in their correlated performance in maltose (and *none* to the same extent as in glucose). For other nutrients, the relationship is likely to be different (Chapter II). This suggests that the complexity of the adaptive landscape depends upon the environment, and that even seemingly small differences in environment (maltose versus glucose) can have profound effects on evolutionary divergence.

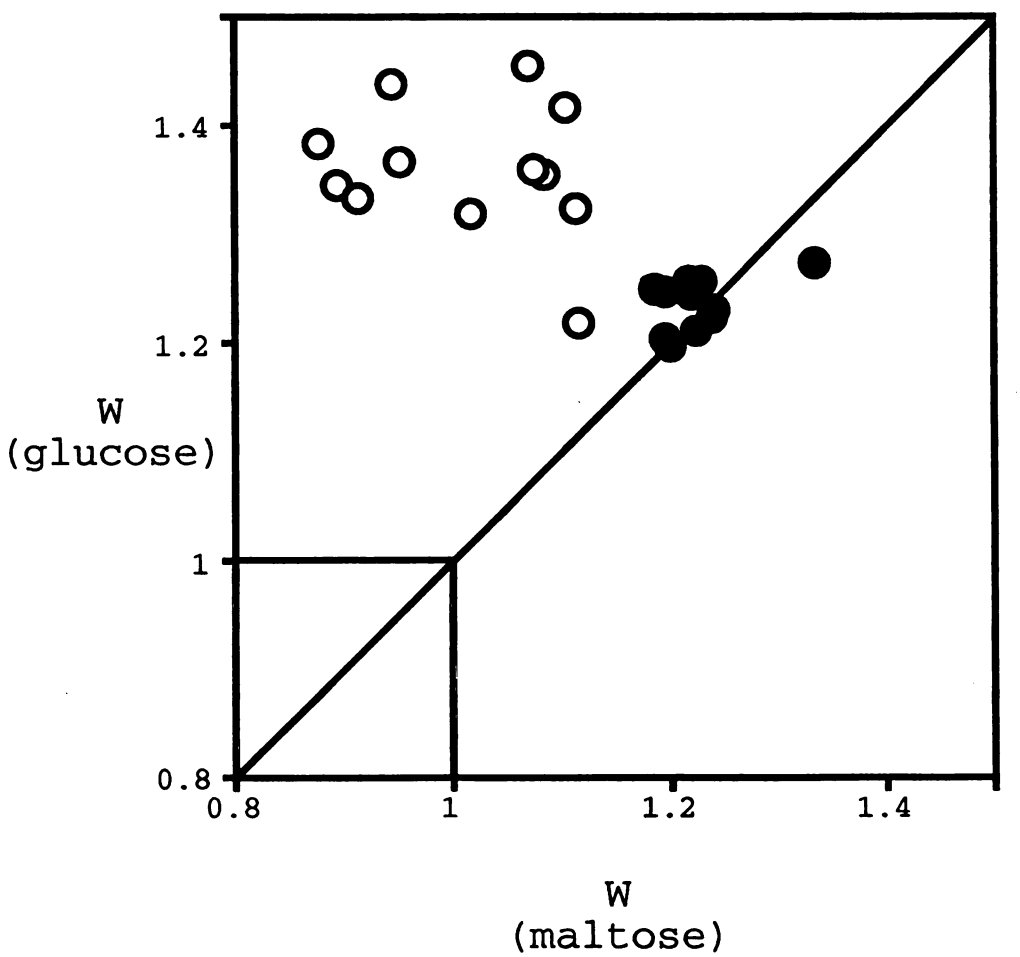
What physiological mechanisms could have given rise to the observed pattern of correlated responses? Previous work indicated that the G populations improved in fitness by improving uptake of glucose

from the environment (Chapters I and II). Because of the differences in uptake mechanisms between glucose and maltose, and yet their similarities in catabolism, it appears reasonable to assume that the M populations also improved in fitness by improving their uptake of maltose. Although the mechanisms of glucose and maltose uptake involve completely different proteins, they do share a common element. Cyclic adenosine monophosphate (cAMP) binds to the promoter regions (in concert with catabolite activator protein) of many genes, including those for maltose and glucose uptake proteins, increasing their expression. Thus, increased transcription of multiple uptake genes can be achieved by increased levels of cAMP within a cell (Magasanik and Neidhardt, 1987). However, the genes for glucose uptake have multiple promoter regions (Postma, 1993), so that increased transcription may occur by a number of different mechanisms (some more cAMP dependent than others), while transcription of the maltose uptake genes is always strongly regulated by cAMP (Schwartz, 1987).

Returning to the four scenarios described in the introduction, it was shown previously that the evolution of the G populations most closely followed scenario four (Chapters I and II) in which replicate populations converge on a fitness ridge or plateau (but not on a single peak). But in this chapter it has been shown that the evolution of the M populations appears to follow scenario one, in which the replicate populations are convergent in their correlated as well as direct responses, suggesting a single fitness peak. This pair of evolutionary results is consistent with the relationship of adaptive mutations in the glucose and maltose nutrient environments (Figure 10, relationship 2), where the adaptations to the maltose environment are a subset of the

adaptations to the glucose environment. This consistency is summarized in Figure 11, where the divergence among the G populations is evident in their corrlelated variation in fitness in maltose, while the M populations show little variation in either nutrient environment.

Figure 11. Graph of the relative fitness of the M and G populations, at 1,000 generations, in maltose and glucose-limited minimal media.



Closed circles indicate M populations, and open circles indicate G populations.

Chapter IV
HISTORICAL EFFECTS ON THE OUTCOME OF SELECTION

The examination, and explanation, of biological diversity has long been a motivation for the study of biology. The practitioners of natural history, from its known origin with Aristotle (circa 344 B.C.), through Linnæus (1758) and Darwin (1859), attempted to describe biological diversity and to understand its origins. Since the rediscovery of Mendel's work and the resulting improved understanding of the mechanisms of heredity, three factors have dominated the discussion of how diversity is generated and maintained. These are phylogeny, adaptation, and chance.

Phylogeny may profoundly constrain the evolutionary potential of distantly related organisms (e.g. peonies and elephants) (see Harvey and Pagel, 1991; Chao and Carr, unpubl. ms.), but the relative effects of phylogeny, adaptation, and chance among more closely related organisms are less well understood. In studies of variation among closely related organisms, adaptation to the current environment is often considered to the exclusion of phylogeny. However, phylogeny may potentially have large effects on variation among populations of the same species if epistatic interactions among genes are important.

The importance of epistasis can be expressed in terms of the "adaptive landscape" envisioned by Wright (1932). The adaptive landscape, as originally described, is the n-dimensional fitness manifold for all possible genotypes, given a particular set of environmental conditions. Wright depicted the adaptive landscape as having numerous peaks and valleys; however the presence of multiple

peaks requires strongly non-linear interactions among genes, or epistasis. Moreover, for there to be multiple fitness peaks, rather than simply a broad plateau a or ridge, epistatic effects must be both negatively and positively synergistic, and not simply sub-additive. In contrast with a multiple peak view, if epistasis is unimportant or simply sub-additive, then the adaptive landscape consists of a single adaptive peak or ridge, up which natural selection pushes all populations regardless of their genotypic composition.

In the framework of a multiple peak adaptive landscape, even small phylogenetic constraints on populations (i.e., with different initial genetic composition) may be evolutionarily important, because different populations may be in the domain of attraction of different adaptive peaks. Natural selection will then push populations up different adaptive peaks.

Lenski and associates (Lenski et al., 1991; Vasi et al., submitted; Chapter I, Chapter II) have experimentally examined the relationship of adaptation and chance in twelve replicate populations of *Escherichia coli* propagated for 2,000 generations in a glucose-limited medium. All twelve populations were founded from a single ancestral genotype, and were therefore initially homogeneous and identical to one another, so that chance events in the appearance and fixation of *de novo* mutants could be the only source of divergence. Three alternative scenarios were postulated for the outcome of this experiment. The three scenarios were distinguished from one another by the trajectory of grand mean fitness of the populations relative to their common ancestor, and the trajectory of genetic variation for mean fitness (see Figure 7, Chapter III). According to scenario 1, the populations eventually

converge to the same adaptive peak, which may be genetically monomorphic or polymorphic. The change in grand mean fitness, relative to the common ancestor, is strictly non-decreasing. However, due to the stochastic nature of the appearance and fixation of adaptive mutants, genetic variation would be expected to increase for a time before eventually declining to zero. According to scenario 2, the adaptive landscape itself varies with time, because of ecological interactions among genotypes mediated by metabolic by-products, which can result in interference and/or syntrophy of the type observed by Paquin and Adams (1983) or Turner, P.E., V.Souza, and R.E.Lenski (pers. comm.). Thus there is no stable outcome for mean fitness or genetic variation for fitness, but instead the populations "chase" temporally varying adaptive peaks. According to scenario 3, sustained divergence of the populations occurs, as the populations evolve towards different adaptive peaks of unequal mean fitness. Mean fitness is again strictly non-decreasing in time, plateauing out eventually, but genetic variation for fitness would not decline to zero.

After 2,000 generations, representative strains isolated from each of the twelve populations had improved in fitness by approximately 35% relative to their common ancestor, but they differed from each other by only a few percent (Lenski et al., 1991). In fact, the dynamics of adaptation and divergence in mean fitness were such that it was not possible to exclude the hypothesis that the twelve replicate populations were converging towards the same adaptive peak (scenario 1). Analyses of demographic components of fitness largely supported the same interpretation, as there were systematic changes in five components of fitness across the twelve strains, but little variation among the

genotypes for any fitness component (Vasi et al., submitted). However correlated responses to selection, as measured by fitness in unselected nutrient media, showed that the populations had diverged into at least six distinct groups (Chapter II). This outcome essentially defines a fourth scenario as it indicated that that the adaptive surface consisted either of a broad plateau or of different peaks of similar fitness.

Nevertheless, the populations studied by Lenski et al. (1991) had not diverged in fitness in the selected environment. Although adaptation led to divergence, the divergence itself appeared to have little or no direct fitness consequence. The lack of such an effect may have resulted from the extremely conservative nature of the experiment. All of the populations were initiated from the same genotype, and only chance events could move the replicate populations to fitness peaks of different height. Thus the populations may have been constrained by their common ancestry (their common starting point on the adaptive landscape) from more significant divergence. Alternatively, even if populations were genetically distinct at the start, adaptation in a uniform environment could result in convergent evolution, rather than divergent evolution.

This chapter describes an experimental test of the relative importance of historical contingencies (i.e. phylogenetic differences) in promoting or constraining subsequent evolution. Using the twelve populations selected by Lenski et al. (1991) as sources for phylogenetically distinct genotypes, another long-term selection experiment was performed in a previously unselected environment, but where the initial populations were now genetically distinct and differed substantially in fitness. The experimental design of this new selection

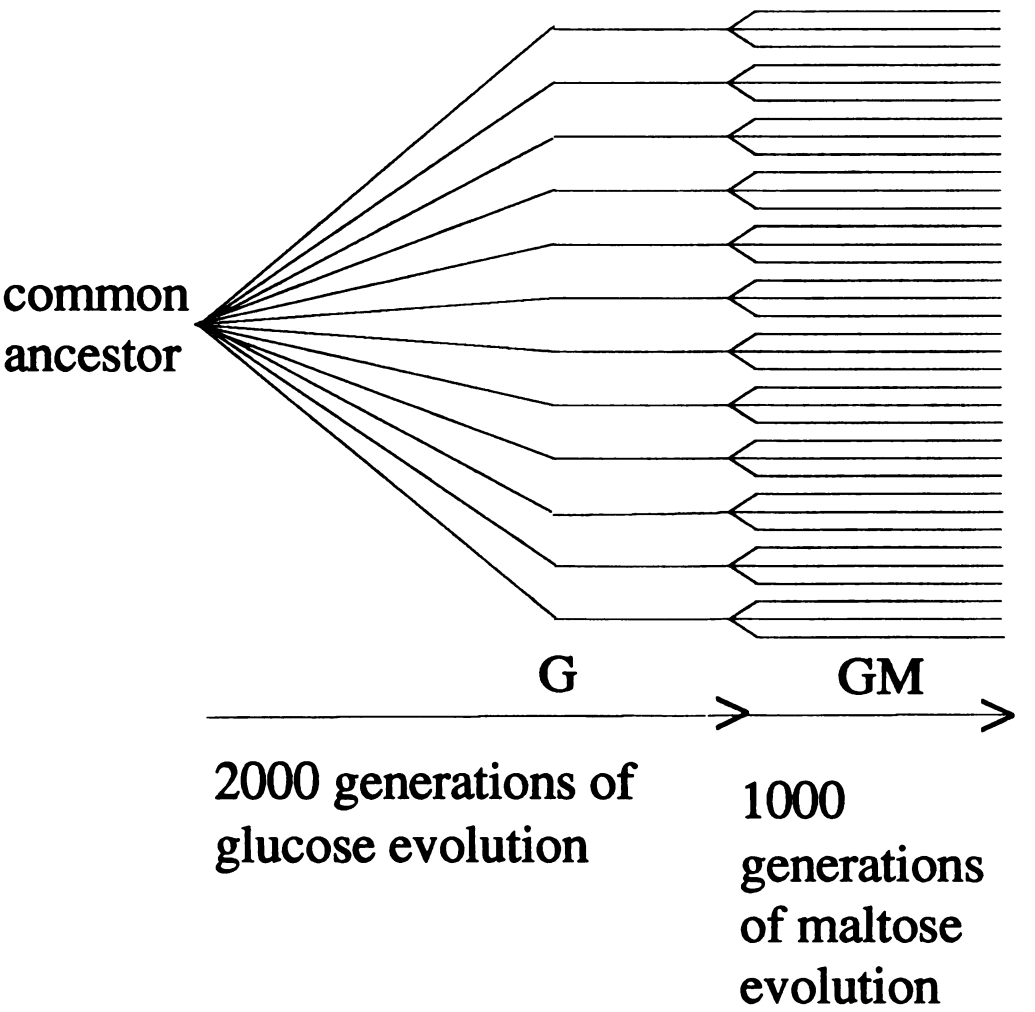
experiment was such that, for the first time, the relative effects of adaptation, phylogenetic history, and chance could be determined by a single manipulative experiment.

EXPERIMENTAL OVERVIEW AND EXPECTATIONS

Lenski et al. (1991) initiated twelve replicate populations that were propagated for 2,000 generations in a resource-limited medium containing glucose as the sole carbon and energy source usable by the bacteria. All populations were initiated from a single ancestor and thus were initially isogenic, except for a neutral marker. The populations were maintained on a 24 hour schedule, where a small sample (0.1 ml) of the previous day's culture was transferred into 9.9 ml of fresh medium. At 2,000 generations, a single clone was isolated from each population to serve as the representative genotype of each parental population. When assayed in maltose-limited medium, the twelve strains showed no systematic change in fitness from the ancestor as a group, but there was considerable fitness variation from genotype to genotype (Chapter I).

The twelve glucose-adapted genotypes (henceforth denoted as G genotypes) were used to initiate thirty-six new populations (henceforth denoted GM), with three replicate GM populations for each G genotype, which were subsequently propagated for 1,000 generations in maltose-limited batch culture (Figure 12). Due to the nested design of the experiment, all of the initial genetic variation among the GM populations was among populations having different parental genotypes, and there was no initial genetic variation among populations having the same parental genotype.

Figure 12. Phylogeny of the GM experimental populations.



The outcome of this experiment was analyzed in terms of five expectations. *Expectation 1* - If the adaptive evolutionary process among the GM populations is convergent, despite their phylogenetic differences and the effect of chance events, then total genetic variation for fitness among the GM populations should decline. If the total genetic variation for fitness is non-zero, then the nature of the genetic variation is examined with respect to expectations 2 - 5. *Expectation 2* - If chance is an important factor affecting adaptive evolution, then the GM populations having the same founding genotype should diverge in fitness during their propagation. *Expectation 3* - If the initial state of a population is an important factor affecting its subsequent adaptive evolution, then the genetic variation for fitness among the GM populations due to having different founding genotypes should be non-zero. If genetic variation due to having different founding genotypes is non-zero, then that variation is further characterized with respect to expectations 4 and 5. *Expectation 4* - If initial fitness was an important historical factor, then fitness of a GM population after 1,000 generations should be dependent on its initial fitness. That is, there should exist a positive correlation between initial and final fitnesses. *Expectation 5* - If the founding genotype, and hence prior history but not simply initial fitness, had an important effect on subsequent adaptive evolution, then the 1,000-generation fitnesses of the GM populations should group by common ancestral genotype, but not simply by initial fitness. In that case, there should be significant deviations from a monotonic relationship between initial and final fitnesses.

MATERIALS AND METHODS

Bacterial strains. -- All of the strains used in this study were ultimately derived from a single clone of *Escherichia coli* B which has been used in a number of other evolutionary studies (Chao et al. 1977; Lenski, 1984; Lenski and Levin, 1985; Bouma and Lenski, 1988; Lenski, 1988a, 1988b; Lenski et al., 1991; Bennett et al., 1992; Vasi et al., submitted). Briefly, this strain carries no plasmids and harbors no functional bacteriophage, and is strictly asexual. It is prototrophic but unable to use L-arabinose as a nutrient source. From this strain, an Ara^+ mutant was selected by plating $>10^9$ cells on minimal arabinose agar (Lenski, 1988a). The Ara^- and Ara^+ marker versions of the ancestral genotype form red and white colonies, respectively, when spread on tetrazolium arabinose (TA) agar, allowing the genotypes to be easily differentiated (Levin et al., 1977). The arabinose marker has been shown to be effectively neutral in a wide variety of carbohydrate-limited minimal media, including maltose (Table 1, Chapter I). These two ancestral clones, as well as their derived descendants, are stored in glycerol based medium at -80°C, making them available for simultaneous comparison with one another at any time.

The twelve G populations had been founded from the two ancestral clones (six Ara^- and six Ara^+) (Lenski et al., 1991). They were propagated for 2,000 generations under conditions identical to those in this study, except that the genotypes were cultured in shaking 50 ml Erlenmeyer flasks, rather than stationary 18 mm tubes as used in this study (see below), and in glucose- rather than maltose-limited medium.

Culture conditions. -- The populations were propagated by daily 100-fold serial dilution from 10 ml cultures containing roughly $5x10^7$

cells ml^{-1} into fresh Davis minimal medium (Carlton and Brown, 1981) supplemented with 2×10^{-6} g ml^{-1} thiamine hydrochloride and 25 μg ml^{-1} maltose. Cultures were maintained in 18 mm tubes at 37°C, and were not shaken. The 100-fold dilution and regrowth results in 6.64 generations ($\log_2 100$) of binary fission per day.

Evolving populations. -- All populations were founded from single colonies, so that there was no initial variation within populations. The thirty-six GM populations were founded from twelve replicate G genotypes (Lenski et al., 1991), with three-fold replication of each founding G genotype. Genetic variation among the GM populations was solely due to genetic variation among founding G genotypes, so that GM populations sharing the same founding genotype initially had no genetic variation among them. Under the selective conditions of this experiment, the founding G genotypes exhibited at most a two-fold range in relative fitness (Table 3, chapter I).

The GM populations were propagated for 150 days, or approximately 1,000 generations of binary fission. Daily transfers of the genotypes into fresh medium was performed by alternating Ara^- and Ara^+ populations, so that any cross-contamination from one population to the next would be detectable by changes in marker state. No cross-contamination was observed.

Sampling. -- Samples of each population were collected approximately every 100 generations. After performing the daily transfer, glycerol was added to a final concentration of roughly 15%, and a sample (~3-4 ml) was removed to a screwcap vial, which was stored at -80°C.

Measurement of fitness in competition experiments. -- Competition

experiments were performed using the same culture conditions in which the populations had been propagated. The two marker versions of the ancestral genotype, which were directly ancestral to the G populations (and more distantly to the GM populations), were used as the common competitors in all of the experiments reported here. The protocol for the competition experiments was as follows. After growing up aliquots of the appropriate genotypes from the freezer for one day, both competitors were separately conditioned for one or two days (one or two cycles of dilution and regrowth) in the standard maltose-limited medium. Each competitor was then diluted 200-fold into a competition culture containing fresh medium (so that the total initial population size was equivalent to the usual 100-fold dilution of the stationary-phase population). This competition culture was then incubated under standard conditions for one day. Initial and final population densities of each competitor were determined by plating aliquots from the competition flask onto TA indicator agar, which allowed Ara^- and Ara^+ competitors to be distinguished by their colony color. However, during the selection experiment, one population developed an extremely low plating efficiency on TA agar. Initial and final densities for this population were determined on minimal maltose-limited agar, which was easily done as the ancestral and derived states differed in their colony morphology on this agar. Fitness (W) was defined as the ratio of the number of doublings for the evolved and ancestral competitors:

$$W = \log_2 (N_f' / N_i') / \log_2 (N_f / N_i)$$

where N_i' and N_i are the initial densities and N_f' and N_f are the final

densities of the derived and ancestral types, respectively.

Experimental design and statistical analysis. -- To test the selective neutrality of the arabinose marker, replicate competition experiments were performed using the reciprocally marked ancestors in maltose-limited minimal medium. To test for possible interactions between the arabinose marker and the derived versus ancestral genetic backgrounds, the average fitness of the Ara^+ versus Ara^- genotypes were compared for the GM populations at 1,000 generations.

Fitness of the evolving GM populations was determined approximately every 100 generations, excepting generations 700 and 900. Five estimates of fitness were obtained for each founding G genotype (i.e. the GM populations at generation 0) and ten estimates of fitness were obtained for each GM population at 1,000 generations. Between 0 and 1,000 generations, one or two fitness estimates were obtained per GM population per sampled generation. Fitness assays were run in complete blocks, except when there was contamination or plating difficulties. Most of the time neither contamination nor plating was a problem: however, for the population that did not plate well on TA agar, only one of the ten fitness assays at 1,000 generations was done as part of a complete block. The remaining nine were preformed as additional replicates in the tenth block.

In general, statistical tests on the above data took two forms, depending upon whether the test was for a mean effect or a variance effect. Tests of mean fitness were performed on the data without regard to block effect. Tests of variance, and determination of variance components, were performed both by including block effects in a two-way analysis of variance or by attempting to remove block effects by

performing analyses on residuals. Residual analysis was performed in an attempt to remove effects of unequal block size in the GM populations. Residuals were calculated by subtracting the mean fitness of a block from each fitness estimate.

Genetic variance was first partioned among populations and then into phylogenetic (ancestral) effects and chance effects. The effects of phylogeny and chance corresponded to among- and within-ancestral genotype classes. The total genetic variation among GM populations was estimated as the difference in population and error mean squares, divided by the number of replicate assays per population. Genetic variation due to chance was estimated as the difference in the chance and error mean squares, divided by the number of replicate assays per population. Genetic variation due to phylogeny was estimated as the difference in the ancestral and chance mean squares, divided by the product of the number of replicate assays per population and the number of replicate populations per ancestor. For the unbalanced ANOVAs, approximations for the number of replicate assays were used, as suggested by Sokal and Rohlf (1981, pp. 293 - 308). Confidence intervals for genetic variance estimates were computed using the methods described by Sokal and Rohlf (1981, pp. 217-218), using approximations for the number of replicate assays where appropriate.

The effects of initial fitness on final fitness of the GM populations was examined by linear least-squares analysis. Two methods of analysis were performed. For both methods, block effects were not included, nor was sampling error of the final fitness values. The first method was a standard least-squares regression, with three values of final fitness (one for each GM population) for each value of initial

fitness (corresponding to the twelve G founding strains). Statistical significance of deviations from the linear regression was determined by an F-test, where the F statistic was calculated as the ratio of the mean square deviation from the regression line to the mean square error.

The second method was a two-stage least squares regression of the final fitness on the initial fitness. This method was performed because the initial and final fitness values of the populations were measured with comparable error, whereas if the initial fitness had been measured with relatively little or no error a standard least-squares regression would have been appropriate. The procedure followed is described by Acton (1959, pp.127 - 171). Briefly, the two-stage regression attempts to fit the data by a three-step procedure. The first step is to perform a standard regression and then transform the axes so that the new X axis is coincident with the regression line. The next step is to attempt to remove the correlated error between the transformed X and Y values. The final step is to perform a standard regression of the transformed Y data onto the transformed X data, and back-transform to have the slope in the original coordinates. The above procedure can be summarized by the following equation (Acton, p. 161, eq. 30):

$$b = \frac{MSBxy - MSWxy}{MSBx - MSWx},$$

where b is the estimated slope, MSB is Mean Square Between, and MSW is Mean Square Within. Statistical significance of the regression line was determined by first determining the variance in the estimate of the slope (Tukey, 1951; Acton, p. 161, eq. 31) as follows:

$$s_b^2 = \frac{\frac{MSBx\ MSB(y - bx)}{DFB} + \frac{MSWx\ MSW(y - bx)}{DFW}}{(MSBx - MSWx)^2}$$

where s_b^2 is the sample variance in the estimate of the slope, DF is degrees of freedom, and MSB(y - bx) is MSB around the fitted regression line. The confidence limits for the slope was determined by the quadratic, putting in the appropriate F-value (Acton, p. 163, eq. 28'):

$$(\text{Limit} - \text{slope})^2 \leq F_{1,11(0.05)}\ s_b^2.$$

Statistical significance for deviations from the linear regression was determined by an F-test, where the F statistic was calculated as the ratio of mean square of deviations from the transformed regression line to the mean square error from the transformed regression line (Acton, p. 160, eq. 28),

$$\frac{MSB(y - bx)}{MSW(y - bx)} \leq F_{11,24(0.05)}.$$

To characterize further the relative effects of chance (which was measured as a variance component for fitness), phylogeny (which was also measured as a variance component for fitness), and adaptation (which was measured as a change in mean fitness), it was useful to put the three quantities into comparable units. This was most easily done by converting the variance components into their corresponding standard deviations.

RESULTS

Selective neutrality of the genetic marker. -- It has been previously shown that the arabinose utilization marker is effectively neutral in the ancestral genotype when grown in test tubes in maltose minimal medium (Table 19, Chapter III). This finding does not necessarily indicate that the arabinose marker remained neutral in the derived populations, and the marker state did have a significant effect on fitness in the GM populations (Table 31). However, subsequent analyses of the GM populations do not include a marker effect as the primary aim of propagating the GM populations was to determine whether phylogeny affected eventual fitness, and the marker is an aspect of phylogeny.

Mean fitness improved over the course of the experiment. -- The trajectory of grand mean fitness relative to the common ancestor for the GM populations during propagation is shown in figure 13. To calculate the grand mean fitness at a specific generation, first the mean fitness of the three populations having the same founding genotype was determined, then the mean fitness over all twelve such estimates was determined. At 1,000 generations, the grand mean fitness for the GM populations, relative to the common ancestor is 1.213. The corresponding 95% confidence interval (based on the twelve mean fitness estimates so that there are n - 1 = 11 degrees of freedom) ranges from 1.186 to 1.239. A pairwise comparison of the initial and final fitnesses of the GM populations shows that the GM populations have improved significantly in fitness in maltose-limited minimal medium after 1,000 generations (Table 32).

The rate of fitness improvement. -- Visual inspection of the

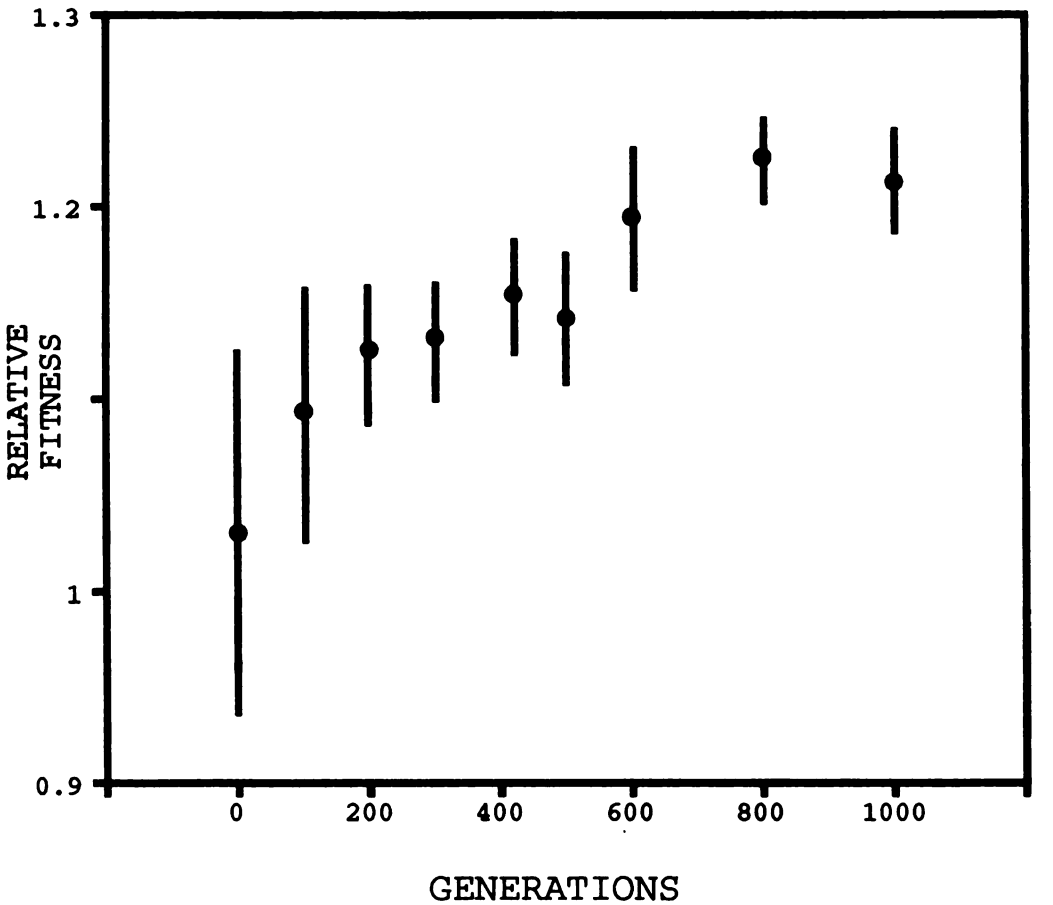
Table 31. Fitness effects of the arabinose marker in the GM derived populations in maltose.

Mean Fitness[1]			
Ara^+ Derived Relative to Ara^- Ancestor	Ara^- Derived Relative to Ara^+ Ancestor	Difference[2] (± SED)	p[3]
1.2417	1.1851	0.0554 (± 0.0183)	0.0132

[1] Difference (and standard error of the difference) in fitness based on six independent set of three populations in each derived marker class.

[2] Two-tailed probability computed from the *t*-distribution with 6 + 6 - 2 = 10 degrees of freedom; the null hypothesis is that difference in relative fitness for the two marker classes of derived populations equals zero, indicating equal changes mean fitness for the Ara^+ and Ara^- derived populations.

Figure 13. Trajectory of grand mean fitness for the GM populations during 1,000 generations in maltose-limited minimal medium



Fitness is expressed relative to the reciprocally marked ancestral genotype. Fitness was estimated for each population, and a grand mean fitness was calculated as the mean of all twelve sets of three GM populations. The error bars show the 95% confidence intervals based on a *t*-distribution with 11 degrees of freedom.

trajectories of mean fitness over time (Figure 13) suggests that populations increased in mean fitness at a more rapid rate during the first 500 generations than in the last 500 generations. To test this, the rate of fitness improvement for the first 500 generations was compared to that of the second 500 generations. Linear regressions of mean fitness versus generation were computed for each population over the entire 1,000 generations and for just the first 500 generations. The regressions were performed by fixing the y-intercept to the initial estimate of fitness for each population (see Lenski et al., 1991; Table 22, Chapter III). The rate of fitness improvement over the second 500 generations was computed by subtracting twice the rate over the entire 1,000 by the rate over the first 500 generations. Mean fitness improved at a rate that was approximately three times as fast during the first 500 generations as during the last 500 generations in the GM populations; however, the difference in rate of improvement was not significant (Table 33). The primary reason for lack of significance is that the populations that had relatively high fitness initially improved slowly throughout the experiment, unlike the populations that had lower initial fitnesses and improved much more rapidly at first. This resulted in a large standard error for the mean rate of improvement over the first 500 generations, and for the mean difference between initial and final rates. During the second 500 generations, the mean rate of improvement was very similar to that observed for other populations whose rate was initially much higher (Table 22, Chapter III; Lenski et al., 1991). This suggests that the GM populations were approaching fitness plateaus, but that some had already nearly 'plateaued' prior to

Table 32. Adaptation of the GM populations to maltose-limited minimal medium after 1,000 generations.

Twelve ancestral genotypes (G) were used to found twelve groups of three populations (GM) that were selected during 1,000 generations in maltose-limited minimal medium.

G Founding Genotype	Fitness Relative to the Common Ancestor[1] Initial[2]	Final[2]	Difference[3]
Ara^-1	0.964	1.218	0.254
Ara^-2	0.695	1.160	0.465
Ara^-3	1.057	1.209	0.152
Ara^-4	1.141	1.193	0.052
Ara^-5	0.965	1.151	0.186
Ara^-6	0.958	1.179	0.222
Ara^+1	1.184	1.261	0.077
Ara^+2	1.157	1.298	0.140
Ara^+3	1.186	1.235	0.048
Ara^+4	0.849	1.199	0.350
Ara^+5	1.064	1.209	0.145
Ara^+6	1.144	1.242	0.098
Mean	1.030	1.213	0.182[4]
SE	0.044	0.012	0.036
P	0.500	< 0.0001	0.0004

[1] Initial fitness estimates based on five assays, while final estimates are based on ten assays for each population, which were then averaged.

[2] Two-tailed probability computed from the *t*-distribution with n - 1 = 11 degrees of freedom; the null hypothesis is that mean fitness is one.

[3] Two-tailed probability computed from the *t*-distribution with n - 1 = 11 degrees of freedom; the null hypothesis is that the difference between the two means is zero, indicating equal fitness before and after propagation in maltose.

[4] The corresponding 95% confidence interval ranges from 0.103 to 0.262.

Table 33. Analysis of trajectories for mean fitness of the GM populations during 1,000 generations in maltose-limited minimal medium.

Rate of Change in mean fitness (per 1,000 generations)		
	Mean ± SE[1]	t_s[2]
A. During all 1,000 gen.	0.198 ± 0.042	4.661***
B. During first 500 gen.	0.296 ± 0.098	3.010*
C. During last 500 gen.[3]	0.100 ± 0.022	4.649***
D. Difference[4]	0.195 ± 0.114	1.713ns

[1] Means and standard errors based on twelve set of three GM populations.

[2] Two-tailed probability computed from the *t*-distribution with 12 - 1 = 11 degrees of freedom. Null hypothesis is that the slope (or difference in slopes) equals zero.

[3] Rate of change during the last 500 generations was calculated as twice quantity A minus quantity B.

[4] Difference calculated as quantity B minus quantity C.

ns $p > 0.05$.

* $p < 0.05$.

** $p < 0.01$.

*** $p < 0.001$.

their propagation in maltose-minimal medium.

Genetic variation among GM populations declined, but not to zero. -- An analysis of variance (based on repeated measures of fitness) for the G genotypes (i.e., GM populations at generation 0 in the maltose-selection experiment) is given in Table 34 and for the GM populations at 1,000 generations in Table 35. Although the populations remained heterogeneous after 1,000 generations, the amount of genetic variation among populations decreased to less than 1/10th of its initial value. The confidence intervals for genetic variance among populations at 0 and 1,000 generations are completely non-overlapping, giving statistical support for the observed decline in genetic variance. The evolutionary process among these populations was therefore primarily convergent, at least as measured by relative fitness.

Chance was not a significant factor causing fitness divergence in the GM populations. -- An analysis of variance for the GM populations at 1,000 generations that separates the effects of chance and phylogeny is given in Table 36. Genetic variation for fitness among populations having the same immediate ancestor was not significantly greater than zero (which was the initial variance). In fact, statistically significant genetic variation due to chance was never observed during the entire propagation of the GM populations. Genetic variance due to chance divergence was estimated at generations 300, 600, and 800 (data not shown) and the estimated variance component was never greater than zero with associated P-values always greater than 0.5. Thus chance did not play a significant role in generating divergence among the GM populations that was selectively important during the 1,000 generations

Table 34. ANOVA for fitness in maltose-limited medium of the G genotypes, from which the GM populations were founded.

Analysis of variance for fitnesses obtained for the twelve independently derived G genotypes relative to their common ancestor, in maltose-limited medium. Genotype and block are both random effects.

Source of variation	df	MS	F	P
genotype	11	0.1137	24.317	< 0.0001[1]
Block	4	0.0485	10.374	< 0.0001
Error	44	0.0047		

[1] Genetic variance among genotypes is 0.0218, with associated 95% CI of 0.0105 - 0.0646.

Table 35. ANOVA for fitness of the GM populations after 1,000 generations.

Analysis of variance for fitness obtained for the thirty-six derived GM populations relative to their common ancestor in maltose-limited medium. Population and block are both random effects; see Materials and Methods for description of two designs.

	Source of variation	df	MS	F	P
Unbalanced Design	Population	35	0.0195	3.579	< 0.0001[2]
	Block	9	0.1846	32.290	< 0.0001
	Error	314[1]	0.0054		
Residuals Design	Population	35	0.0195	3.670	< 0.0001[3]
	Error	323[1]	0.0053		

[1] There is a missing value.

[2] Genetic variance among populations is 0.001416, with associated 95% CI of 0.000735 to 0.002800.

[3] Genetic variance among populations is 0.001421, with associated 95% CI of 0.000745 to 0.002800.

Table 36. Nested ANOVA for fitness for the GM populations after 1,000 generations.

Analysis of variance for fitness obtained for the thirty-six derived GM populations relative to their common ancestor in maltose-limited medium. Ancestor, chance, and block are all random effects; see Materials and Methods for description of two designs.

	Source of variation	df	MS	F	P
Unbalanced Design	Ancestor	11	0.0477	7.312	< 0.0001[2]
	Chance	24	0.0065	1.199	0.2399[3]
	Block	9	0.1846	32.290	< 0.0001
	Error	314[1]	0.0054		
Residuals Design	Ancestor	11	0.0475	7.164	< 0.0001[4]
	Chance	24	0.0066	1.249	0.1971[5]
	Error	323[1]	0.0053		

[1] There a one missing value.

[2] Genetic variance due to different ancestors is 0.001385, with associated 95% CI of 0.000564 to 0.004403.

[3] Genetic variance due to chance is 0.0001094, with associated 95% CI of -0.000169 to 0.000731.

[4] Genetic variance due to different ancestors is 0.001366, with associated 95% CI of 0.000534 to 0.00422.

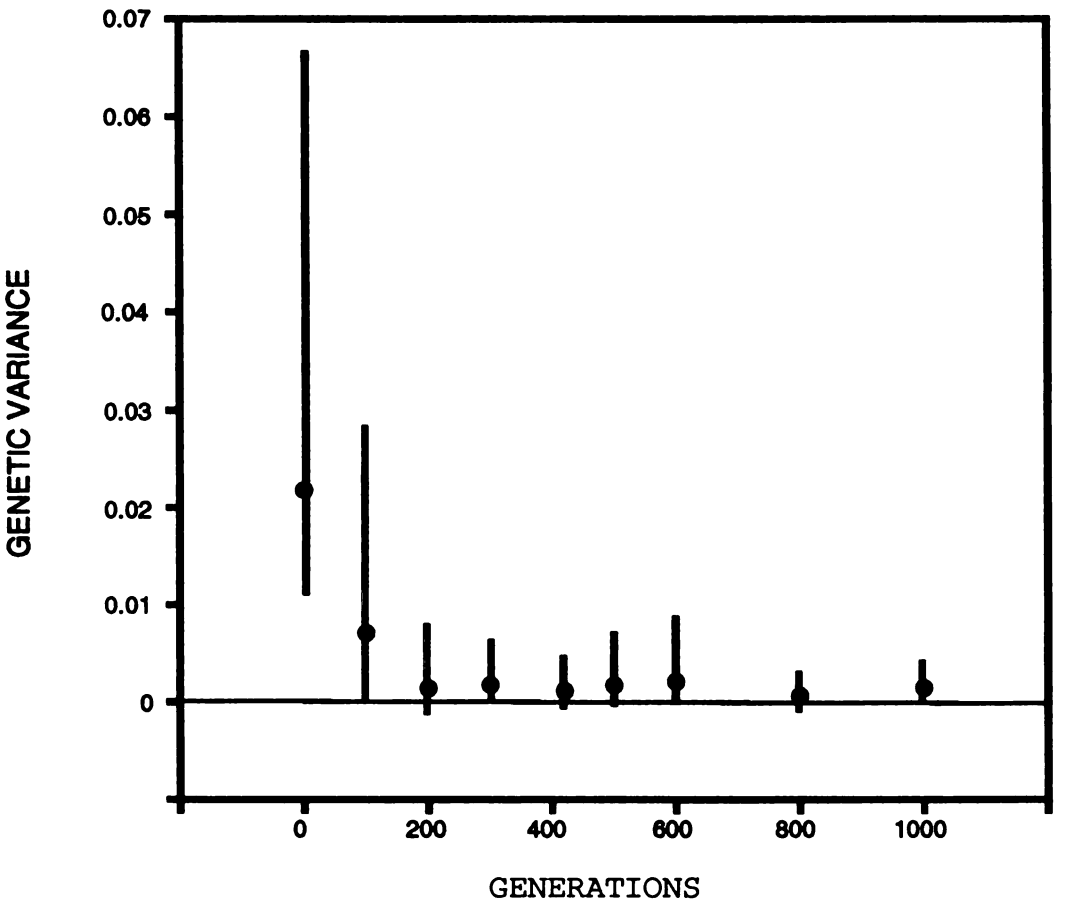
[5] Genetic variance due to chance is 0.0001328, with associated 95% CI of -0.000146 to 0.000760.

of propagation.

Ancestral state has a significant effect on fitness at 1,000 generations in the GM populations. -- As indicated in Table 36, phylogenetic effects on fitness were still important in the GM populations after 1,000 generations in the maltose-limited environment. However, as the evolutionary process was generally convergent, the differences are much smaller among the derived populations than their ancestors. The phylogenetic component of the among-population genetic variation declined quickly during the experimental evolution, as shown in Figure 14, and does not appear to have played a major role in generating selective differences among the GM populations at any time during the propagation. Nevertheless, phylogenetic effects on fitness at 1,000 generations were significant and can be partioned into linear and non-systematic effects. The former corresponds to an effect of the ancestor's initial fitness, whereas the latter implies an effect due to the ancestral genotype that is *not* reflected in its fitness.

Initial fitness of the GM populations affects fitness at 1,000 generations. -- The standard least-squares regression of the final versus initial fitness of the GM populations gives a slope of 0.196 (P = 0.0104, Table 37). The two-stage regression (see Materials and Methods) also gives a statistically significant slope of 0.217 (P = 0.0034)(Table 38, Figure 15). In both cases, a substantial portion of the variation in fitness at 1,000 generations results from differences in initial fitness. Partioning the variance between explained (linear) and unexplained (other) components, in the two-stage regression, yields 0.000895 and 0.000591, respectively. Thus, about 60% of the genetic

Figure 14. Trajectory of the phylogenetic component of the among-population genetic variation for fitness for the GM populations.



Error bars indicate 95% confidence intervals. Methods by which genetic variation and error were estimated are given in the text.

Table 37. Linear regression analysis of the phylogenetic component of fitness variation in the GM populations.

Standard Regression	Source of variation	df	MS	F	P
	Ancestor	11	0.0053	6.6025	< 0.0001[1]
	Regression[2]	1	0.0287	9.8859	0.0104[3]
	Deviations	10	0.0029	3.6522	0.0045[4]
	Error		24	0.0008	

[1] Consistent with ANOVA shown in Table 36.

[2] The regression line has slope 0.1956 and intercept 1.0112.

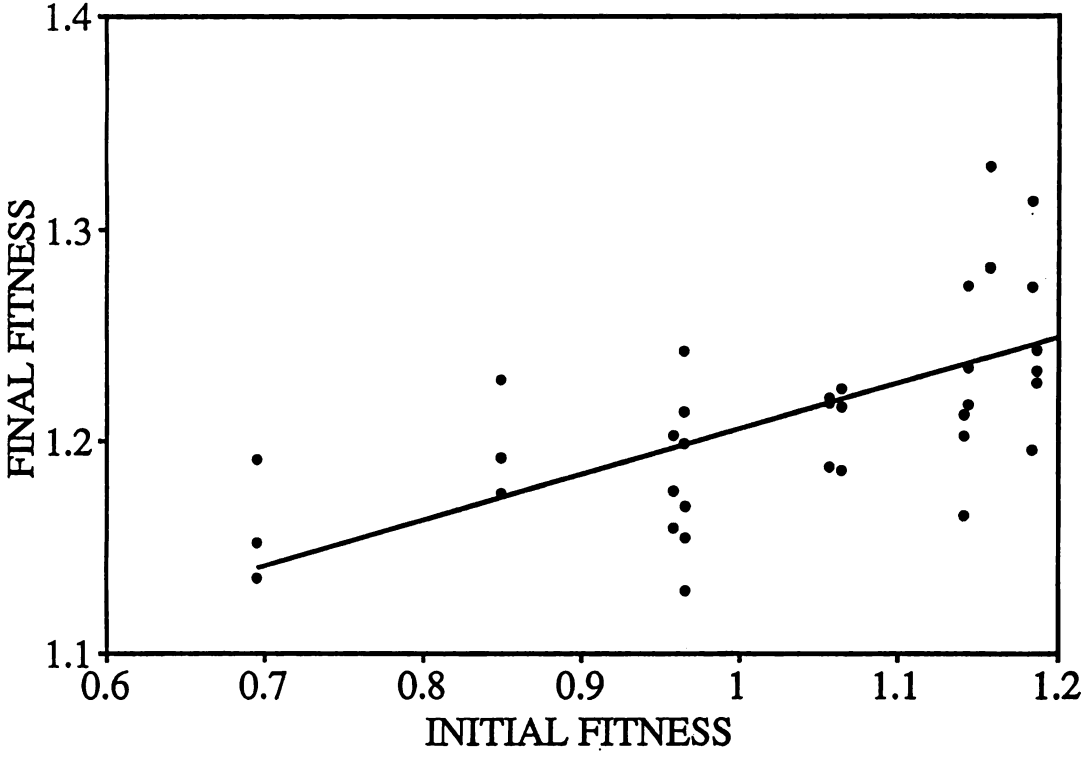
[3] Genetic variance component is 0.000783.

[4] Genetic variance component is 0.000704.

Table 38. Mean squares for the two-stage linear regression analysis of the phylogenetic component of fitness variation in the GM populations.

Source of variation	MSW	MSB
Initial fitness (X-axis)	0.0067	0.0682
Final fitness (Y-axis)	0.0008	0.0053
Covariance (XY)	0.0	0.0045

Figure 15. Two-stage regression of final versus initial fitness of the GM populations.



This regression adjusts for the fact that both X and Y variables are measured with error (see Materials and Methods for details). Fitness is expressed relative to an ancestral strain. Because each G strain founded three GM populations, there are three estimates of final fitness for each estimate of initial fitness. The slope is 0.217 and is significantly different from zero (P = 0.0034). Systematic deviations from the line are only marginally significant (P = 0.0774).

variance due to phylogenetic differences at 1,000 generations can be apportioned to a linear relationship with initial fitness. Roughly half of the total phylogenetic variance component is explained by the standard linear regression as well (Table 37).

Other aspects of ancestral genotype also have a small effect on fitness at 1,000 generations. -- Table 37 also indicates significant deviation from the fitted line for the standard regression (P = 0.0045). Deviations from the two-stage regression line are only statistically suggestive (P = 0.0774) (Figure 15). The significant (or at least suggestive) lack-of-fit of the regression models reflects that GM populations having the same ancestor tend to fall together on one side of the regression line or the other, rather than any suggestion of a curvilinear relationship between initial and final fitness. Approximately 40% of the genetic variance due to phylogenetic differences at 1,000 generations appears to result from aspects of the founding genotype distinct from its initial fitness.

Relative effects of adaptation, phylogeny and chance. -- Because adaptation was measured as a mean, while phylogeny and chance were measured as variances, the relative importance of each is difficult to compare unless they are converted into comparable units. The standard deviation of variance components most easily provides values in units comparable to adaptation.

The improvement in grand mean fitness after 1,000 generations in maltose-limited medium was 0.182, with 95% confidence limits of 0.103 and 0.262 (Table 32). After 1,000 generations, the genetic variance among populations due to phylogenetic differences was 0.001366, with 95%

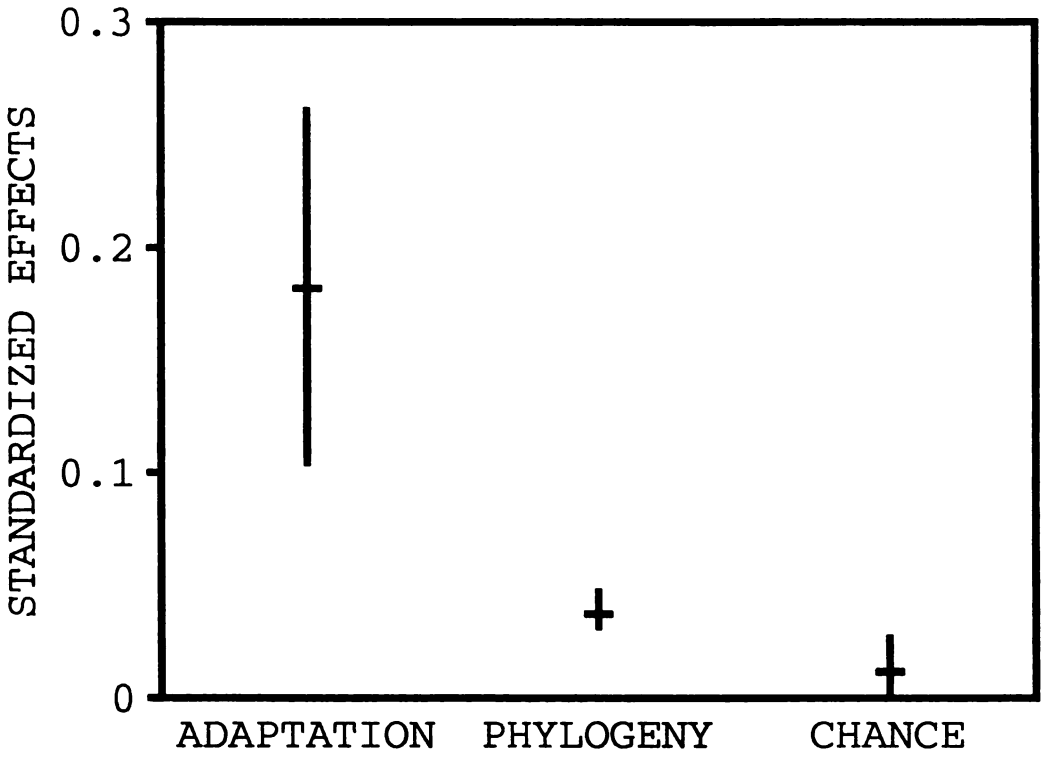
confidence limits of 0.00091 and 0.00228 (Table 36). The corresponding standard deviation is 0.0369, with confidence limits ranging from 0.0302 to 0.0478. For chance, after 1,000 generations the genetic variance among populations was 0.0001328, with 95% confidence limits of 0.0 and 0.000760 (Table 36). The corresponding standard deviation is 0.0115, with confidence limits ranging from 0.0 to 0.0276.

Adaptation to the current environment was the most important factor affecting the evolution of the GM populations (Figure 16). Comparing the point estimates, the effect on fitness due to adaptation was almost 5-fold (0.182/0.0369) greater than that of phylogeny, and over 15-fold (0.182/0.0115) greater than chance. Although the effect of phylogeny is relatively small compared to that of adaptation, phylogeny did have a significantly greater effect on fitness at 1,000 generations than did chance, as the confidence intervals for each do not even overlap.

DISCUSSION

The major findings of this study can be summarized as follows. (1) Mean fitness increased in all GM evolving populations by ~18%, on average, relative to their corresponding G ancestral populations. (2) During the second 500 generations the rate of fitness improvement was small, suggesting that all of the populations were approaching fitness plateaus. (3) Genetic variation for fitness among the GM populations declined substantially over time, indicating that the evolutionary process was primarily convergent for mean fitness. (4) Chance was not an important factor in generating divergence in fitness among the GM populations. (5) Phylogenetic differences among the GM populations

Figure 16. Relative contributions of adaptation to the selection environment, phylogeny, and chance to the eventual fitness of the GM populations.



Methods by which mean and 95% confidence interval estimations were performed are described in the text.

affected fitness; however variation among populations due to ancestry declined to less than 10% of its initial value. (6) Phylogeny influenced fitness through both linear and non-systematic effects, suggesting that different founding genotypes imposed constraints on subsequent adaptation beyond those implied simply by their initial fitness. (7) Adaptation to the imposed environment was largely responsible for the fitness of the GM populations after 1,000 generations.

Diversity

In the introduction (after Lenski et al., 1991), four different scenarios were presented for the adaptation and divergence of replicate populations propagated in uniform environments. In the first scenario, all the populations eventually converge to the same adaptive peak, although there may be some transient divergence due to the stochastic nature of the appearance and fixation of mutants. According to scenario two, complex ecological interactions among genotypes within populations result in a temporally varying adaptive landscape; there may or may not be any phylogenetic constraints on these complex evolutionary dynamics. In the third scenario, the populations do not eventually converge to the same adaptive peak, but rather diverge to different adaptive peaks of unequal mean fitness. In the fourth scenario, the populations converge to the same mean fitness; however, they do not converge to the exact same adaptive peak. The first and fourth scenarios cannot be distinguished solely by measurements of mean fitness and its genetic variance. In this study, the GM populations were initiated with substantial among-population genetic variance for fitness. The

experimental evolution of the GM populations therefore enabled an examination of whether genetic variation declined to zero, as in scenarios 1 & 4, or if genetic variation among populations was maintained or even enhanced, as in scenario 3. The great reduction in genetic variation and the slow rate of fitness improvement during the last 500 generations suggest that the GM populations are converging to very similar mean fitnesses.

Factors affecting diversity in fitness. -- Phylogenetic constraints have been put forth as an important determinant of responses to natural selection, and some have argued that biological diversity may be more affected by phylogeny than by adaptation to the current environment (Brooks and McLennan, 1991). A critical issue with respect to the relative importance of phylogeny and adaptation is the amount of phylogenetic differentiation necessary to produce divergent adaptive responses to the same environmental conditions. In this study, adaptation during 1,000 generations was able to overcome almost completely very large phylogenetic differences that had arisen during the preceding 2,000 generations. In fact, within only 200 generations (Figure 9) the phylogenetic effect on fitness in the new environment was reduced by more than 90%.

Adaptation to the current environment was overwhelmingly the most important factor affecting the eventual fitness of a population and not phylogeny or chance. This dominance of adaptation results from the combination of two factors. The first factor was the very large mean adaptive response of the populations. For example, the average fitness improvement would have to be decreased by more than 50% for there to be no statistically significant difference between the effects of

adaptation and phylogeny.

The second factor was the relatively small amount of fitness variation, due to either phylogeny or chance, after 1,000 generations. This small residual variance among populations was despite the fact that the initial genetic variation among populations (or rather the corresponding standard deviation) was almost as large as the mean change in fitness due to adaptation. By 1,000 generations, only ~4% of the variation attributable to initial fitness variation remained. Other constraints, indicated by the non-systematic deviations from the linear regression of final on initial fitness, generated only ~3% as much variation as was initially present, and chance contributed <1%. Thus, very little genetic variation for fitness was maintained or generated during the 1,000 generations.

Although phylogeny was unimportant relative to adaptation, phylogeny did nonetheless play a larger role than chance in the eventual fitness of the GM populations. This finding indicates that initial variation in genotype resulted in a larger sampling of the adaptive landscape than chance alone would have. Moreover, genetic variation among GM populations was due to genotype, and not simply initial fitness, suggesting that there are subtle constraints that distinguished the G populations' potential to adapt to the maltose-selected environment. Given that the source genotypes were likely to have differed from one another in at most eight adaptive mutations (see Lenski et al., 1991), and that the initial divergence occurred under the same environmental conditions, it may not be too surprising that the populations did not diverge to a greater extent.

<u>The Nature of the adaptive landscape.</u> -- There are two aspects of

the adaptive landscape that can affect the divergence of populations: the closeness of peaks in terms of genetic distance, and the relative height of different peaks. Previous studies suggest that different adaptive solutions to a common environment are readily accessible by only a few genetic changes, and that those different adaptive solutions are of roughly similar height (Bull and Molineux, 1992; Chapter I; Chapter II). The results of this study are consistent with those previous studies, and extend their generality over a greater range of the adaptive landscape. However, whether the different adaptive solutions represented different adaptive peaks (i.e., with maladaptive hybrids), or different genotypes on a single adaptive plateau (i.e. with equivalent hybrid fitnesses), was not readily determined.

Given these results, one may ask if multiple peaks on the adaptive landscape, and hence epistasis, are important factors affecting biological diversity? Chao and Carr (unpubl. ms.) suggest that differences and similarities among species are perhaps due more to niche conservation than to phylogenetic constraints. Although organisms may not be completely free (of constraints) to respond to selection in an infinite variety of ways, they may be generally flexible enough (free of constraints) to respond adaptively, given enough time. For example, there are numerous examples of habitat invasions in which the organisms have substantially altered morphology, and yet still retain (diagnostic) aspects of their ancestral state (whales, true seals, sea lions, penguins, ichthyosaurs, plesiosaurs, etc.). It may be that phylogenetic constraints often do not limit the fitness that an organism can reach in a particular environment, but may affect the dynamics of the fitness improvement and the specific genotypic composition and phenotypic

attributes that result. Thus, organisms that are initially less well adapted to a particular environment may take longer to reach a certain level of fitness, but they will eventually do so nonetheless (provided that they are isolated and not driven extinct by other populations that are initially preadapted). This argument does imply that there are multiple adaptive trajectories, but not that adaptation involves traveling up distinct adaptive peaks or moving from one peak to another. Thus, many of the limits to adaptive evolution may result from inexorable physical and chemical constraints, while phylogenetic differences may primarily affect the dynamics of adaptation and the nature of non-adaptive corrlelated responses.

APPENDIX

APPENDIX A

Table 39. Relative fitness of the twelve glucose-selected genotypes in carbohydrate limited media.

G[1] Derived Genotype	Selection Rate Constant Against the Common Ancestor in:											
	Glc	Fru	Gtl	Man	Mtl	NAG	Tre	Gal	Gly	Lac	Mal	Mel
Ara^-1	1.098	1.033	1.652	1.055	1.691	1.501	0.931	-0.984	1.875	1.595	-0.229	-2.308
Ara^-2	1.415	-0.028	1.291	-0.193	1.517	1.137	-2.416	-0.813	0.518	1.226	-1.637	-2.029
Ara^-3	1.430	1.327	1.691	0.405	1.674	1.391	0.925	-0.071	2.184	1.738	0.250	-0.256
Ara^-4	1.244	0.946	1.355	0.792	2.007	1.636	1.002	-0.558	0.888	4.517	0.670	-1.874
Ara^-5	1.168	1.294	1.135	1.111	1.686	1.370	-0.149	-0.908	2.182	-1.388	-0.346	-3.358
Ara^-6	1.446	1.386	0.917	0.605	1.901	1.621	-0.795	-0.464	2.174	-1.077	-0.169	-0.809
Ara^+1	1.475	0.934	1.509	-0.191	1.687	1.576	0.153	-0.342	1.903	1.586	0.526	-1.987
Ara^+2	1.229	1.319	1.445	0.953	1.636	1.744	2.030	0.106	2.271	2.875	0.394	-2.948
Ara^+3	1.431	1.675	1.101	0.650	1.501	1.697	1.047	-0.412	2.015	4.737	0.637	-2.667
Ara^+4	1.398	1.192	0.618	0.841	1.397	1.562	-0.720	-1.261	-1.953	-0.743	-1.420	-2.417
Ara^+5	1.187	1.516	1.554	-0.427	1.667	1.385	-0.378	-0.338	1.884	1.771	0.386	-2.454
Ara^+6	1.292	1.391	-0.511	-0.466	1.049	1.429	0.330	-0.405	-0.691	5.464	0.940	-4.030
Mean	1.318	1.166	1.146	0.428	1.618	1.504	0.163	-0.537	1.271	1.858	0.000	-2.262
SE	0.037	0.126	0.176	0.170	0.070	0.049	0.336	0.113	0.391	0.651	0.235	0.295
t_s[2]	8.508	1.315	0.832	-3.363	8.820	10.240	-2.489	-13.606	0.693	1.319	-4.258	-11.073

[1] Derived genotypes isolated from twelve initially isogenic populations after 2,000 generations in glucose-limited minimal medium.

[2] Test statistic to be compared to the *t*-distribution with n - 1 = 11 degrees of freedom; the null hypothesis is that the Selection Rate Constant is zero.

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