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LONG-TERM EXPERIMENTAL EVOLUTION IN *ESCHERICHIA COLI*. III. VARIATION AMONG REPLICATE POPULATIONS IN CORRELATED RESPONSES TO NOVEL ENVIRONMENTS

MICHAEL TRAVISANO[1], FARIDA VASI, AND RICHARD E. LENSKI
Center for Microbial Ecology, Michigan State University, East Lansing, Michigan 48824

Abstract.—Twelve populations of *Escherichia coli* were founded from a single clone and propagated for 2000 generations in identical glucose-limited environments. During this time, the mean fitnesses of the evolving populations relative to their common ancestor improved greatly, but their fitnesses relative to one another diverged only slightly. Although the populations showed similar fitness increases, they may have done so by different underlying adaptations, or they may have diverged in other respects by random genetic drift. Therefore, we examined the relative fitnesses of independently derived genotypes in two other sugars, maltose and lactose, to determine whether they were homogeneous or heterogeneous in these environments. The genetic variation among the derived lines in fitness on maltose and lactose was more than 100-times greater than their variation in fitness on glucose. Moreover, the glucose-adapted genotypes, on average, showed significant adaptation to lactose, but not to maltose.That pathways for use of maltose and glucose are virtually identical in *E. coli,* except for their distinct mechanisms of uptake, suggests that the derived genotypes have adapted primarily by improved glucose transport. From consideration of the number of generations of divergence, the mutation rate in *E. coli,* and the proportion of its genome required for growth on maltose (but not glucose), we hypothesize that pleiotropy involving the selected alleles, rather than random genetic drift of alleles at other loci, was the major cause of the variation among the derived genotypes in fitness on these other sugars.

Key words.—Adaptation, correlated response, divergence, genetic drift, latent selection potential, pleiotropy, resource competition, selection.

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Gould and Lewontin (1979, p. 593) have remarked that "subpopulations within a species often develop different adaptations as solutions to the same problem." For example, North American and European populations of the two-spotted spider mite evolved different mechanisms of malathion resistance (Matsumura and Voss 1964). (For an extensive listing of many other such examples, see Cohan 1984a, p. 496.) In principle, these divergent adaptations may be caused by (1) subtle differences in environments, (2) differences in the initial genetic composition of populations, or (3) the stochastic nature of the processes involved in the generation and loss of genetic variants. These alternative explanations are not mutually exclusive, nor are they easily disentangled without an appropriate experimental design. In this paper, we address the third explanation: To what extent do isolated populations diverge from one another even when their environments and initial genetic composition are identical?

Selection may oppose the divergence of populations in identical environments, but its power to prevent divergence may be limited by the restricted range of genotypes that are present within any finite population (Cohan 1984a; Clarke et al. 1988; Mani and Clarke 1990). Selection may even accelerate the divergence of initially identical populations living in identical environments, if selection favors different mutant alleles in the separate populations. Moreover, the tendency for divergence is enhanced by the historical nature of evolution. Once populations have begun to differentiate, their further divergence may become more likely, because the same allele may not have the same selective value in all populations as a consequence of epistatic effects of that allele with the different genetic backgrounds (Wright 1932, 1982, 1988).

The comparative approach often is useful for identifying the adaptive significance of differences between populations and species. But this approach is less useful for evaluating the relative roles of *chance* and *necessity* (Monod 1971) in promoting divergence, because the units of comparison invariably have been subject to somewhat different environments. Hence, it is virtually impossible to exclude a purely adaptive explanation when trying to understand the contributions of random processes and historical contingencies to adaptation. Some rigorous experimental studies have been performed with *Drosophila* populations and have have sought to examine the issue of divergence of replicate populations under uniform selection (Cohan 1984b; Cohan and Graf 1985; Cohan and Hoffmann 1986; Hoffmann and Cohan 1987). Although these experiments have contributed to our understanding of the effects of chance and necessity in adaptive evolution, such studies are limited by the generation time of the study organism. Moreover, replicate populations in these experiments are founded from base populations that contain abundant genetic variation, which will be present in each of the replicate populations (except for founder effects). Consequently, adaptive evolution is likely to be parallel among the replicate populations because, over the short term, selection in identical environments will favor the same alleles. Although drift may cause some divergence, the role of random mutation in promoting divergence is likely to be overlooked. Over the short term, new mutations may not contribute very much genetic variation relative to that which is already present, but mutation may be an important stochastic process in promoting divergence over longer evolutionary time scales.

Because of their rapid generation times, bacteria such as *Escherichia coli* permit experimental studies of evolutionary

[1] Corresponding author.

processes that extend for hundreds and even thousands of generations (reviewed by Dykhuizen 1990; Lenski 1992). Moreover, given their asexual mode of reproduction, one can perform an experiment in which replicate populations are initially homogeneous and identical. Hence, selection can act only upon variation that has arisen de novo, by mutation, in the replicate populations. Large population sizes ensure that mutations occur frequently, so that the absence of genetic variation at the outset of an experiment does not preclude rapid and extensive adaptation. Moreover, populations of *E. coli* are easily propagated under tightly controlled environmental conditions; they can be stored at −80°C, so that ancestral and derived populations can be simultaneously evaluated and even directly competed against one another; and there is a wealth of information about their physiology, biochemistry, and genetics on which to draw when trying to understand the mechanistic bases of their adaptation.

Lenski et al. (1991) serially propagated 12 replicate populations of *E. coli* in a glucose-limited medium. (By glucose-limited, we mean that the stationary-phase population density can be increased by raising the concentration of glucose in the medium. However, the per capita growth rate cannot be increased appreciably by raising the glucose concentration, because growth rate levels off at a low concentration.) All of the populations were founded from a single ancestral genotype, and they were therefore initially homogeneous and identical to one another (except for a neutral marker, which was used to distinguish competing genotypes in assays of relative fitness). After 2000 generations, all of the replicate populations had improved in fitness in this environment (relative to their common ancestor) by 35%, on average; however, they differed from one another by only a few percent, indicating a high degree of parallelism. The replicate populations were also very similar in life-history traits and demographic parameters that confer the higher fitness on the derived genotypes (Vasi et al. 1994). However, Lenski et al. (1991, p. 1337) pointed out that "It is possible that the populations have evolved to similar mean fitnesses, but by different physiological adaptations," and they suggested that "it may be possible to distinguish the populations from each other on the basis of differences under other environmental conditions."

We seek to address these possibilities in this paper by comparing the performance of genotypes from each of the replicate populations in environments that contain limiting nutrients other than glucose. In particular, we competed the derived genotypes against their common ancestor, and against one another, in medium supplemented with either maltose or lactose instead of glucose. From these data, we estimated the genetic variation among independently derived lines in fitness on these sugars. If the similar fitnesses of the derived lines in glucose masked underlying heterogeneity in the physiological bases of their adaptation, then we might expect to see more genetic variance for fitness in maltose or lactose.

Maltose provides an especially interesting contrast with glucose, insofar as maltose is a disaccharide of glucose. Although degradation of glucose and maltose for energy requires the same enzymes, glucose and maltose are taken up from the environment by two distinct mechanisms that are mediated by two different sets of gene products (see Schwartz 1987), as illustrated in figure 1. Hence, we expect relative fitnesses to be similar in maltose and glucose if the physiological bases of the adaptations responsible for improved fitness on glucose involve metabolic activities downstream of glucose transport. But if the physiological basis of adaptation to the glucose-limited environment is improved transport of glucose, then such adaptations are not expected to be beneficial in an otherwise identical environment that contained maltose instead of glucose. Lactose is a disaccharide of glucose and galactose, and it shares with glucose one of the steps involved in transport.

We also performed an experiment to examine the concordance of fitnesses inferred by competing derived genotypes against one another and against their common ancestor. If frequency-dependent competition mediated by secondary metabolites was important, then we would expect relative fitnesses to be nonadditive and perhaps even nontransitive (Paquin and Adams 1983; Helling et al. 1987).

Materials and Methods

Bacterial Strains and Culture Conditions

The bacteria used in this study have been described previously (Lenski et al. 1991). Briefly, all genotypes were derived from a strain of *E. coli* B that is strictly asexual. This strain is unable to use L-arabinose as a nutrient (i.e., it is Ara^-), but an arabinose-utilizing mutant (Ara^+) was obtained (Lenski 1988). The Ara^+ mutant forms white colonies, whereas Ara^- colonies are red, on tetrazolium arabinose (TA) indicator agar (Levin et al. 1977; Lenski 1988).

Twelve populations (six each of Ara^- and Ara^+) were founded from separate colonies and, hence, from single cells. These populations were serially propagated in Davis minimal (DM) medium supplemented with 25 μg of glucose per ml (~0.14 mM), which supported $\sim 5 \times 10^7$ cells per ml (Carlton and Brown 1981; Lenski 1988; Lenski et al. 1991). Culture flasks were held in a shaking incubator at 120 rpm and 37°C. Each day, 0.1 ml of the previous day's culture was transferred into 9.9 ml of fresh media; this 100-fold dilution and subsequent regrowth permitted ~6.6 ($\log_2 100$) generations of binary fission each day. After 300 d (~2000 generations), clones derived from single cells were randomly picked from each population and stored at −80°C. All experiments reported in this paper were performed using these derived clones and their Ara^- and Ara^+ ancestors.

Measurements of Selection-Rate Constants in Competition Experiments

Competition experiments were performed under the same culture conditions used to obtain the derived genotypes, except that maltose or lactose was sometimes substituted for glucose (as indicated). The same concentration (25 μg/ml) was used for all three sugars, and the resulting yields (cells per ml at stationary phase) were comparable. Competition experiments were run in a pairwise fashion, always using reciprocally marked (Ara^- and Ara^+) genotypes. Competing genotypes may be both ancestral, both derived, or one of

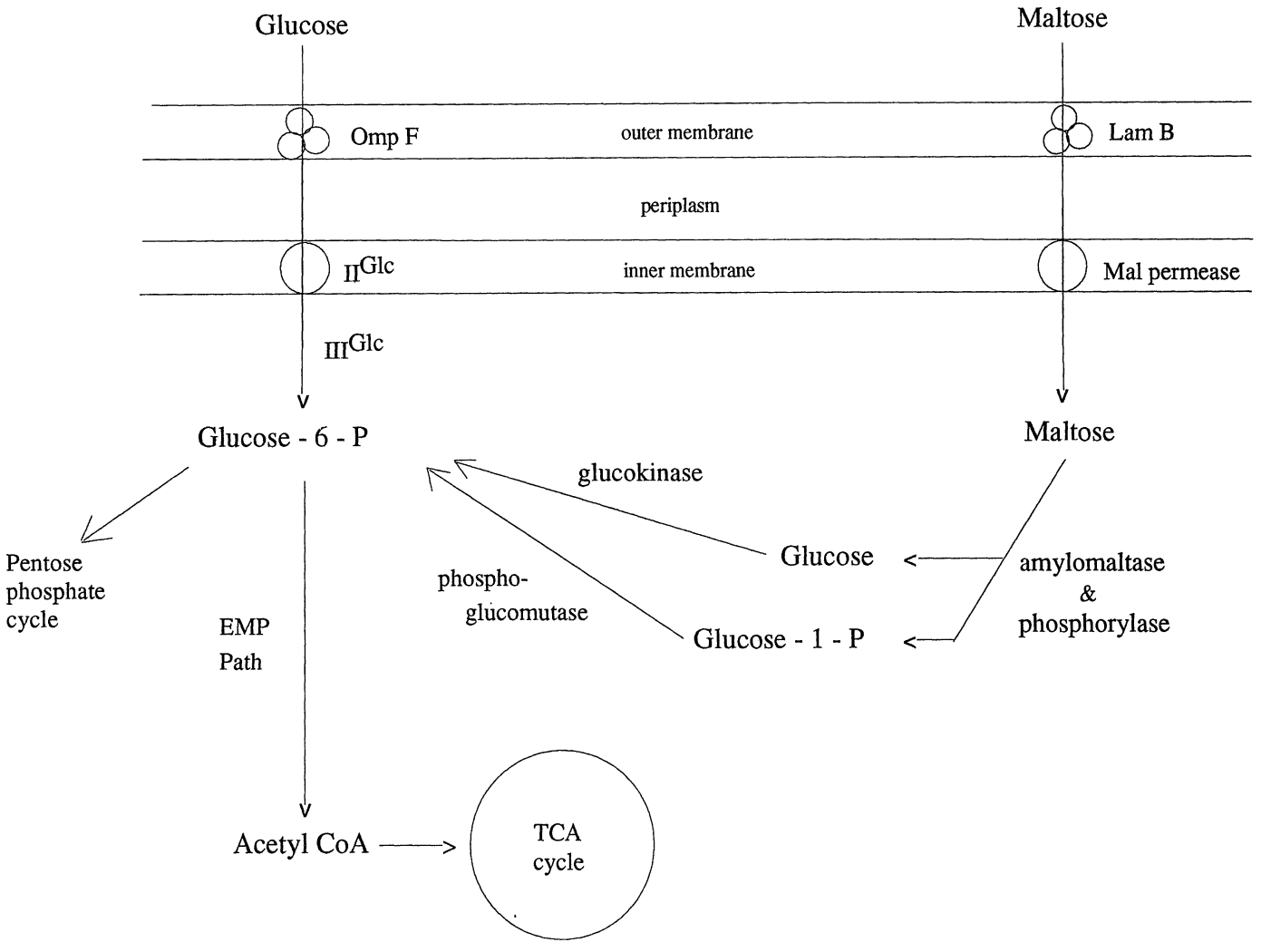

FIG. 1. Glucose and maltose differ in the gene products involved in their transport into the *Escherichia coli* cell, but the gene products used in their subsequent degradation for energy are almost identical. Maltose also diffuses through OmpF, but at low concentrations it does so at a lower rate than through LamB (Szmelcman and Hofnung 1975).

each (as indicated), depending on the purpose of the experiment.

The protocol for the competition experiments was always as follows. After removing aliquots of the appropriate genotypes from the freezer, both competitors were separately conditioned for 1 d (one complete cycle of dilution and regrowth) in the same type of medium (containing the same sugar) in which they would subsequently compete. Each competitor was then diluted 200-fold into a competition flask 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 flask was incubated under standard conditions for one day. Initial and final population densities of each competitor were determined by plating samples (containing ~500 cells) from the competition flask onto TA indicator agar, which allowed Ara$^-$ and Ara$^+$ competitors to be distinguished by their colony color.

We use selection-rate constants, r_{ij}, to quantify the relative performance of competing genotypes:

$$r_{ij} = \frac{\ln [N_i(1)/N_i(0)] - \ln [N_j(1)/N_j(0)]}{1 \text{ d}},$$

where $N_i(0)$ and $N_j(0)$ are the initial densities of the two competitors and $N_i(1)$ and $N_j(1)$ are their corresponding densities after 1 d. The selection-rate constant is thus equal to the difference in the two competitors' Malthusian parameters (Nagylaki 1977; Lenski et al. 1991). This difference may, in principle, reflect differences in reproduction or survival during any phase of population growth (lag, growth, and stationary) in the serial transfer regime. Any difference between the two competitors in plating efficiency will not affect estimates of r_{ij}, provided that the plating efficiency of each competitor is the same in the initial and final samples (which is expected because they are in stationary phase at both times).

To compare the performance of competing genotypes, Lenski et al. (1991) used the *ratio* of Malthusian parameters (= relative fitness, W_{ij}), instead of the *difference* in Malthusian

parameters (= selection-rate constant, r_{ij}) that we use here. For consistency, we would have preferred to use relative fitness in this study as well. However, because relative fitness is a ratio of two quantities, it is more sensitive to pure sampling error when one competitor's Malthusian parameter is very small, as was sometimes the case for one of the sugars (lactose) used in this study. Thus, the selection-rate constant provided a more suitable measure of relative performance for this study. A selection-rate constant can be converted into a corresponding relative fitness, W_{ij}, as follows:

$$W_{ij} \cong 1 + (r_{ij}/\bar{m}),$$

where $\bar{m}$ is the average Malthusian parameter (here, $\bar{m} = \ln 100 = 4.6$ per d). This conversion is only approximate, however, owing to the difference between the average Malthusian parameter (which is the same in all experiments because of the consistent 100-fold dilution and subsequent regrowth) and the actual Malthusian parameter for the genotype that appears in the denominator in calculating relative fitness (which varies depending on genotype and medium).

Experimental Designs and Statistical Analyses

We never assign statistical significance to any particular estimate of the selection-rate constant, regardless of the magnitude of the difference in the competitors' apparent Malthusian parameters. Instead, we base all of our statistical inferences on multiple measures of the selection-rate constant, with the degrees of freedom reflecting either the number of independently replicated competition experiments or the number of independently derived genotypes (as appropriate to the particular hypothesis).

To test the selective neutrality of the arabinose marker, replicate competition experiments were performed using the reciprocally marked ancestors in each medium. We also tested for possible interactions between the arabinose marker and the derived versus ancestral genetic backgrounds by comparing the average fitnesses of the two classes (Ara^- and Ara^+) of derived genotypes in each medium.

Five, six, and four estimates of the selection-rate constant for each of the 12 derived genotypes (relative to the reciprocally marked common ancestor) were obtained in media containing glucose, maltose, and lactose, respectively. Replicate assays were run in sets of complete blocks. Inferences about the grand mean selection-rate constant for a given sugar were based on the number of derived genotypes ($n = 12$), as were paired comparisons of relative performance across two sugars. The genetic variance in fitness (on a particular sugar) among independently derived genotypes was tested by a two-way ANOVA, in which genotype and block 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. Confidence intervals for V_G were computed using methods given by Sokal and Rohlf (1981, pp. 217–218). Although the 12 independently derived genotypes are regarded as random rather than fixed entities for most purposes, we performed a Bonferroni-corrected T test for multiple comparisons (Miller 1981; SAS Institute 1988, pp. 593–595) to ask how many genotypic classes could be distinguished on the basis of fitness in the experimental sugars.

To test for possible nonadditivity in relative fitness of genotypes, we also competed each of the six Ara^- derived genotypes against each of the six Ara^+ derived genotypes, with twofold replication, in medium containing lactose. Lactose was chosen because genotypes exhibited the greatest genetic variation in fitness on this sugar (see Results section), thus providing the largest signal for possible deviations from additivity. These data were analyzed by a two-way analysis of variance, with the interaction term providing a test for the significance of nonadditivity of fitness effects.

TABLE 1. Effective neutrality of the arabinose-utilization marker in the ancestral genotype in medium containing the three different sugars used in this study.

Sugar	Selection-rate constant (d^{-1}) (Ara^+ relative to Ara^-)	
	Mean (± SE)*	P†
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

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

† 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^- strains.

RESULTS

Neutrality of the Arabinose-Utilization Marker in Glucose, Maltose, and Lactose

The Ara^+ and Ara^- variants of the ancestral genotype were competed against one another in DM medium supplemented with the three different sugars used in this study. The arabinose-utilization marker is effectively neutral in all three sugars (table 1). 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 lines (as a group) are no more or less fit then the six Ara^- derived lines (as a group) in any of these sugars (table 2). These results, coupled with the fact that all experiments were fully balanced with respect to marker, lead us to pool the data for Ara^+ and Ara^- lines in our subsequent analyses.

Parallel Fitness Improvements in Glucose among Replicate Lines

In glucose, the grand mean selection-rate constant for the 12 independently derived genotypes, relative to their common ancestor, is 1.318 per day (table 3, first column). The corresponding 95% confidence interval (based on the t-distribution with 11 df) ranges from 1.236 to 1.400 per day. An ANOVA (based on the repeated measures of the selection-rate constant for each of the derived lines) shows no significant heterogeneity among the 12 genotypes in their selection-rate constants (table 4). The estimated genetic variance component, V_G, is 0.0050; the upper limit of the 95% confidence interval for that variance is 0.0366. The correspond-

TABLE 2. Effective neutrality of the arabinose-utilization marker in the derived genotypes in medium containing the three different sugars used in this study.

Sugar	Mean selection-rate constant (d^{-1}): Ara$^+$ derived relative to Ara$^-$ ancestor	Mean selection-rate constant (d^{-1}): Ara$^-$ derived relative to Ara$^+$ ancestor	Difference (± SED)*	P†
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

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

† 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 genotypes equals 0 (indicating equal changes on average in the fitness for the Ara$^+$ and Ara$^-$ derived genotypes).

ing standard deviations ($[V_G]^{1/2}$) are 0.071 and 0.191 per day, 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 lines, consistent with the results reported by Lenski et al. (1991).

Genetic Adaptation to Glucose Does Not Increase Average Fitness in Maltose

We also measured the selection-rate constants for the 12 derived lines in DM medium containing maltose instead of glucose. Although these lines showed substantial 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, all 12 derived lines are more fit, relative to the common ancestor, in medium containing glucose than in medium containing maltose.

Derived Genotypes Vary in Their Fitnesses in Maltose

The derived lines, as a group, do not show any significant change in fitness relative to the ancestor in medium containing maltose (table 3). However, an ANOVA (based on the repeated measures of the selection-rate constant in maltose for each line) indicates highly significant variation in fitness among the lines for 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 per day, respectively. The genetic variation for fitness in maltose is significantly greater than the corresponding variation in glucose; in fact, the 95% confidence limits for V_G are completely nonoverlapping. Among the 12 independently derived lines, a Bonferroni-corrected multiple-range test indicates at least three distinct classes in terms of fitness in maltose.

TABLE 3. Differential adaptation to glucose and maltose. The bacteria evolved for 2000 generations in medium that contained glucose as the sole carbon source. Glucose and maltose enter the cell by very different mechanisms, but maltose is a glucose disacharride and the subsequent breakdown of these two sugars for energy is virtually identical (see fig. 1).

Derived genotype	Selection rate constant (d^{-1}) relative to the common ancestor in: 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†	< 0.001	0.998	< 0.001

* The selection rate constant for each genotype is based on five or six assays for glucose or maltose, respectively.

† Two-tailed probability computed from the t-distribution with $N - 1 = 11$ degrees of freedom (where $N = 12$ is the number of independently derived genotypes); the null hypothesis is that the mean selection rate constant (or difference between two means) is 0, indicating equal fitness for the average derived genotype and the common ancestor (or equal relative fitness for the two sugars).

Thus, the relationship between mean change in fitness and divergence in fitness among the independently derived lines 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 lines 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 lines.

Additional Variability Is Observed in Lactose

Based on fitness in medium containing glucose, the 12 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 mea-

TABLE 4. Analysis of variance for the selection-rate constants obtained for the 12 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		

TABLE 5. Analysis of variance for the selection-rate constants obtained for the 12 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		

sured in medium containing yet another sugar. To that end, we estimated the selection-rate constants for each of the 12 derived lines (relative to the common ancestor) in lactose. The grand mean selection-rate constant is 1.858 per day; the corresponding 95% confidence interval (based on the t-distribution with 11 df) ranges from 0.426 to 3.290 per day, indicating that the derived lines, as a group, show significant improvement relative to their ancestor on lactose.

An ANOVA (based on repeated measures of the selection-rate constant) also indicates highly significant variation among the lines 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 per day, respectively. The genetic variance for fitness on lactose is significantly greater than that on glucose (and even greater than that on maltose). Thus, during the 2000 generations of experimental evolution, there arose significantly greater variation among the independent lines in their fitnesses in both novel environments (maltose and lactose) than in the environment in which the bacteria were selected (glucose) (fig. 2).

Among the 12 independently derived lines, a Bonferroni-corrected multiple-range test indicates at least two distinct classes in terms of fitness in lactose. Although the correlation between fitness on lactose and fitness on maltose is significant ($r = 0.673$, df = 10, $P = 0.015$), 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 experimentwise type I error rate of 0.05. When we introduce a further Bonferroni correction because we performed two separate multiple-range tests (thus using an experimentwise 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 12 genotypes selected for performance on glucose can be divided into at least four distinct groups based on their fitnesses in maltose and lactose.

TABLE 6. Analysis of variance for the selection-rate constants obtained for the 12 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		

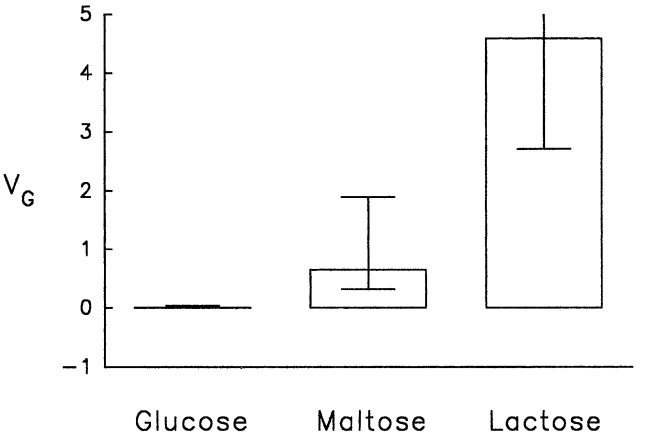

FIG. 2. Estimated genetic variances, V_G, among derived genotypes in their selection-rate constants on three sugars. Error bars indicate 95% confidence intervals around V_G. The derived genotypes were isolated from populations that had independently evolved for 2000 generations in medium containing glucose. The genetic variation in fitness on two other sugars, maltose and lactose, was much greater than the variation in fitness on glucose.

Relative Fitnesses Are Ecologically Additive and Transitive

In all of the preceding experiments, we measured the fitness of the derived genotypes relative to their common ancestor. We assumed that any difference in fitness resulted from the ability of genotypes to use the sugars (glucose, maltose, and lactose) that we added to the medium, rather than to use (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 nontransitive interactions and other forms of frequency-dependent selection. Therefore, we sought to examine the possible importance of such complex interactions by competing derived genotypes against one another, as well as against their common ancestor. We ran these additional competition experiments in lactose because the genetic variation in fitness was greatest, therefore providing the largest signal for possible deviations from additivity. In these experiments, we competed all 36 (6×6) pairs of derived genotypes that differed in their arabinose-utilization marker states.

We observed significant heterogeneity among the six Ara^+ derived genotypes and among the six Ara^- derived genotypes; but there was no indication whatsoever of any statistical interaction (nonadditivity) in the selection-rate constants (table 7). Moreover, the fitnesses of the derived genotypes relative

TABLE 7. Analysis of variance for the selection-rate constants 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		

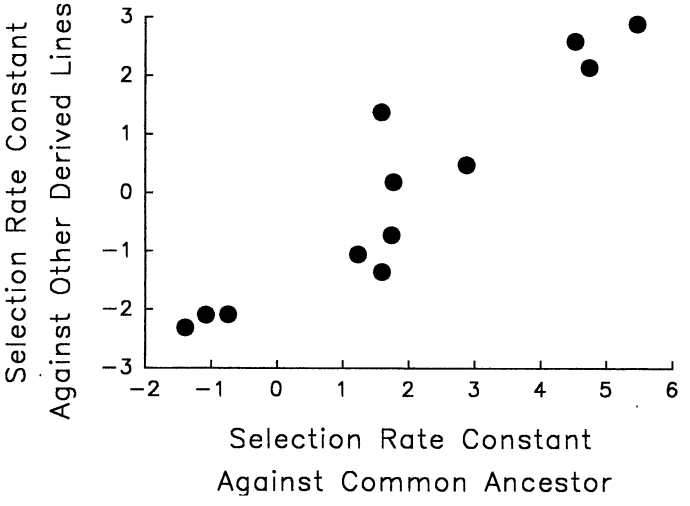

FIG. 3. Correlation between selection-rate constants (d^{-1}) obtained relative to the ancestor and relative to other derived genotypes, in lactose. Each of the twelve independently derived genotypes was competed against the reciprocally-marked common ancestor (with fourfold replication) and against each of the six reciprocally marked derived genotypes (with twofold replication). $r = 0.933$, df = 10, $P < 0.001$.

to their common ancestor and relative to one another were highly correlated ($r = 0.933$, df = 10, $P < 0.001$; fig. 3). 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 with one another. Thus, we find no evidence for ecological nonadditivity or nontransitivity in the interactions of the genotypes, and hence no support for the supposition that competition other than for the resources that we deliberately added to the medium was important for the observed fitnesses.

DISCUSSION

Populations of *Escherichia coli* that were propagated for 2000 generations in a glucose-limited environment greatly improved their fitness in this environment relative to their ancestor, but replicate populations diverged very little from one another in mean fitness. Lenski et al. (1991) suggested that the replicate populations might have achieved the same phenotypic solutions to the challenges imposed by the experimental environment; however, by using data only on relative fitnesses, they could not exclude the alternative hypothesis that the replicate populations had achieved similar fitness by quite distinct phenotypes. Vasi et al. (1994) further showed that the replicate populations had undergone similar changes in demographic parameters and other life-history traits (measured in the glucose-limited environment). Relative to their common ancestor, the derived genotypes had higher maximal growth rates, shorter lag phases after transfer to fresh medium, larger individual cells, and fewer cells at stationary phase.

In contrast to their high degree of similarity in the glucose-limited environment, we have demonstrated here that the independently derived genotypes diverged considerably from one another in their competitive fitness in novel environments. The genetic variance for fitness on two sugars (maltose and lactose) that the bacteria had not encountered for 2000 generations increased by more than 100-fold, relative to the increase in genetic variance for fitness on the sugar (glucose) that was provided during that period (fig. 2). Evidently, the similar fitness of the replicate lines in the glucose-limited environment masks much greater heterogeneity in their adaptation to other environments.

Drift versus Pleiotropy

In principle, the much greater fitness variation observed under conditions different from those in which the bacteria had been evolving for 2000 generations could result from either of two distinct population genetic processes. (1) The heterogeneity in fitness on maltose and lactose might be due to mutation and random drift of alleles at loci that affect performance on these two sugars, but which are neutral in the glucose-limited environment. (2) Mutations that have similar beneficial effects on fitness in the glucose-limited environment might be heterogeneous in their pleiotropic effects on fitness in environments that contain maltose or lactose instead of glucose.

We will present circumstantial evidence that heterogeneous pleiotropic effects were more important than drift in promoting the divergence in fitness on maltose and lactose. This evidence concerns metabolic couplings in utilization of these sugars by *E. coli* as well as the dynamics of genetic drift.

All 12 independently derived genotypes were less fit in maltose than in glucose (table 3). If this differences between maltose and glucose was caused by random genetic drift, then each of the derived genotypes must have suffered one or more substitutions in a gene that affects fitness in maltose but not glucose. Only about 12 genes in *E. coli* are involved in maltose metabolism that are not also involved in glucose metabolism (including mostly transport and associated regulatory genes). These maltose-specific genes are *lamB, malE, malF, malG, malI, malK, malM, malP, malQ, malT, malX,* and *malY* (Ferenci et al. 1980; Clement and Hofnung 1981; Gilson et al. 1982, 1986; Duplay et al. 1984; Froshauer and Beckwith 1984; Dassa and Hofnung 1985; Cole and Raibaud 1986; Palm et al. 1987; Schwartz 1987; Reidl et al. 1989; Reidl and Boos 1991). Otherwise, we would expect any mutation that reduced fitness in maltose to have been detrimental in glucose as well, and hence to have been selected against during the evolution experiment. These maltose-specific genes together comprise about 20,000 nucleotide base pairs, or ~0.5% of the *E. coli* genome (Riley and Krawiec 1987). If mutations in the maltose-specific genes were responsible for the derived genotypes' uniformly poorer performance in maltose than in glucose, then this implies a minimum rate of substitution by random genetic drift of one substitution per 20,000 base pairs per 2000 generations, or $\sim 2.5 \times 10^{-8}$ substitutions per base pair per generation. (The inferred rate would be higher if we accounted for multiple hits and silent substitutions in the maltose-specific genes.) The expected rate of substitution for neutral alleles in a lineage is equal to the rate of neutral mutations (Kimura 1983). This expectation is independent of effective population size, and it is not influ-

enced by selection at other loci. (Especially in asexual organisms [Levin 1981], selection affects the dynamics of the substitution process for any given neutral allele, which may either be purged or hitchhike to fixation. However, selection does not affect the expected overall rate of neutral substitutions.) Therefore, the calculated rate of substitution implies a corresponding minimum estimate for the mutation rate of $\sim 2.5 \times 10^{-8}$ per base pair per generation, which is ~50-fold higher than the generally accepted base-pair mutation rate in *E. coli* (Drake 1991). Thus, it seems unlikely that random genetic drift can account for the differential adaptation of the derived genotypes to glucose and maltose.

Maltose and glucose differ in their mode of transport through the *E. coli* cell envelope, but they are virtually identical in their subsequent catabolism, maltose being a glucose disaccharide (fig. 1). Therefore, the higher fitness of the derived genotypes in glucose than in maltose probably indicates that adaptation to the glucose-limited environment was achieved primarily by improvements in glucose transport. The fact that the derived genotypes were, on average, more fit in lactose than in maltose is also consistent with this argument: glucose and lactose (but not maltose) have OmpF as their primary route for diffusion across the outer membrane. The transport of sugars and other nutrients into the *E. coli* cell is governed by complex regulatory feedbacks; for example, the use of other sugars (including both maltose and lactose) is repressed when glucose is available. Given these complex regulatory mechanisms, as well as potential interactions between the actual membrane proteins involved in transport, one might expect that many mutations that increase glucose transport would have pleiotropic effects on maltose and lactose transport and, moreover, that these effects would be heterogeneous depending on their precise molecular basis. Thus, subtle differences in the genetic basis for the same phenotype (improved glucose transport) may cause profound differences in performance in other sugars.

Adaptations in Transport versus Downstream Metabolism

Glucose is the preferred resource for *E. coli,* such that the use of other available substrates is repressed when glucose is abundant. It is perhaps surprising, therefore, that improvements in its transport could have been achieved. However, Dykhuizen et al. (1987) have shown that lactose transport by *E. coli* is also submaximal; mutants can be isolated that have higher transport activity and thereby higher fitness in a lactose-limited environment. The cell envelope must protect *E. coli* from a wide variety of environmental agents (including viruses, toxic compounds, etc.), while also allowing uptake of a wide range of nutrients depending on availability. Dykhuizen and Dean (1990) hypothesized that these functional constraints affecting the cell envelope could prevent *E. coli* from achieving in nature the same level of transport that would be optimal in a constant laboratory environment, where these constraints are relaxed. (By contrast, catabolic enzymes active inside the cell perform under relatively more homogeneous conditions and might therefore be subject to fewer constraints.) Our results support this interpretation.

Mikkola and Kurland (1992) recently studied adaptation to the laboratory environment of *E. coli* strains isolated from nature. In contrast to our results, they found that changes in ribosomal properties accounted for the improved performance of their derived lines. If that had been the case in our experiments, we would expect the derived genotypes to have high and uniform fitnesses in maltose as well as glucose. (Ribosomal adaptations could exhibit specificity depending on growth rate; however, growth rates of our ancestral *E. coli* strain on maltose and glucose were similar [M. Travisano and F. Vasi unpubl. data].) We would also expect the derived genotypes of Mikkola and Kurland (1992) to be more fit than their wild-caught ancestors regardless of the growth substrate (assuming similar growth rates), given that the protein synthetic machinery was the primary basis of adaptation in their strains.

This difference in the apparent targets of selection between these studies could be the result of differences in the starting genotypes, the environments imposed, or some combination thereof. For example, Mikkola and Kurland (1992) hypothesized that *E. coli* strains taken from nature are adapted to environments that permit only slow growth; therefore, a shift to laboratory conditions that allow rapid growth favors increased translational efficiency, which can be achieved by an altered ribosomal phenotype. However, our ancestral strain had already been in the laboratory for several decades; although there was demonstrable room for improvement of this strain in the particular glucose-limited medium that we supplied, the ancestor was presumably already well adapted to conditions that allow rapid growth.

Genetic Architecture of Fitness

Evolutionary biologists often base their understanding of adaptive evolution on the selective value of a particular phenotype and implicitly ignore the underlying genetic details of that phenotype. The results of our study, as well as several others (Hall 1983; Lenski 1988; Bull and Molineux 1992), indicate that independently evolving populations may acquire similar adaptations that differ in their underlying genetic details. In this study, all 12 lines adapted to the glucose medium to a similar extent (Lenski et al. 1991; see also table 4 in this paper), and they underwent similar changes in demographic parameters and other life-history traits (Vasi et al. 1994). Moreover, all 12 lines were more fit in glucose than in maltose, suggesting that they all improved primarily in terms of glucose transport. Nonetheless, the replicate lines differ dramatically in their fitnesses on maltose and lactose, indicating an underlying heterogeneity in the genetic bases of their adaptation. Thus, we agree with Bull and Molineux (1992, p. 892) 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."

Given this multiplicity of genetic states and the resulting heterogeneity in correlated responses, one might reasonably ask, How does this affect our understanding of adaptation by natural selection? We believe that these findings bear on at least four important issues related to adaptive evolution. First,

such findings clearly show that one must be cautious in assigning adaptive explanations to differences between populations and species (Gould and Lewontin 1979). Even striking differences in phenotypes and measures of performance (e.g., fitness in maltose) may not be adaptive per se but instead may be caused by heterogeneous pleiotropic effects of alleles that have similar beneficial effects (e.g., improved glucose transport). Hence, it must be established that the phenotypic properties and performance measures are relevant to reproductive success in the appropriate environment.

Second, the multiplicity of genetic states producing similar phenotypes provides a source of variation that is available to selection in changing environments. This variation has been described as a latent selection potential (Dykhuizen and Hartl 1980, 1983; Hartl and Dykhuizen 1981, 1985; Hartl et al. 1985; Stebbins and Hartl 1988; Kimura 1991; Silva and Dykhuizen 1993), and it is discussed further below. Previous discussions of latent selection potentials have focused primarily on the role of genetic drift of *neutral* alleles in generating this variation (Hartl et al. 1985; Kimura 1991). The results of our experimental studies indicate that heterogeneity in pleiotropic effects of similar *beneficial* alleles may also contribute significantly to latent selection potentials.

Third, the divergent genetic bases of adaptation to identical environments may be quite important for subsequent evolution even without environmental change. With respect to Wright's fitness surface (1932, 1982, 1988), genetic differences among populations that arise by chance during selection in identical environments may cause the populations to enter the domains of distinct evolutionary attractors (see also Mani and Clarke 1990). Thus, adaptations that are initially equivalent to one another may engender different constraints on subsequent evolution because of genetic interactions.

Finally, these findings suggest that many different mutations have functionally equivalent effects on some primary phenotype, which may be subject to selection. Such mutations might be expected to interact epistatically, but in a subadditive manner. That is, a mutation at one locus may confer a 10% advantage by increasing a certain rate process, as may another mutation at a different locus; but the two mutations together would provide an advantage of $< 20\%$ because of the effect of diminishing returns. (See Hartl et al. [1985] and Dykhuizen and Dean [1990] for relevant discussions of the relationship between metabolic flux and fitness. See Lenski et al. [1991] for evidence that the rate of adaptive evolution in these bacterial populations slowed down in manner consistent with this view.)

Latent Selection Potentials in Novel Environments

According to Fisher's fundamental theorem, the rate of change in a population's mean fitness in a constant environment is proportional to its (additive) genetic variation for fitness (Fisher 1930; Nagylaki 1977; Ewens 1989; Frank and Slatkin 1992). (In the case of asexual organisms, such as the bacteria used in this study, it is not necessary to specify "additive" because all genetic variation is able to respond to selection [Nagylaki 1977; Ewens 1989; Lenski et al. 1991].) The 12 independently derived lines exhibit only very slight genetic variation in fitness on glucose. Therefore, a population constructed from an admixture of the derived lines, and propagated in the glucose-limited environment, would change rather slowly in its genetic composition and hence its mean fitness. However, if a comparable admixture were propagated instead in medium containing either maltose or lactose, then the initial rate of change in mean fitness in that environment would be $\sim$100 or $\sim$1000 times greater, respectively, then in the glucose-limited environment, because of the different genetic variances for fitness in each of these environments. Thus, a latent selection potential may provide the "raw material for adaptive evolution" (Kimura 1983, p. 270), and evolutionary advances may often occur by "capitalizing on preexisting latent selection potentials in the presence of novel ecological opportunity" (Stebbins and Hartl 1988, p. 5141).

In most of the experiments reported here and by Lenski et al. (1991), fitnesses of derived lines were measured relative to the common ancestor rather than relative to one another. Genetic admixtures of the sort described in the previous paragraph might not respond as expected if there were nontransitive interactions or other forms of frequency-dependent selection. For example, the admixture might evolve more rapidly than expected in glucose if genotypes having similar fitnesses relative to their common ancestor were dissimilar in their fitnesses relative to one another. Or the admixture might evolve more slowly than expected in maltose or lactose if genotypes having very different fitnesses relative to their ancestor were similar relative to one another. Such outcomes could occur if cross-feeding, allelopathic, or detoxification interactions are important in addition to scramble competition for the exogenously supplied resource (Levin 1972; Chao and Levin 1981; Fredrickson and Stephanopoulos 1981; Paquin and Adams 1983; Lenski and Hattingh 1986; Helling et al. 1987; Levin 1988). For example, *E. coli* excretes acetate during rapid growth on glucose; after the glucose is exhausted, additional growth can be achieved on the excreted acetate (Holms and Bennett 1971; Bennett and Holms 1975). Helling et al. (1987) showed that ecologically differentiated genotypes evolved and coexisted during long-term propagation of *E. coli* in a chemostat; stable coexistence occurred because one genotype grew better on acetate excreted by another genotype, which grew better on glucose (Rosenzweig et al. 1994). Other complex interactions may even result in nontransitive relative fitnesses, such that genotype *A* is competitively superior to *B,* and *B* is superior to *C,* but *A* is inferior to *C* (Paquin and Adams 1983). However, such complications do not appear to be important in the present study, based on supplementary experiments designed to test these possibilities. Lenski et al. (1991) showed that the among-population genetic variance for mean fitness in the glucose-limited environment was small whether the derived lines were competed against their common ancestor or against one another. And in this study, we found no evidence for a significant interaction component for fitness measured in lactose (table 7, fig. 3); genotypes that performed well relative to one competitor (derived or ancestral) also performed well relative to other competitors, and to a degree consistent with ecological additivity of differences in competitive fitness.

Silva and Dykhuizen (1993) also recently demonstrated

the effect of novel environments in amplifying genetic variation for fitness, but in a rather different way from our study. They transformed the *lac* operon from wild strains of *E. coli* into a common genetic background and competed the resulting genotypes against one another in medium containing either lactose or one of several other substrates for the *lac*-encoded β-galactosidase. Lactose is relatively common in the environments that *E. coli* inhabits, but these other substrates are either absent or quite rare. Silva and Dykhuizen (1993) found that variation in fitness was greater on these rare substrates than on lactose. We believe that our results complement those of Silva and Dykhuizen (1993); their study had the virtue of relating to genetic variation present in natural populations, but consequently they could not entirely exclude the possibility that the genotypes they studied had somewhat different selection histories. By contrast, our study dealt exclusively with evolution in the laboratory, so that one can be quite confident that all of the populations grew on glucose, but were not exposed to maltose or lactose, during their 2000 generations of divergence.

Future Directions

The finding that the independently derived genotypes are all more fit in glucose than in maltose suggests that they have adapted by improving uptake of glucose from the environment. Glucose diffuses passively into the periplasmic space (between the inner and outer cell membranes) through the OmpF porin and then is actively transported across the inner membrane using the enzymes of the phosphotransferase (PTS) system; maltose uses neither OmpF as its primary porin nor enzymes of the PTS system for active transport (fig. 1). However, certain other substrates use OmpF for diffusion across the outer membrane, the PTS system for active transport across the cytoplasmic membrane, or both (Klein and Boos 1993; Kornberg 1990; Lin 1987; Nikaido and Vaara 1987; Postma 1987; Saier 1989; Saier and Reizer 1992; Saier and Stiles 1975). By examining fitnesses of the derived genotypes in competition for these other substrates, we may be able to ascertain what step in glucose transport has improved, and thereby narrow the search for candidate loci involved in the adaptive evolution.

We also can use these 12 genotypes, which have identical selection histories but which diverged as the result of chance events, to found new populations and propagate them in identical maltose-limited environments. We can then ask whether the populations converge towards similar mean fitnesses in the new environment, regardless of their prior ancestry, or whether each population's adaptive potential is constrained by its initial genetic state. With such an experiment, we may be able to rigorously quantify the contributions of chance, phylogeny and environment to evolutionary change in this system (Travisano et al. 1995).

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