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EVOLUTIONARY ADAPTATION TO TEMPERATURE. V. ADAPTIVE MECHANISMS AND CORRELATED RESPONSES IN EXPERIMENTAL LINES OF *ESCHERICHIA COLI*

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Abstract.—We previously demonstrated temperature-specific genetic adaptation in experimental lines of *Escherichia coli.* Six initially identical populations were propagated for 2000 generations under each of five regimes: constant 20°C, 32°C, 37°C, and 42°C, and a daily switch between 32°C and 42°C. Glucose was the sole carbon source in all cases. Here, we examine the physiological bases of adaptation to determine whether the same mechanisms evolved among the replicate lines within each thermal regime and across different regimes. Specifically, we investigate whether changes in glucose transport may account for the temperature-specific adaptation. We compared each line's direct response of fitness to glucose with its correlated response to maltose; glucose and maltose enter the cell by different pathways, but their catabolism is identical. Except for lines maintained at the ancestral temperature (37°C), almost all derived lines had significantly different fitnesses (relative to their common ancestor) in glucose and maltose, supporting the hypothesis that adaptation involved changes in glucose transport. An alternative explanation, that maltose transport decayed by genetic drift, appears unlikely for reasons that are discussed. Although most lines showed evidence of temperature-specific adaptation in glucose transport, several different mechanisms may underlie these improvements, as indicated by heterogeneity in correlated responses (across temperatures and substrates) among replicate lines adapted to the same regime. This heterogeneity provides a latent pool of genetic variation for responding to environmental change.

Key words.—Adaptation, bacteria, correlated responses, *Escherichia coli*, fitness, glucose, maltose, pleiotropy, preadaptation, sugar transport, temperature.

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This study is part of an ongoing project examining evolutionary adaptation of experimental lines of the bacterium *Escherichia coli* to different thermal environments (Bennett et al. 1990, 1992; Bennett and Lenski 1993; Lenski and Bennett 1993; Leroi et al. 1994; Travisano et al. 1995a; Mongold et al. 1996). An important aspect of the study of evolutionary adaptation is the elucidation of the mechanisms underlying differential performance and reproduction (e.g., Endler 1986; Grant 1986; Dykhuizen and Dean 1990). A study of the adaptive process should ideally specify not only the extent of fitness advantage attained during evolution but also the biochemical, physiological, and genetic features that mediate that improvement in fitness (Clarke 1975; Powers 1987). This present study investigates one mechanism, nutrient transport, that is potentially very important in the adaptation of bacteria (e.g., Dykhuizen et al. 1987; Dykhuizen and Dean 1990).

A simple yet potentially powerful method for examining adaptive changes in nutrient transport in bacteria has been developed by Travisano et al. (1995b). This method involves measurement of the relative fitness of derived and ancestral genotypes in nutrients that differ in the mechanisms of their transport into the bacterial cell. Of particular interest in the context of our experiments is a comparison of the fitnesses of *E. coli* strains when grown on the sugars glucose and maltose (a glucose dimer). Glucose and maltose have different mechanisms of transport through both the outer and inner cell membranes (reviewed in Nikaido and Saier 1992). Glucose diffuses across the outer membrane of *E. coli* via the major porin OmpF, whereas maltose induces synthesis of a usually repressed, larger diameter porin, LamB. Glucose is then actively transported across the inner membrane via the phosphoenolpyruvate: sugar phosphotransferase system (PTS), whereas maltose enters through a specific binding protein-dependent transport system. These systems have been well characterized both biochemically and genetically (reviewed in Saier 1985; Postma 1987; Nikaido and Saier 1992). After entry, however, both sugars are converted to glucose-6-phosphate and are thereafter catabolized identically. Glucose was the only carbon and energy source available to our experimental populations during their recent evolution at different temperatures, and hence adaptations to glucose utilization may be considered "direct" responses to selection. Maltose is one of many nutrients apparently encountered in nature by *E. coli* (judging from the presence of a specific transport system), but our lines have not encountered maltose for at least 4000 generations (and probably much longer than that) in their recent laboratory evolution. Hence, maltose can be considered a novel nutrient, and changes in fitness in maltose can be regarded as *correlated* responses to selection in our experiments.

Correlated responses to novel environments will vary according to the mechanism of adaptation to the selective environment. Adaptive mechanisms may be elucidated from these patterns of differential fitness (Travisano et al. 1995b). Specifically, adaptations related to nutrient transport can be identified by differential fitness in nutrients with different mechanisms of uptake (Fig. 1). If the mechanism involves a functional process unrelated to transport, then the extent of fitness improvement in two different nutrients should be indistinguishable, even though their mechanisms of transport may be different (Fig. 1A). For instance, if adaptation occurs through changes in ribosomal properties (e.g., Mikkola and Kurland 1992), patterns of amino-acid synthesis (e.g., Ron and Davis 1971), or stress protein activation (e.g., Neidhardt

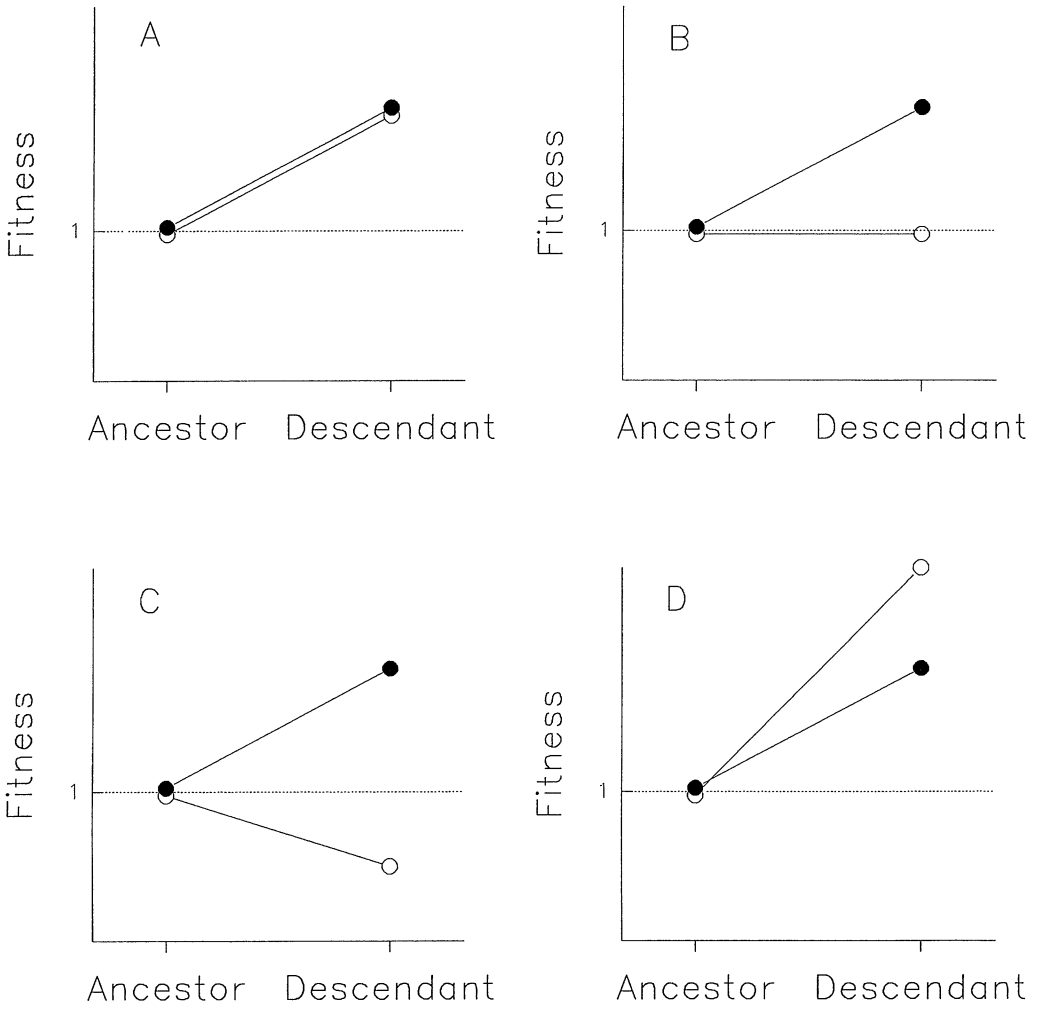

FIG. 1. Patterns of direct and correlated responses of fitness in a selective (filled circles) and a nonselective (open circles) environment. (A) Fitness gains are equal in the two environments. (B) Fitness improves in the selective environment but is unchanged in the nonselective environment. (C) Fitness improves in the selective environment but declines in the nonselective environment due to a pleiotropic trade-off. (D) The correlated fitness gain in the nonselective environment exceeds the direct response in the selective environment. In this study, the selective environment is medium containing glucose, whereas the nonselective environment contains maltose. Pattern A is consistent with adaptations that are unrelated to mechanisms of glucose transport. Patterns B–D indicate that adaptations to the selective environment are probably associated with enhanced glucose transport (for explanation, see text and Travisano et al. 1995b).

and VanBogelen 1987), then these adaptations should result in comparable fitness improvements in different nutrients. However, if the mechanism of adaptation specifically involves the enhancement of transport of the selective nutrient (glucose, in our experiments), then one would expect that relative fitness measured in that nutrient should differ from that in other nutrients with different transport mechanisms (e.g., maltose). Several different patterns of fitness might result, depending on the specific adaptive mechanism. Fitness in the alternative nutrient might be unaltered from ancestral levels (Fig. 1B); it might decrease if the mechanism of adaptation involves antagonistic pleiotropy, that is, a trade-off (Fig. 1C); or it might even surpass that in the selective nutrient (Fig. 1D). By measuring relative fitness in glucose and maltose, we can thereby assay experimental lines selected at different temperatures for evidence of adaptations involving glucose transport. (Note that measurements of relative fitness compare performance of ancestral and derived genotypes in each nutrient, not the relative performance between nutrients. Similar relative fitnesses between nutrients do not therefore imply that rates of transport, growth, and other dynamic variables are equal in glucose and maltose but only that the "relative" change from the ancestral condition has been similar in each nutrient.)

The foregoing method for elucidating adaptive mechanisms depends critically on the absence of drift effects on correlated responses. As performance on maltose was not of selective importance in the experimental evolutionary environment, it is possible that divergence from the ancestral state might have been due to the accumulation of neutral mutations (i.e., mutations that affect fitness in maltose but not glucose). Such divergence would undermine our ability to associate specific mechanisms with patterns of fitness change. However, we do not believe that drift played a significant role in diversification of fitness in maltose in these experiments for three reasons, which are described more fully in the Discus-

sion. First, some of the derived lines are actually more fit (relative to their ancestor) in maltose than in glucose; drift, being a decay process, is unlikely to produce such a result. Second, the control (37°C) group, which exhibited the least extensive adaptation to glucose, showed the least divergence among the replicate lines in their correlated fitness responses to maltose, consistent with an explanation based on pleiotropy but not one based on drift. Third, Travisano et al. (1995b) calculated from known mutation rates in *E. coli* that the duration of the evolution experiment (2000 generations) is too short to accommodate substantial drift in the limited portion (<1%) of the genome affecting performance in maltose but not glucose. We therefore believe that the observed differences in fitness between maltose and glucose were not due to chance fixation of neutral mutations but rather, in many cases, were pleiotropic effects of adaptations to glucose.

In addition to examining mechanisms of adaptation, this study also addresses two other important evolutionary patterns (e.g., Cohan 1984; Lenski 1988; Travisano et al. 1995b): the diversity of adaptations to a common environment and the correlated consequences of such changes for performance in other environments. Does the genetic constitution of the common ancestor predispose evolution toward a single adaptive mechanism in response to identical environmental challenges so that the same solution occurs again and again if replicate populations are similarly challenged? And are different patterns of genetic correlation produced by different adaptive mechanisms, such that some populations are fortuitously preadapted to novel environments? Experimental populations of bacteria are ideal for investigating these issues, because replicated populations founded by a single genotype can be propagated in a defined environment and monitored for thousands of generations (e.g., Lenski et al. 1991; Lenski and Travisano 1995b). Genetic variability within and among the replicate populations is initially absent, and all genetic changes must occur de novo, by mutation, in each population. Thus, a comparison of replicate lines permits examination of the diversity of adaptive mechanisms that arise as well as their consequences for performance in both the selective and novel environments.

Materials and Methods

Organisms and Their History.—The bacteria used in these experiments are asexual lines of *E. coli* B, the selective histories of which are described in Bennett et al. (1992) and Mongold et al. (1996). Briefly, all lines share a common ancestor, which itself was derived from a population previously maintained at 37°C for 2000 generations (Lenski et al. 1991). The ancestral bacterium in our experiment was cloned to produce six replicate populations in each of five evolutionary treatment groups, which were then exposed to different thermal regimes for 2000 generations. One group was maintained at the historical temperature of 37°C. Three others were propagated at novel but constant temperatures of 20°C, 32°C, and 42°C. The fifth group was alternated daily between 32°C and 42°C.

During their selective histories, all populations were maintained by serial transfer in Davis minimal medium (Carlton and Brown 1981) supplemented with 25 μg/ml glucose (see Lenski et al. 1991). Each day, cultures were diluted 100-fold into fresh medium, and the bacterial populations underwent ~6.6 ($\log_2 100$) cell divisions in all temperature regimes prior to depleting the glucose and entering a quiescent stationary phase.

Measurement of Relative Fitness in Different Environments.—The fitness of each derived line relative to the common ancestor was estimated using procedures given by Lenski et al. (1991), except that fitness was assayed in either of two media: DM medium containing 25 μg/ml glucose or DM medium containing 25 μg/ml maltose. These media provide comparable amounts of energy for the bacteria. The ancestor has two forms, differing in a marker (+/−) for arabinose utilization, and each evolutionary treatment group contains three lines derived from each ancestral marker state. The neutrality of this marker in both nutrient media was tested across the range of experimental temperatures by direct competition between the reciprocally marked ancestral genotypes. All other fitness assays were performed with a derived line and the ancestral genotype with the opposite marker state. Both the ancestral and derived lines are maintained frozen at −80°C. After removal from the freezer, both competitors were separately cultured on the first day in LB medium (Miller 1972) at 37°C, on the second day in DM medium containing either glucose or maltose at 37°C, and on the third day in the same DM medium at the experimental temperature to acclimate the bacteria to the competition conditions (see Leroi et al. 1994). Both the ancestor and derived line were then diluted 200-fold into fresh DM. Initial densities were estimated by plating onto tetrazolium-arabinose agar. The competition culture was then incubated at the experimental temperature (±0.5°C) for one day, and the final density of competitors was estimated by plating. Fitness of the experimentally derived line relative to the ancestor is given by

$$W = \log(E_f/E_i)/\log(A_f/A_i)$$

where subscripts i and f denote initial and final values, respectively, for the population densities of the evolutionarily derived (E) and ancestral (A) genotypes. Relative fitness therefore corresponds to the ratio of the genotypes' Malthusian parameters during head-to-head competition.

Fitnesses in both glucose and maltose were measured for each evolutionary treatment group at its selective temperature. For certain groups, fitnesses were also measured at other temperatures to examine correlated responses across thermal environments. Assays to test the neutrality of the arabinose marker state were replicated 10-fold. Assays for each derived line were replicated sixfold, and each replicate was paired in the incubator with a replicate in the alternate sugar (glucose or maltose). As our primary interest is in the fitness differential between glucose and maltose for each line, this pairing ensures that any uncontrolled environmental variables affect performance in each sugar equally. Experiments were also blocked by marker state and experimental temperature, so that lines +1, +2, and +3 of an evolutionary treatment group at one temperature were blocked in one experiment and lines −1, −2, and −3 of the group were measured at that temperature in another experiment. However, block effects were generally not observed (consistent with marker neutrality and

TABLE 1. Effective neutrality of the arabinose-utilization marker in the ancestor in glucose or maltose at different temperatures.

	Fitness of Ara+ Relative to Ara−			
	Glucose		Maltose	
Temperature	Mean (± SE)*	Significance†	Mean (± SE)*	Significance†
20°C	1.012 (± 0.006)	0.10	1.031 (± 0.015)	0.06
37°C	1.001 (± 0.019)‡	0.96	1.013 (± 0.013)	0.34
41°C–42°C	0.993 (± 0.012)‡	0.59	0.994 (± 0.044)	0.90

* Mean (and standard error of the mean) based on ten assays at each temperature.
† Two-tailed probability, using the t-distribution with $N - 1 = 9$ degrees of freedom; the null hypothesis is that mean fitness equals 1.
‡ Data from Bennett et al. 1992.

assay repeatability) and were not included in the statistical analyses.

In the assays at 42°C, relative fitnesses of certain derived lines in maltose were so great that final densities of the ancestral competitor were too low to measure with accuracy. These experiments were therefore repeated (with sixfold paired replication) with three treatments differing in the initial relative frequencies of the competitors, as follows: 1 derived:1 ancestral in glucose; 1 derived:9 ancestral in glucose; and 1 derived:9 ancestral in maltose. The possibility of frequency dependent fitness effects was tested by comparing the paired replicates of the two glucose treatments. No significant frequency-dependence was detected by paired t-test for any of the experimental lines (data not shown). Therefore, relative fitnesses at 42°C in both glucose and maltose were estimated using the 1:9 initial relative frequencies.

Statistical Analysis and Hypothesis Testing.—Selective neutrality of the arabinose marker was evaluated by comparing the relative fitness of the + and − states to the neutral expectation of 1.0 by t-test. For each derived line (in a given nutrient and temperature), the change in relative fitness from the ancestral condition ($W = 1$) was evaluated by t-test. Differences in relative fitness between glucose and maltose were determined by paired t-tests. All t-tests were two tailed. Differences among lines within an evolutionary treatment group were evaluated by ANOVA; if significant differences were indicated, then the minimum number of statistical groupings of lines was determined by the Tukey-Kramer multiple comparisons test. Mean fitness and its confidence limits for each evolutionary treatment group were determined from the mean values for the six selected lines so that the corresponding degrees of freedom are five.

The hypothesis that glucose transport changed during the evolution of any particular line was evaluated by comparing relative fitnesses in glucose and maltose. The hypothesis was accepted if fitnesses were significantly different. The hypothesis that different mechanisms were involved in adaptation was tested by comparing fitnesses among lines within an evolutionary treatment group. The hypothesis was accepted if there were significant differences among the lines within a group.

RESULTS

Effective Neutrality of the Marker.—Table 1 reports the relative fitnesses of the two arabinose marker states in the ancestor in different media and at different temperatures. In both sugars and at all temperatures tested, relative fitness is unaffected by the marker state. The marker can therefore be considered to be effectively neutral in the environments used in this study. Moreover, all evolutionary treatment groups and fitness assays are balanced with respect to the marker.

Fitness Responses at Selective Temperatures.—Table 2 reports the fitnesses obtained in both glucose and maltose of all derived lines, measured at their selective temperatures; the corresponding mean fitnesses of all evolutionary treatment groups are summarized in Table 3. The 37°C group, which continued exposure to the historical thermal regime of the ancestor and thereby served as a control for the thermal specificity of adaptation (see Bennett et al. 1992), experienced a small (2.5%) but significant increase in fitness (as previously reported in Bennett et al. 1992). In none of the six lines, however, could this adaptation be ascribed to specific improvements in glucose transport, because relative fitnesses were comparable in glucose and maltose (Table 2, 37°C group, p Glu = Mal row). In each of the four groups that evolved under a novel thermal regime, the mean fitness improvement in glucose at their corresponding selective temperatures exceeded that of the 37°C group (Table 3). Moreover, each of the groups that evolved under the novel regimes contained multiple lines that apparently adapted via improvements in glucose transport (Table 2, p Glu = Mal rows). In both the 32°C and 42°C groups, all six lines have different relative fitnesses in glucose and maltose. In the 20°C group, three of the six lines have different fitness responses to these two sugars, and in the 32–42°C group, four of the six lines do. Summing over all four treatment groups that adapted to novel thermal regimes, 19 of the 24 lines show evidence for improved glucose transport as a mechanism of adaptation.

Although the general mechanism of adaptation (glucose transport) appears similar for many of the lines, the specific mutational alterations and their pleiotropic effects are often very different. The fitness responses of the lines within each evolutionary treatment group are heterogeneous in the 42°C and 32–42°C groups in glucose and in all five groups in maltose (Table 2, p ANOVA column). A posteriori tests identify a minimum of two different fitness clusters in maltose for the 32°C, 37°C, and 42°C groups and three for the 20°C and 32–42°C groups. The number of statistically distinct clusters provides a minimum estimate of the number of different specific mechanisms involved in adaptation within an evolutionary treatment group. A more detailed examination of the fitnesses in maltose of the 19 lines with different relative fitnesses in glucose and maltose reflects the diversity of their correlated responses (Table 2). In three lines, fitness in mal-

TABLE 2. Relative fitness of the six lines in each evolutionary treatment group in comparison to their common ancestor at their selective temperature.

Group and temperature	Sugar	Lines*						p ANOVA†	Minimum no. of groupings‡
		+1	+2	+3	−1	−2	−3		
20°C group @ 20°C	Glucose	1.134 ± 0.066	1.074 ± 0.031	1.055 ± 0.066	1.083 ± 0.051	1.088 ± 0.043	1.088 ± 0.062	0.21	1
	Maltose	1.127 ± 0.066	1.052 ± 0.080	0.925 ± 0.044	1.183 ± 0.017	0.946 ± 0.017	1.100 ± 0.062	<0.001	3
	p Glu = Mal	0.87	0.43	0.006	0.003	<0.001	0.77	<0.001	2
32°C group @ 32°C	Glucose	1.088 ± 0.028	1.085 ± 0.057	1.131 ± 0.058	1.085 ± 0.052	1.147 ± 0.026	1.103 ± 0.033	0.07	1
	Maltose	1.008 ± 0.021	0.803 ± 0.039	1.039 ± 0.029	0.982 ± 0.052	0.869 ± 0.023	0.970 ± 0.074	<0.001	2
	p Glu = Mal	0.003	<0.001	0.01	0.002	<0.001	0.01	<0.001	2
37°C group @ 37°C	Glucose	1.026 ± 0.049	0.998 ± 0.032	1.022 ± 0.032	1.058 ± 0.054	1.020 ± 0.036	1.028 ± 0.040	0.24	1
	Maltose	1.070 ± 0.068	0.977 ± 0.021	1.069 ± 0.045	1.011 ± 0.060	1.028 ± 0.034	1.003 ± 0.068	0.02	2
	p Glu = Mal	0.15	0.10	0.16	0.14	0.77	0.46	0.07	1
42°C group @ 42°C	Glucose	1.250 ± 0.145	1.200 ± 0.097	1.293 ± 0.066	1.650 ± 0.324	1.329 ± 0.128	1.288 ± 0.157	0.001	2
	Maltose	1.488 ± 0.314	1.369 ± 0.212	1.430 ± 0.183	2.074 ± 0.539	1.168 ± 0.083	1.752 ± 0.333	<0.001	2
	p Glu = Mal	0.04	0.03	0.04	0.009	0.01	0.01	<0.001	2
32°C–42°C group @ 32°C–42°C	Glucose	1.190 ± 0.048	1.253 ± 0.106	1.236 ± 0.082	1.155 ± 0.036	1.090 ± 0.038	1.118 ± 0.028	<0.001	2
	Maltose	1.119 ± 0.020	1.057 ± 0.028	1.089 ± 0.050	0.970 ± 0.013	1.070 ± 0.014	1.135 ± 0.009	<0.001	3
	p Glu = Mal	0.003	0.005	0.002	<0.001	0.27	0.17	<0.001	2

* Mean values of six replicate assays and 95% confidence limits are reported. *p* Glu = Mal is the probability by two-tailed paired *t*-test (with df = 5) that fitnesses in glucose and maltose are equal (i.e., the probability that the two estimates would differ by as much due to chance). Note that different line designations among groups (e.g., +1 of the 20°C, 32°C, etc. °C groups) have no paired relationship to each other, and all lines in all groups share the same common ancestor.

† *p* ANOVA is the probability that the fitness responses are heterogeneous among the lines of each evolutionary treatment group in glucose, in maltose, and in the paired differences between them.

‡ Minimum number of groupings is the statistical estimate of the fewest indistinguishable sets of lines by Tukey-Kramer multiple comparisons test.

TABLE 3. Mean relative fitness of each evolutionary treatment group at their selective temperature and other assay temperatures.

Group	Assay temperature	Sugar	Group mean*
Responses at selective temperatures			
20°C	20°C	Glucose	1.087 ± 0.027
		Maltose	1.056 ± 0.107
32°C	32°C	Glucose	1.107 ± 0.028
		Maltose	0.945 ± 0.095
37°C	37°C	Glucose	1.025 ± 0.020
		Maltose	1.026 ± 0.039
42°C	42°C	Glucose	1.335 ± 0.168
		Maltose	1.547 ± 0.336
32°C–42°C	32°C–42°C	Glucose	1.174 ± 0.068
		Maltose	1.073 ± 0.061
32°C–42°C	32°C	Glucose	1.049 ± 0.016
		Maltose	1.020 ± 0.017
32°C–42°C	42°C	Glucose	1.267 ± 0.079
		Maltose	1.597 ± 0.469
Responses at other temperatures			
20°C	37°C	Glucose	0.967 ± 0.117
		Maltose	0.971 ± 0.123
32°C	20°C	Glucose	1.024 ± 0.021
		Maltose	0.970 ± 0.164
32°C	37°C	Glucose	1.024 ± 0.068
		Maltose	0.966 ± 0.095

* ± 95% confidence limits, with df = 5.

tose is unchanged from its ancestral value ($W = 1$). In five lines, it has significantly decreased; that is, adaptation to glucose has produced a correlated trade-off in performance in maltose. In another five lines, fitness in maltose increased above the ancestral value but not to the same extent as in glucose. And in another six lines (including five in the 42°C-selected group), fitness in maltose increased even more than fitness in glucose.

The variable thermal regime, in which the 32–42°C group evolved, consisted of rapid transitions between two component temperatures, 32°C and 42°C. The fitnesses reported in Table 2 for this group were measured in a full cycle consisting of exposure to both of these temperatures and rapid transitions between them. The fitness responses of this group to the two constant temperature components are given in Tables 3 and 4. As previously reported (Leroi et al. 1994), the fitness improvement of this group in glucose is much greater at 42°C than at 32°C ($P = 0.001$ by two-tailed paired *t*-test); it is also greater at 42°C in maltose ($P = 0.02$). At 32°C, analysis of variance (ANOVA) indicates no significant heterogeneity among the six lines for fitness in glucose, maltose, or the difference between glucose and maltose. By contrast, at 42°C, there is significant heterogeneity among lines for fitness in maltose and for the difference between glucose and maltose; at least two of the lines have greater fitness in maltose than in glucose (Table 4).

Correlated Fitness Responses to Novel Temperatures.—Relative fitnesses for both the 20°C and 32°C groups were examined at temperatures other than those corresponding to their selection histories (Table 5). Of particular interest is the constancy (or lack thereof) of each line's fitness differential between glucose and maltose across the thermal niche. If the differential were constant, then fitness in maltose could be predicted at any temperature, given only the fitness in glucose

TABLE 4. Relative fitness of the six lines of the 32°C–42°C group in the two constant temperature components of its variable thermal environment.*

Group and temperature	Sugar	Lines						p ANOVA	Minimum no. of groupings
		+1	+2	+3	−1	−2	−3		
32°C–42°C group	Glucose	1.060 ± 0.028	1.029 ± 0.025	1.060 ± 0.047	1.040 ± 0.060	1.039 ± 0.042	1.067 ± 0.037	0.49	1
@ 32°C	Maltose	1.016 ± 0.022	1.031 ± 0.020	1.024 ± 0.041	1.006 ± 0.045	1.000 ± 0.068	1.045 ± 0.046	0.44	1
	p Glu = Mal	0.04	0.84	0.17	0.36	0.24	0.50	0.81	1
32°C–42°C group	Glucose	1.280 ± 0.398	1.360 ± 0.142	1.169 ± 0.082	1.318 ± 0.133	1.186 ± 0.079	1.288 ± 0.213	0.51	1
@ 42°C	Maltose	1.810 ± 0.800	2.319† ± 1.197	1.756 ± 0.410	1.308 ± 0.232	1.211† ± 0.183	1.179† ± 0.365	0.02	2
	p Glu = Mal	0.03	0.06	0.02	0.91	0.51	0.68	0.003	2

* See Table 2 for explanation of entries.
† $n = 5$

TABLE 5. Relative fitness of the six lines of the 20°C and 32°C groups at other temperatures.*

Group and temperature	Sugar	Lines						p ANOVA	Minimum no. of groupings
		+1	+2	+3	−1	−2	−3		
20°C group	Glucose	0.998 ± 0.057	0.744 ± 0.096	1.030 ± 0.053	1.036 ± 0.040	0.975 ± 0.051	1.021 ± 0.031	<0.001	2
@ 37°C	Maltose	0.905 ± 0.031	1.187 ± 0.091	1.001 ± 0.052	0.917 ± 0.055	0.964 ± 0.058	0.852 ± 0.034	<0.001	3
	p Glu = Mal	0.02	<0.001	0.31	0.001	0.76	<0.001	<0.001	3
32°C group	Glucose	1.043 ± 0.014	0.998 ± 0.056	1.005 ± 0.044	1.019 ± 0.058	1.037 ± 0.025	1.039 ± 0.028	0.28	1
@ 20°C	Maltose	1.127 ± 0.062	0.756 ± 0.031	1.006 ± 0.024	1.031 ± 0.031	0.800 ± 0.083	1.102 ± 0.017	<0.001	3
	p Glu = Mal	0.02	<0.001	0.96	0.71	<0.001	<0.001	<0.001	2
32°C group	Glucose	0.911 ± 0.058	1.099 ± 0.024	1.039 ± 0.045	0.995 ± 0.036	1.063 ± 0.024	1.037 ± 0.023	<0.001	3
@ 37°C	Maltose	0.833 ± 0.022	1.034 ± 0.032	0.946 ± 0.022	0.948† ± 0.040	1.097 ± 0.044	0.937 ± 0.069	<0.001	3
	p Glu = Mal	0.008	0.02	0.009	0.07	0.19	0.02	0.003	2

* See Table 2 for explanation of entries.
† $n = 5$.

TABLE 6. Sets of lines within the 20°C and 32°C groups that are statistically indistinguishable in their fitnesses (W) in either glucose or maltose at any temperature assayed.

Group	Set of lines	Distinguishing characteristics
20°C	+1, −1, −3	Elevated $W_{maltose}$ at 20°C, depressed $W_{maltose}$ at 37°C
	+3, −2	Depressed $W_{maltose}$ at 20°C, ancestral $W_{maltose}$ at 37°C
	+2	Depressed $W_{glucose}$ at 37°C, elevated $W_{maltose}$ at 37°C
32°C	+3, −1, −3	Ancestral $W_{glucose}$ and $W_{maltose}$ at 37°C
	+2, −2	Depressed $W_{maltose}$ at 20°C and 32°C, elevated $W_{glucose}$ and $W_{maltose}$ at 37°C
	+1	Depressed $W_{glucose}$ and $W_{maltose}$ at 37°C*

* This line was also previously shown to have severely depressed $W_{glucose}$ at even higher temperatures of 40°C–42°C (fig. 7, Bennett and Lenski 1993).

at that temperature and the differential at another temperature. In fact, this differential is not constant and the cross-temperature relationships are quite unpredictable. In the 20°C group, the glucose-maltose fitness differentials themselves differ significantly between 20°C (Table 2) and 37°C (Table 5) for five of the six lines (all five $P < 0.03$ by t-test, with df = 10 per line); only in line +1 is the differential indistinguishable at these two temperatures ($P = 0.13$). In the 32°C group, all six lines show significant differences across the assay temperatures of 20°C, 32°C, and 37°C (Tables 2, 5) in their glucose-maltose fitness differentials (all six $P \leq 0.01$ by ANOVA, with 2 and df = 15 for each line). A detailed examination of these data indicates that not only are quantitative differences between fitnesses in these two sugars variable across temperatures, but even the signs of the differences are sometimes changed. Evidently, the fitness differential between glucose and maltose of these derived lines is neither constant nor predictable across the thermal niche.

To further examine the extent of differences in the underlying mechanisms of adaptation among the lines within an evolutionary treatment group, we simultaneously consider patterns of fitness across both nutrients and temperatures. An analogy can be made here to differences in the discriminatory power of one- and two-dimensional gel electrophoresis. By separating along two different gradients, resolution of different components can be achieved that would be impossible using only a single factor. Using fitnesses estimated in each sugar and at each assay temperature, we performed all pairwise comparisons among the six lines in both the 20°C and 32°C groups (Tables 2, 5). These pairwise comparisons were Bonferroni-corrected (Rice 1989) for *both* the number of line pairs ($6 \times 5/2 = 15$) and the number of different assay environments (2 sugars × 2 temperatures = 4 environments assayed for the 20°C group; 2 sugars × 3 temperatures = 6 environments assayed for the 32°C group). These analyses produce sets of lines that are statistically distinct in their fitness responses across the nutritive and thermal environments. Thus, an evolutionary treatment group comprised of six replicate lines may contain between one and six distinct sets. In the former case, all lines behave similarly across the assay environments, and perhaps reflect a common physiological mechanism (or at least mechanistic differences cannot be distinguished on the basis of fitness in any of the assay environments). In the latter case, all lines behave differently and therefore presumably have different underlying mechanisms. In the 20°C evolutionary treatment group, this two-dimensional analysis yielded three distinct groups (Table 6). A similar analysis of the 32°C group also revealed three statistically distinct patterns of fitness across temperatures and sugars (Table 6). These findings imply that at least three different specific mechanisms were involved in the evolutionary adaptation of both the 20°C and 32°C groups. At the same time, the noticeable similarities of certain lines in their correlated responses (Table 6) suggest that some of them may have adapted by very similar means. Further mechanistic and genetic analyses will be required to substantiate these associations.

DISCUSSION

In this study, we have examined correlated responses of fitness to novel nutrients and temperatures in lines of *E. coli* that had adapted evolutionarily to growth on glucose under several different thermal regimes. These correlated responses provide information that may be used to address three different evolutionary issues: (1) the nature of the physiological mechanisms underlying the observed adaptation; (2) the similarities or differences in the adaptive mechanisms achieved by replicate lines; and (3) the extent of cryptic variation among lines, which may provide a latent pool of genetic variation for adapting to changing environments.

Our results indicate that evolutionary adaptation to temperature in these experimental lines may frequently have involved modifications of glucose transport. At least three-quarters of the populations that adapted to one of the novel thermal regimes showed differences in relative performance between glucose and maltose, as would be expected for changes in the regulation and/or expression of glucose transport proteins (but not for glucose catabolism or other aspects of cell metabolism). It is quite possible that additional mechanisms unrelated to glucose transport also evolved in some or all of these lines, and we will continue to explore that possibility. The specific mechanisms underlying the modifications in glucose transport appear to be diverse, even among lines with identical selection histories. For the reasons given below, we believe this physiological diversity to have arisen as pleiotropic side effects of beneficial mutations, rather than by genetic drift. Interestingly, these adaptations in glucose transport are remarkably temperature specific, improving fitness in glucose at each line's selective temperature but generally having little impact, either beneficial or adverse, on fitness in glucose at other temperatures (Bennett et al. 1992; Bennett and Lenski 1993).

Pleiotropy versus Drift.—Were the widespread differences in fitness between maltose and glucose caused by the chance fixation of mutations that were neutral (or nearly so) in glu-

cose, but which had measurable effects on performance in maltose? If the derived lines had adapted to their selective temperatures by some means *other than* glucose transport, then we would expect the fitness improvement caused by the beneficial mutations to be comparable in glucose and maltose. In that case, any difference in relative performance between glucose and maltose would have to arise by random genetic drift. Or are these differences the pleiotropic consequences of the very same mutations that produced adaptation to the selective environment? If the derived lines adapted to their environments by improving glucose transport, then different mutations conferring such improvements may have had different pleiotropic side effects on performance in medium containing maltose. We favor the latter interpretation on three grounds.

First, several of the derived lines are actually better (relative to the common ancestor) in maltose than in glucose (Tables 2, 4). But improvements are very unlikely to occur by drift, which is essentially a decay process. Moreover, almost all cases in which fitness was greater in maltose than in glucose occurred among the groups selected at high temperature (42°C and alternating 32–42°C); drift is not expected to produce such environmental specificity. Second, the group selected at the ancestral temperature, 37°C, exhibited the smallest fitness gains in glucose of any group and also showed an absence of fitness differences between glucose and maltose (Table 2). If glucose-maltose fitness differentials were due to genetic drift acting on performance in maltose, then there is no reason for them to be any less common at the temperature where there was the least adaptation to glucose. However, if glucose-maltose fitness differentials were instead by-products of the mutations producing adaptation to glucose, then one would expect such differentials to be uncommon in any environment where beneficial mutations were infrequent. The third line of evidence against the drift hypothesis is based on estimates of the rate of mutations that would affect performance in maltose but not glucose. Travisano et al. (1995b) estimated that mutations in $<1\%$ of the *E. coli* genome should affect performance in maltose but not in glucose. Using typical mutation rates estimated at 37°C for *E. coli*, a 2000 generation evolution experiment is much too short to accommodate substantial drift in such a small portion of the genome. Therefore, unless one invokes much higher mutation rates at *both* lower and higher temperatures, genetic drift cannot adequately explain the prevalence of glucose-maltose fitness differentials at all temperatures except 37°C.

Mechanisms of Adaptation.—Before considering the evolution of transport mechanisms in this study, we will review the historical adaptation of the common ancestor of our evolutionary treatment groups. This ancestral genotype was isolated from one (Ara$^-$1) of 12 replicate lines that had been propagated for 2000 generations in glucose-limited medium at 37°C (Lenski et al. 1991). During that time, the mean fitness in glucose of these 12 lines relative to their founder increased by ~35%. Although there was little heterogeneity in fitness in glucose among these lines, they were very heterogeneous in their fitnesses in maltose (Travisano et al. 1995b). In all 12 lines, fitness in glucose differed significantly from that in maltose, consistent with adaptation in their glucose transport mechanisms; and in each case, fitness in maltose was less than that in glucose (Travisano 1993). In particular, in line Ara$^-$1, fitness in maltose declined significantly below the ancestral value ($P = 0.005$), indicating a prior trade-off between performance in maltose and glucose in the common ancestor for our evolutionary treatment groups.

In this study, the 37°C group continued the historical thermal environment for another 2000 generations. Although fitness improvement in this environment continued during this time (see also Bennett et al. 1992), its extent was small and did not demonstrably involve further improvements in glucose transport. That is, further fitness gains carried over to an environment in which maltose was substituted for glucose, a result that would be expected for adaptations other than improved glucose transport. In contrast, in the evolutionary treatment groups adapted to the novel thermal environments, about three-quarters of the lines apparently adapted by temperature-specific changes in glucose transport. In the 32°C and 42°C groups, all of the lines adapted by this means.

The inference that adaptation to the novel thermal regimes involved glucose transport mechanisms is supported by further observations on the fitnesses of the 32°C group when competing for several other carbon sources (unpubl. data). Fitnesses in fructose and mannitol, which share with glucose the use of PTS transport across the inner membrane, are indistinguishable from fitness in glucose ($P = 0.55$ by repeated measures ANOVA, with one replicate for each of six lines in three nutrients). The average fitness in these three PTS sugars ($W = 1.11$) is significantly greater (paired t-test, $P = 0.02$ with df = 5) than the average fitness ($W = 0.91$) in maltose, galactose, and melibiose, all of which cross the inner membrane via other mechanisms (Saier 1985; Nikaido and Saier 1992).

An important feature of these transport adaptations is that they are temperature specific; they are not simply improvements in glucose transport that enhance fitness in glucose in all thermal environments. The specificity of the fitness improvement at selected temperatures (Tables 2, 5; see also Bennett et al. 1993, and Mongold et al. 1996) suggests that the beneficial mutations in these groups are qualitatively different from those experienced in the original adaptation of the ancestral line to glucose at 37°C (Lenski et al. 1991; Travisano et al. 1995b).

The precise mechanisms that underlie these temperature-specific adaptations in glucose transport are currently unknown and will be the topic of further study. Some of the patterns of differential fitness in the two sugars, such as enhanced fitness in glucose and no change from the ancestral condition in maltose (Fig. 1B), are not surprising given the independent transporters involved in uptake of these nutrients. That is, changes in the glucose transport pathway that would not affect maltose uptake can be readily envisioned. Also, as previously discussed, we anticipate that adaptations that are not mechanistically related to nutrient transport will impact fitness in the two sugars equally (Fig. 1A). Less easily interpreted, however, are the trade-offs seen in some lines, in which fitness in maltose actually decreased below ancestral values as fitness in glucose increased (Fig. 1C). At least three of the 12 lines in the original experiment involving adaptation to glucose at 37°C (Lenski et al. 1991) showed a similar trade-off (Travisano 1993). Also difficult to interpret are the

fitness gains in maltose that are even greater than the gains in glucose (Fig. 1D). This "hyper-maltose-fitness" response was seen most frequently (five of six lines) in the 42°C group (Table 2), which was exposed to a stressfully high temperature (Lenski and Bennett 1993), and it also occurred in one line of the 20°C group (Table 2), at the lower boundary of the ancestral thermal niche (Bennett and Lenski 1993; Mongold et al. 1996). It also occurred in at least two of the lines in the 32–42°C treatment group when their fitnesses were assayed at 42°C but not at 32°C (Table 4). The hyper-maltose-fitness response was never observed at more intermediate selective temperatures (e.g., 32°C or 37°C) in this experiment nor among the 12 lines evolving at 37°C in the original experiment at 37°C (Lenski et al. 1991; Travisano et al. 1995b).

Although glucose and maltose rely on different proteins for their uptake, their transport pathways are functionally interconnected in several ways with respect to regulation and activation (reviewed in Saier 1985, 1987, 1989). These interactions may explain the complex and unexpected patterns of trade-off and hyper-maltose-fitness observed in some of our derived lines. In regard to the former, PTS enzyme IIA^{Glc} (Saier and Reizer 1992) can be a potent inhibitor of non-PTS transport systems, including that for maltose (Dean et al. 1990). In the presence of glucose, maltose transport capacity (as well as that of other non-PTS sugars) is greatly inhibited (Saier and Roseman 1976; Saier 1989). Mutations affecting the expression of PTS enzymes such as IIA^{Glc} may have occurred in some of our experimental lines that simultaneously up-regulated glucose transport and down-regulated maltose transport. The hyper-maltose-fitness response may result from the mutual dependence of gene activation of both the PTS (Rephaeli and Saier 1980) and maltose transporters (Raibaud et al. 1989) on cyclic AMP in a complex with its receptor protein (cAMP-CRP). This system regulates the expression of many inducible transport and catabolic operons in *E. coli*, as well as those operons expressed during starvation (reviewed in Botsford and Harman 1992). Mutations up-regulating this system might not only increase glucose transport but simultaneously activate maltose transport to an even greater extent.

If this latter hypothesis is correct, then the derived lines showing the hyper-maltose-fitness response should have a shorter lag phase (prior to the commencement of growth) than their ancestor in medium containing either glucose or maltose and, importantly, the reduction in the duration of lag phase should be even greater in maltose than in glucose. To test this prediction, we monitored the population growth kinetics in glucose and in maltose of the ancestor and two derived lines from the 42°C group (−1 and −3) that showed the hyper-maltose-fitness response at 42°C (Table 2). Both derived lines had shorter lag phases than their common ancestor in both sugars (Fig. 2). Moreover, for both derived lines the improvement in comparison with the ancestor was substantially greater in maltose (where lag was reduced from 8+ hr to ~2 hr) than in glucose (where lag was reduced from ~2 hr to ~1 hr). These growth kinetics are thus consistent with the hypothesis that enhanced activation of the cAMP-CRP system, having arisen as an adaptation to glucose, might differentially activate growth in maltose and thereby produce a greater fitness gain in maltose than in glucose.

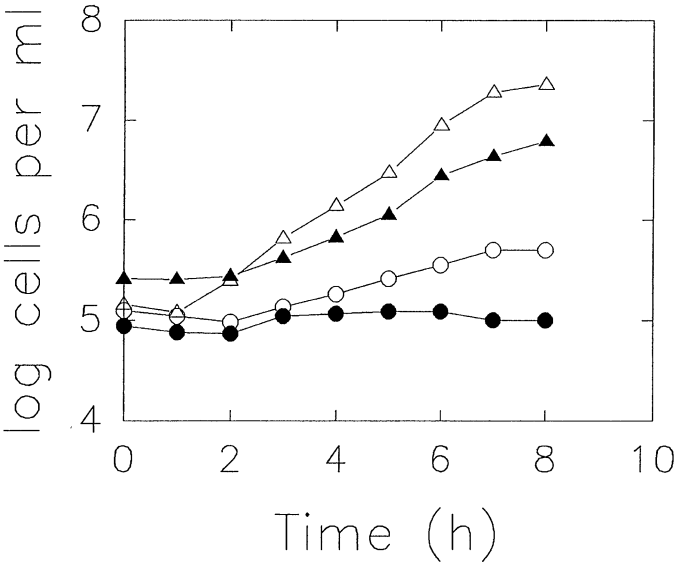

FIG. 2. Growth kinetics of the ancestor and a derived line exhibiting the "hyper-maltose-fitness" response, measured at 42°C in both glucose and maltose. Bacteria were inoculated into medium containing 25 μg/ml of either glucose (open symbols) or maltose (filled symbols); densities were estimated hourly by diluting samples onto agar plates and subsequently counting colonies. Circles indicate the ancestor (mean of four replicates) and triangles indicate line −1 of the 42°C group (mean of two replicates). Results similar to the latter were obtained for line −3 of the 42°C group.

We do not claim that the physiological mechanisms of adaptation discussed above are in fact those responsible for the patterns of fitness response observed in this study. However, these patterns are consistent with previously described effects of regulatory factors that govern expression of the distinct transport pathways for glucose and maltose. These mechanisms would be logical points of departure for further physiological investigations of adaptive changes in the derived lines. In effect, patterns of fitness responses to different nutrients may have been useful in narrowing the search for temperature-specific mechanisms of adaptation and focusing that search on the glucose transport pathway.

Diversity of Adaptive Mechanisms.—In each evolutionary treatment group, six replicate populations were founded from a common ancestral genotype and then propagated in identical environments. Thus, initial genetic variation was eliminated by design, as were environmental differences. Under these stringent restrictions, we may then ask to what extent the independently evolving lines within a treatment group adapted by the same physiological mechanism.

A superficial examination of the results might lead one to conclude that there was a high degree of parallelism of adaptive mechanism throughout the experimental evolution: 19 of 24 lines in the four novel thermal environments apparently adapted by temperature-specific modifications of glucose transport. In two evolutionary treatment groups (32°C and 42°C), all six lines in each group adapted in this manner. A closer examination, however, reveals that several different specific mechanisms underlie the improvements in glucose transport. Statistical analyses of fitness in the two sugars at each group's selective temperature (Table 2) indicates a min-

imum of two and sometimes three different patterns within each group. Heterogeneity among the independently derived lines within a group is also observed when fitnesses are measured in a single sugar (glucose) across a range of temperatures (Bennett and Lenski 1993; Leroi et al. 1994). One might expect, therefore, that each line could be distinguished from all others within its evolutionary treatment group if performances across both sugars and temperatures were considered simultaneously. However, the two evolutionary treatment groups thus examined (the 20°C and 32°C groups) each include sets of lines with noteworthy similarities in their correlated responses (Table 6). This parallelism in fitness responses across temperatures and sugars suggests that a limited number of physiological mechanisms may be involved in the adaptation of each of these groups to their respective environments (for a similar situation, see Lenski 1988). These phenotype similarities may, of course, mask underlying genetic differences at the molecular (DNA) level. Also, we do not suggest that these are the only patterns of correlated response that could have evolved. If more replicate lines had been included in the evolution experiment, then more patterns would probably have emerged. What we do suggest, however, is that there may be only a finite number of ways of adapting to these thermal regimes (given the ancestral genetic state) and that the same physiological mechanisms of adaptation may have evolved independently in some of the replicate populations.

Preadaptation as a Correlated Response to Adaptation.— One of the most interesting results of this study is the diversity of correlated responses associated with the direct response to any given regime. Glucose was the sole source of carbon and energy available to the populations during their evolutionary adaptation to the various thermal regimes employed in this study. Their common ancestor had not encountered maltose for at least 2000 generations (and probably much longer than that). After another 2000 generations, several different correlated fitness responses to maltose had evolved within a given evolutionary treatment group. In the 20°C and 32°C groups, the genetic variance for fitness among the replicate lines was significantly greater in maltose than in glucose, and similar but nonsignificant trends were observed for the 37°C and 42°C groups (Fig. 3). All possible fitness responses to maltose (Fig. 1) were observed, including decreased fitness relative to the ancestral condition, no change in fitness from the ancestral value, and fitness increased above the ancestral state. In fact, two of the derived lines at high temperatures had relative estimated fitnesses in maltose above 2, which is much larger than any of the fitnesses estimated in glucose. In our notation, a relative fitness of 2 indicates that a derived line grew at twice the rate of its ancestor during competition and left approximately ten times as many descendants after a single day.

By whatever population genetic processes and physiological mechanisms it arose, it is clear that there was a great deal of genetic variation for fitness in maltose after experimental evolution in glucose. The quantitative and even qualitative nature of the correlated fitness response to maltose varied among the different lines in an evolutionary treatment group. When the correlated responses to other temperatures (besides the selective temperature) are taken into consider-

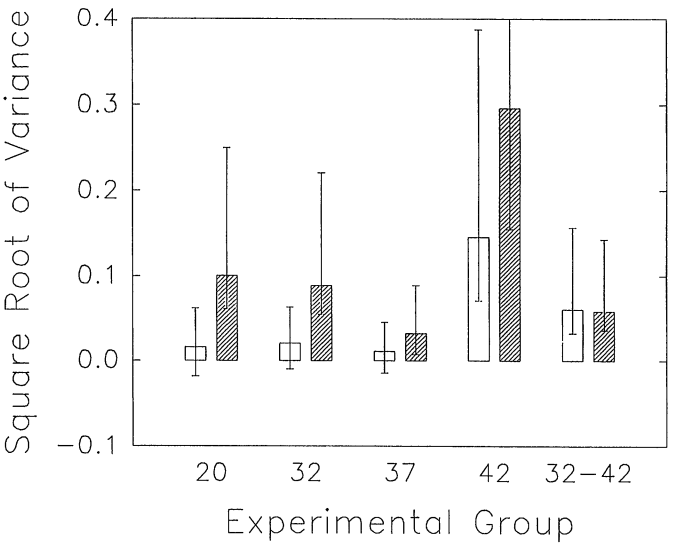

FIG. 3. Genetic variance of fitness among the replicate lines in each evolutionary treatment group, in either glucose (open bars) or maltose (hatched bars). The among-line variance component was estimated by ANOVA as $(MS_{line} - M_{error})/n$, where $n = 6$ is the number of replicate fitness assays performed per line. Error bars show approximate 95% confidence limits calculated according to Sokal and Rohlf (1981, p. 217–218).

ation, even more diversity is apparent. If these derived lines were to face new environments, in which the available nutrient or ambient temperature (or both) were altered, then they would be very differently positioned to function and prosper in these changed circumstances. Some lines would be at a disadvantage relative to their ancestor. Others, however, would be preadapted to these environments and would be able to function exceptionally well, even though their ancestors had not previously encountered those particular conditions for thousands of generations (if ever). This preadaptation would have been achieved as a by-product of selection in another environment. Our results indicate that even subtle differences in the mechanistic bases of functionally similar adaptations may have dramatically different consequences for performance in novel environments. The resulting diversity of correlated responses provides a reservoir of genetic variation for adapting to changing environments.

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