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EVOLUTIONARY ADAPTATION TO TEMPERATURE. III. ADAPTATION OF *ESCHERICHIA COLI* TO A TEMPORALLY VARYING ENVIRONMENT

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Abstract.—Six lines of the bacterium *Escherichia coli* were propagated for 2,000 generations in a temporally varying environment. The imposed environmental regime consisted of alternating days at 32°C and 42°C, with rapid transitions between them. These derived lines are competitively superior to their ancestor in this variable temperature regime. We also measured changes in the fitness of these lines, relative to their common ancestor, in both the constant (32°C and 42°C) and transition (from 32°C to 42°C and from 42°C to 32°C) components of this temporally varying environment, to determine whether the bacteria had adapted to the particular constant temperatures or the transitions between them, or both. The experimentally evolved lines had significantly improved fitness in each of the constant environmental components (32°C and 42°C). However, the experimental lines had not improved in making the sudden temperature transitions that were a potentially important aspect of the temporally variable environment. In fact, fitness in making at least one of the transitions (between 32°C and 42°C) unexpectedly decreased. This reduced adaptation to the abrupt transitions between these temperatures is probably a pleiotropic effect of mutations that were responsible for the increased fitness at the component temperatures. Among the six experimental lines, significant heterogeneity occurred in their adaptation to the constant and transition components of the variable environment.

Key words.—Acclimation, adaptation, bacteria, *Escherichia coli,* fitness, temperature, variable environments.

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Organisms may evolve diverse phenotypes in response to fluctuating environments (Lewontin 1957; Levins 1968). Such diversity could be due to phenotypic plasticity, wide performance breadth, genetic polymorphisms, or other factors (Lewontin 1957; Levins 1968; Orzack 1985; Lynch and Gabriel 1987; Gillespie and Turelli 1989; Stearns 1989; Gillespie 1991; Schlichting and Pigliucci 1993; Scheiner 1993; Via 1993; Lenski et al. 1994). Which of these factors will evolve depends on the particular characteristics of the environment, the relationship of the phenotype to fitness, and the genetic system (Orzack 1985; Gillespie 1991). Also potentially very important, but rarely discussed, are the fitness consequences of the *transitions* between component environments, that is, the ability of organisms to live and reproduce in a temporal sequence of different environments as opposed to the ability to live and reproduce in a range of environments, each of which is held constant.

An example of a temporally varying environment is an alternation between two temperatures. A population subjected to such a varying environment might adapt to either or both component temperatures, or to a particular sequence of temperatures, or to all of these aspects simultaneously. (If one environment is particularly aversive, dormancy is a common avoidance strategy, but one that we do not consider further here.) Levins (1968, 1969), Feder (1978), and Tsuji (1988) argue that thermally variable environments should select for increased "flexibility" or "acclimation ability." Despite the considerable attention that physiologists have paid to acclimation (Prosser and Brown 1961; Precht et al. 1973; Prosser 1973; Rome et al. 1992), the studies of Levins, Feder, and Tsuji are among the few that have been concerned with its evolution (see also Bradley 1978a,b). Unfortunately, insofar as these studies are based on natural populations, their comparisons inevitably confound differences in the variability of the thermal environment with differences in average temperature (as well as other unmeasured environmental variables).

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To address the nature of evolution in temporally variable environments by rigorous manipulative experiments, we have studied the effects of temperature—including changes in temperature—on the competitive fitness of populations of *Escherichia coli* that have been propagated under defined temperature regimes for thousands of generations (Lenski et al. 1991; Bennett et al. 1992; see *Materials and Methods* section).

Experimental Overview

The experimental lines used in this study consist of (1) an ancestral "A" genotype adapted to constant 37°C for 2000 generations (see Lenski et al. 1991), and (2) six "V" lines (called "32/42" in Bennett et al. 1992) derived from the A genotype but propagated for an additional 2000 generations in a thermally variable environment. This regime consisted of daily alternation between 32°C and 42°C in serial dilution culture, which permitted 6–7 generations per day. The transitions between temperatures were brief (i.e., minutes); thus, this variable environment may be viewed as consisting of two constant temperatures and the rapid transitions between them.

Relative fitness of the derived and ancestral genotypes is estimated by competition experiments (see next section). These competition experiments have two steps: an acclimation step, in which each of the two competitors is grown separately for 1 d, and a competition step, in which the two genotypes are mixed and allowed to compete for 24 or 48 h. This procedure allows the possibility of acclimating lines to one temperature and letting them compete either at that temperature or another one. The effects of temperature, as well as transitions in temperature, on relative fitness can thus be directly measured. Leroi et al. (1994), found that acclimation temperature has large, and sometimes unexpected, effects on the fitness of the ancestral "A" genotype. When the ancestor was acclimated to 32°C, it had a substantial fitness advantage in subsequent competition at 32°C and, paradoxically, also at 42°C, relative to acclimation at 42°C. In this paper, we examine the change in this ancestral acclimatory pattern during evolution of the V lines.

We investigate the fitness properties of the V lines relative to their ancestor in various thermal regimes to answer the following questions:

1. Have the experimental lines adapted to their variable thermal regime? Has fitness improved in the experimental environment of daily alternating temperatures?

2. Have the experimental lines adapted to each of the constant components of their environment? Are the V lines better adapted to constant 32°C, constant 42°C, or both than is their ancestor?

3. Have the experimental lines adapted to the transition components of the temporally varying environment? The fitness of the ancestor is extremely sensitive to the effects of acclimation temperature. During their evolution, the V lines were subjected daily to sudden transitions between 32°C and 42°C or vice versa. What are the effects of acclimation temperature on the competitive fitness of the V lines? In particular, have the V lines become better adapted to these transitions?

4. How similar or different are independently derived lines in their adaptations to a varying environment? Adaptation of the six V lines depended solely on mutations that occurred de novo during their experimental evolution. These random events may have caused divergence among replicate lines even though they were initially identical genetically and were subject to identical environments. Do the six V lines differ in the degree of their overall adaptation to the variable environment? Do these replicate lines differ in their relative adaptation to the constant and transition components of the temporally varying thermal regime?

Materials and Methods

Bacterial Strains and Culture Conditions.— General culture conditions and the origin of the ancestral and derived genotypes are described in detail by Lenski et al. (1991) and Bennett et al. (1992). The ancestral genotype exists in two forms, differing only in arabinose-utilization marker state (Ara^- and Ara^+). There are six V lines, three Ara^- and three Ara^+; each was founded from a single colony, and hence a single cell, of either the Ara^- or Ara^+ ancestral line. The six V lines were propagated for 2000 generations (300 days) in a glucose-limited minimal medium (DM), alternating daily between 32°C and 42°C, with an average of 6–7 generations sequentially at each temperature. At 2000 generations, each line was plated on tetrazolium-arabinose (TA) indicator agar, from which a single colony was randomly selected. All of the clones were stored at −80°C, and aliquots were subsequently used in these experiments.

Measurement of Relative Fitness in Competi-

tion Experiments.—All of the experiments reported here estimate the competitive fitness of one of the six V lines relative to the ancestral genotype of the opposite marker state. The arabinose marker permits genotypes to be distinguished by their colony color on TA indicator agar. The marker has previously been shown to be effectively neutral during competition in DM medium at a variety of temperatures, including constant 32°C, 37°C, and 42°C, as well as a daily alternation between 32°C and 42°C (Bennett et al. 1992). Moreover, the experiments reported here are balanced with respect to the association between marker state and evolutionary history. All hypothesis testing of possible differences between the V lines and their ancestors uses degrees of freedom based upon the number of independently derived replicate V lines (six).

The fitness assays include two sequential steps, acclimation and competition. In the acclimation step, two genotypes are separately grown in DM medium at either 32°C or 42°C for 24 h. [Incubator temperatures were controlled to ± 0.5°C. Because the upper thermal limit of the genotypes used in these experiments is approximately 42.2°C–42.3°C (Bennett and Lenski 1993), incubator temperatures designated "42°C" were actually maintained between 41.2°C and 42.0°C.] One day's (several generations') exposure should be sufficient to induce a phenotypic acclimation response in these clonal populations, but it is not enough time for new mutations to reach densities where they measurably affect population properties (Leroi et al. 1994). Except as noted otherwise, the two competing genotypes were acclimated to the same temperature as one another.

The competition step may last 1 d (24 h) at either 32°C or 42°C; or this step may last 2 d, 1 d at each of these temperatures. In the 1-day competition experiments, the competition temperature may be the same as the acclimation temperature, in which case fitness is estimated in a constant thermal environment (i.e., without including the effects of a sudden shift in temperature); or the competition temperature may be different from the acclimation temperature, in which case the fitness measurement includes the effects of a sudden transition in temperature. In the 2-day competition experiments, the temperature during the acclimation step was always the opposite of that during the first day of competition. These 2-day competition experiments thus include the effects of both sudden transitions in temperature.

Aliquots of the two separately acclimated and reciprocally marked populations are simultaneously diluted (1:200 each) into a common flask of fresh medium, whereupon an initial sample is plated on TA indicator agar. In a 1-day competition, the mixed population is then incubated for 24 h at the appropriate temperature, when a final sample is plated on TA indicator agar. In a 2-day competition experiment, following incubation at one temperature for 24 h, the mixed population is diluted 1:100 into fresh medium and incubated for another 24 h at the other temperature; at the end of these 48 h, a final sample is plated on TA indicator agar.

The relative fitness, W, of a derived genotype relative to its reciprocally marked ancestor is calculated from the change in their relative abundances between the initial and final samples in the competition step:

$$W = \log_e(V_f/V_i)/\log_e(A_f/A_i),$$

where V and A denote the densities of the derived and ancestral genotypes, respectively, and subscripts i and f indicate initial and final samples, respectively. In the case of a 2-day competition experiment, the actual densities estimated from the final plate count are multiplied by 100 to reflect the 1:100 dilution between the first and second days of the competition step. Thus defined, W is equivalent to a ratio of the realized Malthusian parameters of the two populations during competition with one another in the experimental environment (Lenski et al. 1991). A value of $W = 1$ indicates that the two competitors had equal fitness. Unequal fitness may reflect a difference in survival, or reproduction, or both during any phase of the competition step (i.e., lag, growth, and stationary phases; Vasi et al. 1994). Note that we always express the fitness of a derived line relative to the common ancestor. Therefore, we expect $W > 1$ when the V line has improved relative to the ancestor in a particular thermal regime.

Results

The six V lines evolved for 300 d in an environment in which temperature alternated between 32°C and 42°C on a daily basis; that is, each day in which they were propagated at 32°C was preceded by a day at 42°C and vice versa. Each day also entailed a rapid transition between these temperatures. First we ask the extent of adaptation to the variable environment itself, which includes all these components. Next we

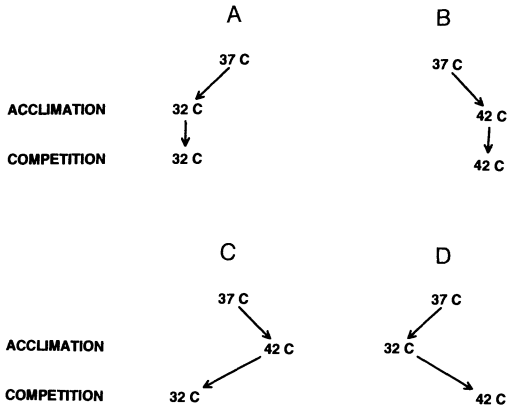

FIG. 1. Diagram of the experimental temperature sequences used to evaluate fitness improvements in the constant (A and B) and transition (C and D) components of the variable thermal environment. Thirty-seven degrees is the synchronization temperature to which all lines were subjected prior to acclimation. The acclimation and competition steps were each of 24-h duration.

examine adaptation to the constant components of the environment, specifically (A), adaptation to constant 32°C, and (B), adaptation to constant 42°C. Then we measure adaptation to the transition components of the environment, that is, with prior acclimation at the alternative temperature, specifically (C), acclimation to 42°C before competition at 32°C, and (D), acclimation to 32°C before competition at 42°C. See figure 1 for a diagram of these experiments. Finally, we examine the evolution of the acclimatory response by considering factors A, B, C, and D simultaneously.

Overall Fitness of V Lines in the Variable Thermal Environment.—To assess the overall adaptation of the derived lines to the temporally varying environment, we used two different treatments of sequential temperature exposure. In one treatment, we acclimated the V lines and the ancestral genotypes to 32°C, competed them at 42°C for 1 d and then at 32°C for a second day. In another treatment, we acclimated them to 42°C, let them compete at 32°C for one day and then at 42°C for a second day. We performed five assays of relative fitness for each of the V lines in each of these temperature sequences. Table 1 shows a two-way mixed-model analysis of variance in which the two temperature sequences and the six independently derived V lines are fixed and random effects, respectively. Temperature sequence has no significant effect on fitness, nor is there

TABLE 1. Analysis of variance for the overall fitness of the V lines relative to the common ancestor in the temporally variable environment (daily alternation between 32°C and 42°C).

Source	df	MS ($\times 10^3$)	F	P
Temperature sequence	1	0.385	0.727	0.411
Derived line	5	7.896	4.776	< 0.001
Interaction	5	0.530	0.320	0.898
Error	48	1.654		
Total	59			

NOTE.—ANOVA is a mixed model, with temperature sequence a fixed effect and independently derived line a random effect.

any significant interaction between temperature sequence and derived line. Significant heterogeneity, however, occurs among the six V lines in the magnitude of their fitness improvement in the alternating 32°C–42°C environment. (In a later section, we will consider whether these lines also differ in their relative adaptation to the constant and variable components of this temporally variable regime.) The grand mean fitness and its standard error, based on the six independently derived V lines, are 1.094 ± 0.012, so that the mean is significantly greater than one ($P < 0.001$, based on a two-tailed t-test with $n - 1 = 5$ degrees of freedom). This result confirms that of Bennett et al. (1992), who reported a mean fitness of 1.116 ± 0.011.

Adaptation of V Lines to the Constant and Transition Components of their Thermal Environment.—We obtained five estimates of fitness for each of the six independently derived V lines relative to the common ancestor, under each of four different thermal treatments: (A) acclimation and competition steps both at 32°C; (B) acclimation and competition steps both at 42°C; (C) acclimation step at 42°C and competition step at 32°C; and (D) acclimation step at 32°C and competition step at 42°C. Table 2 and figure 2 summarize these data. Columns A and B indicate that the V lines showed significant improvement in fitness, as a group, at both of the constant component temperatures. The average improvement at 42°C, ~20%, is much greater than the average improvement at 32°C, ~4%, and this difference is significant ($P = 0.031$ based on a two-tailed Wilcoxon signed-ranks test of the six line differences; $P < 0.001$ based on a two-tailed t-test for paired comparisons). Column D shows that the V lines also improved signifi-

TABLE 2. Fitness of V lines relative to common ancestor in constant and transition components of the variable thermal environment.

Derived line	Acclimation → Competition temperatures (°C) 32 → 32 (A)	42 → 42 (B)	42 → 32 (C)	32 → 42 (D)	(C + D) − (A + B)
V−1	1.053	1.163	1.008	1.185	−0.022
V−2	1.011	1.153	0.968	1.163	−0.033
V−3	1.022	1.216	1.005	1.130	−0.104
V+1	1.019	1.164	1.015	1.096	−0.072
V+2	1.059	1.243	1.124	1.149	−0.029
V+3	1.074	1.275	1.001	1.081	−0.267
Mean	1.040	1.202	1.020	1.134	−0.088
SE	0.011	0.022	0.022	0.016	0.042
P*	0.031	0.031	0.188	0.031	0.031
P†	0.015	0.0003	0.405	0.0004	0.090

NOTE.—The final column indicates the relative adaptation to the two variable and two constant component environments.

* Two-tailed significance based on Wilcoxon signed-ranks test ($n - 1 = 5$ df). The null hypotheses for each of the first four data columns are that mean fitness equals one; the null hypothesis for the final column is that the difference equals zero.

† Two-tailed significance based on t-test ($n - 1 = 5$ df). Null hypotheses are as in the previous footnote.

cantly, as a group, in their fitness at 42°C measured following a sudden transition from 32°C. However, column C indicates no significant adaptation by the V lines, as a group, to competition at 32°C following a sudden transition from 42°C. The average improvement at 42°C (following acclimation to 32°C), ~13%, is much greater than the average improvement at 32°C (following acclimation to 42°C), ~2%, and this difference is again significant ($P = 0.031$ based on a two-tailed signed-ranks test of the six line differences; $P = 0.011$ based on a two-tailed t-test for paired comparisons).

Partitioning Adaptation of the V Lines to the Constant and Transition Components of their Thermal Environment.—The previous analyses establish that the V lines were better adapted genetically to both of the component temperatures when they were acclimated to those temperatures. However, during their experimental evolutionary history, the V lines were subjected to a temperature each day that was different from the temperature to which they had been acclimated the previous day. Did the V lines also become better at making the transitions between temperatures? We compute an index of relative adaptation to the constant and transition components of the environment as the difference between quantities (C + D) and (A + B) (the sum of the transition fitness components minus the sum of the constant fitness components; see fig. 1 and table 2). This difference indicates that the V lines have not improved in their ability to make the transitions between the dissimilar temperatures. In fact, they have apparently gotten worse at making the two temperature transitions (table 2, final column: $P = 0.031$ for a nonparametric test; $0.05 < P < 0.1$ for the corresponding parametric test; the marginal failure of the parametric test to yield statistical significance is apparently due to the extreme negative value of line V + 3).

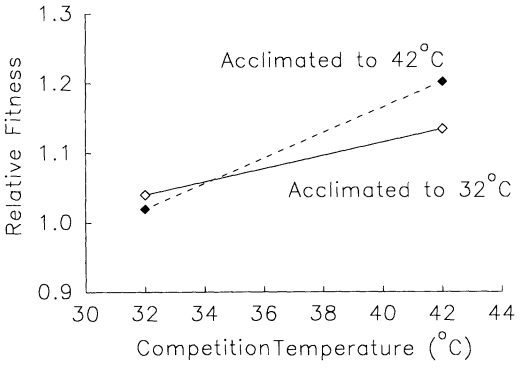

FIG. 2. Effect of temperature during the acclimation and competition steps on the mean fitness of the V lines relative to their common ancestor. Both competitors are separately acclimated to the same temperature, and then mixed at the competition temperature (which may be the same as or different from the acclimation temperature). The hollow symbols and solid line are for acclimation to 32°C; the filled symbols and dashed line are for acclimation to 42°C. See tables 2 and 3 for statistical analyses.

TABLE 3. Heterogeneity among V lines in relative adaptation to constant and transition components of their thermal environment.

Source	df	MS (×10²)	F	P
Among lines	5	4.325	2.870	0.036
Error	24	1.507		
Total	29			

NOTE.—Relative adaptation calculated as (C + D) − (A + B), where these quantities are defined in table 2. For each of the six V lines, we obtained five estimates of relative adaptation, with each estimate based on an independent set of these four quantities.

Heterogeneity Among V Lines in Relative Adaptation to the Constant and Transition Components.—Table 3 shows significant heterogeneity among the V lines in their relative

adaptation to the constant and transition aspects of the alternating 32°C–42°C regime. In particular, line V + 3, while the best adapted of the six V lines to the constant components, is actually the most poorly adapted to the transition components. Over all six lines, however, there is not a significant correlation between these two aspects, (A + B) versus (C + D) ($r = 0.124$, $n - 2 = 4$, $0.5 < P$).

Discussion

Adaptation of the experimental populations of *Escherichia coli* occurred during their propagation for 2000 generations in a temporally varying thermal environment. Such adaptation is not particularly surprising and was previously documented by Bennett et al. (1992). What is unexpected is the way in which the derived lines, as a group and individually, adapted to the various constant and transition components of this environment.

Adaptation to the variable environment was mediated principally through a fitness increase in its constant components.—The V lines are clearly superior in fitness relative to their ancestor when acclimation and competition temperatures are the same (table 2, columns A and B). This increase in fitness, which is manifest at both 32°C and 42°C, explains the overall fitness improvement of the V lines in the variable environment. Bennett and Lenski (1993) also found that the V lines have gained in fitness over a range of temperatures from 22°C to 42°C. These findings indicate that the V lines have truly become "jacks-of-all-trades" (or "jacks-of-all-temperatures," Huey and Hertz 1984). Fitness improvement was not, however, equal across temperatures. In particular, adaptation to 42°C was far greater than adaptation to 32°C. Similarly, fitness improvement of the ancestral genotype over 2000 generations to constant 42°C was found to be greater than to constant 32°C (Bennett et al. 1992).

No adaptation occurred to the transition components of the variable environment.—To the contrary, the V lines are apparently less able to take advantage of the sudden transitions between temperatures than is their common ancestor (table 2, final column). In particular, the ancestral fitness advantage of acclimation to 32°C, instead of acclimation to 42°C, prior to competition at 42°C (~20%, Leroi et al. 1994) has been diminished in the V lines. If the V lines had become better (or at least not worse) at making the transition from 32°C to 42°C, we expect that competition experiments at 42°C between V lines acclimated to 32°C and their ancestors acclimated to 42°C would yield fitnesses at least commensurate with combining the benefits of their *evolutionary adaptation* to 42°C (table 2, column B) and their *phenotypic acclimation* to the lower temperature (Leroi et al. 1994). In fact, when these experiments were conducted (Leroi 1993), it was observed that the fitnesses of the V lines relative to their ancestor were significantly below this expectation. This additional experiment therefore supports the conclusion from the experiment reported here (table 2) that the V lines have actually become worse in making the temperature transitions.

What could account for this unexpected diminution in acclimation ability? One possibility is that there may be pleiotropic tradeoffs between adapting to the constant and transition components of the temporally varying environment. Clearly, a mutation that conferred a competitive advantage at one temperature (irrespective of acclimation temperature) would be favored in a transition environment, even if the advantage happened to be larger in the corresponding constant environment than in the actual transition environment. Because the V lines showed much greater adaptation to 42°C than to 32°C (table 2), we decided to examine six additional lines that had evolved for 2000 generations at constant 42°C (Bennett et al. 1992) for evidence of a pleiotropic loss of performance in making the 32°C to 42°C transition. Preliminary experiments with these 42°C-selected lines indicate that the ancestral benefit of acclimating to 32°C prior to competition at 42°C had been substantially diminished in this group as well (Leroi, Lenski, and Bennett unpubl. data). These parallel results therefore support the hypothesis that adaptation to 42°C entails a loss of the ancestral acclimatory response. These results support Via's (1993) view that selection on direct responses to an environment may affect (negatively in this case) phenotypic plasticity as a correlated character.

Levins (1968, 1969), Feder (1978), and Tsuji (1988) argued that temporally variable environments should select for increased acclimation ability, or phenotypic flexibility. They found that the magnitude of phenotypic responses to different acclimation temperatures, when assayed at a common temperature, was greater in organisms from variable climates than those from constant climates. However, we observed unexpectedly that the effect of acclimation temperature

on the phenotype (as measured by its effect on competitive fitness) diminished in the populations that had evolved in a temporally variable regime. There are several possible explanations for this difference in result, including that earlier published comparisons were confounded by environmental factors other than the difference between variable and constant temperature regimes. Another possibility is that the temporally varying thermal regime used in this study was not heavily enough weighted towards the transition components. Although the transition components were quite frequent by human standards (every day), the bacteria nonetheless underwent six or seven cell generations at each temperature between these transitions. Perhaps if the transitions had been even more frequent, or if they had been gradual rather than sudden, we might have found evidence for an improvement in acclimation ability. More subtly, the evolution of acclimation ability may depend on the kind of trait under study. One school of thought stemming from cybernetics (Ashby 1956) and physiology (Cannon 1932) holds that, if fitness is to remain constant in the face of environmental variation, then other aspects of an organism's phenotype must change by way of compensation (Lewontin 1957; Caswell 1983).

Replicate lines differ in the basis of their fitness improvement in the thermally variable environment.—The significant between-line variation in table 1 indicates that the six independently derived V lines are heterogeneous in their overall fitness in the temporally varying environment. But that analysis cannot reveal whether the lines differ in their relative adaptation to the constant and transition components of the alternating 32°C–42°C regime. One possibility is that some lines simply had larger fitness gains because certain classes of mutation happened, by chance, to occur earlier in some replicate populations than in others (see Lenski et al. 1991). In that case, we might expect that, with enough time, all of the populations would converge on a similar overall fitness, even if their fitnesses are significantly heterogeneous at a particular time.

A more interesting possibility is that the replicate V lines diverged in the phenotypic bases by which they achieved their improved competitive fitness in the variable environment. In particular, some might be relatively better adapted to making the transitions between temperatures, and others better in the constant component temperatures. The analysis shown in table 2 indicates that the V lines, as a group, are significantly better adapted to the constant component temperatures than to the transition components. There is, however, also significant heterogeneity among lines in their relative adaptation to these aspects of their environment (table 3). This heterogeneity in the relative importance of different phenotypic components of performance indicates the possibility of multiple physiological routes by which adaptation can occur to a temporally varying environment, some of which involve adaptation to transition components while others do not.

In summary, the experiments reported here do not support the hypothesis of Levins (1968, 1969), Feder (1978), and Tsuji (1988) that evolution in a temporally varying environment will favor increased acclimation ability or phenotypic flexibility. Instead, it appears that bacterial adaptation to the constant component temperatures was more important than, and may even have traded off with, adaptation to sudden transitions in temperature. Nonetheless, our results do indicate that phenotypic acclimation ability itself has an underlying genetic basis and so may respond evolutionarily. However, the direction of evolution in acclimation ability may be more complex than simple models would suggest, so that predicting the evolution of acclimation ability may depend on detailed knowledge of genetic and environmental correlations.

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