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EVOLUTION OF THERMAL DEPENDENCE OF GROWTH RATE OF *ESCHERICHIA COLI* POPULATIONS DURING 20,000 GENERATIONS IN A CONSTANT ENVIRONMENT

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Abstract.—Twelve experimental populations of the bacterium *Escherichia coli* evolved for 20,000 generations in a defined medium at 37°C. We measured their maximum growth rates across a broad range of temperatures and at several evolutionary time points to quantify the extent to which they became thermal specialists with diminished performance at other temperatures. We also sought to determine whether antagonistic pleiotropy (genetic trade-offs) or mutation accumulation (drift decay) was primarily responsible for any thermal specialization. Populations showed consistent improvement in growth rate at moderate temperatures (27–39°C), but tended to have decreased growth rate at both low (20°C) and high (41–42°C) temperatures. Most loss occurred early in the experiment, when adaptation was most rapid. This dynamic is predicted by antagonistic pleiotropy but not by mutation accumulation. Several populations evolved high mutation rates due to defects in their DNA repair, but they did not subsequently undergo a greater decrease in growth rate at thermal extremes than populations that retained low mutation rates, contrary to the acceleration of decay predicted by mutation accumulation. Antagonistic pleiotropy therefore is more likely to be responsible for the evolution of thermal specialization observed in maximum growth rate.

Key words.—Antagonistic pleiotropy, bacteria, mutation accumulation, specialization, thermal niche.

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Temperature is one of the most important physical factors that defines a species' fundamental niche. Temperature limits some species' distributions (Stevens and Fox 1991; Hoffmann and Watson 1993; Clarke 1996) because both extreme heat and cold can adversely affect metabolic and life-history traits (Hochachka and Somero 1984; Cossins and Bowler 1987; Prosser 1991; Ingraham and Marr 1996). Several important physiological changes that involve differential expression of various proteins (e.g., the heat-shock response) have also been found to be associated with changing temperature (Herendeen et al. 1979; Gross 1996; Ingraham and Marr 1996). Such effects should lead to optimization of metabolic and demographic traits over the range of temperatures most often experienced by a population; if this range is narrow, then thermal specialization may evolve. This hypothesis frequently presumes an evolutionary trade-off between capacity to tolerate a broad range of temperatures and peak performance over a narrow range, but this trade-off lacks empirical support (Huey and Hertz 1984).

Laboratory populations of the bacterium *Escherichia coli* have been employed to study the evolution of thermal performance because of the feasibility of performing long-term selection experiments (Bennett et al. 1992; Bennett and Lenski 1993; Mongold et al. 1996, 1999). These studies have examined genetic adaptation to various temperatures as well as the correlated effects of this adaptation on performance at other temperatures. Not surprisingly, experimental populations always became better adapted to the temperatures at which they evolved. However, their correlated responses at other temperatures varied depending on the selective temperature and even varied among the replicate populations maintained at the same selective temperature. For example, populations that evolved at 20°C systematically became inferior competitors at temperatures above 40°C (Mongold et al. 1996), whereas five of the six populations that adapted to 42°C did not become inferior competitors at low temperatures (Bennett and Lenski 1993). These evolutionary experiments therefore provide ambiguous support for the existence of trade-offs in performance across the thermal niche. However, because thermal tolerance varies greatly among natural taxa, the idea that selection at one temperature will reduce performance at other temperatures remains popular.

In this study, we examine the performance of 12 experimental populations of *E. coli* that have evolved at 37°C for 20,000 generations (Lenski et al. 1991; Lenski and Travisano 1994; Cooper and Lenski 2000). We test whether they became temperature specialists by comparing their maximum growth rate with that of the ancestor across a range of temperatures. Our study parallels other research on these same populations that examines whether they became resource specialists during their evolution in a medium in which glucose was their sole source of carbon and energy (Cooper and Lenski 2000). By examining performance breadth across a range of temperatures and resources, we test the general theory that evolutionary adaptation to one particular environment leads to loss of performance in alternative environments (Futuyma and Moreno 1988; Holt 1996), and we address whether adaptation to a constant environment similarly affects these two different dimensions of the niche.

The physiological functions that govern thermal performance and resource usage are, of course, quite distinct. Both functions, however, should be subject to the same fundamental population-genetic processes that can lead to specialization: antagonistic pleiotropy and mutation accumulation. Under antagonistic pleiotropy, adaptation to the selective environment and loss of function in other environments are caused by the same mutations, which mediate a trade-off between performance across environments. In contrast, under mutation accumulation, adaptation and loss of function are caused by different mutations. Adaptive mutations are fixed by selection but do not themselves cause any loss of function

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in the other environments; such losses are caused instead by mutations that spread by random drift in genes no longer under positive selection.

These two distinct processes have long been recognized, but their relative contributions have only rarely been examined because they are difficult to disentangle (Rose and Charlesworth 1980; Futuyma and Moreno 1988; Sgrò and Partridge 1999). However, the two processes should lead to different dynamics of functional decay (Cooper and Lenski 2000). Antagonistic pleiotropy predicts a temporal association between improvement in the selective environment and decay in alternative environments. Thus, if adaptation to a selective environment is initially fast but decelerates over time, then so too should the rate of functional decay be initially more rapid. In contrast, functional decay arising from mutation accumulation occurs by stochastic drift of neutral alleles; it should therefore occur at a constant rate that depends only on the mutation rate in relevant genes (Kimura 1983). In an asexual population, selection at other loci will cause periodic bottlenecks that influence the dynamic of any particular neutral mutation but not their overall rate of substitution, which is independent of effective population size. If the mutation rate itself should increase (see below), then the rate of mutation accumulation will increase correspondingly, even if the rate of adaptation is unaffected.

Materials and Methods

Experimental Overview

The 12 populations studied here are all derived from a single clone of *E. coli* B (Lenski et al. 1991). The populations evolved in and adapted to a constant 37°C environment for 20,000 generations (3000 days). We isolated three clones at random from each population at five time points (2000, 5000, 10,000, 15,000, and 20,000 generations), giving a set of 180 clones (12 populations × 5 time points × 3 clones) plus two ancestral clones that differ only by a neutral mutation used as a marker. We measured the maximum growth rate, V_{max}, of each clone at nine temperatures, ranging from 20°C to 42°C; growth rates were measured in microtiter plates to handle all the clones simultaneously. (V_{max} is a microbiological term; it is equivalent to r or r_{max}, which is more commonly used in the ecological literature.) Maximum growth rate is not the sole component of fitness in the serial transfer regime in which the bacteria evolved, but both theoretical and empirical analyses indicate it was the most important component (Vasi et al. 1994).

The 12 populations experienced different mutation rates for part of their history, a circumstance that, as noted above, has implications for the rate of functional decay expected under mutation accumulation. Sniegowski et al. (1997) demonstrated that three populations evolved defects in DNA mismatch repair and, as a consequence, had roughly 50-fold higher genomic mutation rates than the other populations or their common ancestor. These populations became mutators around 2500, 3000, and 8500 generations (Sniegowski et al. 1997), and all three remained mutators at generation 20,000 (P. D. Sniegowski, pers. comm.). Therefore, they experienced substantially elevated mutation rates for the majority of the experiment. A fourth population also became a mutator, but only much later; this mutator was first seen at generation 16,500 and did not become fixed for another 1500 generations (P. D. Sniegowski, pers. comm.). Thus, this line spent most of its history with the ancestral mutation rate.

Bacteria

Genetically heterogeneous samples from each experimental population were obtained every 500 generations. From the samples obtained at generations 2000, 5000, 10,000, 15,000, and 20,000 generations, aliquots of cells were spread on nutrient-rich agar plates and three clones were randomly chosen from each. The two ancestral variants differ only by a spontaneous mutation that affects arabinose utilization. This mutation was used as a marker in the evolution experiment and in competition assays, in which the mutation is selectively neutral (Lenski et al. 1991). Six of the evolving populations were founded from the Ara^- ancestor and six from the Ara^+ variant. The ancestors as well as the genetically heterogeneous samples and clones from the evolving populations were stored frozen at −80°C.

Assays

Clones were inoculated into 30-ml test tubes containing 10 ml of Davis minimal medium (Lenski et al. 1991) supplemented with 500 μg glucose/ml (DM500), and the cultures were then incubated for 24 h at 37°C in a shaking incubator. Each culture was then diluted 1:100 into fresh DM500 and incubated for 24 h at the relevant assay temperature, such that all clones were comparably acclimated to the temperature and growth medium in which their growth rates would be measured. The acclimated cultures were diluted 1:100 into fresh DM500, and 250 μl of each new culture was then transferred to one of two 100-well microtiter plates that fit simultaneously into a Bioscreen (Labsystems, Helsinki, Finland). The Bioscreen is a spectrophotometric plate reader that allows precise temperature control and agitates the plates to maintain aeration. In pilot experiments for this study, the performance of the ancestor was assessed at various well locations to test for site-dependent variation, but none was detected. Six replicates of both ancestral variants were interspersed among the 180 derived clones at each assay temperature, as were eight blank wells. The optical density (OD) of each well was measured every 10 min for 16 h.

The environment in which the experimental bacterial populations evolved consists of shaken 50-ml flasks that contain 10 ml of minimal medium with 25 μg glucose/ml. The scale of these experiments necessitated two modifications: a greater nutrient concentration (500 μg glucose/ml) was required to produce concentrations of bacteria that could be accurately read by the Bioscreen, and growth was measured in microtiter plates rather than flasks to enable greater replication. A separate investigation of these populations found that adaptation was concentration specific, with significantly increased fitness relative to the ancestor at both higher (250 μg/ml) and lower (2.5 μg/ml) concentrations of glucose, although the greatest improvement was seen at the concentration of 25 μg glucose/ml experienced during the evolution experiment (Cooper 2000). However, we found no association between performance at foreign temperatures (this study) and fitness

at high glucose concentrations from that earlier study (data not shown). Therefore, it seems unlikely that this aspect of the assay environment would bias our estimation of the thermal niche of these populations in any systematic manner. Nonetheless, the results presented here represent only an approximation of the consequences of adaptation to a specific temperature, rather than a direct measure of fitness.

Numerical and Statistical Analyses

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

We computed the mean V_{max} at a given temperature and generation of the three clones from each population; we also averaged the six replicate measurements for each ancestral variant. A two-tailed *t*-test was then performed to test whether the 12 derived populations, on average, grew significantly faster or slower than the two ancestors at a particular temperature. We used Welch's approximate *t*-test, which assumes unequal variances, because the ancestors are genetically homogeneous whereas the evolved populations have diverged from one another. In principle, one might adjust the significance levels of these tests to reflect the fact that multiple tests were performed across many generations and temperatures. However, we have chosen not to do so because the results across generations are generally consistent with one another, and the results across temperatures indicate that the differences fall consistently into only three distinct thermal ranges: low (20°C), moderate (27–39°C), and high (40–42°C). We also employed Welch's approximate *t*-test to compare average V_{max} values for the mutator and nonmutator populations at a given temperature and generation. We excluded the population that became a mutator late in the experiment from the 20,000 generation comparison, because of the ambiguity of assigning it to either class with respect to the possible effect of mutation accumulation. The among-population genetic variance was estimated using the VARCOMP procedure in SPSS (ver. 8.0, SPSS, Inc., Chicago). All other statistical analyses were also conducted using SPSS.

RESULTS

Figure 1 reports the V_{max}, as a function of temperature, of the ancestral *E. coli* and the mean values of the derived lines after 20,000 generations. Figure 2 shows the average V_{max} of the evolving populations across generations and temperatures; these averages are all expressed relative to the ancestral value at each temperature.

Growth Rates Improved at Moderate Temperatures

V_{max} increased in the evolving populations at all five assay temperatures from 27°C to 39°C (Figs. 1, 2). After 20,000 generations, the greatest improvement of V_{max} in the derived populations relative to the ancestor occurred at 37°C, the selective temperature during the evolution experiment (Fig. 1). However, growth rate in these populations improved almost as much at slightly lower and slightly higher temperatures (Fig. 2). All but one of the 25 statistical comparisons between the derived populations, as a group, and the ancestors were significant at $P < 0.05$ across this range of temperatures and at each generation sampled (Fig. 3). Most of these correlated improvements in growth rate arose during the first 5000 generations, as indicated by the overall flattening of the temporal curves for these temperatures (Fig. 2).

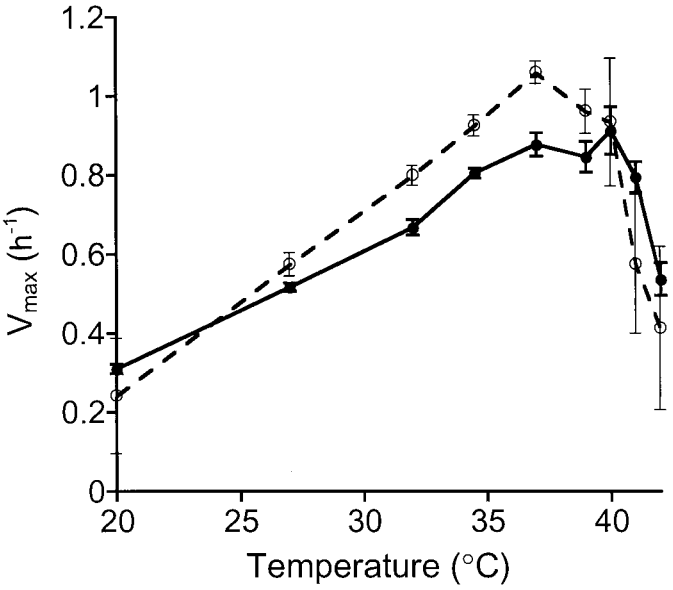

FIG. 1. Maximum growth rate, V_{max}, of the ancestral *Escherichia coli* (filled circles, solid line) and evolved populations at generation 20,000 (open circles, dashed line) as a function of temperature. Error bars represent 95% confidence intervals, based on 12-fold replication (ancestors: six assays for each of the two neutrally marked variants; evolved: 12 populations).

Growth Rates Declined at Extreme Temperatures

V_{max} measured at more extreme temperatures, both low (20°C) and high (41°C and 42°C), declined in many of the evolving populations (Fig. 2). As with the gains seen at moderate temperature, most of the reductions in V_{max} at the thermal extremes occurred in the first few thousand generations (Fig. 2). The decline at 20°C was significant at $P < 0.05$ at three of the five generations sampled, and the decline at 41°C was significant at two of these time points (Fig. 3). At each extreme temperature, three or more of the derived populations had higher growth rates than the ancestor (Fig. 3), but these populations were only marginally better than the ancestor and influenced the mean value less than the more substantial declines in some other populations. Although the proportional decay measured at 42°C was similar in magnitude to that at 41°C, the difference at the higher temperature was nonsignificant in each generation. As shown in the next section, the inconsistency of the decline at extreme temperatures—in contrast to the consistency of the improvements at moderate temperatures—reflects greater genetic variation among the derived populations at the extreme temperatures.

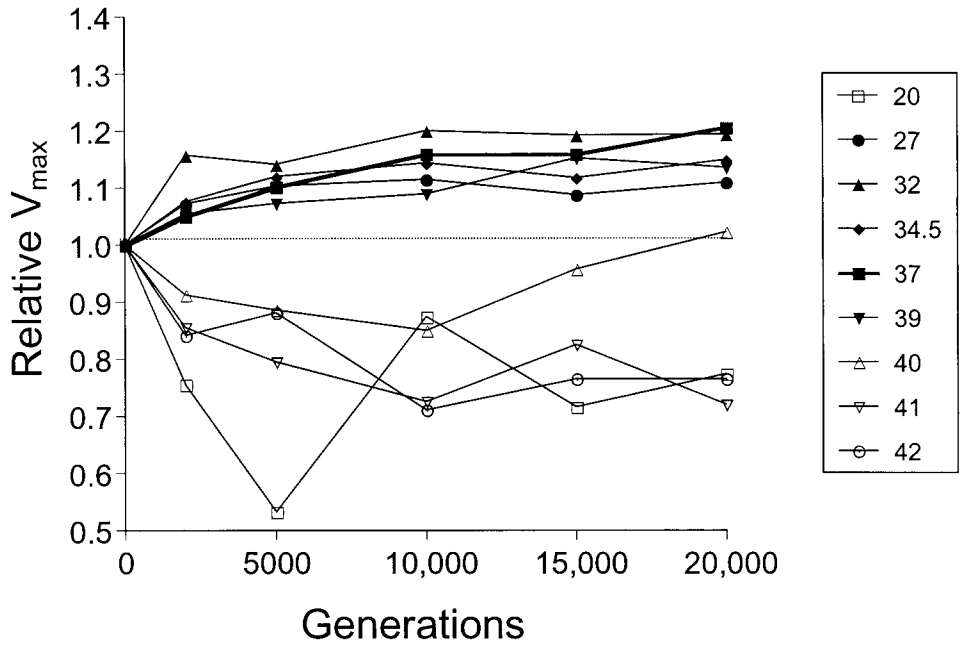

FIG. 2. Evolution of maximum growth rate, V_{max}, scaled relative to the ancestor, measured at nine different temperatures (see boxed legend). Each point is the grand mean of 12 replicate populations, with each population's mean based on three clones.

More Genetic Variance among Derived Populations at Extreme Temperatures

We estimated the among-population genetic variance at each time point and temperature assayed (Fig. 4). The roughly U-shaped pattern indicates that the replicate derived populations generally had more similar growth rates at moderate temperatures, with genetic variation in growth rates increasing at both low and high extreme temperatures.

The greater variance among the derived populations at extreme temperatures resulted, in large part, from certain clones that failed to attain measurable growth rates. In some cases, only one or two of the three clones from a particular population sample failed to grow; we cannot say whether this clonal variation reflects within-population genetic variation or experimental noise, because we assayed each clone only once per temperature. However, there were 13 cases in which all three clones from a population sample failed to grow, and all of these cases occurred at extreme temperatures (20°C, 41°C, or 42°C).

Spurious Difference between Mutator and Nonmutator Populations at Extreme High Temperatures

We compared growth rates of the mutator and nonmutator populations to examine the impact of increased mutation rate, and hence mutation accumulation, on the lower growth rates at extreme temperatures. We used the 20,000-generation sam-

	Generations				
Temperature (°C)	2000	5000	10,000	15,000	20,000
20	-1.96	-3.56	-2.26	-2.32	-1.05
27	7.52	4.07	2.68	2.34	4.19
32	10.51	7.41	10.39	10.83	10.81
34.5	14.38	13.98	9.98	6.54	9.53
37	3.17	5.60	7.95	7.13	10.33
39	3.34	4.68	2.87	8.91	4.16
40	-1.32	-1.76	-1.58	-0.62	0.26
41	-1.62	-3.14	-2.10	-1.46	-2.26
42	-1.02	-0.77	-1.75	-1.32	-1.31

FIG. 3. Statistical summary of the effects of evolution at constant 37°C on maximum growth rates measured at nine temperatures. For each generation and temperature, the mean of the 12 replicate populations was compared with the ancestral mean using a two-tailed *t*-test that assumes unequal variances. Black shading indicates that the derived populations, as a group, grew significantly more slowly than the ancestor ($P < 0.05$). Gray shading indicates the derived populations, as a group, grew significantly faster than the ancestor ($P < 0.05$). Each cell also shows the corresponding *t*-statistic; positive values indicate that the evolved populations grew faster, on average, and negative values indicate that evolved populations grew slower, on average, than did the ancestors.

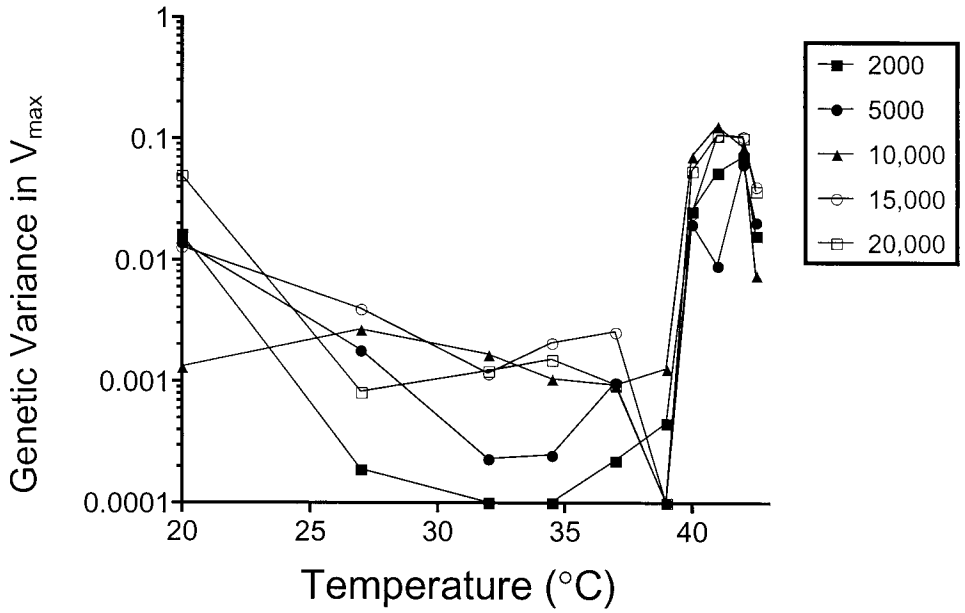

FIG. 4. Genetic variance among populations in V_{max} measured across temperatures. The different symbols represent variances estimated in different generations (see boxed legend). Note the logarithmic scale for the vertical axis. Estimated variances ≤ 0.0001 are shown as 0.0001. The among-population genetic variance component was estimated as the variance among derived populations in excess of the average clonal variance within each population (Sokal and Rohlf 1995).

ples, because they should provide the maximum signal associated with mutation accumulation in the three populations that became mutators between 2000 and 10,000 generations. The mutators performed significantly worse than did the nonmutators at 40°C, 41°C, and 42°C (Fig. 5A). The mutators did not have significantly lower growth rates than the nonmutators at any other temperature tested, including the low extreme of 20°C (two-tailed $P = 0.463$). However, the deficiency in V_{max} of the mutator populations at high temperatures cannot be attributed to their mutator status per se, because the growth-rate differences between the three populations that would become mutators and the other nine populations had already arisen by generation 2000 (Fig. 5B), which was prior to the evolution of the mutator genotype in any of the derived lines. Therefore, the association between mutator populations and reduced growth rate at high temperatures appears to be spurious rather than indicative of any direct causal relationship.

DISCUSSION

We studied genetic changes in the thermal dependence of maximum growth rate of *E. coli* populations during 20,000 generations of experimental evolution at constant 37°C. Our main results can be summarized as follows. (1) Populations showed consistent increases in V_{max} at moderate temperatures, ranging from 27°C to 39°C. (2) Most of this improvement evolved during the initial period of rapid adaptation. (3) Some populations tended to decay in V_{max} at both low and high thermal extremes. (4) Most of this decay also occurred during the initial period of rapid adaptation. (5) There was more genetic variation among the derived populations in V_{max} at both thermal extremes than at moderate temperatures. In some populations, there was no measurable growth at one of the extreme temperatures. (6) There appeared to be a positive association between the decline in growth rate at high temperature and the evolution of an elevated mutation rate, which occurred in several populations. However, this association is evidently spurious because the decline in growth rate at high temperature was already manifest in these same populations even before they became mutators.

These results support, in broad outline, the hypothesis that adaptation to a constant temperature leads to thermal specialization, with reduced performance at extreme temperatures but enhanced performance at intermediate temperatures. It is important to keep in mind, however, that this analysis is based on a single fitness component, maximum growth rate. In spite of the presumptive importance of V_{max} for fitness (Vasi et al. 1994), further studies on relative fitness (based on direct competitions), thermal niche breadth, and stress resistance in these derived lines are required before the consequences of these changes are fully known. Moreover, it is unclear whether the increased glucose concentration used for these assays or the microtiter plates themselves might have altered the temperature dependence of growth rate in these populations. (Some indirect evidence mentioned in the Materials and Methods section indicates that the effect of glucose concentration was probably not responsible for the patterns reported in this study.) These qualifications serve to emphasize the value of precise experimental manipulation to isolate the effect of a single environmental variable on fitness.

One might be surprised by how little performance was affected at the thermal extremes, and by the breadth of temperatures over which the correlated improvements occurred. These quantitative associations presumably depend on the underlying physiology of the organism as well as the evolutionary duration of environmental constancy. We might

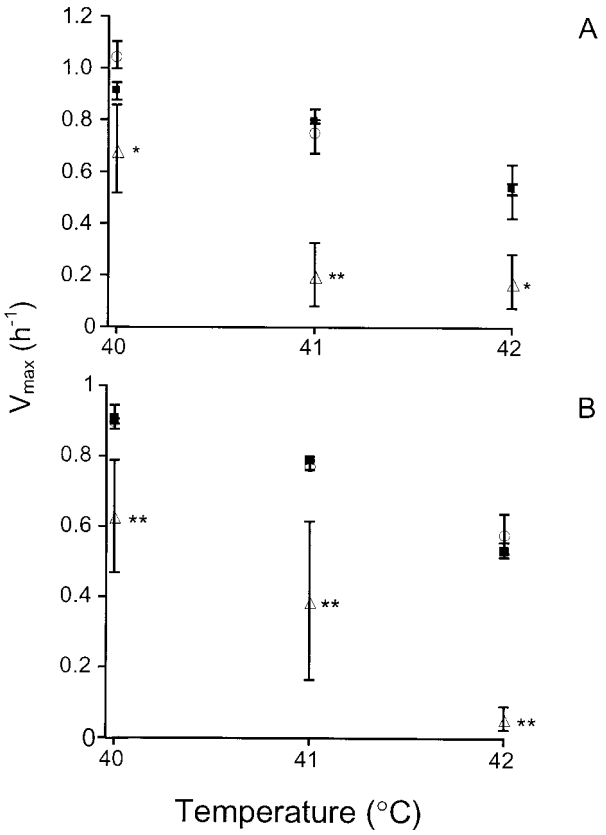

FIG. 5. Differences in V_{max} between mutator and nonmutator populations at high temperatures. Black squares, ancestral bacteria; open circles, derived populations that retained the ancestral mutation rate; open triangles, derived populations that became mutators between generations 2500 and 8500. Error bars are standard errors. (A) Data at generation 20,000. (B) Data at generation 2000, which was prior to the evolution of the mutators. Mutator and nonmutator means were compared using a one-tailed Welch's approximate *t*-test (* $P < 0.05$, ** $P < 0.01$).

have observed greater loss of performance at the thermal extremes, and perhaps losses over a wider range of temperatures, during a much longer period of evolution.

We now consider the population-genetic processes that can best explain the observed pattern of evolutionary change in V_{max}. A fundamental issue in population genetics—but one that remains poorly resolved—is whether the same beneficial mutations that enhance performance in a selective environment (here, 37°C) are, through their pleiotropic effects, also responsible for the changes in performance in foreign environments (here, all other temperatures). Alternatively, other mutations that are neutral in the selective environment may accumulate by drift and be responsible for changes in performance in the foreign environments.

Focusing first on the correlated improvement in performance at moderate temperatures, it seems obvious that these must be beneficial pleiotropic effects of the mutations that enhanced growth at 37°C. In any environment, it is generally accepted that many more mutations are deleterious than are advantageous. If the random accumulation of mutations that are neutral at 37°C were responsible for the performance gains at other moderate temperatures, then we would have to suppose that many more unselected mutations were beneficial than deleterious at these temperatures. It is much more likely that many of the mutations that adapt the bacteria to 37°C also improve performance at somewhat higher and lower temperatures. Exactly how far in either direction this positive correlation extends may depend, as noted above, on physiological details and evolutionary circumstances. In addition to this intuitive reasoning, three lines of evidence support the conclusion that the performance gains across moderate temperatures were pleiotropic effects of the mutations that enhanced fitness at 37°C. First, the magnitude of improvement in growth rates between 32°C and 39°C was quite similar to the improvement at 37°C itself (Fig. 2). Second, the gains at moderate temperatures were temporally correlated with genetic adaptation; that is, these gains tended to occur early in the evolution experiment, as did adaptation to 37°C (Fig. 2). Third, the low genetic variance among populations in growth rates at moderate temperatures (Fig. 4) is consistent with parallel selection on the traits that underlie performance in this thermal range.

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

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

Turning back to the interpretation of our experimental data, we have shown previously, by competing ancestral and derived bacteria, that the rate of fitness increase during the first few thousand generations was an order of magnitude faster

than during the later generations of this experiment (Lenski and Travisano 1994; Cooper and Lenski 2000). The losses of function at extreme temperatures also generally occurred early in the experiment (Fig. 2), a temporal association consistent with antagonistic pleiotropy but not mutation accumulation. At 20°C, the average growth rate declined by 25% (from 0.310 to 0.234 per hour) by generation 2000 but did not decline further over the next 18,000 generations. At 42°C, the average growth rate declined by 16% (from 0.536 to 0.451 per hour) in the first 2000 generations but declined only 9% more (from 0.451 to 0.410 per hour) between 2000 and 20,000 generations.

Antagonistic pleiotropy and mutation accumulation are not mutually exclusive, and therefore evidence in support of antagonistic pleiotropy does not exclude the possibility that mutation accumulation might also contribute to the evolution of specialization. If mutation accumulation were a significant force, then its effect should have been much greater in those populations that acquired defects in DNA repair and thus became mutators. Three populations evolved into mutators before generation 10,000, leaving more than 10,000 subsequent generations for their 50-fold higher mutation rates to generate mutations that harmed performance at the thermal extremes (but which were neutral at 37°C). At generation 20,000, the mutator populations grew more slowly at high temperatures (40–42°C) than did the nonmutators (Fig. 5A), seemingly consistent with a role for mutation accumulation. However, on closer examination, the difference between the two sets of populations is spurious, because the lower growth rates of the mutators were already manifest at generation 2000 (Fig. 5B), before any of the populations had become mutators (Sniegowski et al. 1997). In other words, the three populations that would later become mutators evolved lower growth rates at high temperatures before they evolved into mutators. The statistical association thus appears to be a false positive, although we cannot exclude the possibility that loss of high-temperature performance somehow predisposes a population to lose its DNA repair at a later time. In the absence of a reasonable mechanism to explain this association, this pattern remains a coincidence, albeit an intriguing one. In any case, these early losses of performance at high temperature cannot be explained by additional mutation accumulation in mutators. Instead, they support antagonistic pleiotropy as the main population-genetic process responsible for specialization, because they reinforce the observation that most of the decay at high temperatures occurred during the period of most rapid adaptation.

A related process that could lead to an association between adaptation and reduced performance in other environments is genetic hitchhiking of slightly deleterious mutations (as opposed to those that are strictly neutral in the selective environment). Slightly deleterious mutations that more severely affect performance at extreme temperatures may have been fixed because they became linked to a second, strongly beneficial mutation. These two separate mutations would therefore be phenotypically indistinguishable from a single beneficial mutation with antagonistic effects at foreign temperatures. This hitchhiking scenario would generate a signal similar to that expected under antagonistic pleiotropy, in which most losses of function would be temporally associated with adaptation. We cannot absolutely exclude this possibility in the present case of thermal adaptation. However, in a related study on the evolution of resource specialization in these very same *E. coli* populations (Cooper and Lenski 2000), two lines of evidence were presented that run to counter to this scenario and instead support antagonistic pleiotropy. First, there was strong parallelism across populations in the extent of decay of their capacity to use 64 foreign resources, whereas such parallelism is not expected under the hitchhiking scenario. (We cannot apply similar reasoning to our results on the thermal niche, because the high and low temperature extremes comprise only two foreign environments.) Second, a set of beneficial mutations in glucose were shown to have antagonistic pleiotropic effects on performance on another resource, ribose (Cooper et al. 2000). Although we lack comparable evidence to exclude this hitchhiking scenario in the case of thermal adaptation, it seems a reasonable working assumption that the same population-genetic processes would govern the evolution of resource and thermal specialization in the very same set of populations.

The high among-population variance in growth rate at extreme temperatures indicates an absence of selection for maintaining performance in these environments (Fig. 4). This finding is consistent with either mutation accumulation or antagonistic pleiotropy, and thus provides no compelling evidence with respect to their relative influence. According to mutation accumulation, divergence of replicate populations would indicate random drift. According to antagonistic pleiotropy, this divergence would imply heterogeneity among beneficial mutations selected at 37°C with respect to their pleiotropic effects on performance at the thermal extremes. Without isolating the particular mutations that produced adaptation to 37°C, we cannot exclude either explanation for the high among-population variance in performance at the extreme temperatures. However, as discussed above, the temporal dynamics of decay suggest that antagonistic pleiotropy was primarily responsible for the losses of performance at the thermal extremes.

In conclusion, our results show that, in terms of maximum growth rates, these bacteria became thermal specialists relative to their ancestor during 20,000 generations of evolution at a constant temperature. In addition, the data suggest that antagonistic pleiotropy—in which mutations that improved performance at moderate temperatures reduced V_{max} at extreme temperatures—was the main population-genetic force leading to ecological specialization. Both of these conclusions run parallel to, and thus reinforce, findings from a recent study of the evolution of catabolic function in these same *E. coli* populations, which found that unused catabolic functions tended to decline and that antagonistic pleiotropy was primarily responsible for catabolic specialization (Cooper and Lenski 2000).

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