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EVOLUTIONARY ADAPTATION TO TEMPERATURE. VII. EXTENSION OF THE UPPER THERMAL LIMIT OF *ESCHERICHIA COLI*

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Abstract.—What factors influence the ability of populations to adapt to extreme environments that lie outside their current tolerance limits? We investigated this question by exposing experimental populations of the bacterium *Escherichia coli* to lethally high temperatures. We asked: (1) whether we could obtain thermotolerant mutants with an extended upper thermal limit by this selective screen; (2) whether the propensity to obtain thermotolerant mutants depended on the prior selective history of the progenitor genotypes; and (3) how the fitness properties of these mutants compared to those of their progenitors within the ancestral thermal niche. Specifically, we subjected 15 independent populations founded from each of six progenitors to 44°C; all of the progenitors had upper thermal limits between about 40°C and 42°C. Two of the progenitors were from populations that had previously adapted to 32°C, two were from populations adapted to 37°C, and two were from populations adapted to 41–42°C. All 90 populations were screened for mutants that could survive and grow at 44°C. We obtained three thermotolerant mutants, all derived from progenitors previously adapted to 41–42°C. In an earlier study, we serendipitously found one other thermotolerant mutant derived from a population that had previously adapted to 32°C. Thus, prior selection at an elevated but nonlethal temperature may predispose organisms to evolve more extreme thermotolerance, but this is not an absolute requirement. It is evidently possible to obtain mutants that tolerate more extreme temperatures, so why did they not become prevalent during prior selection at 41–42°C, near the upper limit of the thermal niche? To address this question, we measured the fitness of the thermotolerant mutants at high temperatures just within the ancestral niche. None of the four thermotolerant mutants had an advantage relative to their progenitor even very near the upper limit of the thermal niche; in fact, all of the mutants showed a noticeable loss of fitness around 41°C. Thus, the genetic adaptations that improve competitive fitness at high but nonlethal temperatures are distinct from those that permit tolerance of otherwise lethal temperatures.

Key words.—Adaptation, bacteria, *Escherichia coli*, fitness, mutation, niche, temperature, thermotolerance.

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All organisms have a limited range of abiotic environments in which they can persist. Some ranges are broad and others are quite narrow, but beyond these limits, reproduction and survival become impossible. A familiar and biologically important example is the range of temperatures tolerated by an organism. Exposure to temperature above the upper limit of the thermal niche typically causes rapid death. How can populations expand their tolerance limits so that they can colonize such lethal environments? According to one hypothesis, as populations gradually adapt genetically to high but nonlethal temperatures, their performance at even higher temperatures will also improve (Huey and Kingsolver 1989). Consequently, their upper limit increases along with their thermal optimum. Hence, we call this the "sliding niche" hypothesis. Many evolutionary and ecological models implicitly accept the validity of this hypothesis by assuming that the total area under the fitness function is constant (Levins 1968; Huey and Slatkin 1976; Lynch and Gabriel 1987; Pease et al. 1989). However, extension of the upper thermal limit in response to selection at high temperature does not always occur (Bennett and Lenski 1993). An alternative hypothesis is that adaptation to high but nonlethal temperature predisposes the evolution of thermotolerance once a population is exposed to a lethally high temperature. In other words, the extension of the thermal niche does not occur during selection at the nonlethal temperature, but the genotypes that are selected there may interact favorably with other mutations that can give rise to thermotolerance at more extreme temperatures. We call this the "stepping stone" model of thermal niche evolution. A third possibility, of course, is that the upper limit of an organism's thermal niche is so highly constrained that a population exposed to a more extreme temperature cannot evolve but instead goes extinct.

This study investigates evolutionary adaptation to a lethal thermal environment in a model system comprising replicated clonal lines of the bacterium *Escherichia coli*. We have previously studied adaptation of populations to temperatures across the ancestral thermal niche (extending from about 19.5 to 42.2°C) in experimental culture conditions (reviewed in Mongold et al. 1996b). The upper thermal limit did not significantly and systematically increase in any group of derived lines, even those that evolved at 41–42°C. Exposure to 44°C was invariably lethal to all of the selected lines. However, during the determination of this lethality, we obtained a single thermotolerant mutant—designated "Lazarus"—that grew during exposure to 44°C (Bennett and Lenski 1993). Having obtained one such mutant, we initiated the present study to obtain others and examine their properties. We imposed a lethal selective screen of 44°C on replicated cultures of six lines that had previously adapted to different temperatures, two each from 32°C, 37°C, and 41–42°C. This study examines the following three questions.

Can additional thermotolerant mutants be obtained with this lethal selective screen? The Lazarus mutant was obtained serendipitously, and similar mutations may be extremely rare. Although one laboratory has reported that such thermotol-

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erant mutants can be readily obtained (Droffner and Yamamoto 1991, 1992), they used very different selective conditions that are not comparable to those that we have employed in our research. Therefore, we sought to isolate additional mutants under controlled and replicated conditions similar to those in our earlier work.

Is the frequency of such mutations influenced by the progenitor's prior selective history? As noted above, significant extensions of the upper thermal limit did not occur in our previous evolutionary experiments. However, it is possible that some selective regimes gave rise to derived lines whose genetic backgrounds now support the expression of thermotolerance mutations, which would not be sufficient on other backgrounds to result in an extension of the thermal niche. Therefore, we investigated whether the propensity of a line to yield thermotolerant mutants depends on its prior selective history. In particular, did adaptation to a high but tolerable temperature (41–42°C) act as a "stepping stone" to allow adaptation to even more extreme high temperatures?

What are the fitness properties, within the ancestral niche, of thermotolerant mutants relative to their progenitors? If thermotolerant mutants exist, then why did they not accumulate in the populations that were adapting to high but nonlethal temperature? One possible explanation is the existence of significant tradeoffs in adapting to nonlethal versus lethal high temperatures, such that thermotolerant mutants are less fit than their progenitors within the ancestral thermal niche, even at high temperature. To determine whether such tradeoffs exist, we measured the competitive fitness of each mutant against its immediate progenitor across the ancestral thermal niche. We also measured the thermal niche (i.e., range of temperatures that allow population persistence) for the thermotolerant mutants and their progenitors.

Materials and Methods

Bacterial Lines and Culture Media

All of the bacteria used in this study were derived from a single ancestral clone. This clone (here designated "Anc") was itself isolated from a population of *E. coli* B that had been serially propagated in glucose-limited medium for 2000 generations at 37°C (Lenski et al. 1991). The Anc genotype was used to found five treatment groups, each one consisting of six replicate lines (Bennett et al. 1992; Mongold et al. 1996a). These lines evolved for 2000 more generations in the same culture conditions, except that each group was propagated under a different thermal regime. Alternating lines within each group were marked with a selectively neutral marker for arabinose utilization. This marker allowed us to exclude the possibility of contamination between lines. The marker also allowed us to measure adaptation in the evolving lines by placing derived clones in competition with the ancestor expressing the opposite marker state. The adaptation of these treatment groups to their selective regimes and their correlated responses in other thermal regimes have been previously reported (Bennett et al. 1992; Bennett and Lenski 1993, 1996; Mongold et al. 1996a). Clones from six evolved lines—two each from the groups maintained at 32°C, 37°C, and 41–42°C—were used as progenitors in the lethal selection experiment described below. The designations of these lines are given in Table 1.

TABLE 1. Death rates and other properties of the six progenitor clones used in the lethal selection at 44°C. Historical temperature indicates the temperature at which the progenitor evolved during the preceding 2000 generations. Upper thermal limit indicates the maximum temperature at which the progenitor can sustain a constant population in the face of 100-fold daily dilution. Death rate is that measured during the first two or three days at 44°C, showing the mean and standard error based on 15 replicate cultures. Number of mutants is the number of 15 cultures that yielded thermotolerant mutants.

Progenitor designation	Historical temperature (°C)	Upper thermal limit (°C)	Death rate per day at 44°C (± SE)	Number of mutants
32−1	32	41.5	4.39 (0.19)	0
32+1	32	42	6.57 (0.50)	0
37−2	37	39–40	5.57 (0.23)	0
37+3	37	41.5	5.47 (0.21)	0
42−1	41–42	42	4.89 (0.36)	2
42+1	41–42	42	5.41 (0.26)	1

The culture medium used throughout this study was Davis minimal (DM) supplemented with glucose at 25 μg/ml. Tetrazolium-arabinose (TA) indicator agar was used to discriminate marker states and determine population densities in the competitive fitness assays. The common ancestor and derived lines, as well as all mutant clones obtained in this study, are stored in a 12% glycerol suspension at −80°C.

Lethal Selection Protocol

Each of the six progenitor clones described above was used to inoculate 15 cultures of LB broth. These 90 cultures were grown overnight at 37°C. They were then diluted $1{:}10^4$ into DM and incubated for 24 h at 37°C, yielding cell densities of $\sim 5 \times 10^7$ per ml. An aliquot of 0.1 ml ($\sim 5 \times 10^6$ cells) was then transferred into 10 ml of fresh DM, and these selection cultures were immediately placed in a water-bath at 44°C with temporal fluctuations ± 0.1°C. Each selection culture was sampled daily for six days, and the density of surviving cells (colony-forming units) was estimated by plating the sample on TA agar. The per capita death rate (d^{-1}) was calculated as the slope of the decline in ln-transformed colony-forming units during two to three days at 44°C. If a culture exhibited growth by the final day, a random colony was chosen from the TA plate. Each selected bacterium was then screened for several distinctive markers (Lenski et al. 1991) that were present in the progenitor, to verify that the bacterium was not a contaminant. Colonies verified not to be contaminants were stored in the freezer for further analysis.

Fresh cultures were then started from these frozen stocks in exactly the same manner as for the original genotypes. We verified that the ability to grow at 44°C (allowing two days to do so) persisted in these clones after they were frozen and then propagated for two serial-transfer cycles at 37°C, whereas this ability was not manifest in their progenitors. This procedure demonstrates that the selected clones were in fact thermotolerant mutants and not merely phenotypically acclimated progenitors.

Measurements of Absolute Fitness and Thermal Niche

Each clone was serially propagated in DM for five days at all of the following constant temperatures: 17.0, 18.0, 19.5, 42.0, 42.5, 43.0, 44.0, and 44.2°C. These experiments were done in a shaking water-bath incubator with thermal fluctuations of ± 0.1°C, and they were replicated sixfold. The cultures were removed from the incubator for only a few minutes each day to sample for population density and transfer to fresh medium. Absolute fitness was estimated by regressing the natural logarithm of each day's final population density against time (Bennett and Lenski 1993). Only 1/100th of the population is transferred each day, and so the population must be able to grow at least 100-fold in 24 h to maintain a constant population size. A population that always reaches its maximum density (for the given resource level) by the end of each day has an absolute fitness of zero. An absolute fitness less than zero indicates the population is growing more slowly than the dilution rate, and so the population will eventually go extinct. The thermal niche of a genotype is defined as the range of temperatures over which it can maintain a stable population in the face of serial dilution (Bennett and Lenski 1993).

Measurements of Relative Fitness

Each thermotolerant mutant and its progenitor necessarily have the same arabinose marker state. Therefore, they cannot be placed in competition with one another to obtain direct estimates of their relative fitness (because the marker is needed to distinguish the competing genotypes in mixed culture by plating on TA indicator agar). Therefore, relative fitness was determined by running separate competitions between each pair of clones—mutant and progenitor—and the ancestral clone, Anc, with the opposite marker state. The marker itself has been shown to be effectively neutral in the ancestral background and culture conditions, including temperatures, used in this study (Bennett et al. 1992; Bennett and Lenski 1993; Mongold et al. 1996a). The resulting fitness values for a mutant and its progenitor were then compared using standard *t*-tests based on sixfold replication of each competition. The competitive fitness assays were performed at the following temperatures, which lie inside the ancestral thermal niche: 20, 32, 37, 40, 40.5, 41, and 41.5°C.

Competing clones were inoculated from freezer stocks into separate LB broth cultures and grown overnight at 37°C. These cultures were then diluted $1{:}10^4$ into DM, where they grew for 24 h at the assay temperature to allow the bacteria to become comparably acclimated to the environment in which they would compete (Bennett et al. 1992; cf. Leroi et al. 1994; Bennett and Lenski 1997a). After this conditioning step, the cultures of the two competitors were mixed 1:1 volumetrically, diluted 100-fold into fresh medium, and placed again at the assay temperature. Samples of the competition mixture were taken immediately and again after 24 h, then diluted and plated on TA agar to estimate the initial and final densities of each competitor. Relative fitness is defined as the ratio of the realized Malthusian parameters of the competitor and the ancestral clone while growing in direct competition (Lenski et al. 1991).

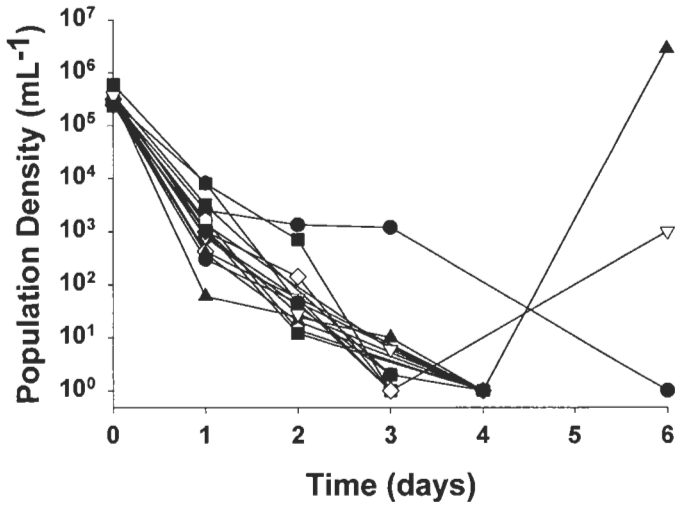

FIG. 1. Mean population density, expressed as colony-forming units per ml, of 15 replicate cultures inoculated with the progenitor 42–1 as a function of time at 44°C. Between days 3 and 6, two of the populations rebounded. Clones from these two populations (42–1/44.A and 42–1/44.B) were analyzed and shown to have heritable changes in their thermotolerance.

RESULTS

Efficacy of Selection

Ninety cultures were exposed to lethal selection at 44°C, with 15 from each of the six progenitor clones. Out of all these, only three contained viable bacteria by the sixth day. Figure 1 shows the precipitous drop in the number of survivors over time for replicates of the progenitor clone 42–1. This decline was followed by the rapid resurgence of population density in two of the 15 cultures by the sixth day. All 90 cultures showed this initial decline in population size, while all three recoveries occurred after the initial decline.

The death rate for each replicate culture was computed by regressing the natural logarithm of the density of viable cells versus time over the first three days (two days if there were no viable cells by the third day). Table 1 shows the mean death rate at 44°C for each of the six progenitors, along with the its historical temperature, upper thermal limit, and the number of its cultures that yielded thermotolerant mutants. An ANOVA indicates significant variation in death rate among the progenitors ($F = 5.564$; df = 5, 84; $P = 0.0002$). There is no clear relationship, however, between death rate at 44°C and either the progenitor's historical temperature or its estimated upper thermal limit for population persistence. For example, one of two progenitors that evolved at 32°C has the highest death rate at 44°C, but the other one with the same history has the lowest rate of any of the six progenitors.

More interestingly, all three thermotolerant mutants obtained from this lethal selection experiment were derived from the two progenitors that had previously evolved at 41–42°C. We performed two statistical tests to determine the significance of this relationship; both are one-tailed tests because we expected a priori that evolution at high temperature would predispose a genotype to further adaptation to even higher temperatures. First, a Fisher's exact test, treating each

culture as the unit of observation, indicates that significantly more thermotolerant mutants were obtained from the progenitors previously selected at 41–42°C than from those selected at 32°C and 37°C ($P = 0.0346$). Second, a simple correlation between the proportion of cultures yielding thermotolerant mutants (using the arcsine-square root transformation; Sokal and Rohlf 1995) and the progenitor's evolutionary temperature, with each progenitor as the unit of observation (and thus more conservative), also indicates a significant association ($r = 0.8311$, $n = 6$, $P = 0.0202$). These results therefore suggest that genotypes adapted to high temperature within the thermal niche are predisposed to produce thermotolerant mutants with an extended upper thermal limit. However, against this conclusion, the thermotolerant mutant that we found serendipitously in an earlier study (Bennett and Lenski 1993), which we designated "Lazarus," was derived from a line that had evolved at 32°C (although not one of the two progenitors used in this study).

Absolute Fitness and Changes in Thermal Niche

As described in the Materials and Methods, a population that maintains itself in the face of serial dilution has a net growth rate, or absolute fitness, of zero. By contrast, a population that cannot sustain itself in the face of serial dilution has a negative value for its absolute fitness. A population that neither grows nor dies, but is simply diluted 100-fold each day, has an absolute fitness of $\ln 0.01 \cong -4.6$; any lower value indicates that the death rate exceeds the growth rate. We estimated, with fourfold replication, the absolute fitness across eight temperatures ranging from 17°C to 44.2°C of the three thermotolerant mutants isolated in this study, the Lazarus mutant we isolated previously, and all of their progenitors.

Figure 2 shows the mean value and 95% confidence limits for these absolute fitnesses. Looking first at the low range of temperatures, it is evident that two of the mutants, 42–1/44.A (panel A) and especially Lazarus (panel D) grow significantly more slowly than their progenitors below 19.5°C. Hence, the lower limit of the thermal niche has shifted upward in these two thermotolerant mutants. In contrast, the other mutants, 42–1/44.B (panel B) and 42+1/44 (panel C), have growth rates at low temperature that are generally indistinguishable from their progenitors.

At the high range of temperatures, all four of the thermotolerant mutants appear to have extended their upper thermal limit relative to their progenitors. Mutant 42–1/44.A (panel A) shows the smallest change; it can persist in serial dilution culture at 42.5°C and has a higher absolute fitness than its progenitor at that temperature ($P = 0.034$, one-tailed t-test), but it cannot persist at 43°C. Mutant 42–1/44.B (panel B) persists at 43.0°C and has an absolute fitness that is marginally significantly greater than its progenitor ($P = 0.079$, one-tailed t-test). Mutants 42+1/44 (panel C) and Lazarus (panel D) are able to maintain stable populations at 43.0°C, whereas their progenitors cannot persist even at 42.5°C. At 44.0°C and 44.2°C, none of the lines, mutant or progenitor, can persist in daily serial dilution culture.

Although all four mutants have extended the upper limit of their thermal niche, it may seem surprising that the three new mutants, which were selected at 44°C, *cannot* maintain a stable population density at that temperature. The explanation for this apparent discrepancy is that the thermotolerant mutants were initially selected for their ability to grow over several days at 44°C, and they were subsequently screened for their ability to grow 100-fold within two days at that temperature (after having been frozen and then propagated for two cycles at 37°C to ensure that the phenotype was heritable). By contrast, in the assays of absolute fitness at 44°C, the bacteria had to grow 100-fold in only *one* day to avoid extinction. Evidently, all three of the new thermotolerant mutants can grow at 44°C, although too slowly to maintain a constant population in the face of 100-fold daily dilution.

Fitness Tradeoffs within the Ancestral Niche

Given the existence of thermotolerant mutants that have the ability to grow at elevated temperatures, why were they not selected in all of the lines propagated for 2000 generations at 41–42°C? Only one of the six lines in this treatment group evolved the ability to persist at 43°C (Bennett and Lenski 1993). One possibility, of course, is that the underlying mutations are very rare. Indeed, we found only three such mutants among 90 cultures. However, it should be noted that each of the six evolving populations in our original selection experiment comprised a temporal sequence of 300 equivalent cultures. Thus, although these mutants are not extremely common, they are likely to have occurred repeatedly in the evolving populations.

Another hypothesis for the failure of thermotolerant mutants to become dominant in the populations selected at 41–42°C is that they might not have any advantage there. Indeed, they might even be selectively disadvantaged if there is a tradeoff between adaptations to lethal and nonlethal high temperatures. To examine this possibility, we measured the fitness in competition of each of the thermotolerant mutants and the progenitor clones from which they were derived, all relative to the common ancestor, Anc, across seven temperatures within the ancestral thermal niche. All assays were replicated sixfold; t-tests were performed to compare the relative fitness of each mutant and its progenitor at every temperature.

Figure 3 summarizes the results of these assays; each panel depicts one thermotolerant mutant and its paired progenitor. Between 20°C and 37°C, three of the four mutants suffered a significant loss of fitness relative to their progenitors. This loss is most striking in the Lazarus mutant, which also had the greatest loss of absolute fitness at temperatures below the lower limit for population persistence. More important with respect to the failure of thermotolerant mutants to have been selected during the 2000 generations at 41–42°C, none of them has any advantage relative to its progenitor at the higher temperatures of the ancestral niche. In fact, all four of the thermotolerant mutants have highly significant disadvantages ($P \leq 0.02$) at one or more of the three assay temperatures between 40°C and 41.5°C. Therefore, the ability to survive and grow at temperatures outside the ancestral niche invariably incurred a significant tradeoff in competitive fitness

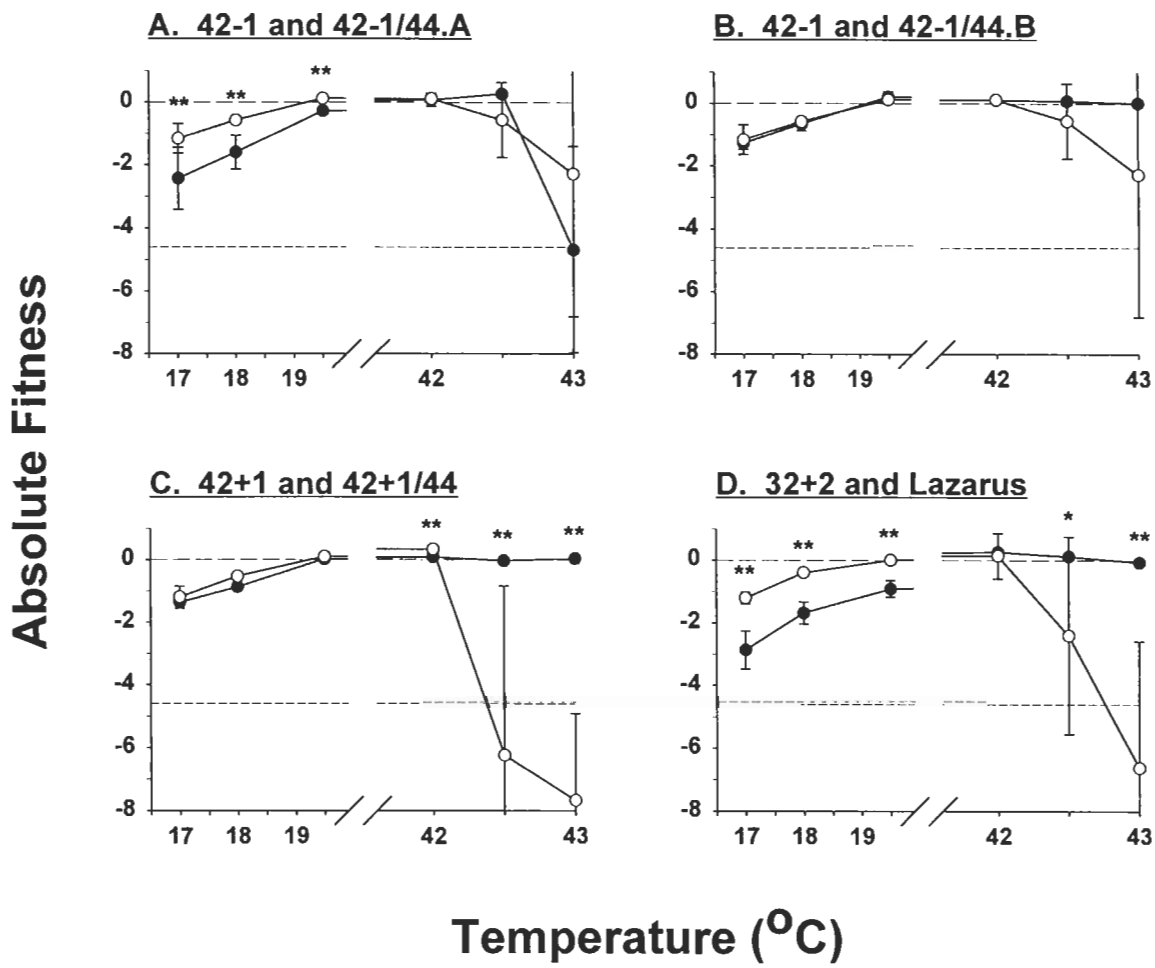

FIG. 2. Absolute fitness of the thermotolerant mutants (filled circles) and the progenitor clones from which they were derived (open circles) across a range of temperatures (note different scales at low and high temperatures). Each panel compares one of the mutants with its immediate progenitor: (A) 42–1 and 42–1/44.A; (B) 42–1 and 42–1/44.B; (C) 42+1 and 42+1/44; (D) 32+2 and Lazarus. Each value is the mean of four replicate assays. The error bars are 95% confidence intervals (based on the *t*-distribution); they are not visible when their range is smaller than the symbol for the mean. The asterisks indicate the significance level of two-tailed *t*-tests comparing the fitnesses of a mutant and its progenitor at that temperature ($*P < 0.05$; $**P < 0.01$). A population that maintains constant density during serial dilution has a net growth rate, or absolute fitness, of zero (long dashed line). A population that cannot sustain itself in the face of serial dilution has a negative value for its absolute fitness. A population that neither grows nor dies, but is diluted 100-fold each day, has an absolute fitness of $\ln 0.01 \cong -4.6$ (short dashed line); any value below this means that the death rate exceeds the growth rate.

within the ancestral environment. This tradeoff was manifest even near the upper extreme of the thermal niche.

DISCUSSION

The rate at which any population can adapt to a changing environment depends on the genetic variation for performance in the new environment. Beneath this truism, however, lie more subtle questions about the structure of that genetic variation, including pleiotropic effects of genes on fitness across an environmental gradient and epistatic interactions among mutants. For example, there are two distinct hypotheses about how an organism's adaptation to an extreme environment might be promoted by adaptation to a moderate environment that lies in the same direction along the gradient. According to one hypothesis, the same alleles that promote adaptation to a moderate change in the environment pleiotropically improve performance in even more extreme environments (Huey and Kingsolver 1989; Huey et al. 1991). This is what we call the "sliding niche" model, because a shift in a population's adaptation along some environmental gradient simultaneously raises or lowers its fitness along the entire gradient while maintaining the same niche breadth and shape. According to the second hypothesis, genetic adaptation to the moderate environmental change does not simultaneously improve fitness in the more extreme environment. However, mutations that facilitate performance in the extreme environment might do so only in genetic backgrounds that have already become adapted to the moderate change, in effect a form of epistatic interaction between mutations. (Lenski [1984] describes such a case of stepwise adaptation by bacteria to moderate versus high concentrations of a bacteriophagous virus.) Imagine, for example, that mutation A

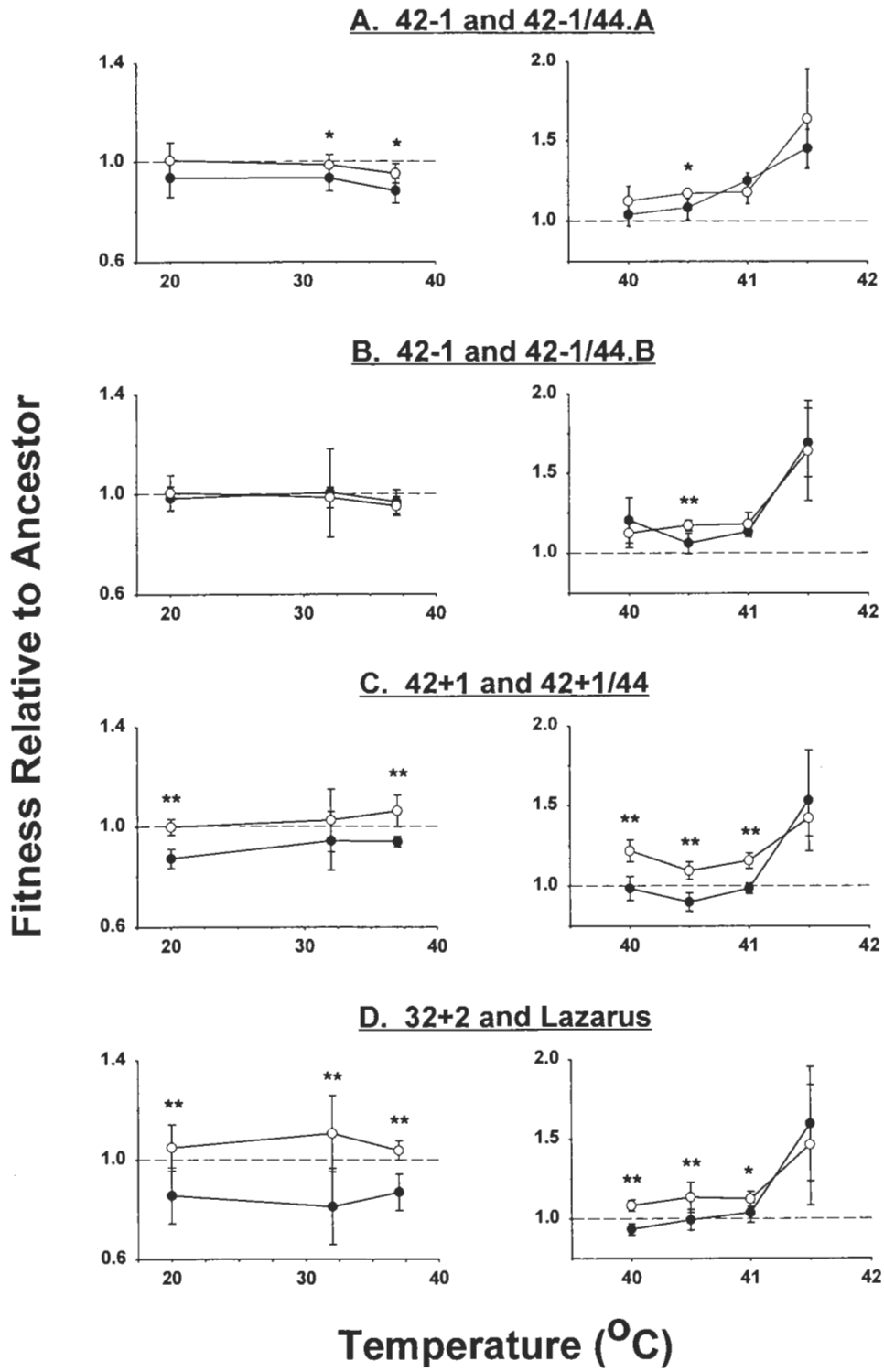

FIG. 3. Relative fitness of the thermotolerant mutants (filled circles) and the progenitor clones from which they were derived (open circles) across a range of temperatures within the ancestral niche: (A) 42–1 and 42–1/44.A; (B) 42–1 and 42–1/44.B; (C) 42+1 and 42+1/44; (D) 32+2 and Lazarus. All of the fitness values were obtained in competition with the common ancestor of all lines and are expressed relative to that ancestor. Each point is the mean of six replicate assays; error bars indicate 95% confidence intervals, which are not visible when their range is smaller than the symbol for the mean. The asterisks indicate the significance level of two-tailed *t*-tests comparing the fitnesses of a mutant and its progenitor at that temperature ($*P < 0.05$; $**P < 0.01$). For better visibility, the x-axis is split and the region from 40–42°C is shown on an expanded scale.

allows an organism to thrive at some elevated temperature, while mutations A and B are *both* necessary for it to survive at a more extreme temperature. In that case, prior adaptation to the elevated temperature predisposes a population to adapt subsequently by mutation and natural selection to the more extreme environment, even though it gains no immediate advantage in the extreme environment. We call this the "stepping stone" model of thermal niche evolution.

We have shown previously that genetic adaptation of *E. coli* to elevated, but nonlethal, temperatures did not generally preadapt the derived lines to survive and grow at slightly higher, but lethal, temperatures (Bennett and Lenski 1993). Thus, we rejected the sliding niche model, at least as a universal proposition. We also serendipitously isolated one thermotolerant mutant during measurements of the death rate at a lethally high temperature (Bennett and Lenski 1993). But we did not know whether such mutations were extremely rare, whether prior adaptation to the elevated temperature predisposed adaptation to the lethal temperature, or why thermotolerant mutants (if they were reasonably common) had not accumulated during the prior selection at the high but nonlethal temperature. The aims of the present study were to address all these issues.

Thermotolerant Mutants Are Not Uncommon

Ninety cultures of *E. coli* were grown to stationary phase at 37°C, then diluted into fresh medium and placed at 44°C. The vast majority of cells died rapidly (Table 1; Fig. 1), but from three of the cultures genotypes emerged that could survive and grow at this temperature (two are shown in Fig. 1). Each culture was founded from a clone, derived from a single cell, so that there was no genetic variation within a culture except that which had arisen by spontaneous mutation during growth of the culture or its subsequent exposure to 44°C.

It is often assumed that mutations occur only in replicating cells, but this is not necessarily the case (Ryan 1955; Mittler and Lenski 1990; Foster 1994; Rosenberg et al. 1994). Mutations can occur in nondividing cells as a consequence of movement of transposons and other mobile genetic elements, whereas other mutations may occur by damage to one of the DNA strands and subsequent misrepair. Our protocol ensures that mutations arose independently in the replicate cultures. The precise stage at which the mutations arose is not relevant to the issues in this paper, except that we must express the mutation rate per cell, rather than per cell-generation, because we have not established that the mutations occurred during cell division. In fact, one might be tempted to infer that they arose during exposure to 44°C (after growth of the progenitor had ceased) from the significant lag in their appearance (Fig. 1). However, this is a questionable inference, given the slow growth of these mutants and the possibility that they might have required some period to become phenotypically acclimated before they could commence growth at such high temperature. Thus, the timing of their appearance remains unresolved.

Each of the 90 cultures had an initial population size at 44°C of $\sim 5 \times 10^6$ cells (i.e., 5×10^7 cells/ml in the source culture grown at 37°C, with 0.1 ml of that transferred into 9.9 ml medium at 44°C). We can estimate the average mutation rate to thermotolerance based on the zero-class of the Poisson distribution (Luria and Delbrück 1943). Given that 87 of the 90 cultures yielded no mutants, we estimate the mutation rate to be $\mu = -(\ln p_0)/N \cong 7 \times 10^{-9}$ per cell. This value is neither exceptionally high nor unusually low. It is about 20-fold higher than the typical base-pair mutation rate for growing *E. coli* cells (Drake 1991), but such rates are highly variable across sites (Moxon et al. 1994; Rosenberg et al. 1994). Moreover, we estimated the mutation rate to a phenotypic class; if 20 different base-pair mutations each yield a thermotolerant phenotype, then this estimate would match the typical base-pair rate.

All four of the thermotolerant mutants can sustain a stable population at temperatures at least 0.5°C above the limit for persistence of their progenitors (Fig. 2). These effects are fairly modest, but they do permit the bacteria to occupy a new thermal environment that was lethal and thus unavailable to their progenitors. They overcame some previous physiological limitation to survival and growth, allowing them to extend their thermal niche by 0.5°C to 2.0°C. Presumably, some other factor now limits their persistence at still higher temperatures.

Ambivalent Support for the Stepping Stone Model

All three of the thermotolerant mutants isolated in this study were derived from bacterial lines that had previously evolved at and adapted to 41–42°C over the course of 2000 preceding generations. None were derived from bacterial lines that had adapted to lower temperatures (i.e., 32°C or 37°C), despite twice the sampling effort (Table 1). This difference in propensity to sport thermotolerant mutants is significant using either the 90 replicate cultures ($P = 0.0346$) or the six independently derived progenitors ($P = 0.0202$) as the unit of observation. Based on several objective criteria, 41–42°C was stressful for the ancestral genotype, Anc, which was adapted to 37°C (Lenski and Bennett 1993; Bennett and Lenski 1997b). The adaptive mutational changes that occurred during the evolution of the bacteria at 41–42°C apparently produced a genetic background that enabled the expression of subsequent mutations that confer tolerance to temperatures at or above 42.5°C. These data thus support the stepping stone model for the evolution of an extended thermal niche, according to which prior adaptation of a population to moderate environmental change genetically predisposes that population to evolve tolerance to a more extreme environment.

However, we qualify this result by pointing out that the thermotolerant mutant, Lazarus, which we serendipitously isolated in our previous work, derived from a progenitor that had adapted to 32°C (Bennett and Lenski, 1993). We cannot combine this earlier result with our present data because of important differences in protocol; specifically, a single replicate of this line was examined and it was serially diluted during exposure to lethal temperatures. One subsequent culture of the progenitor genotype failed to yield another thermotolerant mutant (Bennett and Lenski 1993, fig. 8b). Given the existence of Lazarus, the support of these experimental results for the stepping stone model must be qualified.

TABLE 2. Summary of changes in the performance abilities of thermotolerant mutants relative to their progenitors. The data for absolute and relative fitnesses are shown in Figures 2 and 3, respectively.

Mutant designation	Absolute fitness		Relative fitness	
	Below 20°C	Above 42°C	At 20–37°C	At 40–41.5°C
42−1/44.A	↓	↑	↓	↓
42−1/44.B	—	↑	—	↓
42+1/44	—	↑	↓	↓
32+2/Laz	↓	↑	↓	↓

Tradeoffs in Adaptation to Lethal and Non-lethal High Temperatures

Mutations that confer thermotolerance clearly exist, and so we must ask why the mutants did not come to numerical dominance during 2000 generations of selection at 41–42°C, which is very near the upper thermal limit for these bacteria. These mutations are not exceptionally common, but neither are they so rare that they would have been absent for such a long period. One plausible explanation is that the thermotolerant mutations selected at lethally high temperatures confer no selective advantage at slightly lower, nonlethal temperatures (Bennett and Lenski 1993). Our data, as summarized in Table 2, strongly support this hypothesis.

All four thermotolerant mutants have higher absolute fitness than do their progenitors at temperatures just above the upper limit of the ancestor's thermal niche (Table 2, column 2). However, slightly below this limit, all four mutants are significantly less fit in competition with the ancestor than are their progenitors (column 4). Evidently, there is a tradeoff between adapting to a lethally high temperature versus one that is slightly lower and nonlethal. Thus, when thermotolerant mutants appear during selection at high but nonlethal temperatures, they are competitively inferior to their progenitors and thus would not persist in the population.

Interestingly, whereas all four of the thermotolerant mutants exhibit this tradeoff at high temperatures, not all of them show deficits at lower temperatures. Only three of the four mutants are less competitive than their progenitors between 20°C and 37°C, which is within the ancestral thermal niche (Table 2, column 3). Also, only two of them have lost absolute fitness at temperatures at or below 19.5°C (column 1), such that the lower limit of their thermal niche shifted upward concomitantly with the change in their upper thermal limit. Thus, tradeoffs in performance may sometimes be stronger at nearby points along an environmental gradient than at more distant points. Although this seems counterintuitive, one can imagine mechanistic explanations. For example, growth and survival at extremely high temperature may involve expression of some protective protein that is costly when it is not needed, but which is unnecessarily expressed at slightly (but not much) lower temperatures due to imprecision in gene regulation. A test of this speculative hypothesis awaits identification of the mutations that confer thermotolerance, but it serves to illustrate the potential complications that organisms may face when adapting to environmental change close to the boundaries of their niche.

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