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EVOLUTIONARY RESPONSE OF *ESCHERICHIA COLI* TO THERMAL STRESS

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Abstract.—We used a clone of the bacterium *Escherichia coli* previously adapted to 37°C to found replicate populations propagated at constant 32°C, constant 37°C, constant 42°C, and a daily alternation between 32° and 42°C. Several criteria indicate that 42°C was stressful for the ancestor, while 32° and 37°C were not. For example, 42°C was within 1°C of the limit for extinction, and yield was substantially reduced at this temperature. Adaptation was assayed by competing derived genotypes against their common ancestor at various temperatures. Bacteria adapted much more rapidly to 42°C than to either lower temperature. Also, bacteria propagated in the alternating environment exhibited greater adaptation to 42°C than to 32°C. Adaptation was temperature-specific in all groups, but adaptation to 42°C entailed little loss of fitness at lower temperatures. Nor did adaptation to 42°C much extend the upper limit for population persistence, although we isolated more thermotolerant mutants by imposing hard selection. Thus, whereas the stressful 42°C environment consistently led to more rapid adaptive evolution than did nonstressful regimes, superstressful temperatures caused either extremely rapid adaptive evolution or extinction. Although defining stress in general terms is difficult, one can evaluate specific criteria and test evolutionary hypotheses using appropriate experimental systems.

Every population of organisms has the potential to exist over a range of physical environmental conditions, for example, in environments of different temperature, osmolarity, or acidity. This range may be broad or narrow, but toward its extremes the population begins to run into trouble. The organism's functional capacities may be compromised and suboptimal (in the sense of Huey and Stevenson 1979). In even more extreme environments, which lie outside its potential niche, the population is unable to maintain itself and becomes extinct if kept there. Such extreme environments are termed stressful because of their detrimental effects on the population and on the individuals that comprise it (Levitt 1980; Hoffmann and Parsons 1991).

Recognizing that some environments are difficult for organisms is not the same, however, as being able to define or quantify stress unambiguously. The concept of stress has proven to be elusive to simple operational definition (for review, see Hoffmann and Parsons 1991). Many common definitions are essentially circular and unhelpful. Others use biochemical or physiological measurements (Ivanovici and Wiebe 1981) that involve analytical precision but have unknown implication for organismal function or population persistence. To us, the most satisfying

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definitions treat stress as a physical environmental factor that causes a reduction in fitness (see, e.g., Koehn and Bayne 1989; Sibly and Calow 1989; Hoffmann and Parsons 1991). Fitness, though its theoretical significance is clear, is operationally difficult to measure in many systems. Consequently, models and discussions of evolutionary responses to stressful environments often become vague with respect to how their assumptions or predictions can be empirically investigated.

One purpose of this article is to point out that empirical investigations of evolution in stressful environments, however these are defined, can be feasible and experimentally tractable. The key is choosing an appropriate biological system for investigation. We describe the particular system that we have been studying, and we consider the applicability of various criteria for ascribing stress to our experimental treatments. We then report the evolutionary responses of experimental populations propagated in stressful and nonstressful environments, and we interpret these responses with respect to the following general questions: First, do populations evolve more rapidly in stressful environments than in nonstressful environments? Second, does adaptation to a stressful environment entail loss of performance in nonstressful environments? Finally, does adaptation to a stressful environment extend the boundaries of an organism's potential niche, which thereby preadapts a population to even more stressful environments?

We conclude by suggesting some future directions for the study of evolutionary responses to stress that can be addressed in this particular system or by using other biological systems that are amenable to experimental analyses of evolutionary processes.

CHOICE OF EXPERIMENTAL SYSTEM

Useful Properties for Studying Evolutionary Responses to Stress

An ideal biological system for investigating evolutionary responses to environmental stress should have the following properties.

Rapid generations.—Discussions of stress often focus on individual tolerance or survival, but the effects of stresses on reproductive output and on the differential success of genotypes are the appropriate objects of an evolutionary investigation. An ideal study system should therefore span many generations, which would permit an examination of not only the responses of extant populations but also changes in the genetic composition of populations during their evolution in stressful and nonstressful environments.

Manipulative and inferential control.—Rigorous analyses of evolutionary responses to stress require that an experimental system be well controlled in two respects. First, the environmental stress of interest must be amenable to precise experimental manipulation and regulation, so that direct and correlated responses can be examined over a range of conditions, including those very close to critical demographic limits. Second, such studies require appropriate control populations for evaluating specific hypotheses. The best possible inferential control population would be the ancestral population itself, which is feasible with organisms

that can be stored as clones at very low temperature (or otherwise in a genetically homogeneous, nongrowing state). And the best possible ancestor would be one that was already well adapted to all aspects of the laboratory environment *except* the stress factor of interest, which would thereby facilitate detection of specific adaptations to stress as opposed to general adaptations to the laboratory regime. This condition can be *approximately* fulfilled by having already propagated the ancestral population in the laboratory under defined environmental conditions, until such time as the rate of further improvement under those conditions becomes negligible. Because this latter condition is difficult to determine precisely (see Lenski et al. 1991), an evolving control treatment is appropriate, in which organisms are further propagated under the ancestral regime. A second desirable control treatment would be one in which organisms are propagated under novel but nonstressful conditions.

Replication.—The experimental system should allow several replicated populations in each control and treatment group. In this way, statistical confidence can be established for particular hypotheses, which would indicate that any effects are, in fact, due to the imposed environmental conditions and not merely to chance divergence that might result even among replicate populations. Such divergence could arise by random mutation or genetic drift, or it might reflect slight but unavoidable differences among replicate environments. If so desired, one could measure relevant properties of individuals (or other population subsets) to test for heterogeneity among replicate populations, which might indicate multiple phenotypic solutions to a particular environmental challenge (see, e.g., Cohan and Hoffmann 1986; Lenski 1988). Ideally, to test general hypotheses about the evolutionary consequences of stress, one would like to investigate several different biological systems or the effects of several distinct environmental stresses in a particular system. Given inevitable limitations of knowledge of organisms, time, and financial resources, such metanalyses are usually beyond the scope of any single laboratory.

Quantification of the extent and specificity of adaptation.—To study adaptation rigorously requires the ability to distinguish genotypes and to assess their performance under different environmental conditions. The modus operandi of many evolutionary biologists is to identify a phenotype of interest and then determine whether the phenotype is heritable, on the one hand, and whether it has consequences for performance, on the other hand. Ideally, such performance measures should be closely related to fitness (e.g., longevity and fecundity), and they should be complete, since the failure to consider any important aspect of performance might lead one to infer incorrectly the relative success of different genotypes in a particular environment or of a particular genotype in different environments. Clonally reproducing organisms provide a special advantage for quantifying performance, because one can create genetically mixed populations and directly estimate the fitness of one genotype relative to another, based on the rate of change in their relative abundances. Of course, relative fitness depends not only on the genotypes used but also on the particular environmental conditions in which it is estimated.

Evolutionary Responses of Escherichia coli *to Temperature*

Experimental studies of evolutionary processes are both possible and feasible using microbial populations, including especially the bacterium *Escherichia coli* (for recent reviews, see Dykhuizen 1990; Lenski 1992). Rapid generations permit experiments that extend for hundreds and even thousands of generations, genotypes can be stored indefinitely at very low temperatures, and the relative fitnesses of genotypes can be reliably estimated by direct competition experiments.

Investigations of the evolutionary responses of microbial populations to stressful environments are no exception and present no special difficulties. Microbial populations (genetically homogeneous or heterogeneous) may be created and tested over any range of environments that can be achieved. Their survival as well as their ability to grow and replicate themselves may be measured. Their potential and realized niches may be determined by assaying their fitness in the absence or presence of competitors, respectively. The immediate ecological effects of environmental stress may thus be evaluated directly. Moreover, the populations may then be allowed to evolve in environments of varying degrees of stress. Adaptation to these environments may be unambiguously demonstrated by measurement of fitness relative to the founding ancestral clone, under both stressful and nonstressful conditions, after each genotype has been acclimated to the particular conditions. Many replicate populations may be propagated simultaneously and statistical confidence in the resulting patterns thereby ascertained.

We have undertaken experiments to examine the evolutionary adaptation of *E. coli* to different thermal environments (Bennett et al. 1990, 1992; Bennett and Lenski 1993). Temperature was chosen as an environmental variable because it has pervasive effects on most biological rate processes (for general reviews, see Prosser 1973; Hochachka and Somero 1984; Cossins and Bowler 1987; for reviews pertaining to bacterial growth, see Ingraham 1987; McMeekin et al. 1988). Temperature varies both spatially and temporally; natural populations are frequently exposed to different and potentially stressful temperatures, and they can be expected to adapt evolutionarily to characteristic thermal environments. Elevated mortality has frequently been reported to result from extreme temperatures (Kinne 1971), and major episodes of extinction in the paleontological record have been associated with temperature changes (Stanley 1984). Temperature, in fact, is perhaps the most commonly cited and investigated example of environmental stress (Parsons 1987; Hoffmann and Parsons 1991). The prospect of global climate change has heightened interest in the ecological and evolutionary responses of biological populations to changing thermal environments (Holt 1990; Lubchenco et al. 1991).

Temperature is permissive over a certain range and inhibitory or lethal beyond that range (Huey and Stevenson 1979; Huey and Kingsolver 1989). The thermal niche of an organism can be defined as the range of temperatures over which a population can be maintained indefinitely, with all other environmental factors held constant (Bennett and Lenski 1993). This niche can be experimentally determined, and evolutionary experiments can be performed in environments of vary-

ing degrees of stress: well within the thermal niche, near the upper or lower limits of the thermal niche, and even at temperatures outside the niche boundaries.

EXPERIMENTAL SYSTEM AND DELINEATION OF STRESSFUL TREATMENTS

In this section, we first outline some important features of our particular experimental system, and then we employ several criteria to assess the stressfulness of the thermal regimes used in our investigations.

Important Features of the Particular Experimental System

Common ancestor.—We used a clone derived from *Escherichia coli* strain B to found all of our evolution experiments. This clone harbors neither plasmids nor functional bacteriophages and so cannot engage in recombination (in the sense of mixis). The common ancestor had been maintained in serial dilution culture at 37°C in a glucose-limited minimal salts medium for 2,000 generations as part of another study, during which time it improved in competitive fitness relative to *its* ancestor by about 35% (Lenski et al. 1991). Moreover, its rate of fitness improvement was significantly slower during the second thousand generations than during the first thousand generations (Lenski et al. 1991). Thus the common ancestor was already rather well adapted to the 37°C laboratory culture environment, which made it easier to detect temperature-specific adaptations (as opposed to adaptations to other aspects of the laboratory culture regime) in our experimental treatments, which are described below.

Fitness assays.—Two genetic variants of the common ancestor were developed to permit identification of genotypes in competitive assays of relative fitness. These variants differed in ability versus inability to catabolize the sugar arabinose, and they can be distinguished by the color of their colonies on an appropriate medium. The neutrality of this genetic marker was verified in each of the experimental thermal regimes (Bennett et al. 1992). Evolutionary (i.e., genetic) adaptation is distinguished from phenotypic acclimation by storing all isolates in the freezer; then growing them for the first day in a nutritionally rich medium at 37°C, for a second day in a minimal salts medium at 37°C, and for a third day (conditioning step) in the same minimal salts medium at the desired assay temperature; and only then competing the two comparably acclimated genotypes to determine their relative fitness at the assay temperature. Relative fitness is calculated as the ratio of the number of doublings achieved by the two competing genotypes during the complete growth cycle that is imposed by the serial dilution culture regime (Lenski 1988; Bennett et al. 1990, 1992; Lenski et al. 1991). A difference in relative fitness between two genotypes may reflect differences in lag times upon transfer to fresh media, maximal growth rates, efficiencies of resource acquisition and conversion, death rates upon exhaustion of the limiting glucose, or any combination of these parameters.

Evolutionary treatments and analyses.—Six replicate populations (*lines*) were propagated under each of four temperature regimes (treatment *groups*) for 2,000 generations (300 d). Each line was founded using a subclone derived from a single

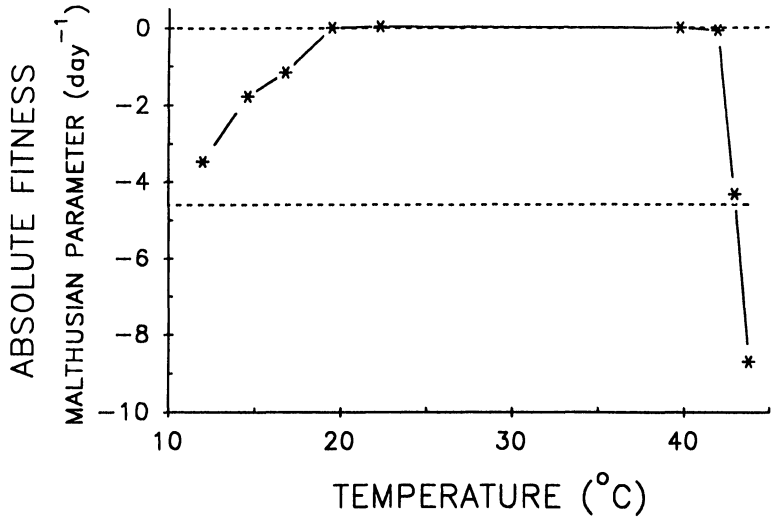

FIG. 1.—Thermal niche of the ancestral genotype. Absolute fitness is calculated by the Malthusian parameter: 0 day^{-1} indicates persistence in serial dilution culture, whereas −4.6 day^{-1} corresponds to imposed dilution (100-fold) without any growth or death. (Data from Bennett and Lenski 1993.)

cell of the common ancestor. Consequently, any evolutionary adaptation can be attributed to selection for new mutations that occurred within a particular population. Changes in the mean fitness of each treatment group relative to the common ancestor were monitored, under the thermal regime in which that group was evolving (*direct* response) as well as at other temperatures that a group had not experienced during its experimental evolution (*correlated* responses). The thermal niche of each line was measured as the range of temperatures over which it was capable of sustaining itself indefinitely by sufficient growth in the standard serial dilution culture.

Applicability of Stress Criteria to Thermal Regimes

Several criteria indicate that 42°C is a stressful temperature for *E. coli*. First, we have determined (Bennett and Lenski 1993) that the thermal niche of the ancestral clone ranges from about 19°C to just over 42°C (fig. 1). That is, this genotype can maintain a stable population indefinitely at these temperatures with sufficient growth to offset the 100-fold daily serial dilution imposed by the culture regime. Its population declines to extinction at either slightly lower (e.g., 17°C) or slightly higher (e.g., 43°C) temperatures. Thus, 42°C is very near the upper thermal limit for persistence of the ancestor.

Second, both the maximum specific growth rate (Ingraham 1987) and the yield of *E. coli* are reduced at 42°C relative to the ancestral temperature, 37°C. Table 1 shows the cell density and total biovolume (cell density × average cell volume) of the ancestral clone at 32°, 37°, and 42°C.

Third, a shift from lower temperature to 42°C induces the expression of so-

TABLE 1

EFFECT OF TEMPERATURE ON BACTERIAL YIELD

	ASSAY TEMPERATURE (°C)		
	32	37	42
Cell density	41.1 (±1.0)	33.2 (±.6)	7.6 (±.4)
Total biovolume	25.4 (±.3)	23.7 (±1.2)	12.8 (±.5)

NOTE.—The ancestral genotype was grown in minimal salts medium supplemented with 25 μg glucose/mL for 24 h, during which time populations reached stationary phase density and exhausted the available glucose. Cell densities (10^6 cells per mL) were estimated electronically using a Coulter counter, excluding any cells having a volume of less than 0.25 fL (= 10^{-12} mL). Total biovolume (nL/mL = 10^{-6}) was calculated as the product of cell density and average cell volume; N = 10 replicates per temperature; 95% confidence limits based on t distribution appear in parentheses.

called stress proteins in *E. coli* (Neidhardt and VanBogelen 1987; Gross et al. 1990). This heat shock response substantially reduces the rate of killing of cells that are subsequently exposed to a still higher temperature (e.g., 50°C), and in the absence of this response cell growth is severely impaired at 42°C (Neidhardt and VanBogelen 1987).

By all three criteria, 42°C must be regarded as a stressful treatment for our ancestral clone of *E. coli*. Continued propagation at 37°C provides a control for further adaptation to the ancestral environment. The 32°C treatment serves as a novel (i.e., nonancestral in the context of this experiment) but nonstressful regime, since the stress criteria used above are largely unfulfilled at this temperature: (1) 32°C is well away (>10°C) from both the lower and upper thermal limits (fig. 1); (2) while maximum specific growth rate is reduced at 32°C (Ingraham 1987), yield is not (table 1); and (3) stress proteins are not induced at 32°C (Neidhardt and VanBogelen 1987). Thus, 32°C is nonstressful, and the 32°/42°C treatment provides an alternation between stressful and nonstressful conditions.

EVOLUTIONARY RESPONSES OF *ESCHERICHIA COLI* POPULATIONS TO THERMAL STRESS

Do Populations Evolve More Rapidly in Stressful Environments?

Among our four experimental thermal regimes, three were novel while one continued the ancestral regime, constant 37°C. The three novel regimes spanned a range of 5°C above and below this ancestral temperature. By the criteria presented in the preceding section, the 42°C and 32°/42°C groups were subject to constant and intermittent stress, respectively. What were the rates of adaptation

TABLE 2

MEAN FITNESS RELATIVE TO COMMON ANCESTOR AFTER 2,000 GENERATIONS OF EXPERIMENTAL EVOLUTION

Treatment Group	Assay Temperature (°C)	Mean Fitness
Propagated at constant 37°C:		
Ancestral regime	37	1.078*
Propagated at constant 32°C:		
Novel but nonstressful regime	32	1.104*
Propagated at constant 42°C:		
Novel and stressful regime	42	1.447†
Propagated at alternating 32°/42°C:		
Novel and intermittently	32	1.037‡
stressful regime	42	1.156‡

NOTE.—The common ancestor was propagated for 2,000 generations at 37°C prior to founding the four treatment groups (Lenski et al. 1991).

* Bennett et al. (1992).

† Bennett and Lenski (1993).

‡ A. M. Leroi, R. E. Lenski, and A. F. Bennett (unpublished data).

(measured by improvements in fitness relative to the common ancestor after both genotypes were comparably acclimated) to these regimes, and did stress affect the rate of adaptation?

The populations propagated in the constantly stressful regime (42°C group) showed more rapid and more extensive genetic adaptation than did the populations propagated in either the continued ancestral regime (37°C group) or the novel but nonstressful regime (32°C group). In fact, within only 200 generations, the mean fitness of the 42°C group relative to the ancestor had increased by about 10% (Bennett et al. 1990). During this same period, there was no discernible improvement among the lines evolving at the ancestral temperature of 37°C.

By 2,000 generations, all three of these groups had significantly improved fitness relative to their common ancestor, when fitnesses were assayed at their respective temperatures (table 2). However, the average rate of improvement in the 42°C group was significantly greater than in the 32°C group, which in turn was significantly greater than in the 37°C group (Bennett et al. 1992). In other words, evolutionary adaptation was fastest in the stressful regime, followed by the novel but nonstressful environment, with the rate of improvement slowest in the continuation of the ancestral regime.

Another interesting contrast is provided by the 32°/42°C group, which was subject to an intermittently stressful regime. After 2,000 generations, all six lines in this group had improved to a greater extent in the stressful component (42°C) than in the nonstressful component (32°C) of their alternating environment (table 2), even though they had spent equal time in each component. The probability that, by chance alone, all six lines would improve to a greater extent in the same component environment is less than 0.05.

Thus, in this experimental system, evolutionary adaptation was consistently more rapid in the stressful environment than in either the ancestral environment

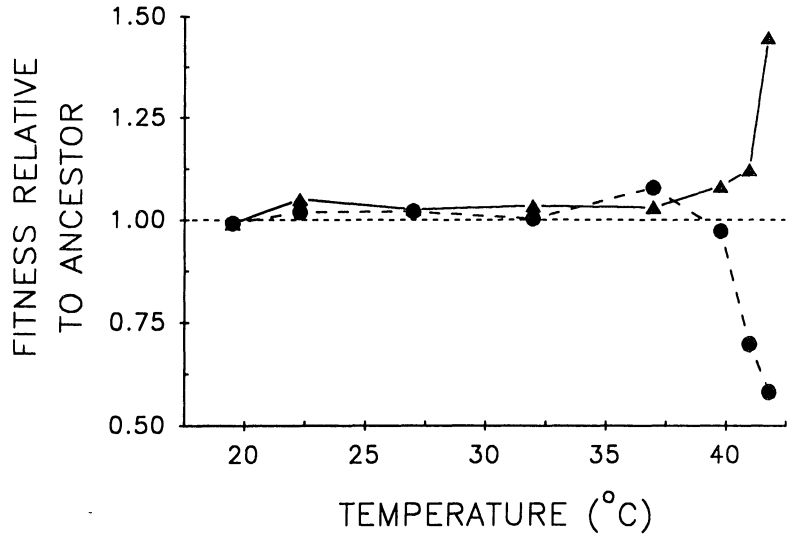

FIG. 2.—Thermal dependence of mean fitness of the lines selected at 42°C (*triangles, solid line*) and at 37°C (*circles, dashed line*), relative to the common ancestor. (Data from Bennett and Lenski 1993.)

or a novel but nonstressful environment. This difference in evolutionary rates was manifest whether the stress was imposed continuously or intermittently.

Does Adaptation to a Stressful Environment Entail Loss of Performance in Nonstressful Environments?

Does the evolution of stress tolerance inevitably cause an associated loss of fitness in nonstressful environments? In other words, are there antagonistic pleiotropic effects of the alleles that enhance stress tolerance on other aspects of performance? Many theoretical models of adaptation to varying environments assume that the area under some "fitness function" is constant, so that adaptation to one regime necessarily entails a fitness loss elsewhere along an environmental gradient (see, e.g., Levins 1968, pp. 14–15; Huey and Slatkin 1976; Lynch and Gabriel 1987; Pease et al. 1989). Empirical evidence from many species indicates that genotypes resistant to certain chemical stresses, such as antibiotics and heavy metals, grow more slowly or are otherwise less fit than susceptible genotypes in the absence of the stressful agent (see, e.g., Hickey and McNeilly 1975; Partridge 1979; McKenzie et al. 1982; Nguyen et al. 1989) . In view of the large increase in fitness at 42°C of the 42°C group, it is of interest to ask whether fitness decreased in other portions of the thermal niche.

The mean fitness of the 42°C group relative to the common ancestor, across the entire thermal niche of the ancestor, is shown in figure 2 (*solid curve*). After 2,000 generations, this group showed significant improvement relative to the ancestor at assay temperatures above about 40°C, but at no temperature was mean fitness *less* than that of the ancestor (Bennett and Lenski 1993). Evidently, the

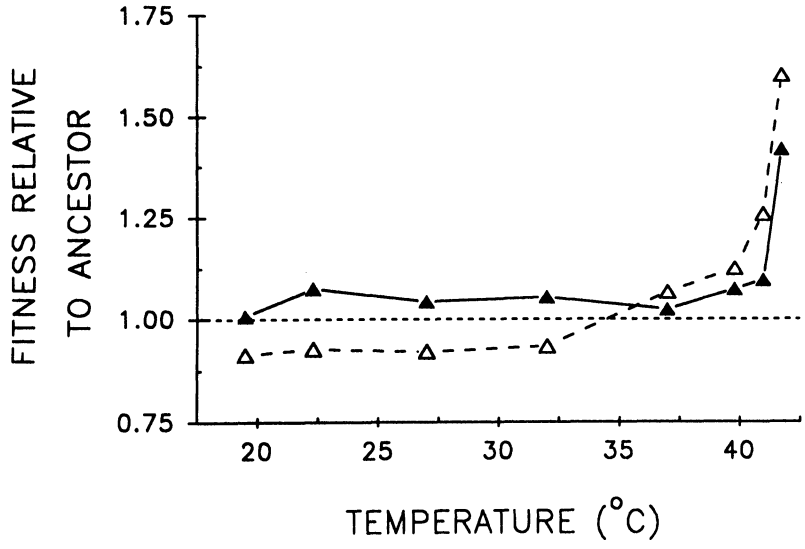

FIG. 3.—Heterogeneity among lines propagated at 42°C in thermal dependence of fitness relative to the common ancestor. *Open symbols* and *dashed line* correspond to one of six populations; *solid symbols* and *solid line* correspond to the mean of the other five populations. (From Bennett and Lenski 1993. Reprinted with permission.)

large increase in fitness under these stressful conditions was not accompanied by any correlated decrement in fitness elsewhere in the thermal niche.

This conclusion might be criticized on the grounds that some of the improvement by the 42°C group may represent continued adaptation to the general culture conditions rather than adaptation to the stressful thermal regime per se. The 37°C group, which evolved for another 2,000 generations at the ancestral temperature, provides a more conservative comparison for the 42°C group than does the common ancestor. Figure 2 also shows the mean fitness of the 37°C group relative to the common ancestor across the same range of temperatures (*dashed curve*). The evolving control lines did improve somewhat at 37°C, but these improvements were also highly temperature specific. Interestingly, some (but not all) of the control lines lost fitness relative to the ancestor at temperatures above about 40°C. This result suggests that continued adaptation to a nonstressful temperature may reduce thermal tolerance (Bennett and Lenski 1993). But consideration of the evolving control group does not alter the conclusion that adaptation to high-temperature stress had, on the average, no discernible detrimental effect on performance at lower temperatures.

Although the 42°C treatment group, as a whole, did not lose fitness at lower temperatures, one of the six replicate lines did, in fact, do so (Bennett and Lenski 1993). Figure 3 shows the thermal dependence of fitness for this one line, along with the mean responses of the other five lines in this group. At each of the assay temperatures below 37°C, this line had the lowest relative fitness of the six lines. (We will see later that this same line is unique among the lines in the 42°C

treatment group in another important respect.) This line therefore meets the expectations of a pleiotropic trade-off: improved fitness under the stressful conditions that obtained during its recent evolutionary history was accompanied by a decrease in fitness in dissimilar environments. However, this line is clearly exceptional in our experiments. None of the other lines in the 42°C group exhibited such a trade-off. In fact, the remaining lines in the 42°C treatment group were consistently more fit at temperatures below 37°C than both their ancestor and the evolving control group, which was continued at 37°C.

In a similar vein, after the first 400 generations, the 42°C group showed a correlated improvement in mean fitness at 37°C, which was significantly greater than the direct response of the 37°C group itself (Bennett et al. 1990). Again, however, there was significant heterogeneity among the lines in the 42°C treatment group, with five of the lines indicating positive correlations between fitness improvements at 42° and 37°C and one indicating a negative correlation.

We conclude that trade-offs in performance at nonstressful temperatures were not a necessary or even a typical consequence of adaptation to the stressful high-temperature regime. This surprising result *cannot* be explained simply as an artifact of continued adaptation to the general laboratory culture conditions. It is evidently possible for these bacteria "to have their cake and eat it too," that is, to increase fitness under high-temperature stress without undergoing a corresponding loss of performance elsewhere along the thermal gradient.

Does Adaptation to a Stressful Environment Extend the Niche?

Does evolutionary adaptation to a stressful environment predispose a population to tolerate even more extreme conditions, ones that were so damaging to the ancestral population as to cause its extinction? In other words, does adaptation to stressful conditions along some environmental gradient increase niche breadth, or at least extend the adjacent boundary of the niche? If so, then adaptation to stressful environments might proceed continuously: a population living under stress becomes adapted to that stress and concomitantly extends its niche, such that its members may then occupy even more extreme environments, becoming further adapted to those stressful conditions, and so on. Many models of evolution in changing environments implicitly assume that entire performance curves, including critical maxima and minima, shift during adaptation to changing conditions (see, e.g., Levins 1968; Huey and Kingsolver 1989; Pease et al. 1989). Such phenotypic shifts are commonly seen during individual acclimation to temperature changes: adjustment to warmer temperatures, for example, often increases subsequent survival at still warmer temperatures, in effect extending the critical thermal maximum (Prosser 1973; Cossins and Bowler 1987; Neidhardt and Van-Bogelen 1987). Similarly, intra- and interspecific comparisons of organisms from different thermal environments frequently indicate greater heat tolerance in organisms from warmer environments (Holland et al. 1974; Huey and Kingsolver 1989; Huey et al. 1991).

The 42°C treatment group spent 2,000 generations in a thermally stressful environment and improved dramatically in its mean fitness relative to the ancestor in that environment (fig. 2). Recall, too, that 42°C was very close (< 1°C) to the

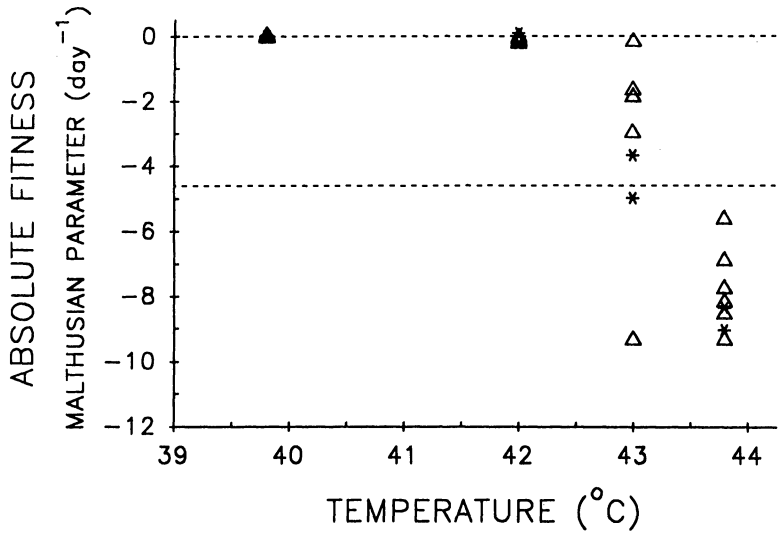

FIG. 4.—Upper thermal limits for population persistence in the common ancestor and in the 42°C treatment group. Absolute fitness is calculated by the Malthusian parameter (see fig. 1). *Asterisks* indicate the reciprocally marked genetic variants of the common ancestor; *triangles,* the six lines selected at 42°C. (Data from Bennett and Lenski 1993.)

upper limit for persistence of the ancestral population (fig. 1). Therefore, it is of interest to know the extent to which the evolution of this group produced a shift in the upper boundary of the thermal niche.

In fact, the upper boundary of the thermal niche changed very little, if at all, in the 42°C treatment group (Bennett and Lenski 1993). None of the six lines in this group could persist at about 44°C, and the rates at which their populations declined to extinction were extremely rapid and statistically indistinguishable from their common ancestor (fig. 4). Even at 43°C, only 1°C above the temperature at which they had evolved for 2,000 generations, five of the six lines in the 42°C treatment group were unable to persist, and their rates of decline to extinction were indistinguishable from the ancestral genotype. One of the lines in this group could, however, reliably maintain a stable population at temperatures about 1°C higher than the ancestor (fig. 5). This was the same line that showed a decrement in fitness relative to the ancestor at lower temperatures (fig. 3).

The 32°/42°C treatment group, which was subject to alternating stressful and nonstressful temperatures, also showed no significant changes in the upper thermal limit for population persistence (Bennett and Lenski 1993). Nor did the 42°C and 32°/42°C groups give any indication of a change from the ancestral condition in the lower critical temperature for population persistence (Bennett and Lenski 1993). However, the groups that evolved exclusively at lower temperatures (32° and 37°C) included some lines that lost the ability to maintain themselves at 42°C (Bennett and Lenski 1993), which indicates again (see fig. 2) that occasional thermal stress could be important in *maintaining* the ancestral ability to tolerate this temperature.

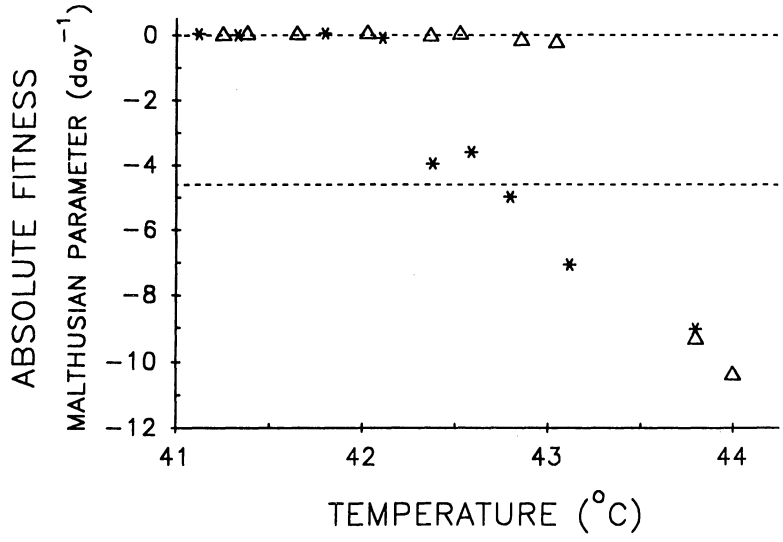

FIG. 5.—Upper thermal limits for population persistence in the common ancestor and in one replicate line from the 42°C treatment group. This particular line had the greatest extension of the upper limit, and it was the only line that had lost fitness relative to the ancestor at lower temperatures (fig. 3). Absolute fitness is calculated by the Malthusian parameter (see fig. 1). *Asterisks* indicate values for the common ancestor; *triangles*, for the line propagated at 42°C. This fine-scale resolution was achieved by taking advantage of a stable thermal gradient that existed in an incubator. (Data from Bennett and Lenski 1993.)

Therefore, we conclude that evolutionary adaptation to the stressful environment in our experimental system produced little or no improvement in the organism's ability to handle still more stressful environments. It is evidently possible to adapt to a very narrow range of stressful conditions along a continuous environmental gradient, such that the organism neither loses performance at points far away on the gradient nor improves its performance under conditions that are very near on the gradient.

It might be argued that the thermal niche in this bacterial strain is physiologically or genetically constrained, so that extensions of the upper temperature for population persistence would require changes in the genome beyond those that could be expected to appear over the course of 2,000 generations. But we are not inclined to this interpretation. During measurements of persistence at high temperatures outside the thermal niche (~44°C), we occasionally observed that a population declining toward extinction suddenly recovered and thereafter maintained itself. Particular lines did not invariably so recover, and bacteria isolated from these recovered populations (which we have dubbed "Lazarus lines") apparently retained their ability to persist at high temperature, even after being frozen and then propagated for many generations at moderate temperatures (Bennett and Lenski 1993). Thus, we interpret these Lazarus lines as being thermotolerant mutants. Interestingly, these mutants sometimes appeared even among lines that had previously evolved under the nonstressful thermal regimes. These

findings suggest that niche extensions may be achieved more often by occasional bouts of hard selection (in the sense of Wallace 1968) in superstressful environments than by prolonged periods of soft selection in stressful but generally nonlethal conditions.

CONCLUSIONS AND INTERPRETATIONS

This study sought to address three questions of general interest. First, we asked whether populations evolve more rapidly in stressful environments than in nonstressful environments. We observed that the rate of adaptation to 42°C, a demonstrably stressful temperature, was much more rapid than to either 32° or 37°C. We also observed that, in populations subject to an alternating 32°/42°C thermal regime, adaptation to the stressful temperature was greater than to the nonstressful temperature. Thus, our results support the hypothesis that stressful environments promote rapid evolution (see review by Parsons 1987). Second, we asked if adaptation to a stressful environment entailed concomitant loss of performance in nonstressful environments. Only one of six replicate lines that adapted to the stressful high-temperature environment became less fit at lower temperatures. Nonetheless, the improvements of the populations that evolved in the stressful regime were quite temperature specific. Thus, our findings are not consistent with the view that adaptation to a stressful environment inevitably trades off with performance under nonstressful conditions. Third, we asked whether adaptation to a stressful environment preadapts a population to even more stressful environments by extending the boundaries of its potential niche. Only one of six replicate lines propagated in the stressful 42°C regime showed even a 1°C extension of the critical thermal limit, above which population extinction results. And yet, by imposing superstressful regimes—temperatures that doomed the majority genotypes to extinction—we sometimes isolated mutants that had extensions of about 2°C in the upper thermal limit for population persistence. Our findings are not consistent with the hypothesis that adaptation to a stressful environment necessarily preadapts a population to further increases in environmental stress. Rather, these results suggest that bouts of selection in superstressful environments may be more efficacious than long-term selection in stressful but nonlethal conditions in promoting niche extensions into extreme environments (see also Parsons 1987).

The evolutionary responses of these bacterial populations to changes in the thermal environment do not accord well with models of evolution in changing environments that assume rigid constraints on a genotype's performance at different positions along an environmental gradient, nor do they accord very well with our own preconceptions. Nevertheless, we believe that these results are compelling: (1) our study extended over a period of thousands of generations; (2) we had tight manipulative control over the variable of interest, while all other aspects of the imposed environment were held constant; (3) we had several important inferential controls, including the founding ancestral genotype itself, as well as populations that were propagated at the ancestral temperature and at another novel but nonstressful temperature; (4) we had multiple populations in each treatment group, so that any differences could be rigorously ascribed to these groups

rather than to divergence of populations from one another that might arise by chance; (5) we distinguished genetic adaptation from physiological acclimation by conditioning steps in our experiments; and (6) we directly ascertained both absolute and relative fitnesses of genotypes for the laboratory environments in which they had evolved.

It is both appropriate and important to ask whether some special feature of our study organism or experimental system might be responsible for the unexpected results. There are important differences between bacteria and multicellular organisms (Andrews 1991), just as there are between different bacterial species or between different plants and animals. One particular feature of our study organism worth bearing in mind is its strict clonality, which tends to slow the evolutionary dynamics by preventing the simultaneous incorporation of multiple beneficial alleles (Crow and Kimura 1965; Lenski et al. 1991). In addition, our experimental populations were initially genetically homogeneous, so that selection acted on new mutations rather than on standing genetic variation. In this respect, our experimental system is more macroevolutionary than microevolutionary, in that reproducibility of findings in replicate populations cannot be attributed merely to selection for alleles that are identical by descent. It is difficult to see how these features of this experimental system could compromise the interpretation of our findings.

One must also be aware of potential artifacts that can arise from studying organisms in a laboratory environment, which might invalidate the applicability of a particular hypothesis or model. Of particular relevance is the concern that genetic correlations between performance measures cannot be properly tested in the laboratory owing to its effects as a novel environment, as elegantly demonstrated by Service and Rose (1985). To avoid this potential pitfall, we took the extraordinary precautions of using as founding genotype in these experiments a strain that had already adapted to the general laboratory culture conditions for 2,000 generations, and we maintained control populations under these conditions for the additional 2,000 generations of our experiment. We were thus able to increase the likelihood of observing evolutionary responses that were specific to the imposed stress and subsequently to demonstrate this specificity.

For these reasons, we believe that our results are compelling and unlikely to be peculiar to the particular system that we have studied. That is not to say, however, that our results are necessarily typical of the evolutionary responses of all organisms to stress. The issue of generality may be best addressed by a mixture of comparative and mechanistic approaches. There are two sorts of comparative analyses that might be performed. First, evolutionary responses of other organisms, both prokaryotes and eukaryotes, to stressful and nonstressful thermal regimes can be examined for concordance of results. Second, responses of this bacterium to other stresses could be investigated to determine the specificity of the observed evolutionary response. As an example of the mechanistic approach, we consider next some alternative explanations for one of our key results, which may shed light on its generality and which we plan to explore in future work.

Evolutionary adaptation proceeded much more rapidly in the stressful thermal regime than in the nonstressful regimes. Why was this so? Adaptation by natural selection requires genetic variability, which in our particular experimental system

was generated solely by mutation. Mutation rates often appear to increase at high temperatures (Lindgren 1972; Parsons 1987), so that high temperature might have increased the amount of genetic variability in our experimental populations on which selection could act. If increased mutation was the sole explanation for the differential responses that we observed, then one might question the generality of the relationship between stress and rates of adaptative evolution. That is, our results might apply to *thermally* stressed populations but not to populations subjected to other types of stress. However, it should also be kept in mind that *many* different types of stress may be mutagenic (Walker 1984; Parsons 1987; see Mittler and Lenski 1990 for a dramatic example of the effect of starvation on the excision rate of a mobile genetic element). We suspect that mutation rates probably did differ in our thermal treatments, although we are doubtful that this effect can account entirely for the observed differential responses for two reasons. First, population densities were substantially lower in the stressful high-temperature treatment (table 1). Thus, any increase in mutation rate would have had to more than offset this difference to account for the results. Second, populations propagated in the alternating thermal regime exhibited significantly greater adaptation to 42°C than to 32°C (table 2). But mutations appearing at either temperature would have been subject to selection at both temperatures, which suggests that the more rapid evolution in the stressful component environment was not entirely due to the thermal dependence of mutation rate.

A second class of explanation is that selection was more intense in the stressful high-temperature environment. This intensity might be due to the proximity of this treatment to the edge of the thermal niche, beyond which fitness drops precipitously (fig. 1). Near such critical limits, small changes in phenotype may translate into larger fitness effects than they would under more benign conditions (cf. Hartl et al. 1985). According to this explanation, one might expect also to see more rapid evolution near the lower boundary of the thermal niche, about 20°C. Yet other possibilities are that selection is more intense because the prior evolutionary history of these lineages has left more room for improvement at higher than lower temperatures, and that more phenotypic avenues are available to resist the damaging effects of higher temperatures than to increase growth rate at lower temperatures.

Without further analyses, it is not possible to know which of these explanations (or which combination of them) is responsible for the differences in evolutionary responses to the stressful and nonstressful conditions. The result remains, however, that adaptive evolution was most rapid and extensive in the stressful high-temperature environment. The beauty of studying evolution experimentally, using an appropriate biological system, is that one can subject these and other hypotheses to rigorous tests.

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