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Phenotypic and evolutionary adaptation of a model bacterial system to stressful thermal environments

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Summary. We studied both phenotypic and evolutionary adaptation to various thermal environments using the bacterium *Escherichia coli* as an experimental model system. We determined that 42°C was stressful to a bacterial clone adapted to 37°C, based on reductions in both absolute and competitive fitness, as well as induction of a heat stress response. This clone was also used to found replicated populations that were propagated for thousands of generations under several different thermal regimes, including 42°C. Evolutionary adaptation of the populations to 42°C resulted in an increase in both absolute and relative fitness at that temperature, measured respectively as an increase in the number of descendants (and their biovolume) and in competitive ability relative to the ancestral clone. The replicated experimental lineages achieved their evolutionary improvement by several distinct pathways, which produced differential preadaptation to a non-stressful nutrient environment. Adaptation to this stressful temperature entailed neither a change in the ancestral thermal niche nor any pronounced tradeoffs in fitness within the thermal niche, contrary to *a priori* predictions. This study system has several important advantages for evaluating hypotheses concerning the effects of stress on phenotypic and evolutionary adaptation, including the ability to obtain lineages that have evolved in controlled and defined environments, to make direct measurements of fitness and to quantify the degree of stress imposed by different environments.

Introduction

Organisms have the ability to tolerate and survive in a range of different thermal environments, which defines their thermal niche. The niche may be very broad in eurythermic species from temperate regions or quite narrow in stenotherms from polar environments (Precht et al., 1973; Cossins and Bowler, 1987). Regardless of the width of the thermal niche, there is typically a pronounced asymmetry in performance and functional capacity associated with its upper and lower extremes (Huey and Stevenson, 1979; Huey and Kingsolver, 1989). Exposure to progressively lower temperatures lowers performance and slows rate processes, often to the point of immobility, but these decrements may not be lethal in themselves. In fact, many organisms, even some vertebrates, can tolerate freezing (Cossins and Bowler, 1987; Storey, 1992). In contrast, exposure to progressively higher temperatures accelerates performance up to a maximal level, and then further temperature increments cause functional capacity to decline

precipitously and result in rapid death. Thus, acute exposure to temperatures at the upper extreme of the thermal niche is liable to be more stressful and damaging than exposure to low temperatures. Since heat stress may become lethal so rapidly, the ability to respond phenotypically to high temperatures may be critical to survival. Over long time periods, the ability of a population to respond genetically and evolve adaptive responses to thermally stressful environments may be crucial to its persistence, particularly in a period of warming climates (Holt, 1990; Karieva et al., 1993). The phenotypic and evolutionary responses of organisms and populations to heat stress are thus of considerable theoretical and practical importance.

Studies of phenotypic and evolutionary responses to heat stress are clearly very desirable. However, discussions and experimental investigations that deal with stress often become bogged down in problems of defining and quantifying the topic of interest (Hoffmann and Parsons, 1991). Many commonsense definitions of stress are so vague that they are not very useful in structuring a quantitative study of its implications for organismal function and survival. A major achievement in studies of stress has been the recognition of the induction of a variety of different gene products (heat shock or stress proteins) in organisms exposed to potentially damaging environments (Morimoto et al., 1990). The production of these proteins may allow an investigator to define operationally whether an environment is indeed stressful and to quantify to some extent the degree of stress imposed. Even so, the organismal consequences of the induction of stress proteins are poorly understood. It is through the differential functioning, survival and reproduction of individual organisms that natural selection operates, and it is the impact of stress on these factors that is ultimately important for evolution. We believe that an analysis of stress, including both its phenotypic and evolutionary consequences, should ultimately be based on these organismal- and population-level consequences. Therefore, we and others (Koehn and Bayne, 1989; Sibly and Calow, 1989; Hoffmann and Parsons, 1991; Lenski and Bennett, 1993; Forbes and Calow, this volume) favor the definition of stress as an environmental factor that causes a reduction in fitness, i.e. the reproductive potential of the individual organism or an entire population. An evolutionary adaptation to stress is one that increases the fitness of a population in the stressful environment.

While quantitative measurements of the impact of stress on fitness are desirable, they are also very difficult to obtain in most kinds of organisms. It may even be difficult to measure the effects of stress on presumptive fitness components, and these difficulties have inhibited progress in investigations of the phenotypic and evolutionary responses to stressful environments, including high-temperature environments. In contrast, it is relatively easy to measure fitness in populations of bacteria. This ability to quantify the impact of stress on fitness in these organisms, along with a suite of other features of their laboratory culture and population structure, makes them ideal subjects for investigating general principles of both

phenotypic and evolutionary adaptation to stress. In this chapter, we report the results of our experimental investigations of the phenotypic and evolutionary responses of a model bacterial system to heat stress. This work is an extension of our previous essay on this topic (Lenski and Bennett, 1993). We discuss below the suite of properties that make bacteria such valuable subject species for investigations of stress, and we outline the general features of our experimental system. We next present our findings on the phenotypic (non-genetic) responses of the ancestral bacterial strain, which is adapted to 37°C, to high-temperature stress in the form of acute exposure to 41.5–42°C. We then discuss the evolutionary adaptation of replicated lineages of this strain to persistent high-temperature stress. Finally, we discuss some general conclusions from our research.

Why bacteria?

It is obvious that bacteria offer many advantages as experimental subjects to a laboratory scientist. They are easy to maintain and propagate in large numbers, and a great wealth of information is available about their molecular and cell biology and genetics. In addition, they offer many special advantages for the experimental evolutionist, in particular someone interested in adaptations to stressful environments. These special features include the following:

1) *Fitness can be readily quantified.* If the best criterion to evaluate the direct phenotypic consequences of stress is fitness reduction (see "Introduction"), then it is necessary to be able to measure fitness accurately and rapidly. For bacterial populations, such measurements are straighforward and can be made in a variety of ways. "Absolute fitness" may be measured by growing replicate cultures in both benign and putatively stressful environments (e.g. moderate and high temperatures) and comparing the impact of the environments on total population production. A reduction in the total number or size (biovolume or biomass) of cells in a population is evidence of stress. Absolute fitness may also be measured simply as the ability of a population to sustain itself in a particular set of environmental conditions. Absolute fitness tests the performance ability of a population of like organisms, in the absence of competition. "Relative fitness" can be measured by experiments involving direct competition between two different populations for a common pool of resources; it is expressed as the differential rate of offspring production during competition. For instance, one can quantify the relative fitness of two different phenotypic (acclimation) states in a defined environment, or one can obtain the relative fitness of genotypes with different evolutionary derivations. If a population is cultured for a long period in a novel environment (e.g. at high temperature), then evolu-

tionary adaptation may lead to increased fitness. This adaptation can be best quantified by direct competition experiments between the ancestral strain and the derived population that was selected in the novel environment. An increase in the rate of offspring production of the derived population relative to that of its ancestor is evidence of evolutionary adaptation, and its mechanistic bases and correlated consequences are then open to further investigation. All these types of fitness measurements are feasible and can be made quickly with bacterial populations.

2) *Experimental evolutionary studies are feasible over relatively short periods of time.* Bacteria may be conveniently propagated in such large numbers that even rare beneficial mutations may occur and be subject to natural selecton (since the frequency of the appearance of a mutation is the product of mutaton rate and population size). It is therefore possible to propagate bacteria in a novel environment (e.g. at high temperature) and to observe genetic change and evolutionary adaptation arising via selection for spontaneous mutations within the population. Because of the large population sizes and rapidity of reproduction, evolutionary changes may occur quite rapidly. For example, we observed evolutionary adaptation in the bacterium *Escherichia coli* to high and stressful temperatures within only 200 generations, which took only 30 days in the experimental regime that we employed (Bennett et al., 1990).

3) *Clonal reproduction facilitates experimental replication and control.* True experiments require rigorus regulation of the variables of interest, replication of treatments, and appropriate controls. These features are all possible in evolutionary experiments using bacteria. The asexual nature of bacterial reproduction facilitates the creation of genetically identical populations, many replicates of which can be cultured simultaneously in a novel (e.g. stressful) environment as well as in the ancestral environment, the latter serving as experimental controls. Since each population is physically separated from the others after its creation, it is an independent replicate of the experiment, and measurements of the performance of replicate cultures can be analyzed statistically to determine whether significant directional change has occurred in the variables of interest. Moreover, all genetic changes arise *de novo* in each population, owing to its clonal origin. Thus, unlike in experiments with most higher organisms, one can be sure with bacteria that parallel responses to selective regimes must have arisen by independent genetic events. Populations propagated in the ancestral environment serve as a control for evolutionary changes that may be associated with features of the culture regime other than those of direct experiment interest to the investigators. Further, the fact that the founding ancestral clone can be frozen and later revived permits it to be used as a comparative basis for measurements of evolutionary change in both the experimental and control lineages. For instance, a direct assessment of evolutionary

adaptation to a stressful environment can be obtained by measuring the reproductive success of an experimentally derived lineage relative to that of its ancestor as they compete with one another in the stressful environment.

4) *Adaptation can be analyzed at all levels of biological organization.* So far in this discussion, we have concentrated on the utility of bacteria for observations at the population and organismal levels of biological organization. In terms of wealth of information, however, it is at the cellular, molecular and genetic levels that bacteria are exemplary experimental organisms. It is no exaggeration to say that in these areas the bacterium *E. coli* is the best investigated and understood organism of all species on earth (Neidhardt et al., 1987). Further, phenotypic responses to heat stress, that is, activation of heat shock proteins, in bacteria are homologous to those in eukaryotes (Neidhardt and VanBögelen, 1987; Gross et al., 1990), indicating that responses to stress are widely shared among very different kinds of organisms. Bacteria have already played an important role in elucidating our understanding of the mechanistic bases of the heat shock response. It should also be possible to use bacteria to investigate the mechanisms underlying the evolutionary response to stressful environments. Clones of bacteria that have been shown to have adapted to stressful environments (in the types of evolutionary experiments discussed above) can be subjected to detailed genetic and molecular analyses to determine the mechanistic bases of that adaptation. The availability of the ancestral clone permits the precise identification of the genetic changes that have occurred in the experimental lineages, even when these changes are few in number. Replicate experimental lineages can be analyzed for the variety of alternative forms and mechanisms of adaptation that may have occurred during the experiment, providing evidence for the diversity (or lack of it) of mechanistic solutions to a common environmental challenge.

5) *Natural diversity in thermotolerance.* The diversity of natural thermal environments occupied by the Bacteria and Archaea is unparalleled. They occur in every environment on earth. Bacteria found in permafrost can be revived and grow when thawed. Psychrophilic bacteria that grow at nearly freezing temperatures occur in Antarctic seas (Straka and Stokes, 1960; Baross and Morita, 1978). Mesophilic bacteria with intermediate temperature optima abound in all temperate and tropical environments, including commensals and pathogens of mesophilic higher organisms. Thermophilic bacteria and archaea from hot springs and hydrothermal vents grow optimally at temperatures of 75 to 105°C (Brock, 1986; Stetter, 1995). Each of these groups has a limited range of growth temperatures: optimal temperatures for mesophiles may be lethally hot for psychophiles and yet too cold to permit growth of thermophiles. Presumably all of these groups have evolved different mechanisms of coping with these incredibly diverse thermal environ-

ments. The comparative potential provided by these groups for the analysis of natural evolutionary adaptation to thermally diverse and stressful environments is unmatched in any other group of organisms.

For a wide variety of reasons, therefore, bacteria provide exceptional opportunities for the investigation of adaptation to thermal stress.

The model study system

We have studied the phenotypic and evolutionary adaptation of lineages of the mesophilic bacterium *E. coli* B to different thermal environments, including thermally stressful environments (Bennett et al., 1990, 1992; Bennett and Lenski, 1993, 1996, 1997; Leroi et al., 1994a, b; Mongold et al., 1996). Here we summarize briefly some important features of the design of these experiments; readers are referred to the original publications for more details. We founded our study system from a single clone (designated here as the "ancestor") of a bacterial lineage that had previously been propagated at 37°C by serial dilution in minimal glucose (25 μg/ml) medium for 2000 generations (Lenski et al., 1991). During this initial period, that lineage underwent extensive evolutionary adaptation, increasing its relative fitness by over 30% (Lenski et al., 1991). With continued propagation under the same experimental conditions, however, subsequent fitness improvement was relatively slight (<3% during the next 2000 generations, Bennett and Lenski (1996)), indicating that further evolutionary adaptation to the basic culture conditions was becoming increasingly difficult. The use of an ancestor that was already well adapted to a defined set of culture conditions increased the likelihood that temperature-specific adaptations, rather than general adaptations to culture conditions, would be seen when experimental lines were propagated in novel environments. A genetic marker (± ability to metabolize arabinose) was incorporated into the ancestral strain that permitted identification of lines in competition experiments, and the selective neutrality of the marker was verified over a wide range of experimental temperatures.

The thermal niche of the ancestral strain was determined to be between 19 and 42°C (Fig. 1), the range of temperatures over which it can persist in culture with daily 100-fold serial dilution (requiring a growth rate of 6.64 generations per day). Temperatures below 19°C are not lethal but sufficiently retard growth rate so that the bacteria are unable to produce 6.64 generations daily and are therefore eventually diluted to extinction by the serial transfer regime. In contrast, high temperatures inhibit growth completely or kill the bacteria. A population of the ancestor is able to sustain itself indefinitely at 42.1°C, but it is unable to grow at all at 42.3°C and is therefore rapidly diluted to extinction. At 43°C, the ancestor not only cannot grow but dies at a significant rate. These data illustrate the pronounced

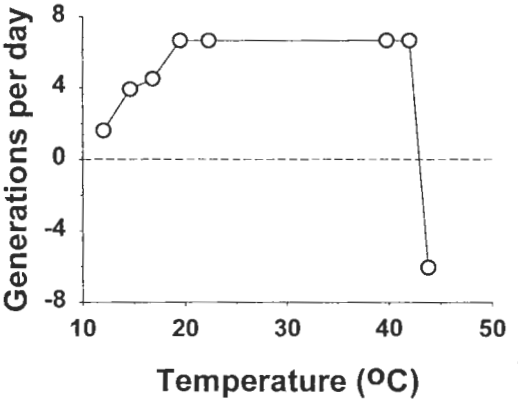

Figure 1. The effect of temperature on population growth rates of the ancestral bacterial clone. Each day, bacterial populations were diluted 100-fold into fresh medium, and the populations grew until nutrients were exhausted. The maximum number of cell divisions possible under this regime is 6.64 ($2^{6.64} = 100$). The thermal niche is defined as the range of temperatures in which this rate of growth occurs. At slower rates of growth, the populations are progressively diluted to extinction. A value of zero indicates no net growth, and negative values indicate cell death as well as dilution. (Data recalculated from Bennett and Lenski, 1993.)

asymmetry of heat and cold stress discussed in the "Introduction": cold temperatures may be inhibiting, but hot temperatures may be rapidly lethal. The thermal niche of this clone is undoubtedly affected by our use of a minimal glucose medium: growth rates are slower and thermal niches consequently narrower in minimal as compared with rich medium (Herendeen et al., 1979). The phenotypic responses of the ancestor to temperatures near its upper lethal limits – but still within its thermal niche – are discussed in the section on "Phenotypic responses to heat stress".

Clones of the ancestral strain were used to found an evolutionary experiment involving groups of populations propagated in four novel thermal environments (constant 20, 32 and 42°C and 32–42°C, the latter being a daily alteration between these two temperatures), as well as continuing propagation in the ancestral environment of 37°C. The ancestral strain lacks plasmids and is strictly asexual, so all groups began with an identical genetic background and all changes in fitness are attributable to *de novo* mutations and subsequent selection. Six experimental lines (3 each of the two genetic marker states for arabinose utilization) were founded for each group (= temperature regime). All 30 lines (five groups with sixfold replication) were propagated by serial dilution in minimal glucose medium for 2000 generations (300 days) under their defined temperature regimes. The absence of cross-contamination among populations and of external contamination by other bacteria was verified by testing appropriate genetic

markers. Adaptation of the experimental and control lines to their respective thermal regimes was measured every 200 generations by direct competition experiments against the ancestral form that possessed the opposite genetic marker state. Ancestral and derived lines were preconditioned separately at the experimental temperature for 1 day before being mixed together in fresh medium, so that both competitors were phenotypically acclimated to the experimental conditions. The fitness of a derived line relative to its ancestor was calculated as the ratio of their growth rates according to the formula

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

where subscripts i and f denote initial and final values, respectively, for the population densities of the evolutionarily derived (E) and ancestral (A) genotypes. Of particular interest in the context of evolutionary adaptation to heat stress are changes in the lines of the 42°C group, which was propagated at a temperature within 1°C of the upper limit of the ancestral thermal niche. The properties of this group are discussed in the section on "Evolutionary responses to heat stress".

Phenotypic responses to heat stress

What are the short-term (phenotypic) and long-term (evolutionary) effects of exposure to high temperature on the functioning and reproductive performance of *E. coli*? Can high temperatures be demonstrated to be stressful to this organism? What are the correlated consequences of that stress for organismal performance in other environments? We begin to answer these questions by examining first the phenotypic responses of the ancestral bacterial strain to exposure to temperatures near the upper limit of its thermal niche. The ancestral strain is well adapted to 37°C, and the upper limit of its niche is approximately 42.3°C. What are the proximate phenotypic effects of exposure to temperatures around 42°C?

1) *Depression of absolute fitness.* The effect of temperature on absolute fitness, the net production of cells, can be measured directly from the number and size of descendants produced at different temperatures given a constant nutrient base. The ancestral strain was grown at several different temperatures, in a medium containing a fixed amount of nutrient (25 µg glucose/ml), for 1 day, during which time the supply of glucose was exhausted. The number of descendent bacteria and their average size was then measured with a Coulter counter, and the total biovolume yield was calculated (Fig. 2; A.F. Bennett and R.E. Lenski, unpublished data). The number of cells produced declines markedly as temperature rises much above 37°C. It is halved at 40°C; at 42.0°C, it is only 11% that at 37°C. Mean cell size increases over this temperature

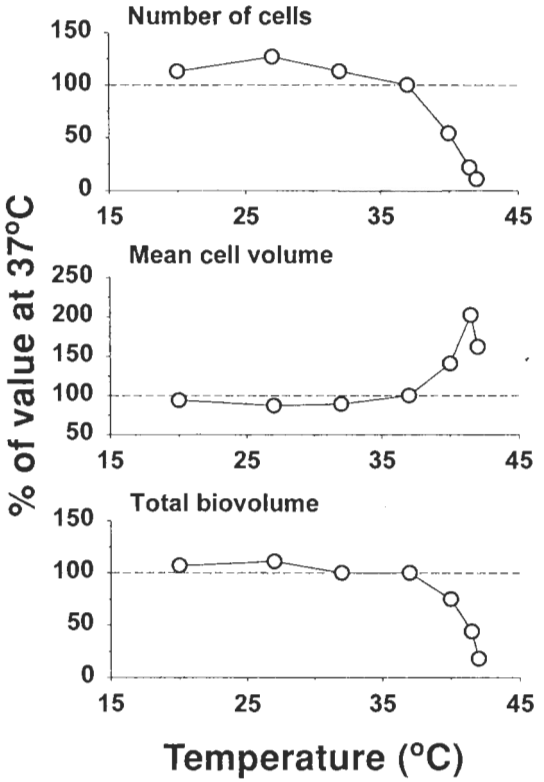

Figure 2. The effect of temperature on total reproductive output of populations of the ancestral clone, given a constant amount of nutrient. Total number of cells produced, the mean cell volume, and their product – the total biovolume produced – were measured with a Coulter cell counter. All values are normalized to values measured at 37°C. (Unpublished data of the authors.)

range (see also Neidhardt and VanBogelen, 1987), at least to some extent because some daughter cells do not separate after replication and thereby form chains of two or more cells (Tsuchido et al., 1986). While the latter effect partially offsets the former effect, overall yield – measured as the total volume of offspring produced – declines, being only 75% at 40°C and 18% at 42.0°C of the biovolume produced at 37°C. These results demonstrate unambiguously that high temperatures are indeed stressful to the ancestral bacterial strain, using our definition of a stressful environment as one that reduces fitness (see Introduction).

2) *Heat shock response.* Growth of the ancestral strain at 41.5°C results in increased thermotolerance at lethally higher temperatures (Leroi et al., 1994a): the death rate of cells at 50°C is only half that of cells that were grown at 32°C. This increased thermotolerance is the original definition of the heat shock response (Neidhardt and VanBogelen, 1987). Exposure to 42°C activates numerous heat shock genes and induces the expression of many heat shock (stress) proteins in *E. coli* (Neidhardt and VanBogelen, 1981, 1987; Gross et al., 1990). The latter are presumably involved in increased tolerance to heat and other forms of stress, but exact causal and mechanistic associations among these factors are still poorly understood (Neidhardt and VanBogelen, 1987; Watson, 1990).
3) *Decreased competitive ability.* It is generally believed that phenotypic adjustments to new environments improve function in those environments (e.g. Hoffmann and Parsons, 1991; Rome et al., 1992). In the case of exposure to different temperatures, a host of different physiological adjustments, termed "acclimation" or "acclimatization", occurs (Hochachka and Somero, 1984; Cossins and Bowler, 1987); at high temperatures, the heat shock response is one of these. According to the "beneficial acclimation assumption" (Leroi et al., 1994a), these resulting phenotypic changes benefit the organism and improve its functional capacities at that temperature in comparison with phenotypes that would result from prior exposure to other temperatures. Contrary to that assumption, however, we found that exposure to heat stress actually handicaps the performance of the ancestral bacterial strain at high but nonlethal temperatures, in comparison with identical bacteria without prior heat exposure (Leroi et al., 1994a). We acclimated cultures of the ancestral strain for one day at either 32 or 41.5°C, then reciprocally cross-competed them the next day and measured their relative fitness according to their acclimation state (Tab. 1). The bacteria acclimated to the competition temperature had prior exposure and underwent various physiological adjustments to that temperature, and so we anticipated that they should be competitively superior to non-acclimated forms. This was indeed the case in competition at 32°C, where the 32°C-acclimated form had higher fitness than the 41.5°C-acclimated form. But at 41.5°C, the 32°C-acclimated form was again competitively superor, with a relative fitness advantage of about 17%. Prior exposure to heat stress in fact disabled the bacteria and made them less able to compete, rather than improving their performance in the acclimation environment. The reduced competitive fitness caused by acclimation to 41.5°C might reflect some physiological handicap associated with expression of stress proteins when they are not actually needed to prevent heat damage (Leroi et al., 1994a). For example, synthesis of most other proteins is repressed by the heat shock response (Neidhardt and VanBogelen, 1987; Watson, 1990). However, further studies at non-

Table 1. The effect of thermal acclimation on relative fitness of the ancestral strain at high temperature

Acclimation temperatures		Competition temperature	Mean fitness	p (W = 1)
32°C	32°C	32°C	1.006*	0.6
41.5°C	41.5°C	41.5°C	0.993*	0.6
32°C	41.5°C	32°C	0.921'	< 0.001
32°C	41.5°C	41.5°C	0.827'	< 0.001

Populations of the ancestral strain expressing reciprocal genetic markers were acclimated to either 32 or 41.5°C for 1 day and then allowed to compete against one another during a second day. Mean fitness (W) of the two different forms is calculated either as the fitness of the Ara+ form relative to that of the Ara− form (*), or as the fitness of the 41.5°C-acclimated form relative to that of the 32°C-acclimated form ('). Differences in fitness between the acclimation states are indicated by statistically significant departures from a mean fitness value of 1. Bacteria acclimated to 32°C are superior to those acclimated to 41.5°C during subsequent competition at either 32 or 41.5°C. (Data from Leroi et al., 1994a.)

stressful temperatures (Bennett and Lenski, 1997) have found other examples of competitive inferiority associated with acclimation, so the mechanistic basis of this fitness depression remains unknown.

Evolutionary responses to heat stress

The ancestral bacterial strain, which had evolved at and adapted to 37°C, is clearly stressed at 41–42°C. As outlined in the previous section, exposure to these temperatures depresses both net production and competitive fitness. What then is the effect of long-term exposure to these thermally stressful temperatures? Do evolutionary changes occur that better adapt the lineage to function at these temperatures? Are there any general patterns in the direct responses to these temperatures or in the correlated responses at other temperatures? What are the mechanisms underlying adaptation to heat stress? We have begun to investigate these questions experimentally by examining the properties of the 42°C group, comprising six replicated experimental lines derived from the ancestral strain that were propagated at 41–42°C for 2000 generations. In this group, we can observe directly the evolution of adaptation to heat stress. The following are some of the main findings from this evolution experiment.

1) *Improvement in absolute fitness.* After 2000 generations of culture at 41–42°C, the absolute fitness, or net productivity, of the lines of the 42°C group had improved substantially in the thermally stressful environment (Fig. 3). At 41.5°C, the number of descendants produced from an equal amount of nutrient is twice that produced by the ancestral strain. Average cell size did not change greatly, so the biovolume yield at 41.5°C is double that of the ancestor. In fact, the mean biovolume of

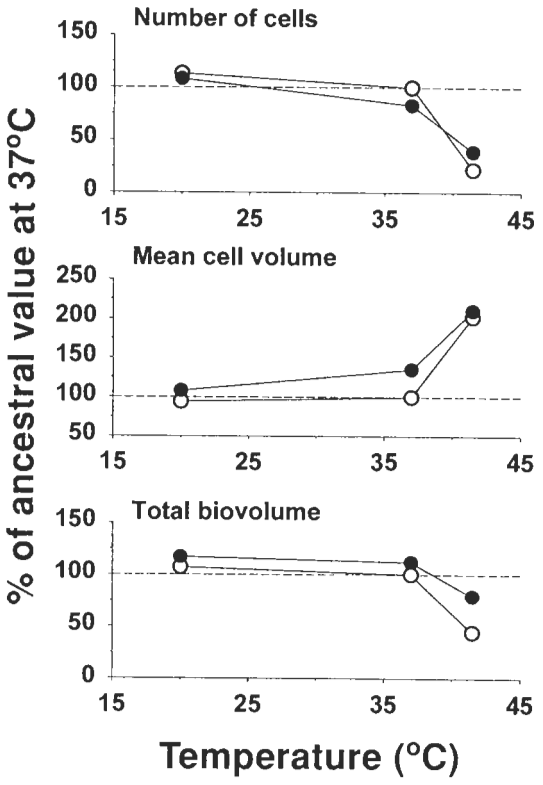

Figure 3. The effect of temperature on reproductive output of the ancestral clone (open circles) and the 42°C experimental group (filled circles). Mean values of the six experimental lines of the latter group are shown. Total number of cells produced, the mean cell volume, and their product – the total biovolume produced – were measured with a Coulter cell counter. All values are normalized to those of the ancestral clone at 37°C. (Unpublished data of the authors.)

the selected lines at 41.5°C is now 80% that of the ancestral clone at is own evolutionary temperature of 37°C. Yield efficiency, the conversion of energy into biomass, has consequently improved substantially during evolution in the thermally stressful environment. This dramatic improvement in productivity at 41.5°C is correlated with more modest but also significant gains at other temperatures in the thermal niche. In the 32–42°C group as well, improvement in biovolume yield at 41.5°C, the thermally stressful component of the environment, was greater than that at 32°C, the non-stressful component (A. F. Bennett

and R.E. Lenski, unpublished data). Clearly, evolution resulted in extensive improvement under heat stress in these experimental groups.

2) *Rapid and extensive improvement in competitive fitness.* Within 200 generations (30 days), the mean fitness of the six experimental lines of the 42°C group had increased about 8.5% at that temperature (Bennett et al., 1990). This 8.5% advantage accrues each and every generation, and so after only 1 day of competition (~6.6 generations of binary fission), the average derived cell has almost 50% more descendants than does a cell of the ancestral strain [exp (0.085 * 6.6 * ln(2)) = 1.48]. No change in fitness at 37°C had occurred in this time in the 37°C group, indicating that the fitness improvement in the 42°C group was probably the result of specific adaptation to temperature and not general adaptation to other aspects of culture conditions. In further support of that conclusion, even after 400 generations, no increase in fitness was observed in any experimental or control group at its selective temperature besides the 42°C group. After 200 generations, the mean fitness of the lines of the 42°C group had increased ~33.5% when assayed at 42°C, a substantially greater improvement than occurred in any other group at its corresponding selective temperature (Tab. 2). Compounding this 33.5% fitness difference as above indicates that the average individual cell in the 42°C group has almost fivefold more descendants than does the ancestor after only 1 day of competition at that temperature. Interestingly, the fitness improvement of the 32–42°C group was also far greater at 42°C than it was at 32°C, even though the group spent the same amount of time (and had nearly equal numbers of generations) at each temperature. Consequently, we conclude that in our study system evolutionary adaptation to environments involving heat stress was far more rapid and extensive than it was to any other thermal environments, including those near the lower extreme of the thermal niche. This finding thus supports the assertion that stressful environments should promote rapid evolutionary change (e.g. Parsons, 1987, this volume; Hoffmann and Parsons, 1991).

Table 2. Fitness of different experimental groups, relative to that of their common ancestor, after 2000 generations in their respective selective environments

Experimental group	Assay temperature	Mean fitness ± 95% C.L.
20°C	20°C	1.087 ± 0.027
32°C	32°C	1.107 ± 0.028
37°C	37°C	1.025 ± 0.020
42°C	42°C	1.335 ± 0.168
32–42°C	32–42°C	1.174 ± 0.068
32–42°C	32°C	1.049 ± 0.016
32–42°C	42°C	1.267 ± 0.079

All fitness values are significantly greater than 1, indicating evolutionary adaptation to the selective environments (Data from Bennett and Lenski, 1996.); C.L.: Confidence Limits.

3) *Evolution of acclimation response.* Did the phenotypic acclimation response of the 42°C group also evolve during adaptation to high temperature? The previous measurements on competitive fitness of this group involved both acclimation and competition at high temperature. Part of the observed fitness improvement may therefore have been due to improvements in the phenotypic acclimation response at high temperature. If the magnitude of the observed fitness benefit were reduced by acclimation to the ancestral temperature prior to competition at high temperature, such a difference would indicate that the acclimation effect itself had evolved in a way that was beneficial during the constant exposure to high temperature for 2000 generations. We ran paired competition experiments between the lines of the 42°C group and their common ancestor (A.F. Bennett and R.E. Lenski, unpublished data). All of the competition experiments were performed at 41.5°C, but in one half of the experiment both competitors were acclimated to 41.5°C and in the other half both were acclimated to 37°C. The difference in relative fitness between the two treatment pairs provides a measure of the evolutionary change in the effect of acclimation (Bennett and Lenski, 1997). Averaging over all six lines, the mean fitness relative to the common ancestor was 1.40 following acclimation at 41.5°C but only 1.24 following acclimation to 37°C (A.F. Bennett and R.E. Lenski, unpublished data). This difference is only marginally significant ($t = 2.161$, 5 d.f., $p = 0.083$) due to considerable heterogeneity among the lines in the effect of acclimation. However, at least two of the lines had much higher fitnesses following acclimation to 41.5°C than they did following acclimation to 37°C. Therefore, in at least some of the lines, a substantial portion of their improved fitness at high temperature depends on acclimation to high temperature. Hence, not only can acclimation influence fitness directly (as shown in the section on phenotypic responses of the ancestor to heat stress), but the acclimation benefit itself can improve during evolution in a thermally stressful environment.

4) *Diversity of adaptive pathways.* Within the 42°C group are six independently derived experimental lines, each of which adapted significantly to this stressful environment. They all began with a common genetic background, and all changes were the result of natural selection acting on mutations that arose *de novo* within the independent lineages. Was there a common adaptive mechanism in all six lines, or did the evolving bacteria "discover" a diversity of adaptive solutions to the problem of heat stress? We do not yet know the physiological mechanisms by which these experimental lines adapted to heat stress, although temperature-specific alterations in glucose uptake appear to be involved in some cases (Bennett and Lenski, 1996). By analyzing heterogeneity in patterns of performance (e.g. fitness in different environments), we can derive minimal estimates for the number of phenotypically distinct lines

within the selected group. For instance, analysis of variance of relative fitness in the selective environment (42°C, minimal glucose medium) indicates two distinct clusters of lines which are statistically different from each other (Bennett and Lenski, 1996). Competition in another nutrient (42°C, minimal maltose medium) also indicates two statistically distinct clusters of lines (Bennett and Lenski, 1996). Analysis of the number of descendant cells and average cell size in the selective environment also indicates two significantly different clusters (A.F. Bennett and R.E. Lenski, unpublished data). Therefore, we conclude that multiple, two or more, adaptive pathways were followed by these lines in their evolutionary adjustment to this stressful environment.

5) *Little change in the thermal niche.* The lines of the 42°C group were maintained within 1°C of their upper limit for persistence in serial dilution culture. During 2000 generations, they experienced considerable adaptive evolution at that temperature. In the course of that evolution, was there an increase in heat tolerance and a shift in the upper boundary of the thermal niche to even higher temperatures? And was there a loss of cold tolerance associated with adaptation to heat stress? Surprisingly, the thermal niche of the 42°C did not change much during its adaptation to high temperature (Bennett and Lenski, 1993). Only one of the six experimental lines increased its upper thermal limit, and that increment was less than 1°C; the mean upper limit of the thermal niche for the group as a whole did not change significantly from that of the ancestor. Likewise, the lower boundary of the thermal niche did not change significantly from its ancestral condition, about 19°C. In spite of fitness gains in the high-temperature environment, the broader thermotolerance of this experimental group was essentially unaltered. In contrast, the 20°C group did experience a downward shift in its thermal niche, decreasing both its lower and upper thermal limits by 1–2°C (Mongold et al., 1996).

Although no thermophilic forms evolved in our main evolution experiments, further experiments at temperatures in excess of 43°C yielded a small number of mutant forms capable of surviving at these temperatures (Bennett and Lenski, 1993; J. Mongold, unpublished observations). Their thermophily persists even after storage and growth at lower temperatures, indicating that the effect is genetic (and not merely delayed phenotypic acclimation to high temperature). We call them "Lazarus" mutants, because they emerge from otherwise dying populations. The upper boundary of their thermal niche is not greatly extended (none can persist in minimal glucose medium above 45°C), but they are capable of growing at temperatures 1–2°C higher than can the ancestral strain. Thus, while it is possible for more thermophilic forms to emerge from the genetic background of the ancestral bacterial strain, none became fixed in our main experiment, even among those lines that experienced 42°C. Preliminary studies indicate that, although

the Lazarus mutants can grow at higher temperatures, their fitness within the ancestral thermal niche is somewhat compromised (J. Mongold, personal communication). Consequently, if one had arisen in an experimental population, it would have been outcompeted by more mesophilic genotypes. These Lazarus mutants can apparently prosper only under the hard selective regime of temperatures that are lethal to the rest of the population.

6) *No tradeoffs in fitness at other temperatures.* Many evolutionary models assume that adaptation to one environment necessarily entails a loss of fitness in some other environments; in the case of thermal environments, improvement in function at one temperature is assumed to be accompanied by poorer performance at temperatures at the opposite end of the thermal niche (e.g. Levins, 1968; Lynch and Gabriel, 1987; Pease et al., 1989). The 42°C group experienced considerable improvement in its absolute and competitive performance at 41–42°C. Although there was essentially no change in its thermal niche, did it lose competitive ability at lower temperatures within that niche? That is, was there a tradeoff in performance, and ultimately fitness, associated with evolutionary adaptation to high temperature? Surprisingly again, no general tradeoff was detected. All six lines in the 42°C group had higher fitness than the ancestor between about 40 and 42°C. One of the six lines in this group showed a modest reduction in fitness at low temperatures, but the mean fitness of the group as a whole did not differ significantly from that of the ancestor between about 19 and 37°C (Fig. 4). Therefore, it is evidently possible for these bacteria to undergo

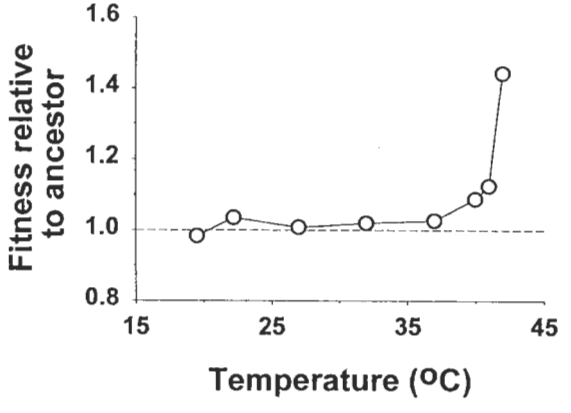

Figure 4. The effect of temperature on the fitness of the 42°C group relative to the ancestral clone, measured during direct competition. Mean values of the six experimental lines are reported. (Data recalculated from Bennett and Lenski, 1993.)

extensive evolutionary adaptation to a stressfully high temperature without simultaneously experiencing a loss of fitness at lower temperatures.

7) *Unexpected preadaptation to other environments.* Expression of heat shock proteins enhances not only thermotolerance but also resistance to other forms of stress (e.g. ethanol exposure, Neidhardt and VanBogelen (1987)). In fact, they are sometimes termed "stress proteins" in recognition of this cross-resistance. Thus, phenotypic responses to heat stress are already known to enhance function in a variety of different stressful environment. What we found in our experiments was that evolutionary responses to heat stress pleiotropically enhanced fitness in other environments. Specifically, during their evolution in minimal glucose medium under thermal stress, five of the six lines in the 42°C group became even more fit in maltose than in glucose, indicating a hyperpreadaptation to this novel nutrient environment (Bennett and Lenski, 1996). Averaging over all six lines, this group had a mean fitness relative to the ancestor of 1.55 in maltose at 42°C, as compared with a mean fitness of 1.34 in glucose at 42°C. The fitness differential in maltose implies that a derived cell has about 12 times as many descendants as an ancestral cell after only 1 day of competition between them. Maltose is a glucose dimer, but it is transported into the bacterial cell through a different pathway from that used for glucose transport (Nikaido and Saier, 1992). Evidently, adaptation to heat stress in one nutrient environment had the correlated consequence of a spectacular inprovement in fitness in another nutrient environment, even though this nutrient was never present during the selective history. This and other correlated responses are presumed to arise as pleiotropic side-effects of the selected mutations, and they may reveal important information about the physiological mechanisms of evolutionary adaptation to heat stress.

Conclusions

The significance of our findings does not lie in the elaboration of the precise patterns and mechanisms by which our experimental organisms adapted to different thermal environments. In the most narrow sense, our results apply only to the single ancestral genotype of *E. coli* that we used to found these experiments. Each experimental organism will have its own particular features and, moreover, be subject to historical constraints and stochastic effects (Travisano et al., 1995). Only comparative studies of widely different kinds of organisms will reveal truly general features and patterns of adaptation to different environments, such as thermal stress.

However, what our experiments can do is to provide an extraordinarily comprehensive view and rigorous analysis of the effects of heat stress in one mesophilic organism and the evolutionary responses to that stress. Our

results are useful in evaluating general assertions about the effects of stress and patterns of evolutionary response to stressful environments. As the first necessary step, we have demonstrated unambiguously that the high-temperature environment is stressful to the ancestral organism by a variety of criteria: activating a stress response, depressing net productivity and reducing competitive fitness. By observing how the experimental lines adapt phenotypically and evolutionarily to this stress, we can evaluate the generality of various assertions about adaptation to stressful environments. We found, for instance, evidence to support the prediction that evolutionary rates in stressful environments should exceed those in non-stressful environments. Contrary to *a priori* expectations, however, we found that an increase in heat tolerance and an upward displacement of thermal niche is not a necessary correlate of evolutionary adaptation to a stressfully high temperature. We also found that tradeoffs (that is, performance decrements in other protions of the niche) are not necessary correlates of adaptation to heat stress: increments in fitness at one temperature extreme were often not accompanied by a loss of fitness at the other temperature extreme. Also contrary to expectations, prior exposure and acclimation to heat stress was shown not to be beneficial in the stressful environment, but rather actually to decrease competitive ability and reproductive success. Thus, many widely held expectations about general patterns of evolution in stressful environments were contradicted by this experimental system. Some of these expectations are explicitly built into models that seek to predict evolutionary responses to environmental change, such as global warming (e.g. (Lynch and Gabriel, 1987; Pease et al., 1989; Bürger and Lynch, this volume). It seems to us that these assumptions have to be re-examined, considering that a rigorous experimental test has failed to lend support to them.

Our study also produced other intriguing results for which there were no particular *a priori* expectations. For instance, the increment in yield efficiency associated with evolutionary adaptation to high temperature is not necessarily anticipated by evolutionary theory (Vasi et al., 1994): differential reproduction is anticipated to be rewarded, but faster growth may occur by either increased or decreased efficiency. How general is the increase in bioenergetic efficiency in adaptive evolution? Another interesting result is the hyper-preadaptation to the novel maltose environment. We had no *a priori* expectation of this result. Are there many other unexpected correlated consequences of adaptation to stressful environments? Are such extreme cases of preadaptation more common during adaptation to stressful than to non-stressful environments? Thus, as with any good experimental system, we are able not only to test existing hypotheses but also to generate new questions and hypotheses of general interest for further examination. The great utility of our system is that it has the flexibility to test both phenotypic and evolutionary hypotheses, generate new ones and then perform further evolutionary experiments to test these too.

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References

Baross, J.A. and Morita, R.Y. (1978) Microbial life at low temperature: Ecological aspects. *In*: D.J. Kushner (ed.): *Microbial Life in Extreme Environments*. Academic Press, New York, pp. 9–71.

Bennett, A.F., Dao, K.M. and Lenski, R.E. (1990) Rapid evolution in response to high temperature selection. *Nature* 346:79–81.

Bennett, A.F. and Lenski, R.E. (1993) Evolutionary adaptation to temperature. II. Thermal niches of experimental lines of *Escherichia coli*. *Evolution* 47:1–12.

Bennett, A.F. and Lenski, R.E. (1996) Evolutionary adaptation to temperature. V. Adaptive mechanisms and correlated responses in experimental lines of *Escherichia coli*. *Evolution* 50:493–503.

Bennett, A.F. and Lenski, R.E. (1997) Evolutionary adaptation to temperature. VI. Phenotypic acclimation and its evolution in *Escherichia coli*. *Evolution* 51:36–44

Bennett, A.F., Lenski, R.E. and Mittler, J.E. (1992) Evolutionary adaptation to temperature. I. Fitness responses of *Escherichia coli* to changes in its thermal environment. *Evolution* 46:16–30.

Brock, T.D. (1986) *Thermophilic Microorganisms and Life at High Temperatures*. Springer-Verlag, New York.

Cossins, A.R. and Bowler, K. (1987) *Temperature Biology of Animals*. Chapman and Hall, New York.

Gross, C., Straus, D.B., Erickson, J.W. and Yura, T. (1990) The function and regulation of heat shock proteins in *Escherichia coli*. *In:* R.I. Morimoto, A. Tissieres and C. Georgopoulos, (eds). *Stress Proteins in Biology and Medicine*. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York, pp. 167–189.

Herendeen, S.L., VanBogelen, R.A. and Neidhardt, F.C. (1979) Levels of major proteins of *Escherichia coli* during growth at different temperatures. *J. Bacteriol.* 139:185–194.

Hochachka, P.W. and Somero, G.N. (1984) *Biochemical Adaptation*. Princeton University Press, Princeton.

Hoffmann, A.A. and Parsons, P.A. (1991) *Evolutionary Genetics and Environmental Stress*. Oxford University Press, Oxford.

Holt, R. (1990) The microevolutionary consequences of climate change. *Trends Ecol. Evol.* 5:311–315.

Huey, R.B. and Kingsolver, J.G. (1989) Evolution of thermal sensitivity of ectotherm performance. *Trends Ecol. Evol.* 4:131–135.

Huey, R.B. and Stevenson, R.D. (1979) Integrating thermal physiology and ecology of ectotherms: A discussion of approaches. *Am. Zool.* 19:367–384.

Karieva, P.J., Kingsolver, J.G. and Huey, R.B. (1993) *Biotic Interactions and Global Change*. Sinauer Associates, Sunderland, MA.

Koehn, R.K. and Bayne, B.L. (1989) Towards a physiological and genetical understanding of the energetics of the stress response. *Biol. J. Linn. Soc.* 37:157–171.

Lenski, R.E. and Bennett, A.F. (1993) Evolutionary response of *Escherichia coli* to thermal stress. *Am. Nat.* 142:S47–S64.

Lenski, R.E., Rose, M.R., Simpson, S.C. and Tadler, S.C. (1991) Long-term experimental evolution in *Escherichia coli*. I. Adaptation and divergence during 2000 generations. *Am. Nat.* 138:1315–1341.

Leroi, A.M., Bennett, A.F. and Lenski, R.E. (1994a) Temperature acclimation and competitive fitness: An experimental test of the Beneficial Acclimation Assumption. *Proc. Nat. Acad. Sci. USA* 91:1917–1921.

Leroi, A.M., Lenski, R.E. and Bennett, A.F. (1994b) Evolutionary adaptation to temperature. III. Adaptation of *Escherichia coli* to a temporally varying environment. *Evolution* 48:1222–1229.

Levins, R. (1968) *Evolution in Changing Environments*. Princeton University Press, Princeton.

Lynch, M. and Gabriel, W. (1987) Environmental tolerance. *Am. Natl.* 129:283–303.

Mongold, J.A., Bennett, A.F. and Lenski, R.E. (1996) Evolutionary adaptation to temperature. IV. Adaptation of *Escherichia coli* at a niche boundary. *Evolution* 50:35–43.

Morimoto, R., Tissieres, A. and Georgopoulos, C. (eds) (1990) *Stress Proteins in Biology and Medicine*. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York.

Neidhardt, F.C., Ingraham, J.L., Low, K.B., Magasanik, B., Schaechter, M. and Umbarger, H.E. (eds) (1987) *Escherichia coli and Salmonella typhimurium: Cellular and Molecular Biology*. American Society for Microbiology, Washington, D.C.

Neidhardt, F.C. and VanBogelen, R.A. (1981) Positive regulatory gene for temperature-controlled proteins in *Escherichia coli*. *Biochem. Biophys. Res. Commun.* 100:894–900.

Neidhardt, F.C. and VanBogelen, R.A. (1987) Heat shock response. *In*: F.C. Neidhardt, J.L. Ingraham, K.B. Low, B. Magasanik, M. Schaechter and H.E. Umbarger (eds): *Escherichia coli and Salmonella typhimurium: Cellular and Molecular Biology*. American Society for Microbiology, Washington, D.C., pp. 1334–1345.

Nikaido, H. and Saier, M.H. Jr. (1992) Transport proteins in bacteria: Common themes in their design. *Science* 258:936–942.

Parsons, P.A. (1987) Evolutionary rates under environmental stress. *Evol. Biol.* 21:311–347.

Pease, C.M., Lande, R. and Bull, J.J. (1989) A model of population growth, dispersal and evolution in a changing environment. *Ecology* 70:1657–1664.

Precht, H., Christofersen, J., Hensel, H. and Larcher, W. (1973) *Temperature and Life*. Springer-Verlag, Berlin.

Rome, L.C., Stevens, E.D. and John-Alder, H.B. (1992) The influence of temperature and thermal acclimation on physiological function. *In*: M.E. Feder and W.W. Burggren (eds): *Environmental Physiology of the Amphibians*. University of Chicago Press, Chicago, pp. 183–205.

Sibly, R.M. and Calow, P. (1989) A life-cycle theory of responses to stress. *Biol. J. Linn. Soc.* 37:101–116.

Stetter, K.O. (1995) Microbial life in hyperthermal environments. *Am. Soc. Micro. News* 61: 285–290.

Storey, K.B. (1992) Natural freeze tolerance in ectothermic vertebrates. *Annu. Rev. Physiol.* 54: 619–637.

Straka, R.D. and Stokes, J.L. (1960) Psychrophilic bacteria from Antarctica. *J. Bacteriol.* 80:622–625.

Travisano, M., Mongold, J.A., Bennett, A.F. and Lenski, R.E. (1995) Experimental tests of the roles of adaptation, chance and history in evolution. *Science* 267:87–90.

Tsuchido, T., VanBogelen, R.A. and Neidhardt, F.C. (1986) Heat-shock response in *Escherichia coli* concerns cell division. *Proc. Natl. Acad. Sci. USA* 83:6959–6963.

Vasi, F., Travisano, M. and Lenski, R.E. (1994) Long-term experimental evolution in *Escherichia coli*. II. Changes in life-history traits during adaptation to a seasonal environment. *Am. Nat.* 144:432–456.

Watson, K. (1990) Microbial stress proteins. *Adv. Microb. Physiol.* 31:183–223.