Experimental Evolution and the Krogh Principle: Generating Biological Novelty for Functional and Genetic Analyses[1]

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ABSTRACT

August Krogh counseled the careful selection of the best subject organism on which to undertake mechanistic physiological research. But what if an organism with the desired properties does not exist? It is now within our power to engineer organisms genetically to achieve novel combinations of traits. I propose that it is a logical extension of the Krogh principle that we use biological methodologies to create novel organisms ideally suited for particular physiological studies. Transgenics may first come to mind as the method for such transformations, but here I suggest that an alternative and complementary technique for generating biological novelty is experimental evolution. The latter has several advantages, including modification of multiple characters in one experiment, the production of advantageous traits, the testing of evolutionary hypotheses, and the identification of previously unsuspected factors involved in adaptation. Three experiments are reviewed, each of which examined the evolution of different physiological characters in different environments and organisms: locomotor performance in mice, desiccation tolerance in fruit flies, and high temperature adaptation in bacteria. While diverse in experimental type and subject, all resulted in the successful production of new variants with enhanced function in their new environments. Each experiment successfully tested hypotheses concerning physiological evolution, and in each case, unanticipated results emerged, which suggests previously unsuspected adaptive pathways and mechanisms. In addition, replicate populations in each experiment adjusted to their common environments by several different means, which indicates that physiological evolution may follow diverse stochastic pathways during adaptation. Experimental evolution can be a valuable method to produce and investigate new physiological variants and traits. The choice of experimental subjects, according to the Krogh principle, is no longer limited to currently existing organisms but is open to our imaginations and our ingenuity.

For a large number of problems there will be some animal of choice, or a few such animals, on which it can be most conveniently studied. (August Krogh [1929])

The Krogh principle has informed and guided mechanistic investigations in comparative physiology for nearly a century. It embodies the recognition that the choice of the study organism is a fundamental (or the fundamental) decision in the design of a biological experiment. The study organism is not to be selected simply because it is readily available, because it is a "model organism," or because it is the one most familiar to the investigator. Rather, it should be chosen because it has unique properties that will facilitate the investigation of the underlying biological phenomenon of interest. In addition to his elegant investigations into specific physiological mechanisms, August Krogh's legacy to comparative physiology has been the forthright declaration of this principle.

But what if an organism with the desired properties does not exist? What if the combination of features desired is not available in any known organism? When Krogh lived, if he had been asked that question, his response might have been a rueful smile and a shrug. Today, however, I believe his response would be another, more knowing kind of smile and the injunction, "Well then, go and create one that does!" That is, we are no longer constrained simply to build better mousetraps to catch and study existing living systems: we can now build a better mouse. These are the days of biological miracle and wonder, when the modification of organisms is not only theoretically possible but is also being undertaken in a variety of laboratories on a daily basis. Organisms can be and are being engineered though a variety of different techniques and for a variety of different reasons. Comparative and evolutionary physiologists have the opportunity to embrace those technologies and the underlying viewpoints that they represent and to create new organisms for study, engineering new variants that can be used

to test fundamental physiological associations by a new application of the Krogh principle.

The Power and Limitations of Transgenics

The bioengineering technologies that first come to mind in regard to organismal modification are those of transgenics: the modification, insertion, or deletion of specific genes. These powerful techniques permit a highly controlled investigation of the functioning of specific genes and gene products and have become a standard methodology in molecular, cellular, and developmental biology. Transgenically modified organisms can then be examined for altered functional capacities. One example of the promise and importance of these techniques for physiologists is the production and study of a mouse lacking myoglobin. Understanding the functional significance of myoglobin has always been problematic (oxygen storage? facilitation of oxygen diffusion?) in spite of its being such an obvious component of vertebrate skeletal and cardiac muscle. Mice were transgenically modified to deactivate the myoglobin gene, and the functional consequences for physiological performance were measured (Garry et al. 1998; Godecke et al. 1999). Very surprisingly, no deficit in cardiac function or locomotor capacity was identified in these studies. Apparently, functional adjustments in other parts of the oxygen transport system (e.g., capillary density) were sufficient to compensate for the elimination of myoglobin. Or perhaps myoglobin is less important to oxygen transport than we thought it was, and alternative functions for the molecule (e.g., nitric oxide scavenging; Brunori 2001) are being investigated.

As powerful and as useful as many of these transgenic techniques are, they are also complicated and require a great deal of training, specialization, and expertise to get them to work. Comparative and organismal biologists might want to take advantage of these technologies by establishing interactive collaborations with molecular and cell biologists to undertake truly integrative and multidisciplinary studies on transgenically modified organisms. In regard to the previous example, I believe the functional and performance studies on the mice without myoglobin could be substantially improved and extended by the addition of integrative physiologists into those research projects. In this regard, an exemplary study of transgenics done in an ecological and evolutionary context is the work of Feder and his colleagues on the functioning of heat shock proteins in fruit flies (e.g., Feder 1999).

Transgenic studies, however, frequently have several limitations that restrict their general interpretation and utility. For instance, they often involve the modification of only a single gene at a time. While such studies can provide considerable insight into the functioning of that gene, they are extremely laborious and do not necessarily provide information about interacting genetic systems, or their effects may even be masked by compensatory actions of other physiological or genetic systems. In addition, the elimination or overexpression of a gene frequently results in deleterious effects for the organism and may be lethal. It is important to study gene modifications that can actually improve function rather than those that handicap or inhibit it. Finally, the application of the approach and the choice of gene for modification are limited by our current understanding of functional relationships. Transgenic studies may test existing hypotheses expeditiously, but they are less useful in developing new ones. The number of genes of most organisms is too large and resources are too limited to begin modifying each and every gene in the entire genome to determine those that might influence a specific physiological system or phenotypic response. Transgenics can help us determine if what we suspect about a gene's function is correct, but it is less useful in uncovering unsuspected relationships.

Experimental Evolution: An Alternative Means of Organismal Modification

A complementary approach to transgenics for producing new organisms for biological study is experimental evolution (see Garland 2003). For those unfamiliar with the term, it may seem somewhat strange, even an oxymoron. How does one experiment with or on evolution? If evolution is taken to mean the historical development of major taxa and their paleobiological diversification and adaptation, then such experiments are not possible. Instead, we rely on comparative, phylogenetically based analyses of their extant descendants (Brooks and McLennan 1991; Harvey and Pagel 1991; Garland et al. 1999). If, however, evolution is regarded as a change through time in gene frequency in a population arising from differential reproduction, then it is indeed possible to undertake evolutionary experiments. These can be done by altering the selective environment of a population, thereby affecting which individuals most successfully reproduce. The descendants of these newly successful reproducers will be more fit in that new environment. Gene frequencies will change from those in the original populations, but the directions and paths of genetic change are unknown and unpredictable. Unlike transgenics, which modifies particular genes, experimental evolution stands back and rewards whatever changes anywhere in the genome make for more successful organisms. Therein lies its ability to surprise us and to uncover previously unsuspected relationships among traits or possible pathways of adaptation.

The essence of any successful experiment is replication and control, and experimental evolution presents important opportunities in those regards, opportunities that are not possible in comparative evolutionary studies. Replication is achieved by simultaneously establishing several populations and monitoring change within each of these through time. Having several populations simultaneously adapting to an altered environment not only permits us to determine the time course and efficacy of the evolutionary change, it also permits an assessment of the

<u>Transgenics</u>   <u>Experimental Evolution</u>

Artificial Truncation   **Laboratory Culling**   **Laboratory Natural Selection**

Modify genotype   *Select phenotype*   *Modify environment: Lethal environment*   *Modify environment: Benign environment*
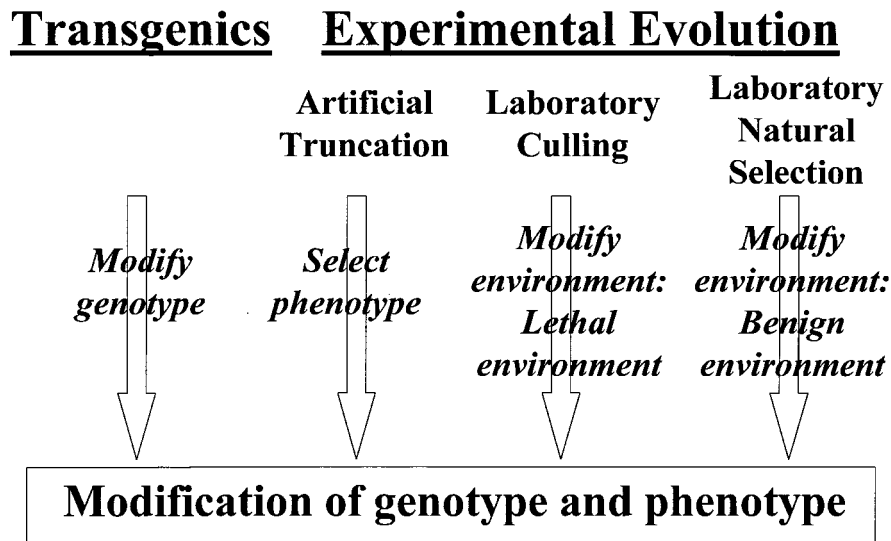
Modification of genotype and phenotype

Figure 1. Diverse methodologies in both transgenics and experimental evolution converge on the modification of genotype and phenotype

diversity of possible adaptive solutions to a common environmental challenge (Cohan and Hoffmann 1989; Lenski et al. 1991). Is there a common pathway of genetic change that is seen in many different experimental populations, or are there as many different solutions to the problem as there are experimental populations? Is there a common pathway up the adaptive peak, or are there numerous different trails?

Control may be achieved in two different and equally important senses. First, the new selective environment itself can be controlled: it can be carefully defined, regulated, and imposed on all of the new experimental populations uniformly. This kind of control permits us to understand and delimit the exact factor or factors to which the populations are adapting. Second, parallel populations of organisms can be maintained in the original (i.e, the ancestral) environmental condition so that any changes seen in the experimental populations can be directly compared with those control populations to assess the reality and amount of change.

The design, applications, and limitations of experimental evolution have been recently described and reviewed by several authors (Rose et al. 1996; Bennett and Lenski 1999; Gibbs 1999; Feder et al. 2000; Harshman and Hoffmann 2000; Bennett 2002; Garland 2003). It is not the purpose of this article to provide another general review. Rather, what is suggested here is that experimental evolution can be an effective tool for creating new functional traits for mechanistic studies. Usually, experimental evolution is used to test general evolutionary hypotheses or assumptions, such as the necessity of adaptive trade-offs or fitness plateaus. It should not be mistaken for a model that is predictive of evolutionary change of similar populations in the natural world. The strict controls and simplified environments that are key elements for successful experimentation preclude the projection of laboratory systems into predictive models for evolution in the variable and stochastic environments of the natural world.

Evolutionary experiments usually take one of three different forms according to the type of selective environment imposed: artificial truncation selection, laboratory culling selection, or laboratory natural selection (Rose et al. 1990). I will briefly discuss each of these types of experiment and the differences between them and give an example from the physiological literature of a study that employed that technique along with some of its results to date. By different means, all three types of experiments share the common goal of modification of the genotype and phenotype of their subject populations. In this, they have the same objectives as transgenics (Fig. 1).

Artificial Truncation Selection: Running Performance in Mice

Artificial truncation selection is probably the most familiar form of evolutionary organismal modification because it is the basis for all animal and plant breeding. Only organisms that possess a desired trait or show a directional tendency toward that trait are permitted to breed and found the next generation; organisms not having the trait are removed from the breeding population. "Artificial" is used to emphasize the human intervention and distinguish this from "natural" selection. Traits to be rewarded and selected are chosen before the experiment, and they could be morphological, physiological, or behavioral. In breeding dogs, for instance, desirable traits in an animal designed to hunt badgers might be a narrow and compact body with short limbs to fit into the badger's burrow, strong forelegs for digging, a pointed snout with sharp teeth, and a fearless hunting disposition. Through artificial truncation selection, out

Artificial Truncation Selection: Running Performance in Mice

4 selected & 4 control lines

20+ generations

Selected trait:

Total distance run

Truncation:

Top 20% breed next generation

Figure 2. Example of artificial truncation selection to study exercise physiology (Swallow et al. 1998*a*, 1998*b*).

of wolves we have engineered dachshunds. Note that with this type of selection, you get precisely what you select for and no more, and you may even accrue undesirable traits in the process. That dachshunds, for instance, are notoriously prone to spinal injuries is an unintended consequence of generations of selection for their elongate body form.

An outstanding example of the use of artificial truncation selection in a physiological study is that of Ted Garland and his coworkers on selection for voluntary running performance in laboratory mice (e.g., Swallow et al. 1998*a*, 1998*b*; Koteja et al. 1999; Girard et al. 2001; Fig. 2). Many rodents, including mice, will voluntarily use exercise wheels and run great distances overnight. As with many traits, there is a great deal of variability in the propensity to run, even within populations of laboratory bred animals. Garland and colleagues gave four selected and four control populations of mice access to running wheels attached to their housing cages. In the selected populations, only the 20% of the mice that ran the furthest in any generation were permitted to breed to found the next generation; in the control populations, mice were permitted to breed at random with regard to running performance. After 20 generations, the selected populations of mice ran nearly 16 km/d, almost three times as far as the controls. This increase in distance was achieved not by spending considerably more time on the running wheel but mainly by running nearly three times as fast, so the selected mice now run close to their maximal aerobic speeds (those eliciting maximal oxygen consumption).

How have the selected mice changed from the controls? Are they physiologically different in characters that support this increased running performance? The ways in which the mice have and have not changed are both informative and interesting (Fig. 3). In spite of their increased running performance, ap-
parently they have not increased their aerobic or circulatory capacity. Evidently, modification of these key aspects of oxygen transport capacity was not involved in achieving this increased performance. The physiological traits that were modified were a decrease in body mass, an increased insulin-stimulated glucose uptake in some hind-limb muscles, an enhanced training effect (a greater increment in hemoglobin concentration and aerobic enzyme activity when given access to exercise), and an altered dopamine sensitivity. Thus, some factors that might have been expected a priori to be involved in the increase in performance were not, and other, unexpected characters turned out to be important.

One trait that emerged in two of the four selected populations was of considerable surprise and interest: a miniaturized triceps surae muscle, a principal retractor of the hind limb (Garland et al. 2002; Houle-Leroy et al., in press). In these animals, the mass of this muscle is only about half that of normal mice, but its aerobic enzyme capacity on a whole-muscle basis is the same, so that the concentration of aerobic enzymes is nearly double that of a normal muscle. This alteration is under the control of a recessive allele that was apparently present in the original founder mouse population at a frequency of about 7%; as a result, <0.5% of the mice were homozygous and possessed this reduced muscle. In two of the selected populations, after approximately 20 generations, the allele frequency had risen to about 70% in one and 50% in the other; in the other two selected populations, the allele has apparently been lost through genetic drift. Two conclusions, both physiologically interesting and revealing, can be drawn from these results. First, this reduced muscle size phenotype is probably an important component of adaptation for increased running performance of two of the populations. Second, it is not a trait that is essential to achieve that increased performance, because the two other selected populations found a pathway to greater running performance that did not involve incorporation of that muscle phenotype.

Physiological Changes in Activity Selected Mice

•Decreased body mass
•Increased training effect
•Altered neurotransmitters
•Decreased limb muscle mass
  (2 of 4 lines)

<u>No</u> change in

•Basal metabolic rate
•Maximal oxygen consumption
•Heart mass
•Sedentary hematocrit or [hemoglobin]

Figure 3. Physiological changes associated with selection for running performance in mice.

Laboratory Culling Selection: Desiccation Resistance in Fruit Flies

5 selected & 5 control lines

100+ generations

Selected trait:

Survival at 0% RH

Culling:

80% lethality

Figure 4. Example of laboratory culling selection for dehydration tolerance (Gibbs et al. 1997).

Laboratory Culling Selection: Desiccation Resistance in Fruit Flies

The second type of evolutionary experiment is laboratory culling selection. In these experiments, selected populations are exposed to a lethal environment every generation. Only the most tolerant organisms, those that best survive in that environment, are permitted to breed and found the next generation. For example, a population might be exposed to a thermal environment that is lethal to all individuals, but some individuals will be less heat tolerant and will die before others. When a large percentage of the population has succumbed, the remaining individuals are rescued and allowed to breed to establish the next generation. This type of selection differs from artificial truncation in that it does not specify a priori a specific organismal trait that is to be measured and rewarded. Rather, it imposes an environmental screen or hurdle, and any combination of traits that prolong survival in the lethal environment is rewarded.

A significant and informative study of this type is that performed by Timothy Bradley, Michael Rose, and their coworkers on desiccation resistance in the fruit fly *Drosophila melanogaster* (e.g., Gibbs et al. 1997; Chippindale et al. 1998; Bradley et al. 1999; Folk et al. 2001). Earlier experiments by Rose and coworkers on fruit fly populations selected life-history characters of early or delayed reproduction. These had the surprising result that seemingly unrelated physiological characters also changed as a consequence of life-history selection (e.g., Service et al. 1985; Graves et al. 1992; Nghiem et al. 2000). These observations led to a new series of experiments in which selection was imposed on the ability of flies to tolerate dry environments (Fig. 4). In each generation, five selected populations of flies were exposed to 0% relative humidity without access to water. After 80% of the flies in each population had died, the remaining 20% were returned to regular culture conditions, and they subsequently reproduced. These experimental populations, along with five control populations that were given access to water during the "desiccation selection" period, were maintained for >100 generations. After that time, the selected populations were able to tolerate desiccation three to four times longer than the controls. In the selected populations, total body water content was increased, and rates of water loss during desiccation were halved; water content at death did not change.

How do the desiccation-tolerant populations differ from the controls (Fig. 5)? Several characters that might have been expected to change as a result of desiccation selection did not. Resting metabolic rate did not decrease, which indicates that the flies did not simply turn down their overall rates of energy processing. Dry mass did not increase, so they did not become more tolerant simply by becoming larger, with a smaller surface-to-volume ratio. Cuticular lipid mass did not increase, so they did not simply apply a thicker coating of waterproofing to prevent water loss. Other physiological characters did change, however. There was an enormous (sixfold) increment in hemolymph volume. This is of course partially responsible for the greater total body water content of the selected populations, but it also raises other interesting physiological questions, such as the possibility of differential hormonal concentrations or receptor sensitivity as a result of this selection. Cuticular lipid composition changed, which indicates that waterproofing may have been achieved by qualitative rather than simple quantitative changes in surface lipids. In addition, selected populations became more quiescent when exposed to dry conditions. While control flies walked around and flew in the desiccation chamber, the selected flies remained more or less immobile. This reduced behavior would of course reduce water loss. Whether these behavioral differences result from altered neurotransmitter profiles (as in the active mice) or some other

Physiological Changes in Desiccation Selected Fruit Flies

•Increased hemolymph volume

•Altered cuticular lipid profile

•Quiescent behavior

•Decreased lipid reserves

•Increased glycogen content (3 of 5 lines)

<u>No</u> change in

•Metabolic rate

•Dry mass

•Cuticular lipid mass

Figure 5. Physiological changes associated with selection for dehydration tolerance in fruit flies.

Laboratory Natural Selection: High Temperature Adaptation in Bacteria

6 selected lines at 42 C
& 6 control lines at 37 C

2000 generations

Selected trait:

Culture at 42 C

Figure 6. Example of laboratory natural selection to study temperature adaptation (Bennett et al. 1992).

factor would be an interesting topic for a study in behavioral genetics.

One of the most surprising results was the entirely unexpected change in energy storage materials in the desiccation-selected flies. It might have been hypothesized a priori that fat reserves would have increased as a consequence of desiccation selection. Metabolic oxidation of fats yields a large amount of water, nearly twice that associated with carbohydrate oxidation on a gram-for-gram basis. Thus, fat might have served as both a compact form of energy storage as well as a source of water during desiccation if that were the metabolic substrate used during that time. Instead, however, fat reserves actually decreased. And completely unexpectedly, total glycogen concentration increased significantly in three of the selected populations, and among the selected populations alone or including the control populations, desiccation resistance is positively correlated with glycogen content. Presently, there is no physiological mechanism that might account for this association. It raises a host of new questions about the distribution of energy storage products with adaptation to different environments.

Laboratory Natural Selection: Temperature Adaptation in Bacteria

The name of the third and final form of experimental evolution, laboratory natural selection, may seem to contain an internal contradiction. Although as physiological ecologists we usually make a distinction between the natural world and the laboratory, natural selection, defined as differential reproduction and survival, may occur in either environment. Laboratory natural selection proceeds through the establishment of a novel but nonlethal environment as the selective agent. There is no other direct intervention by the experimenter, and intrapop-

ulation competition alone determines which traits are favored in the new environment. No particular characters are therefore selected or favored by the experimenter, in contrast to artificial truncation selection. This form of experimental evolution, in contrast to laboratory culling, proceeds by soft rather than hard selection. In physiological terms, it may be expected to produce capacity adaptations rather than resistance adaptations.

An experiment done by Richard Lenski, me, and our co-workers on temperature adaptation in the bacterium *Escherichia coli* (e.g., Bennett et al. 1992; Bennett and Lenski 1993; Mongold et al. 1996; Fig. 6) is an example of laboratory natural selection. A clone adapted to 37°C was used to produce six replicate populations each for a variety of novel thermal environments, including 42°C, as well as a control group of replicates at the ancestral temperature of 37°C. These were propagated in serial dilution culture for 2,000 generations. Because the ancestral founder clone can be frozen and revived, it is possible to make direct comparisons with its descendants in regard to fitness, genetic constitution, and functional properties.

Adaptation to 42°C is of particular interest because this high temperature is highly stressful to the 37°C ancestor (e.g., yield is reduced by >80%) and is close to its upper thermal limit under these culture conditions. As a result of adaptation to 42°C, growth rate increased >30%, yield nearly doubled, and fitness improved >30% at that temperature. Heat shock protein levels and time of survival at lethally high temperature both increased. However, the increased performance at high temperature did not result in an increased thermal niche breadth, and the upper and lower thermal limits of serial dilution culture temperature did not change. Likewise, fitness relative to the ancestor did not decrease at the ancestral temperature of 37°C or even at lower culture temperatures. Thus, adaptation to high temperature did not result in a thermal niche shift or a trade-off in performance at lower temperatures (Fig. 7).

It is now possible to determine the genetic basis of that high temperature adaptation in these bacteria. With microarray tech-

Physiological Changes in High Temperature Selected Bacteria

•Increased growth rate, yield and fitness

•Increased survival at lethal temperature

•Increased expression of heat shock genes

•Duplication of common gene region
(3 of 6 lines)

No change in

•Upper or lower culture temperatures

•Thermal niche breadth

•Fitness at ancestral or lower temperatures

Figure 7. Physiological changes associated with selection at high temperature in bacteria.

nology, it was determined that the same region of the bacterial chromosome had duplicated in three of the six populations (Riehle et al. 2001). These changes occurred independently and involved a duplication of approximately one dozen genes out of a genome of approximately 4,300. This small set of genes included two operons containing the genes *rpoS, nlpD, pcm*, and *surE* (Fig. 8). Thus, several new candidate genes for high temperature survival and adaptation were revealed for further examination. None of these are heat shock genes. Expression of these genes is enhanced substantially as a result of this duplication (Fig. 9). Interestingly, however, in the other three replicate populations, expression of this set of genes was not enhanced by this or other means. These three populations lacking the duplication are equally well adapted to high temperature and therefore must have evolved increased fitness by other mechanisms. A common pathway of adaptation was used in half of the populations; that is, a mechanism of adaptive evolution was replicable, but that is apparently not the only means for achieving high temperature adaptation.

These high temperature selected populations can also be used to test hypotheses concerning stress protein function. *Escherichia coli* has 33 genes that are activated by high temperature stress, eight of which are the classical "heat shock" (molecular chaperone) genes. Expression levels of these latter genes might be hypothesized to decrease after continuous exposure to high temperatures for 2,000 generations because their phenotypic expression is known to decrease growth and deactivate the expression of some other genes. In other words, after exposure to such continuous heat stress, is expression of the heat shock genes diminished, and is this high temperature no longer seen as being so stressful in that regard? Perhaps surprisingly, the heat shock genes are in fact still very highly expressed (about seven times higher than the average gene, even in the absence of rapid thermal changes), and furthermore, their level of expression has significantly increased >20% above that in the ancestor at 42°C (M. Riehle, unpublished data). Evolution at high temperature has not diminished their expression at all.

Common Themes and Conclusions

The three studies cited are diverse in the sort of evolutionary experiment undertaken, the type of organisms involved, and the physiological systems examined. Nevertheless, they share some important common elements.

Choice of Subject Organisms and Transgenics

The Krogh principle dictates that the organism is to be chosen by the requirements of the experiment. It is antithetical to this principle that an experiment be undertaken only on types of organisms that are used extensively for other types of research. Likewise, however, it is not a part of the principle that only highly unusual and poorly known organisms should be the

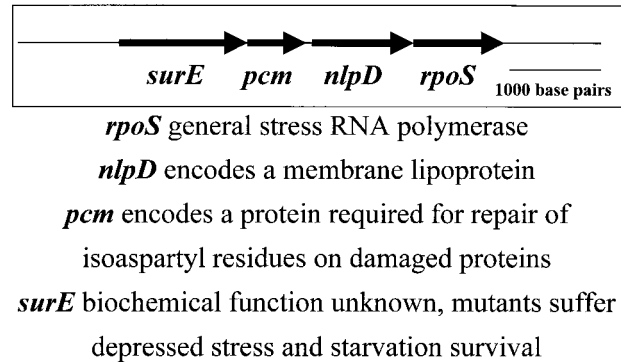## Candidate Genes Present in the Region of the Common Duplication

Figure 8. Four candidate genes for improved performance at high temperature in the bacterium *Escherichia coli* along with their functional consequences.

only subjects of physiological investigations. If an organism possesses a desirable series of traits, it is a desirable subject for investigation, whether it is well or poorly known. In the studies cited here, all of these experimental subjects were model organisms. They were chosen not because of this status but because they possessed a suite of biological traits, such as ease of laboratory culture and rapid reproduction, that are necessary components of this type of evolutionary experiment.

The additional benefit accrued in each of the cases presented is that the genome has already been or will shortly be sequenced for each of these species, and molecular technologies are or will shortly be available for their investigation. In the case of the bacterium, not only is the genome completely sequenced, the functions of approximately two-thirds of the genes are already known. It may therefore be possible to determine expeditiously the genetic basis for the evolved adaptive changes observed. Delimiting these is greatly facilitated by comparing the selected populations to their controls: since most of the genes and their expression are unaltered, the signal-to-background noise ratio of adaptive changes is expected to be very high.

There is also a very fruitful interplay possible with transgenic studies, the other means of engineering biological novelty. Once presumptive adaptive genetic changes are identified by experimental evolution, their putative functions can be verified by transgenically modifying the control (ancestral) population to see whether the anticipated phenotypic effects and changes in fitness in fact occur. In reverse, the evolutionary experiments can suggest new, previously unsuspected adaptive pathways that can then expand the repertoire of genes of interest to molecular biologists.

Testing of Evolutionary Hypotheses

In each case, the experiment permitted the rigorous (i.e., statistical) testing of a variety of hypotheses concerning physio-
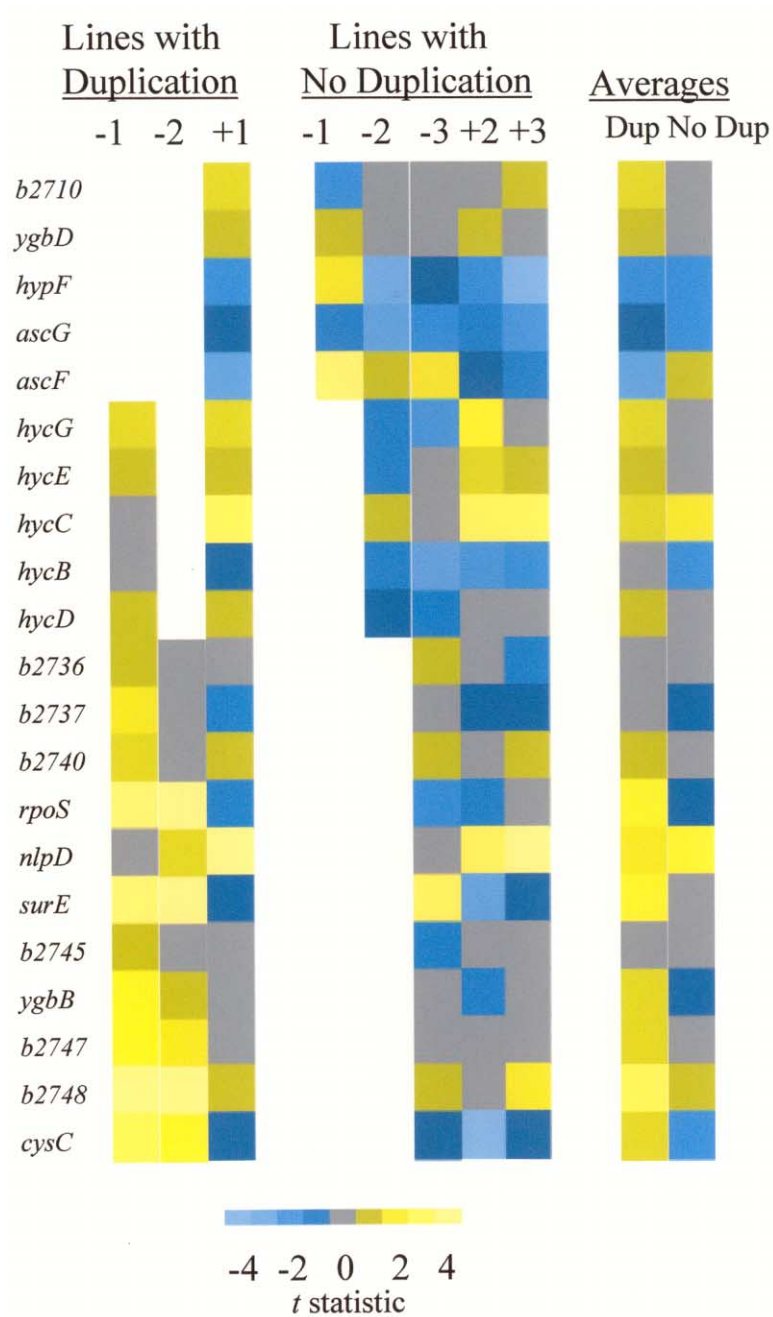
Figure 9. Gene expression during log growth phase at 42°C in the duplicated regions of the *Escherichia coli* chromosome in lines −1, −2, and +1 compared with the lines lacking the duplication. Duplicated chromosomal regions were determined by genomic DNA comparisons to the common ancestor by microarray analysis (Riehle et al. 2001). Gene names in genome order are given on the left, and line designations are reported at the top of the figure. Average expression of the lines with duplication is compared with that of the lines lacking the duplication in the columns on the right. Blue boxes indicate decreased expression, yellow boxes indicate increased expression relative to the ancestor, and gray boxes indicate no change from ancestral condition. Duplication of a chromosomal region significantly increases the expression of the genes within the duplicated region (*P* = 0.006, one-tailed paired *t* comparison of lines possessing and lacking duplication; M. Riehle, A. Long, and A. Bennett, unpublished data).

logical evolution. These were a priori hypotheses, usually derived from comparative investigations, that could then be tested and supported or rejected in an experimental framework with appropriate control and replication. Adaptational biology has in the past been severely criticized as a posteriori rationalization and story telling (e.g., Gould and Lewontin 1979). Experimental evolution provides one method to meet those objections and to investigate adaptive evolution in a prospective rather than retrospective context. The purpose of this type of evolutionary study is not to demonstrate that adaptive change can be produced experimentally (i.e., that selected populations will improve function in their new environments); it is obvious that such adaptation will occur. Rather, the question is whether the evolution occurs in predictable directions and whether there are particular necessary pathways or trade-offs involved in adapting to a particular environment. The method is most powerful in its ability to falsify general hypotheses. In each case investigated here, some anticipated adaptive changes did not occur (e.g., decrements in minimal metabolic rate, increments in aerobic capacity, thermal niche shifts), and therefore these cannot be completely general responses of adaptation to their respective environments.

Emergence of Unanticipated Features

Each experiment produced some surprises. At least one novel relationship emerged in each experiment that identified a pathway of adaptation not previously anticipated. These include the miniaturized but highly aerobic limb muscle in the active mice, the increment in glycogen storage in desiccation-tolerant fruit flies, and the identification of several candidate genes involved in high temperature adaptation in the bacteria. This is a particular strength of experimental evolution in comparison to transgenics: it lets organisms tell us what are the important features and genes involved in adaptive change rather than our having to guess at them. And with the emergence of these unanticipated features, several additional lines of inquiry and questioning are opened up for further investigation.

Diversity of Adaptive Pathways

One of the most interesting questions about evolution concerns the degree of inevitability of its outcomes. Are the adaptive patterns that we see in organisms only one of many possible forms that could have been taken, or are there preferred pathways of adaptation such that under similar starting conditions, the same forms and solutions would emerge over and over again? Regarding nature, we can only speculate about alternative adaptive solutions. In experimental evolution, we can specifically address the probability of the emergence of any adaptive feature by conducting the experiment on many replicated populations simultaneously. In very simple viral systems, for example, the same genetic changes and adaptations emerge repeatedly during experimental evolution at high temperature (e.g., Bull et al. 1997). The experiments discussed here were not designed with a sufficient number of replicated populations to address those issues satisfactorily. However, it is apparent even from the relatively small number of replicates that several of the populations adopted a common feature while others adapted by other means. Two of the four mouse experimental replicates developed miniaturized muscles; the other two did not. Three of the five fruit fly populations increased glycogen content during desiccation selection; two did not. Three of the bacterial populations evolving at high temperature duplicated a common set of genes and increased their expression; three did not. A tentative conclusion based on these admittedly fragmentary results is that there may be common or preferred pathways that several or even many of the populations will adopt. But these are not exclusive solutions. Other populations may find other ways, perhaps equally good ways, to solve the adaptive problem. To return to the analogy of climbing a mountain on an adaptive landscape, it appears that there may be a main trail up to the peak with many smaller, less traveled trails that still reach the top of the mountain.

In the context of this discussion, however, the first and foremost point about these studies is that each of them was successful in producing new kinds of organisms for biological study. Specific questions were asked in regard to the evolution of activity capacity, desiccation resistance, and high temperature adaptation. In each case, as part of the application of the Krogh principle in their investigation, evolutionary variety was created in an appropriate group of organisms to enhance the experiment. All of these organisms are now available for further biological study, and others can be expected to emerge from future evolutionary experiments. The choice of experimental subjects according to the Krogh principle is no longer limited to existing variants but is open to our imaginations and our ingenuity.

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