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The Origin of Mutants under Selection: Interactions of Mutation, Growth, and Selection

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INTRODUCTION

Mutation rates are kept low by multiple mechanisms evolved to protect genetic information and these rates are not regulated in response to the physiological needs of the cell. This broadly held consensus rests on classical bacterial experiments interpreted as evidence that selective stress does not alter the mutation process (42, 46). This is not to say that all repair mechanisms are equally active at all times (12, 31). For example, mutation-repair mechanisms with different substrate specificities, such as MMR and VSR, are differentially active under different conditions of bacterial growth, although both use the MutL protein (5, 11). The interaction between mutation and selection is complex and has been central to the history of biology. Before publication of Darwin's theory of natural selection, induction of genetic change by environmental stress was a popular idea (50). Translated into microbiological terms, this idea posited that the stress imposed by growth limitation increases the rate at which new mutations arise (stress-induced mutagenesis). Darwin may have accepted the idea of stress-induced mutation to explain the origin of the variability required for natural selection (50). However, the lack of experimental evidence on this point contributed to the importance of the classic work of Luria and Delbrück (46) and of the Lederbergs (42) demonstrating that laboratory selections can detect mutant organisms without causing their formation. These experiments validated the use of positive selection, which is fundamental to the whole field of bacterial genetics. The experiments also provided a way to measure the formation rate of mutations during nonselective growth. Later, these classic experiments were more broadly interpreted as support for the current consensus that selective stress does not induce mutations in natural populations.

Later, a flaw in the evidence for the broad interpretation was pointed out by Cairns, Hall, and Shapiro (8, 22, 60). The classic experiments had used lethal selections to detect mutants whose resistance phenotype appeared only several generations after the formation of the mutation. Thus, their selection could only detect mutants that arose well within the prior period of nonselective growth. Any nonmutant cell exposed to selection would be killed before a new mutation could arise and acquire a resistance phenotype. The experiments could not have detected mutations generated in response to stress on the selection plate. This reopened the question of stress-induced mutation in a slightly different form: “Do *all* mutations arise independently of selection (as did those studied in the classic experiments), or could some other mutations

(missed by the classic experiments) arise in response to growth limitation?" The question raised doubts regarding the widely held underpinnings of evolutionary genetics and required experimental attention.

Direct evidence that growth limitation might increase mutation rate has been sought in many microbial systems by using nonlethal selections. In most cases, the results were negative. However, a few systems showed behavior that has been interpreted as support for the idea of stress-induced mutagenesis, reviewed in references 16 and 20. In these systems, populations of cells were plated onto a selective medium that prevents growth but does not kill. Mutant colonies appeared over time and have been attributed to stress-induced mutagenesis (16, 20). The selection conditions superficially resemble standard stringent laboratory selection, and (as for laboratory selections) increases in mutant number are attributed to increases in mutation rate. To make such a conclusion possible, the systems must adhere to the rules for laboratory selection validated by Luria and Delbrück and Lederberg. That is, conditions must ensure that the increase in mutant frequency reflects new mutation events rather than the expansion of a preexisting mutant population during growth under selection.

Below we describe how the relationship between mutation rate and mutant frequency can be obscured in natural populations. First, we introduce the players—growth, selection, and the vastly different formation rates of various mutant types. Then we outline the "Seven Pillars"—the essential rules that allow the field of laboratory microbial genetics to infer mutation rates from mutant frequency. Finally, a series of experimental systems are described and critically analyzed. Each of these systems has been used as support for "stress-induced mutation." Evidence is presented that each system violates one or more of the "pillars" and thereby allows selection to increase the observed phenotype frequency (without mutagenesis). This is not to say that there are not conditions under which mutation rates will change (for example, UV irradiation or growth with an alkylating agent). Neither do we rule out that, in principle, a biological system with an environmentally regulated mutation rate might exist. However, we will argue that the experimental systems and phenomena that have been proposed to support stress-induced increases in mutagenesis in nongrowing cells can better be explained as the result of growth under selection.

MUTATION AND SELECTION

Each mutational event adds a new genetically distinct cell to the population. The phenotypic consequences of mutations vary from none (most synonymous substitutions) to lethality (loss of an essential function), but many of these mutations are expected to generate a phenotype that can be detected by selection. One can experimentally measure the frequency of mutant cells in a culture, but it is more difficult to assess the actual rate at which the responsible mutations arise, especially those with small phenotypic effects. It is especially difficult to measure the formation rate of adaptive mutations in cells growing under selection.

If all other factors are kept constant, an increase in mutation rate should cause an increase in the frequency of cells with abnormal phenotypes. The reverse correlation is more difficult—and underlies the whole field of bacterial genetics. Does an increase in mutation frequency indicate mutagenesis? Genetics of bacteria relies on selection to detect rare mutants in huge populations—it uses the frequency of detected mutations to infer the rate of mutant formation. This procedure underlies measurement of mutation rate by fluctuation test, mutagen specificity, and recombination rates. The reliability of this procedure depends on successful elimination of the many factors (especially selection) that obscure the relationship between mutation rate and mutant frequency.

In contrast, the field of population genetics does not try to establish a simple relationship between mutant frequency and rate but deals primarily with the interfering factors. In natural populations, interference caused by selection and drift is so great that changes in phenotype frequency are almost never attributed to mutagenesis.

Thus, these two areas of genetics use selection in very different ways. In a natural population, cells grow and are subject to selection before, during, and after formation of a new mutation, while laboratory procedures sharply separate selection from growth and mutation. In a natural population, a new mutant is immediately subject to selective conditions that can make its growth rate faster or slower than its parent. Selection can have huge consequences for phenotype frequency since selection initially causes exponential increases in phenotype frequency over time. In contrast, the process of mutation (in the absence of selection) has a linear effect on mutant frequency over time (see

Fig. 1). It should be noted that we can never eliminate all selective effects from a growing population, but we can find conditions that remove selection from particular mutant phenotypes.

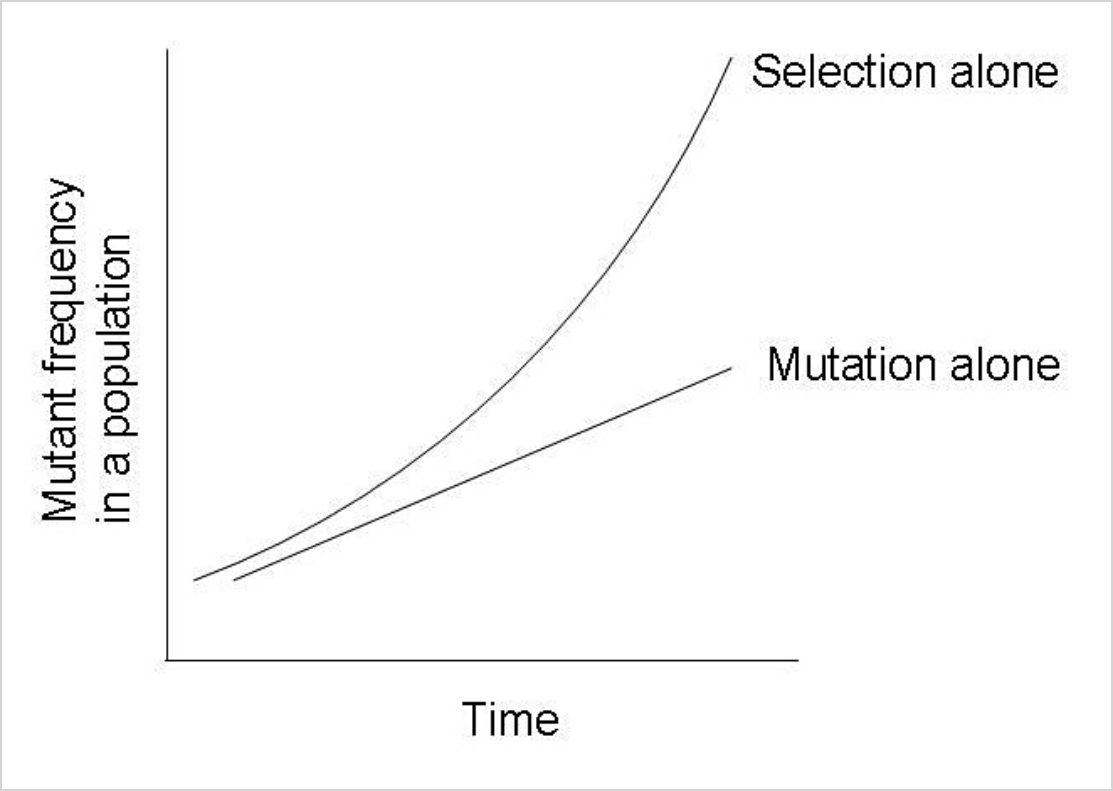

Figure 1Effects of selection and mutation on mutant frequency. In the absence of selection, new mutants accumulate linearly with time. When selection acts, it causes an exponential increase in the frequency of a beneficial mutation. Thus, in a natural population, where both selection and mutation occur together, the frequency of a favorable mutant is more heavily impacted by selection than by recurring mutational events. It is often difficult to appreciate how powerful exponential increases can be.

Natural selection can detect minuscule differences in growth rate and thus can act on the most common mutations, which have small effects on phenotype (see below). Thus, adaptation can occur rapidly in natural populations because selection causes exponential increases in the frequency of mutation types that arise at a high rate. In contrast, laboratory genetics uses restrictive conditions to ensure that selection detects only rare preexisting large-effect mutations and has no effect on their frequency. Prejudices developed because of our experience with restrictive laboratory selections may make it difficult to appreciate the speed at which genetic adaptation can occur once these restrictions are lifted.

Figure 2 depicts the behavior expected of natural populations under various conditions. The vertical axis represents a list of different cell lineages in a population, and the width of each line represents the logarithm of the frequency of each lineage. With neither mutation nor selection, the frequency of each lineage remains constant over time (Fig. 2, top). With mutation but not selection, mutations can alter the genotype of any lineage but the frequency of the (now altered) lineage does not change vis-à-vis others in the population (Fig. 2, middle). By affecting one lineage after another, mutation causes a linear increase in the frequency of mutant cells.

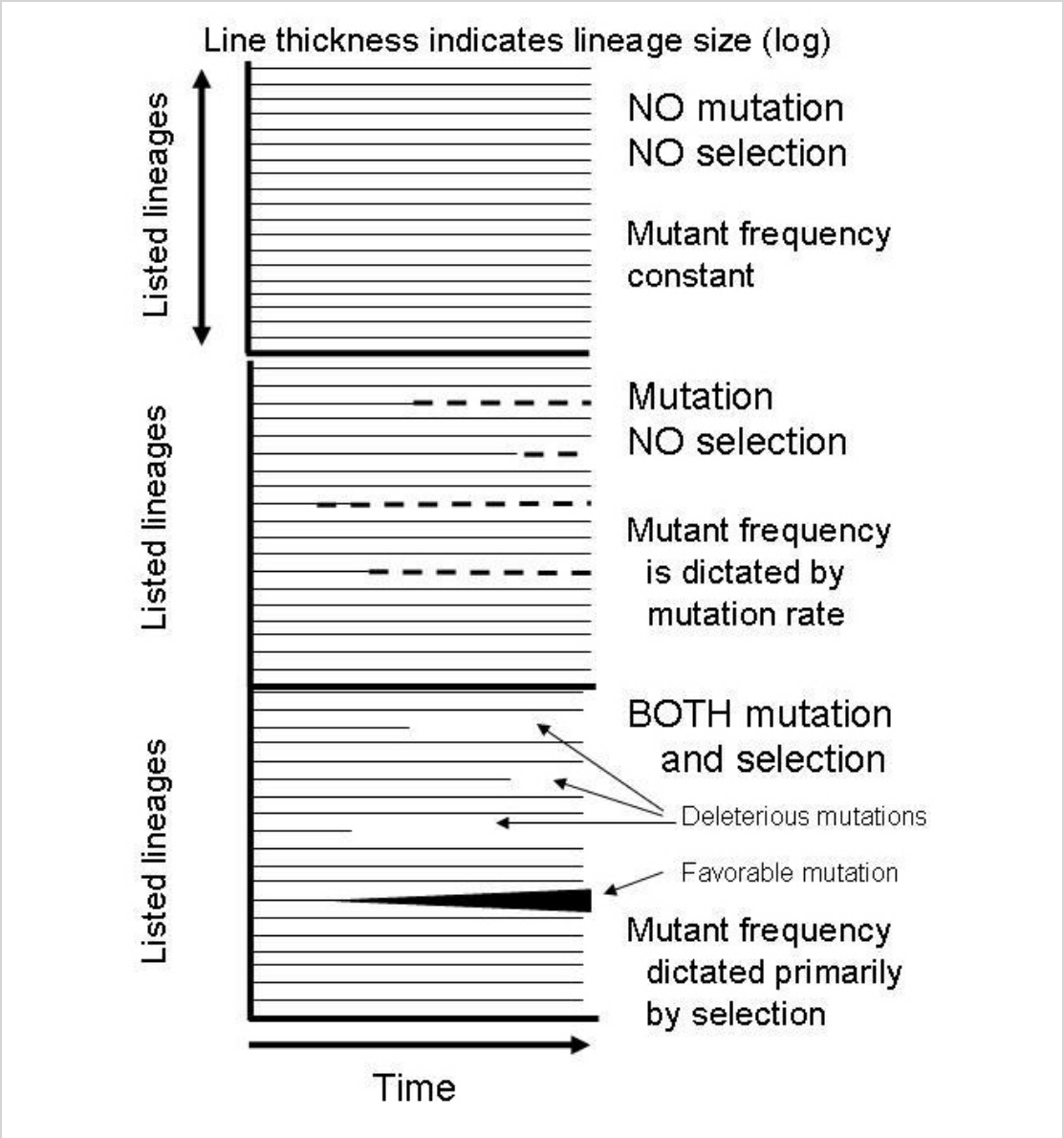

Figure 2Effects of selection and mutation on mutant frequency. The vertical axis is a list of all the genotypes present when a population is initiated. Mutation may change the nature of a particular lineage and the relative size of each lineage may change because of differences in their relative growth rate.

Lineage size (log cell number) is depicted by the thickness of horizontal lines—some dwindle and some increase exponentially. Dashed lines indicate lineages altered by mutation but unaffected in growth rate.

When mutation and selection occur together, some lineages acquire deleterious mutations and are removed from the population by selection (line becomes vanishingly thin). Lineages that acquire beneficial mutations are favored by selection and expand exponentially, increasing the size of the lineage and thereby the frequency of mutant types in the population (Fig. 2, bottom).

The Most Frequent Mutation Types Have the Smallest Phenotypic Effect

There is a wide distribution in the magnitude of mutant phenotypes and a wide variation in the rate of formation of different mutation types. The mutation types arising at the highest rates are ones with the smallest phenotypic effect. For example, one might consider changes in some enzyme activity and the fraction of the parent activity added or lost by a mutation—mutations that cause small changes are more common. This may be in part a consequence of how selection has shaped the genetic code (18) and the several systems that prevent mutations and repair DNA damage. Mutation types that most often escape cellular quality-control systems are those with the smallest phenotypic consequences. Natural selection can detect vanishingly small differences in growth rate and can therefore act on a larger number of mutations. In contrast, experimental genetics focuses on large-effect mutations that are relatively rare (among conventional point mutations). The experience of a laboratory geneticist with stringent selections may generate an intuitive feel for the speed of genetic adaptation that is at odds with the situation in natural populations. A very high rate of genetic adaptation is possible when natural selection acts on growing populations and causes exponential increases in the frequency of common small-effect mutations. We suggest that these considerations may underlie some of the problems inherent in interpreting experiments on mutation under selection.

The basic idea is outlined in Fig. 3 (see below), which classifies 100 spontaneous mutations that might affect a single gene. This table attempts to convey the nature of genetic variability at the moment it arises and before frequency has been acted upon by selection. The data presented summarize results of a variety of studies and must remain a crude estimate since every experimental situation used to generate such data uses a different sequence and detects a different spectrum of mutant types. These frequency estimates are derived from many sources plus our own experience in characterization of spontaneous mutations (19, 43, 44, 49, 51, 52, 55). In most of these studies, frequencies were estimated for cells growing in either rich medium or minimal medium with glucose.

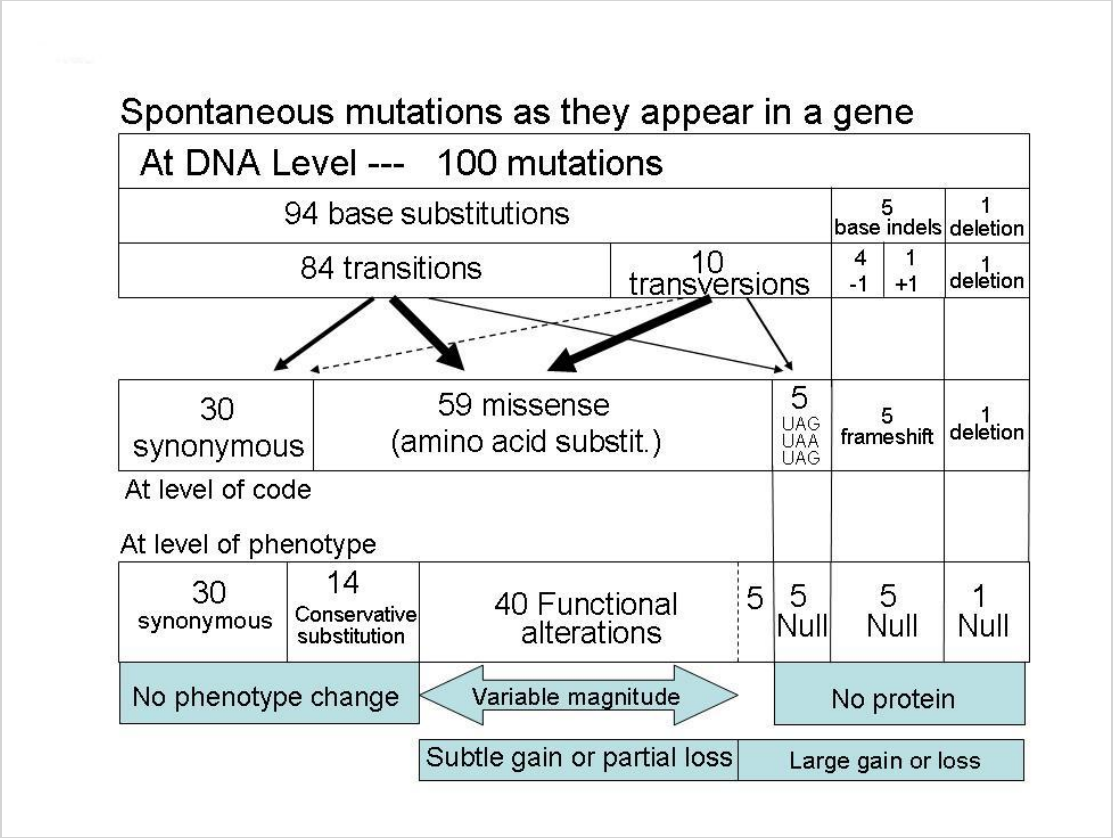

Figure 3The frequency of mutation types as they arise.

It can be seen that, among base substitutions, transitions (A/T ← → G/C) are more common than transversions (all other substitution types). This seems reasonable since transitions involve a failure to discriminate between two purines (or two pyrimidines), while transversions exchange a purine for a pyrimidine. Because of the structure of the code, transitions are prone to cause synonymous codon changes with no effect on the encoded proteins or conservative substitutions of structurally related amino acids (18).

Transversions are less common than transitions, but more prone to alter protein structure. If one considers loss-of-function mutations, transitions are likely (30%) to be synonymous and therefore silent. Relatively few base substitutions (3/64) result in the creation of nonsense codons with an essentially null phenotype. Frameshift mutations (like nonsense) are likely to cause large loss-of-function phenotypes but are rarer than base substitutions. For gain-of-function mutations, the same gradient of effects are expected—large-effect mutations are rare. Missense mutations that improve an existing function or create a novel function are restricted to particular changes at a small number of sites. Small improvements are likely to be more common than

those with a large positive effect. The general tendency is for common mutations to cause small phenotypic changes—whether losses or gains in function.

The same trend is seen for mutations that cause changes in copy number—deletions that eliminate a gene are rather rare, but duplications are among the most common of mutations. Perhaps the most common mutations are copy number changes in genomes with two or more copies of a region. These events contribute to amplification or deamplification by unequal recombination between identical copies in sister chromosomes.

Figure 4 diagrams these relationships. Note, in particular, that duplications (causing a twofold increase in gene dosage) arise at a vastly higher rate than the large-effect mutations in common use in the laboratory. Even more frequent are changes in gene copy number, which can add or remove copies. This copy number changes almost always involve repeats located adjacent to each other in the same orientation (in tandem), but some copy number variants have inverse-order repeats (E. Kugelberg and J. R. Roth, unpublished data). Because common small-effect mutations arise (and recur) frequently they are present at highest frequency in a population before selection. Natural selection can detect these small-effect mutations making them major players in genetic adaptation. Common small-effect mutations are not often detected in experimental genetics and therefore tend to be ignored in thinking about the process of adaptation. (As seen below, laboratory genetics deliberately avoids these mutations.) Yet during growth under selection these mutations can initiate huge phenotypic changes that occur by a cascade of successive small improvements whose frequencies increase exponentially during growth under selection (see below).

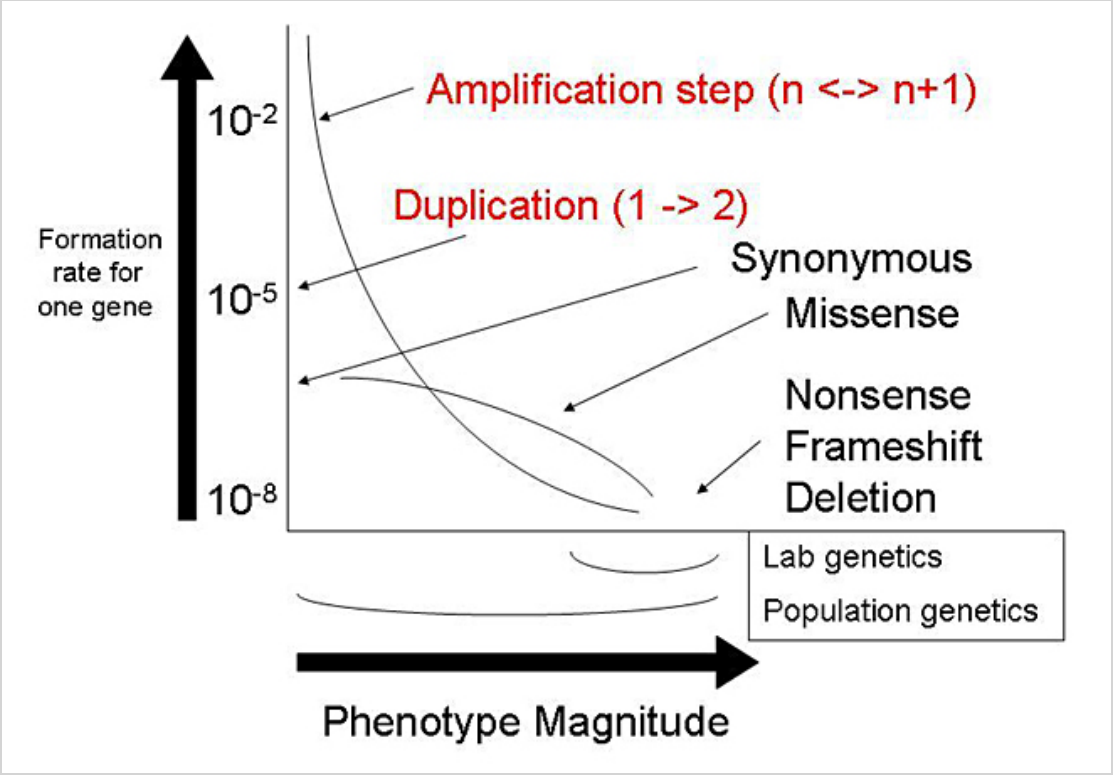

Figure 4Rate of formation of different types of mutations and their phenotypic effects.

The response of selection to copy number variants is particularly rapid because the frequency of these mutants comes rapidly to a very high steady-state frequency even before selection is imposed. Typical loci are duplicated in about 1 cell in a 1,000 of an unselected culture. This steady state is reached because formation rates are high (10^{-5}/cell per division) and loss rates are even higher (10^{-2}). In addition, duplications can have very significant fitness costs, which contribute to the rapid approach of frequency to a steady state. While duplications are common before selection, higher copy number variants are expected to also reach steady-state frequencies at a somewhat lower level (A. B. Reams and J. R. Roth, unpublished data).

When Selection Acts on Common Mutations, Improvement Follows Pathways of Genetic Adaptation

It is suggested above that the nature of the mutation process and the structure of the code dictate that genetic adaptation is likely to occur by a series of minor improvements. This seems likely because (i) the most frequent spontaneous mutations are those of small phenotypic effect and (ii) natural selection can detect these small differences and (iii) cause their frequency in the population to rise exponentially.

Series of improvements are likely because increases in the frequency of one mutation can produce a subpopulation large enough to permit a second common small-effect mutation to arise and provide an additional improvement. The most common secondary mutations (like the initial improvement) are likely to provide small fitness improvements. Each improvement allows expansion of a subpopulation in which the next event can occur. As the frequency of improved cells rises, the probability of subsequent improvement also rises, simply because there are more cells in which they can occur. These selective pathways allow a population to respond quickly. This cascading process is diagrammed in Fig. 5. It will be argued below that such sequences of events underlie many phenomena that have been interpreted as evidence for stress-induced mutation.

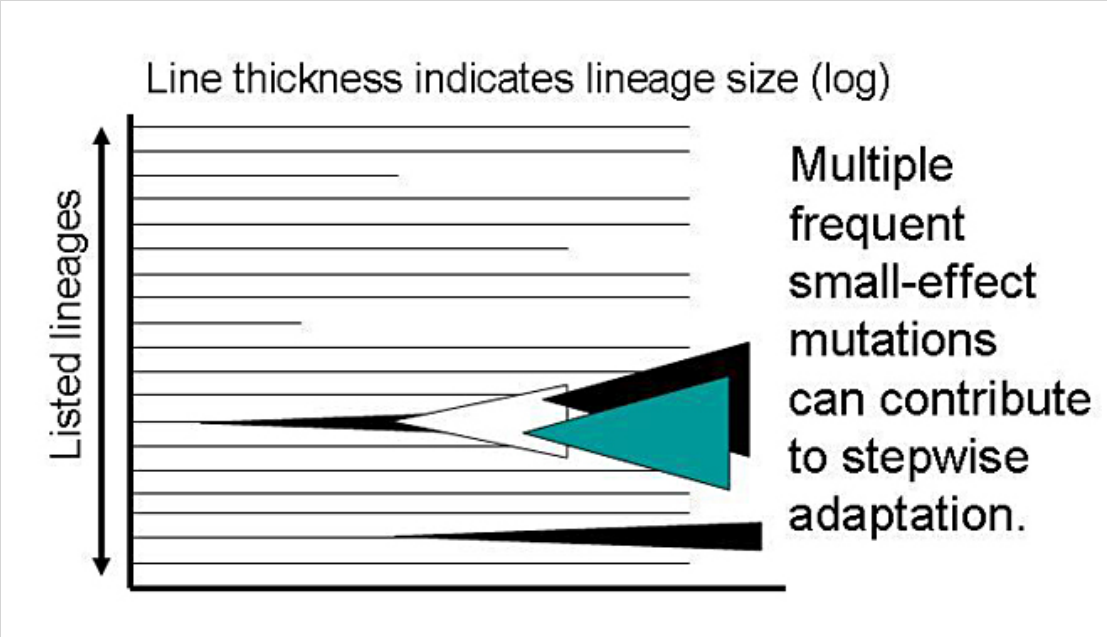

Figure 5Adaptive evolution proceeds by pathways of sequential frequent, small-effect mutations. During growth under selection, common small-effect mutations increase in frequency exponentially and expand clones large

enough to allow secondary improvements to occur.

A point to note in Fig. 5 is that several multistep pathways of improvement may operate in parallel and some genotypes may arise by multiple routes. When a population adapts in this way, the final product may be very hard to analyze genetically, since many mutations have contributed to the final phenotype. Small-effect mutations early in the process may allow a population large enough for a large-effect mutation to arise later. In some cases the final phenotype may no longer require the early events that allowed it to arise. The difficulty of reconstructing these pathways is one that underlies the problem of understanding the origins of cancer and understanding natural selection.

USES OF SELECTION IN LABORATORY GENETICS

Bacterial genetics relies on stringent positive selection to detect rare mutants in astronomically large populations (>10^9 cells). The frequency of detected mutants is used to estimate the rates at which the mutants formed. This procedure is central to assessment of mutation rates, recombination rates, and mutagen activity. To achieve these ends, selection is used in a special way that detects mutants without contributing to their frequency. This essentially eliminates natural selection and is difficult to achieve because growth under selection is such a potent force for changing mutant frequency (as outlined above). However, elimination of natural selection is essential to the practice of experimental bacterial genetics.

To defeat natural selection, laboratory genetics separates selection from growth and mutation. Mutations arise during an initial period of nonselective growth. This is followed by a sharply separated period of very strong selection on solid medium in which parent cells cannot grow and no new mutations can arise (Fig. 6). The selection is made so strong that it also prevents growth of the many small-effect mutants that might otherwise grow slowly and improve to produce a visible colony. When selection is too weak, the process of detecting preexisting mutants goes awry and the number of detected mutants is inflated by common small-effect mutations whose probability of being detected is enhanced by growth under selection. Use of very strong selection prevents the detection process from getting enmeshed in the process of rapid adaptation diagrammed in Fig. 5. The two-stage process used in the laboratory (Fig. 6) allows mutant frequencies to reflect only mutation rate (prior to selection) and prevents selection from influencing mutant frequency (as it does in natural populations).

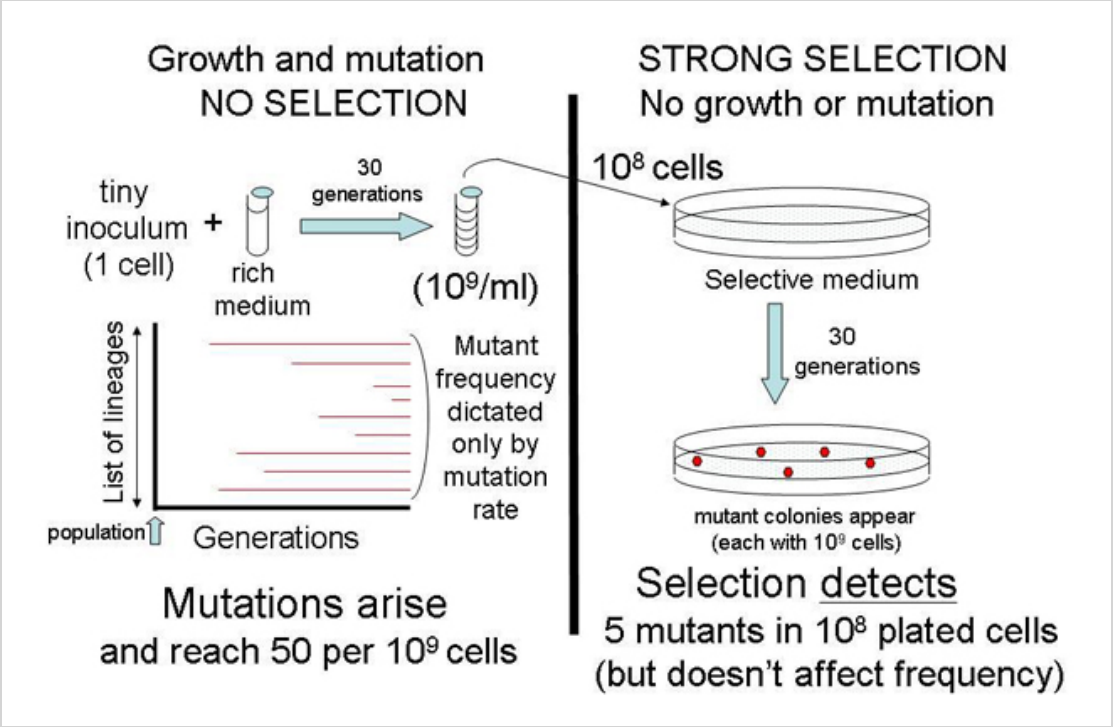

Figure 6The laboratory procedure that allows mutant frequency to indicate mutation rate. Selection is cleanly and completely separated from growth and mutation. Mutations are allowed to accumulate (linearly) during the initial nonselective pregrowth period. Precautions eliminate the effect of fluctuation or stochastic distribution of times at which mutations arise. Samples of nonselective growth culture are plated under strong selective conditions that block all growth of the parent type and all of the common small-effect mutations. Only rare large-effect mutations are allowed to grow under selection. These mutations are assumed to be fully fit and require no adaptive change to be detected on the selection plate.

A caveat should be noted. Whenever cells grow and mutations can occur, natural selection is expected to act (e.g., any deleterious mutation will be disfavored). For the purposes of this discussion, the assumption is made that one can eliminate the effects of selection on the particular phenotype being studied. For example, rich medium is assumed to eliminate selection on auxotrophic mutants.

During the nonselective pregrowth period (Fig. 6, left), mutants with some particular selectable phenotype accumulate linearly in the population; their frequency is dictated by mutation rate, by the timing of mutational events and by the target size for mutations with that particular phenotype (compare with Fig. 2, middle). The imposed strong selection (Fig. 6, right) prevents growth of parent cells and small-effect mutants, but allows large-

effect mutant cells to grow and form visible colonies. In this way, selection detects the fully fit mutations that arose during prior nonselective pregrowth, but does not contribute to increasing the frequency of detectable mutants.

The next section ("Seven Pillars of Laboratory Selection") describes the considerations described above as a set of rules that support the whole edifice of bacterial genetics. These rules are seldom stated, but they are the underpinning of bacterial genetics because only with these rules can one selectively detect mutants in a culture without influencing the answer. The rules make it possible to analyze genetic events in huge bacterial populations. They allow phenotype frequency to be a reliable indicator of mutation rate. Extensive routine use of this procedure may have led to trivialization of the underlying rules, thereby leading to misinterpretation of some experimental results. The rules are not legal obligations. They must be obeyed only if you need to reliably and accurately measure mutant frequency and rates.

SEVEN PILLARS OF LABORATORY SELECTION

Assumptions That Underlie the Use of Phenotype Frequency To Estimate Mutation Rate

The following seven assumptions underlie the use of phenotype frequency to estimate mutation rate:

1. **No preexisting mutants** Inoculate the preselection culture with a population too small to include any of the mutant types of interest. This eliminates founder effects and assures that all of the mutants that are detected later by selection were formed during the previous nonselective growth period

2. **No growth defect or advantage during growth before selection** Assure that the mutant types to be detected grow at the same rate as the parent cells during the preselection period—their frequency in that population is dictated only by mutation.

3. **No lag in expression** Assume that the mutant phenotype appears immediately when the mutation forms (no phenotypic lag). A delay in phenotype appearance allows late-arising mutations to escape detection. Half of the mutation events that occur in the pregrowth culture are expected to occur in the final generation of the pregrowth culture. A corollary assumption is that mutant cells arising during pregrowth can (when plated on selective medium) form a visible colony with 100% efficiency. Thus, the phenotype frequency in the unselected population is the ratio of observed colonies on selection medium to the viable cells placed under selection.

4. **There is no stochastic fluctuation in mutation events** Assume that the mutant frequency perfectly reflects the mutation rate. This rule is difficulty to obey, but can be approximated. Although each mutation arises with the same probability, the exact moment of its formation is subject to stochastic variation. Mutants that happen to arise early in the history of the culture will contribute more heavily to the final mutant frequency. As outlined below, the Luria-Delbrück theory predicts that identical cultures will accumulate different numbers of mutants simply because of variation in the timing of mutations. One can correct for this in a variety of ways (56). A rough, short-hand correction is to simply use of the median phenotype frequency of multiple cultures and thereby avoid the major “jackpots” or “low-pots” cultures in which mutation timing has caused an abnormally high or low phenotype frequency.

5. **Each mutant generates one colony** Perform selection on solid medium. By immobilizing cells of the pregrowth culture, it is assured that each mutant cell in that population forms one distinct countable colony.

6. **No parental growth** Use selection conditions that completely prevent growth of parent strain and any preexisting small-effect mutants. This prohibits new mutations from arising during the period of selection and prevents growth under selection from enhancing the number of detected mutants. While this pillar supports the whole history of bacterial genetics, it is a sticking point for several systems used as evidence for stress-induced mutagenesis. These systems assume that (contrary to this rule) nongrowing cells are subject to mutagenesis. The question of whether or not mutations occur in nongrowing cells will be discussed below.

7. **No heterogeneity in mutant colonies** Each mutant colony is assumed to be a homogeneous population of cells with the same genotype as the cell that initiated it. None of the counted colonies has been initiated by a common small-effect mutant whose ability to form a visible colony depends on acquisition of additional mutations during growth under selection.

How Growth Complicates Determination of Mutation Rate from Mutant Frequency (Even without Selection)

Mutation rates are generally expressed as mutation/cell/division since it is usually assumed that most mutations arise as errors in chromosome replication. Therefore when a laboratory selection is used to estimate mutation rate, it is essential to know how many generations (acts of DNA replication) occurred at which a mutation might have occurred. For example, a mutant would be defined as common if it arose during 100 acts of replication, but as rare if it arose only once in 10^9 acts of division. This calculation is rather easy if one knows the inoculum size and the size of the final population in which mutants are detected. To fairly estimate a mutation rate one must know how many divisions occurred.

Estimating the number of divisions that serve as the basis for the estimated mutation rate can be difficult especially in a population whose net size is not increasing. Several such systems are used as evidence for stress induced mutagenesis. Mutations are said to arise in a time-dependent manner with no acts of cell division. If this is a prominent phenomenon, many conclusions of bacterial genetics are called into question. If this is not true, then the evidence for stress-induced (stationary phase) mutagenesis is called into question.

Cell growth and the occurrence of mutations.

DNA replication provides an opportunity for mutation, and mutation rates are generally expressed per cell/per generation. Thus if a population is heterogeneous and includes a subset with a faster growth rate, that subpopulation is expected to acquire a disproportionate number of new mutations over a given time span simply because it undergoes more acts of replication and includes an increasing proportion of the parent population. This is expected even if all cells have the same probability of making an error during one act of replication. Thus interpreting increases in mutant frequency (assuming no selection) depends on knowing how many acts of replication actually occurred to provide the cells with the assayed mutant phenotype.

Growth can be a problem even in populations showing little net growth.

In several of the experimental systems discussed below, growth of the parent population is blocked (as is true in a standard laboratory selection). However, in some cases these populations are incubated for extended periods of time before the number of mutant colonies is counted. Interpretation of these

experiments has assumed that all colonies appearing are due to large-effect mutations that either (i) arose during pregrowth of the culture, or (ii) were induced in nongrowing cells by a novel (stress-induced) mechanism after plating of the culture. That is, some mutations are assumed to arise in a nongrowing population and to accumulate in a time-dependent manner, without any scheduled DNA replication. Whenever a mutation is attributed to nongrowing cells in this manner, the problem of growth becomes profound. It is difficult to eliminate the possibility that the new mutants actually arise in a growing subpopulation of cells. Establishment of the idea of "stationary-phase" mutagenesis requires elimination of the possibility of this cryptic growth. The difficulty of detecting a growing subpopulation is illustrated in Fig. 7.

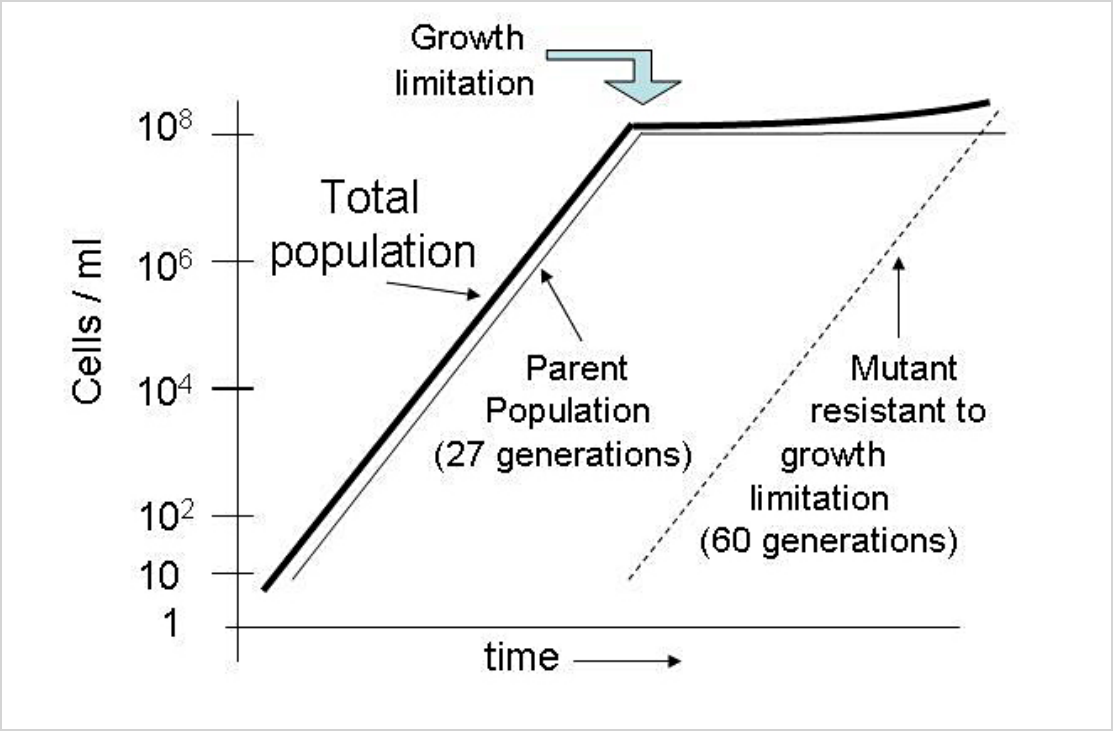

Figure 7Growth without apparent population size increase. The population diagrammed here reaches a plateau (10^8/ml) when some resource is exhausted. A mutant formed during the prior growth continues to divide (in this example for an additional 27 generations, resulting in a doubling of the total population).

Suppose that selection limits growth of a population at 10^8 cells, and a resistant mutant arises during the growth of that population. The new mutant can grow exponentially after growth of the parent cell type has ceased. When the mutant subpopulation reaches 10^8 cells, the total population will have doubled which may be difficult to detect experimentally. Thus an apparently

resting population shows a substantial increase in mutation frequency. It appears that a nongrowing population was mutagenized by some novel (growth-independent, stress-induced) mechanism. Once one is aware of the existence of a growing subpopulation, no mutagenesis is required to explain the frequency increase, which is due entirely to natural selection. It should be noticed that at the end of the experiment in Fig. 7, half of the population will have gone through 27 extra generations of growth and have a 27-fold higher probability of acquiring any mutation.

Another potential problem is seen in a population of constant size in which cells are dying and being replaced by slow growth of the population at large. This is diagrammed in Fig. 8. Here a constant population may be replicating continuously (with different subpopulations possibly reproducing at different rates) and accumulating mutations that appear to form in a stationary-phase population.

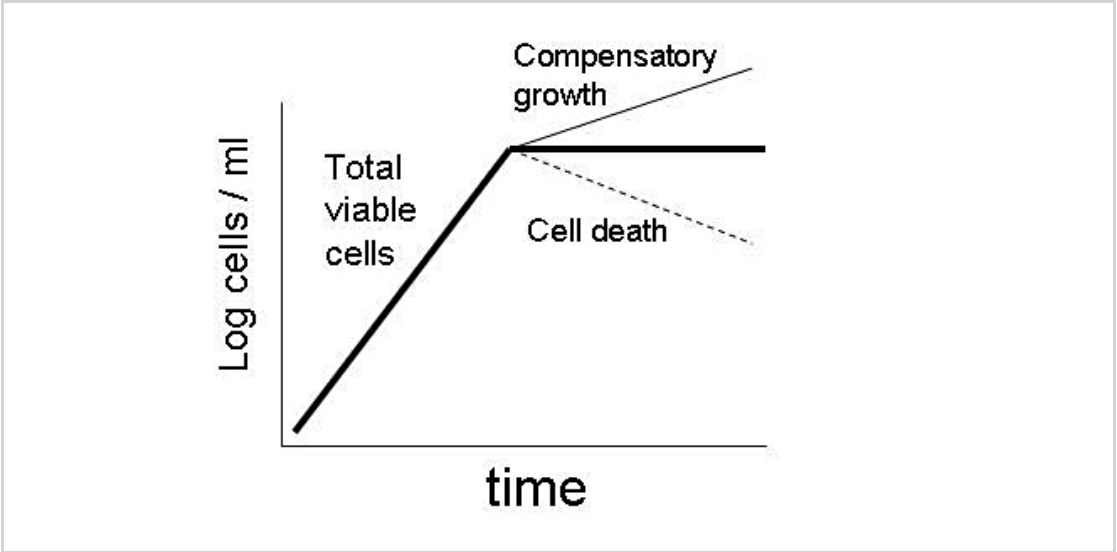

Figure 8A bacterial population of constant size in which cells are dying and being replaced by slow growth of the population at large.

Growth (and replication) contributes to the appearance of the phenotype.

After a DNA sequence change, some time may be required before the mutant phenotype appears (phenotypic lag). *Escherichia coli* cells growing under different growth conditions can have from 1 to 8 chromosomes per cell (1). This time lag may allow a recessive new mutant allele to segregate from parental copies present in a sister chromosome or second bacterial nucleoid. It may allow new mutant enzymes to dilute out preexisting parental protein. If appearance of the mutant phenotype requires two cell divisions, then the

phenotype frequency assessed at any moment will fail to detect mutations that arose within the final two generations. If the population is growing exponentially, more that 75% of the mutational events will have arisen during that period and will escape detection. (Half of the acts of chromosome replication occur at the final cell division, and another quarter occur at the previous cell division.) This phenomenon is particularly relevant in the context of the debate on “stress-induced mutation” because the classic experiments demonstrating that mutations arise before selection all used phenotypes with a very long lag (streptomycin resistance and phage resistance). In both situations, the selected phenotype appears several generations after the mutation arises. Any new mutation arising under selective conditions, would be immediately counterselected and could never get through the lag period and attain a resistant phenotype. Thus phenotypic lag biased these experiments in favor of mutations that arose several generations prior to exposure to selection. All growth of the parent under selection was prevented by the lethal selection and any preexisting mutant that had not escaped lag (or any new mutant arising after plating) was subject to the same fate.

Growth allows mutations to arise at variable times in the history of the population.

The work of Luria and Delbrück clarified the kinetics of phenotype accumulation in unselected populations by demonstrating how the stochastic distribution of mutation occurrence times contributes to the frequency of mutant phenotypes (46). The rate at which any mutation arises is essentially a probability statement, expressing the number of mutations expected to form per act of cell division. In practice, any particular population growing from a small number of nonmutant cells will realize its first mutation at a range of times, distributed around that predicted by the mutation rate. The phenotype frequencies in a series of such cultures can vary widely (fluctuate) based on the time of first mutation. The expected statistical distribution was described by Luria and Delbrück. The result of this fluctuation is that independent cultures with the same mutation rate are expected to contain widely different phenotype frequencies. There are a variety of ways to compensate for this problem experimentally (56) and thereby to determine the actual mutation rate.

How Can One Be Sure that the Pillars of Wisdom Have Been Successfully Followed?

The classical test to ensure that a selection is detecting preexisting mutations is through a Luria-Delbrück fluctuation test. In doing this test, series of independent cultures are all initiated using a small inoculum. Each culture is independently plated on selective medium. If colonies appearing on the selective plate reflect colonies present in the plated population, then you expect to see a frequency fluctuation from one culture to the next. The way to perform this test and treat the data has been described in detail (56).

If one sees fluctuation, the question is whether all mutant colonies were initiated by preexistent cells or only some fraction (enough to cause the observed fluctuation). A way to test the observed fluctuation has been described (56). In this test, one plots the log of the number of mutants/tube (N) versus the log of the probability of a tube containing N or more mutants. If this plot gives a straight line of slope = -1, then the distribution of mutants/tube in your series of tubes reflects a Luria-Delbrück distribution and all observed colonies were initiated by mutants that arose during the pregrowth culture. If some mutants arise on the plate this plot shows a plateau followed by a sharp drop—essentially a narrower variance.

There is a difficulty here in the case of copy number variants. The above test was performed for the Cairns selection and no fluctuation was noted. The conclusion was made that all mutants arose on the plate (providing support for the idea of stress-induced mutation). However, in the case of duplications, fluctuation is not expected because the frequency of duplications approaches a steady-state frequency before selection (51). That is, any differences in the time of appearance of the first duplication are reduced because frequencies all approach the same steady-state value. This prevented the fluctuation test from demonstrating that the revertants appearing (over 6 days of selection) are actually initiated by mutant cells (with a *lac* amplification) that arose before selection. A test has recently been devised which shows that the number of revertants in the Cairns system is in fact determined by the number of cells in the pregrowth culture that have a *lac* amplification before selection (E. Sano and J. R. Roth, unpublished data).

Contribution of Genetic Drift and Founder Effects to Differences in Phenotype Frequency

If a population is initiated by an inoculum that already contains mutant cells, these founders can provide a higher phenotype frequency than one might expect to see if all mutations arise only during the history of the population. This can complicate interpretation of phenotype frequencies and is systematically avoided by one of the rules of laboratory selections described.

Founder effects are similar to consequences of genetic drift. In small populations, the frequency of mutant types can vary from generation to generation, simply by random success in reproduction or stochastic killing that is not influenced by genetic differences. Drift will be ignored here because large microbial populations reduce its importance.

KEY SYSTEMS USED TO STUDY THE EFFECTS OF SELECTION ON MUTATION

Our understanding of the origins of mutations has been strongly influenced by a few key experiments. Luria and Delbrück's experiment, and the Lederbergs' follow-up experiments, showed that mutants could preexist selection in a bacterial culture. This was critically important in establishing bacteria as a valid genetic system for analysis of mutation rates. The Cairns-Foster experiments raised serious questions about the general validity of conclusions based on Luria and Delbrück's experiment. Cairns-Foster raised the issue of whether under nonlethal selection bacteria were capable of "directing mutations" or of selectively increasing mutation rates in response to environmental pressure. The question of whether bacteria could respond to stress by increasing mutation rates was taken further by Radman, Taddei, and Matic when they showed that bacteria apparently increased mutation rates in response to nutrient limitation and aging. Below we examine each of these critical experimental systems to explore their strengths, limitations, and possible weaknesses.

1. The Luria-Delbrück and Lederberg Experiments—the Classic Evidence that (Some) Mutations Are Not Induced by Stress

In some sense, the Luria-Delbrück experiment established the “seven pillars” of laboratory selection (46). The experiment was designed to demonstrate that laboratory selections detect preexisting mutants and do not contribute to the creation of mutants. This experiment validates the use of selection as a means of detecting mutants and is therefore fundamental to bacterial genetics. If exposure to selection actually caused mutations, the whole field of bacterial genetics would be called into question.

The basic observation in the Luria-Delbrück experiment was that the number of detected mutants in a series of independent preselection cultures varies in a manner that can be predicted mathematically if one assumes that the variability reflects a stochastic distribution in the timing of mutational events during nonselective pregrowth of the plated cultures. This variability demonstrates that the mutations arose before exposure to selection. The same conclusion was reached by the Lederbergs' using a more graphic, nonmathematical method involving replica printing a master cell lawn to multiple plates of selective medium and seeing the same pattern of resistant clones on each replica (42).

Use of lethal selections to detect mutants with phenotypic lag.

Both Luria-Delbrück and Lederberg used lethal selections (phage resistance and streptomycin resistance, respectively) in which the detected mutant phenotype appears only several generations following occurrence of the DNA sequence change. The selection conditions made the results particularly clear because they enforced a broad separation of selection from growth and mutation. (They follow the "seven pillars" to the letter of the law.) No growth was allowed under selection (conditions were lethal). Large-effect mutations are detected. Mutations had to arise several generations before plating and thus were certain to arise within the nonselective growth period. Mutant frequency will be subject to the fluctuations in frequency analyzed by Luria and Delbrück. The long phenotypic lag also ensures that, in the Lederberg experiment, cells resistant to streptomycin are likely to be present as sizable clones within the lawns of unselected cells that were replica printed. Phenotypic lag made it extremely unlikely that any mutations arise following plating on the lethal selection plates—cells would have to escape killing for several generations if a new resistant mutant were to be detected.

Avoiding the caveat inherent in the classic experiments.

While use of a lethal selection and mutations with phenotypic lag allowed one to separate mutation from selection, it reduced the power of the experiments to demonstrate that all mutations arise independent of stress. The design of the classic experiments ensured that only preexisting mutations could be detected. This caveat was pointed out by several investigators (8, 23, 60).

To avoid the shortfall of the classic experiments one must somehow measure mutation rates in growth-limited (stressed) cells. This can be done if one limits growth in one way and follows accumulation of mutations that are neutral under those conditions. Such experiments have not demonstrated a general increase in mutations rates during growth limitation (34). It is much more difficult to limit growth and measure the rate of formation of adaptive mutations that are positively selected as soon as they arise. (In principle, this could be done by fluctuation tests using the “zero-tube” method, but the growth and improvement of small-effect mutations makes this technically difficult.) The Cairns-Foster experiment was devised to fill the gaps in the classic experiments.

2. The Cairns-Foster Experiment—an Attempt To Assess Mutation Rates under Selection

The possibility that some fraction of mutations are stress induced has been most aggressively addressed using a system designed by Cairns and coworkers (8) and modified by Cairns and Foster (7). In this experiment the conditions used by Luria and Delbruck were varied in hopes of detecting mutations induced by stress. The selection is nonlethal, and the mutations scored show no phenotypic lag. Parent tester cells are mutants unable to use lactose. These cells are plated on selective medium (lactose), which prevents their growth but does not kill. Over 5 to 6 days, revertant (Lac^+) colonies appear above a lawn of nongrowing parent cells (Fig. 9).

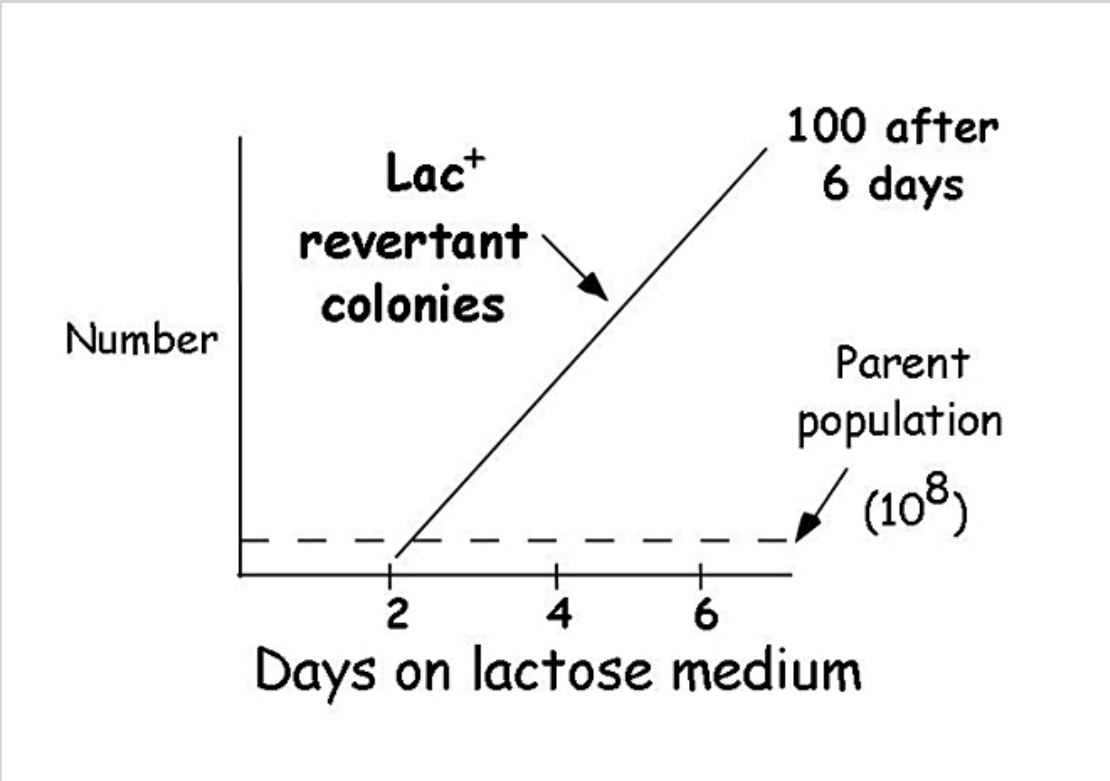

Figure 9The basic form of the Cairns experiment. As in the Luria-Delbrück experiment, parent cells are plated on selective medium mutant (revertant) colonies appear with time. These colonies have been attributed to mutations induced by stress in the nongrowing parent population. The main features of the Cairns-Foster experiment are described in the text and then discussed in terms of models offered to explain them.

In the Cairns experiment, as in the Luria-Delbrück experiment, parent cells are plated on selective medium mutant (revertant) colonies appear with time. These colonies have been attributed to mutations induced by stress in the

nongrowing parent population. The main features of the Cairns-Foster experiment are described below and then discussed in terms of models offered to explain them.

1. The plated Lac^- tester cells (10^8) carry a revertable +1 frameshift mutation.

2. The tester *lac* allele provides about 2% of the β-galactosidase level found in Lac^+ revertant strains. This residual function allows a single mutant cells to form a colony on standard lactose medium.

3. Growth of the tester strain is prevented by scavenger cells having a *lac* deletion. A 10-fold excess of scavenger cells is plated with testers and restricts growth of the tester cells, probably by consuming excreted metabolites.

4. The testers are poised (by the titrated excess of scavenger cells) on the brink of growth, such that any tiny increase in β-galactosidase can allow them to grow.

5. During prolonged incubation (6 days on selective medium with scavengers), the lawn of tester cells grows very little.

6. Within 1 to 2 days under selection, a few colonies appear which are attributed to mutant cells that arose during the growth before selection (as in a standard laboratory selection). These day 2 colonies show a Luria-Delbrück distribution and can be used to determined the unselected formation rate of full Lac revertants as 10^{-8}/cell/division.

7. Over the following 4 days, about 100 additional colonies accumulate above the cell lawn. In some experiments, the accumulation is linear with time—in others it shows some upward inflection.

8. The colonies arising after 4 days, require mutational events occurring after exposure to selective medium. (When independent cultures are tested, the number of revertant colonies does not show the fluctuation predicted by Luria-Delbrück.)

9. The stressed parent population in the lawn is not mutagenized during the period of revertant accumulation. Samples taken from the lawn (between Lac^+ revertant colonies) show no increase in genome-wide mutant frequency.

The described accumulation of Lac^+ revertants above a nongrowing lawn has been interpreted as evidence that stress induces a mechanism for genome-wide mutagenesis. This proposed mechanism chooses a random subset (about 1/1,000) of the population and mutagenizes it heavily and nonspecifically. The mechanism is thought to have evolved under selection because the mutations it provides help cells adapt to stressful conditions. Support for these conclusions have been reviewed recently although it should be noted that there are different versions of the model (16, 20). Most of this evidence assumes the validity of the basic experimental observations—that mutations are in fact induced by stress. Mechanisms to explain this mutagenesis have been proposed and tested, but the basic assumption—stress-induced mutation—has not been questioned or experimentally tested. Can the basic results be explained by selection alone rather than mutagenesis? To evaluate this question, we consider the basic experiment in the light of the general considerations outlined above and then explore alternative explanations.

Comparing the Cairn-Foster experiment to a standard laboratory selection.

The Cairns-Foster experiment resembles a standard laboratory selection in that (i) parent cells are plated on selective medium and (ii) the number of colonies arising under selection is used to estimate a mutation rate. Inferring mutation rate from mutant frequency demands that the rules (Pillars of Laboratory Selection) be obeyed. One must prevent selection from influencing the number of detected mutants.

The Cairns-Foster experiment differs radically from a standard laboratory selection in that (i) mutations arise under selective conditions, (ii) mutations appear to arise in nongrowing cells, and therefore (iii) mutation rates are expressed per unit time not per number of cell divisions. These features make it difficult to comparing mutation rates under selection with those measured during unrestricted growth. The only barrier to selective enrichment of mutant frequency is that the tester population appears unable to grow. This leaves open the possibility that some subpopulation grows and contributes to mutant yield.

The Cairns-Foster experiment violates the rules of laboratory selection.

This experiment deviates from the rules for selection in that the selective conditions are not strong enough to assure that only large-effect mutations (compensating frameshifts) are detected (rules 6 and 7). Selection is weak because the parent tester cells have 2% of the revertant level of β-galactosidase before being exposed to selection. They are poised on the brink of growth by added the scavenger cells. Any small-effect mutant with a slight increase in β-galactosidase activity can upset this poise and initiate a slow-growing colony. Small-effect mutations are especially common since duplications and amplifications can serve to initiate a cascade of selectable events (see below). Small-effect mutations can contribute to the number of counted visible revertant clones if they can improve and form a visible colony. This is facilitated because cells with residual β-galactosidase are held under selection for a very long time period (6 days). These extra colonies can inflate the revertant frequency on which the mutation rate estimation is based.

Selection (without mutagenesis) can enhance the yield of mutants in this experiment because it contributes to the improvement of plated common small-effect mutants. During nonselective growth, these small-effect mutations would escape detection. The frequency of the colonies appearing under selection may be dictated by the frequency of these small-effect mutants and the probability that they can improve so as to form a colony within 6 days—a very long selection period. If the experiment could ensure that every colony is initiated by a single, rare ($lac \rightarrow lac^+$) frameshift mutation formed on the plate, then some induced mutagenesis would appear to be needed. However, if common small-effect mutants can initiate clones, then growth under selection can explain the enhanced revertant yield.

As long as colonies can improve by common small-effect mutations occurring at standard mutation rates, the phenomenology can be explained by selection alone —rapid multistep genetic adaptation during growth under selection using variants that are available under all conditions. If this is true, the unusual set of assumptions needed to explain this system in terms of "stress-induced" mutagenesis are no longer needed.

1. No need to propose induced mutagenesis.

2. No need to propose mutagenesis of nongrowing cells.

3. No need to propose mutagenesis induced in a random subset of the population.

4. No need to propose an evolved mechanism to contravene the elaborate known mechanisms that minimize mutation rates.

A model to explain the Cairns-Foster results by selection (without induced mutagenesis).

The basic observations listed above and diagrammed in Fig. 9 can be explained without recourse to stress-induced increase in the standard mutation rates. Duplications and further increases in copy number are mutations that occur at very high frequencies under all growth conditions (see Fig. 3). More than 0.1% of cells in an unselected population typically carry a duplication of any specified point in the chromosome (3). This high duplication frequency is a steady state achieved because of the high formation rate, higher loss rate, and fitness cost of duplications (described above). Unselected population also carry higher copy number variants at lower steady-state frequencies (55). The *lac* operon used in the Cairns experiment is duplicated in approximately 0.5% of the plated cells. Such mutations double the small amount of β-galactosidase produced by the leaky mutant *lac* allele such. Thus, 10^5 to 10^6 of the 10^8 plated cells have two or more *lac* copies. Further changes in copy number (increases or decreases) occur at a rate of about 0.1/cell/division by unequal recombination between duplicated copies in sister strands. Thus, extremely common copy number increases may serve as small-effect mutations allowing frequent colony initiation under selective conditions. These considerations underlie a model whose basic features are outlined below and diagrammed in Fig. 10.

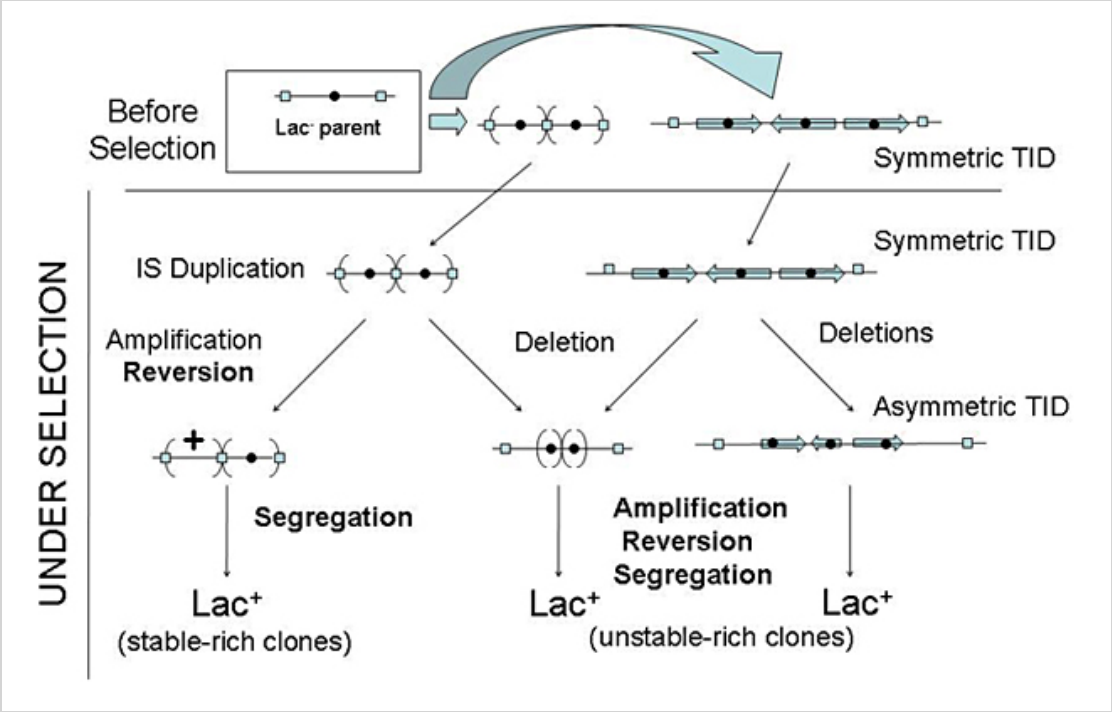

Figure 10Explaining the Cairns system with growth under selection (no mutagenesis).

1. A duplication of the mutant *lac* operon is carried by about 1 in 200 of the 10^8 plated tester cells. This *lac* allele is leaky, producing about 2% of the enzyme active found in a *lac*$^{\pm}$ revertant. Thus, 10^6 of the plated cells have at least two *lac* copies; some may have more, since the steady state duplication frequency is expected to come to a steady state with higher amplifications present at lower frequency (55). It is estimated that roughly 100 of the plated cells may have 5 *lac* copies. (A. B. Reams, M. Savageau, S. Maisnier-Patin, and J. R. Roth, unpublished results).

2. Cells with a sufficient number of *lac* copies initiate colonies. Growth of cells within these colonies can lead to a visible colony if one *lac* allele reverts or attains a sufficiently high copy number by amplification under selection.

3. The duplication and amplification events are frequent events under all conditions, even without selection.

4. The probability of a reversion (frameshift) event to *lac*$^+$ is a function of the number of *lac* alleles on the selection plate. The allele number increases within any developing colony because of increases in the number of *lac* alleles per cell and the number of cells in the colony. For

example, if 10^5 duplication-bearing cells initiate clones that grow to reach 1,000 cells with 10 *lac* copies, a total of 10^8 acts of cell division and 10^9 replications of the *lac* allele occur. This is sufficient to generate 10 reversion events on a plate with no increase in mutation rate. (The standard *lac* reversion rate is 10^{-8}/cell/division.)

5. When a reversion event occurs, one fully functional *lac*$^+$ allele is generated within an array of tandem mutant *lac* copies. Selection favors cells that retain the *lac*$^+$ allele and lose the remaining mutant copies. The extra copies impose a significant fitness cost and increase the likelihood of losing the *lac*$^+$ allele. Ultimately a cell arises that is haploid for a *lac*$^+$ allele and is fully Lac$^+$ in phenotype and that suffers no loss due to segregation or no fitness cost. These haploid revertant types overgrow the original colony. The steps listed above are described on the left side of Fig. 9.

6. Colonies can form without reversion if they sufficiently amplify the mutant *lac* allele. This can occur only if a deletion mutation reduces the size and the fitness cost of the *lac* amplification. A deletion of the IS*3* duplication join point element places *lac* within a smaller repeated unit with a lower fitness cost (Fig. 9, middle). Such cost reduction allows higher *lac* amplification and faster growth on lactose.

7. Deletion events are common since each end point can lie anywhere in a 50-kb region of one copy of the duplicated region. Any higher amplification of the large IS*3*-duplication provides additional pairs of copies between which an effective deletion can occur. The number of paired copies that might form is expected to increase as a function of copy number (n) by the function $D = n(n - 1)/2$.

8. Clones growing by virtue of amplification alone achieve sufficient *lac* copy number that some revertant alleles are expected to arise in every clone. If a clone reaches 10^8 cells by having 100 copies of the *lac* region, each population doubling is expected to add 100 *lac*$^+$ cells to the colony. Cells with such revertant alleles are likely to grow faster than cells with a highly amplified mutant *lac* allele.

9. Two types of revertant colonies arise by the events above. Colonies rich in stable Lac$^+$ cells form when reversion occurs early in one of the many small clones with large, low-copy amplification. In such colonies, *lac*$^+$ haploid cells are able to form and overgrow to produce a colony dominated by *lac*$^+$ haploid cells. Colonies rich in unstable Lac$^+$ cells arise when deletion mutations allow high amplification. These clones are rich in unstable Lac$^+$ cells that grew by virtue of amplification alone. These bifurcating pathways are described at the left side of Fig. 10 and correspond to the pathways of adaptation diagrammed in Fig. 4.

10. A third pathway also appears to contribute to reversion. The events in this pathway are not yet fully elucidated, but a model is described at the right side of Fig. 10. It is duplication frequently arise as a symmetrical tandem inversion duplication (sTID), with extended palindromes at each of two junctions (39). These duplications are likely to be unstable and deleterious, but they provide multiple *lac* copies and are subject to amplification by exchanges between flanking direct repeats. While being maintained by selection for increases in *lac* copy number, these rearrangements can acquire deletions that render the junctions asymmetric and reduce the fitness cost of amplification. Amplifications of this type are estimated to underlie about 30% of the unstable-rich Lac$^+$ revertant colonies. The junctions of asymmetric derivatives (aTID) have been identified and sequenced (38).

Summary of the amplification model.

This model suggests that the Cairns-Foster experiment allows selection to affect the number of detected mutants, because the basic rules of laboratory selection have been skirted. In particular, selection is too weak to prevent growth of extremely common small-effect mutants with multiple copies of *lac*. These cells initiate colonies that sometimes succeed in generating a visible colony. Most of the observed colonies are initiated by common small-effect duplication mutations. The rare frameshift mutations, which are assumed to underlie these colonies, are not required for colony initiation, but they may arise and even predominate in the final colony because of selective growth. The frequency of observable colonies is dictated by common events that initiate the clone that becomes visible in the course of 6 days on selective medium.

As expected for a natural population, genetic adaptation occurs by a pathway of successive common events, each providing a minor growth improvement. Here the succession of events that improve growth is (i) duplication of the mutant *lac* allele, (ii) stepwise further *lac* amplification, (iii) reversion of one copy to lac^+, (iv) stepwise loss of nonrevertant *lac* alleles, and (v) appearance of a haploid cell with a revertant lac^+ allele. As predicted for clones that improve in this way, each colony is a mixture of cell types, some with an amplification of the *lac* region, and some that have reverted to lac^+ and segregated to haploid.

Very slight changes in experimental conditions of the Cairns-Foster experiment can reestablish the laboratory selection conditions (the Seven Pillars) and allow detection of only rare, large-effect frameshift revertants arising during the nonselective pregrowth period. For example, if the parent frameshift mutation is rendered nonleaky by a ribosomal mutation that reduces error frequency or by addition of a competitive inhibitor of β-galactosidase (4), then the tester cells are no longer leaky. This makes selection strong enough to prevent growth of the common small-effect duplications. Under these conditions, the Cairns experiment shows highly reduced accumulation of revertants with time, and detects primarily the preexisting, large-effect revertants.

The Selection Model explains results cited as support of "stress-induced" mutagenesis.

The results most frequently cited in support of the several models for stress-induced mutations (16, 20, 32) are consistent with growth under selection associated with *lac* amplification. Below we discuss and evaluate the evidence provided in support of stress-induced mutagenesis.

1. The stress-induced mutagenesis is attributed to the error-prone DinB bypass polymerase. The sequence changes that generate a lac^+ allele under selection include a high proportion of +1 frameshift mutations in base runs; this is characteristic of mutations caused by DinB (37). Revertants arising under selection in the Cairns system are more likely to carry associated, unselected mutations (in other genes) than revertants arising during nonselective growth.

In the Selection Model these results reflect the fact that the *dinB* gene happens to be located close to *lac* on the F′ plasmid and is included in all of the large IS-dependent *lac* duplications and in about 30% of the smaller high-copy *lac* amplifications. This interpretation is supported by the observation that associated mutations require that *dinB* is located close to *lac*. Alleles of *dinB* located far from *lac* do not contribute to revertant yield or to associated mutations. While the effect of amplifying *dinB* contributes about fourfold to the yield of revertants under selection, selection still enhances reversion to lac^+ in strains devoid of *dinB* and that residual enhancement depends on recombination. The observed mutagenesis is in essence an artifact due to the proximity of *dinB* to the *lac* allele under selection.

2. Stress-induced mutagenesis is attributed to error-prone recombinational replication. This conclusion is used to explain the fact that mutations arise in the apparent absence of chromosome replication and that the yield of mutants under selection requires recombination proficiency. In the Selection Model recombination is responsible for duplication, amplification, and segregation events that allow small-effect mutations to improve growth under selection.

3. Some revertant colonies include cells whose Lac^+ phenotype is unstable. These cells include amplifications of the *lac* operon. Amplifications are attributed to a separate stress-induced event that is independent of frameshift mutation. In the Selection model, the amplification cells are precursors to reversion that allow growth and enhance the probability of a reversion even by providing more target sequences.

4. Short-junction amplifications and asymmetric TID mutations are common among unstable Lac^+ revertants in the Cairns system, but are rare in unselected populations. The stress-induced mutation model suggests that these events occur only when stress induces a novel mechanism that does not operate in unstressed cultures. The Selection Model proposes that these mutations form by a series of genetic events that seldom goes to completion without selection, because short-lived and deleterious intermediates are usually lost from the population. Under long-term selection for increases in *lac* copy number, these precursors are held under selection long enough to acquire the deletion events needed to complete the process.

5. The accumulation of mutants under selection in the Cairns system requires that the *lac* mutation be leaky. The stress-induced mutagenesis model proposes that this residual function provides energy that is needed to permit a costly mechanism to create mutations in nongrowing cells. The Selection Model proposes that this basal level allows copy number variants to increase *lac* enzymes and initiate clones under selection.

3. The Shapiro System—Appearance of Mu-Mediated *ara-lac* Deletions under Selection.

Possibly the first system interpreted as evidence for stress-induced mutagenesis was one described by Shapiro and coworkers (60, 61). In this system, a promoter-less silent copy of the lactose (*lac)* operon is inserted near the arabinose (*ara*) operon of *E. coli* with a Mu prophage present between them. The strain was plated on lactose medium, and Lac^+ colonies appeared with time. Each Lac^+ revertant reflected a deletion mutation that allowed the *lac* operon to be expressed from the promoter of the *ara* region. The deletions were formed by events induced by the Mu prophage. It was initially suggested that these revertants were induced by stress in the form of lactose limitation, since they seemed to be rare in populations that were not exposed to selection. Later work by Cairns and Foster (17) demonstrated by fluctuation tests that the number of selected revertant colonies shows a Luria-Delbrück fluctuation and thus must be determined during the nonselective growth period prior to plating on selective medium and could not be initiated by stress.

This would appear to eliminate stress-induced mutagenesis, but a question remains open. When Lac^+ clones were isolated from the pregrowth culture by sib selections (no stress involved), the nature of the deletions was different from that characterized in revertants isolated after prolonged selection (47, 48).

The results have not been pursued but can be explained by the selection model described here. That is, if mutants with modestly improved ability to grow on lactose arise prior to selection, these mutants may initiate colonies on selective medium within which the population adapts and improves. The improvements may lead to the deletion types ultimately characterized. If these deletions are generated by multistep improvement, the process is expected to operate with or without selection but be driven by mass action when each successive change allows improved growth on lactose. That is, without growth limitation, the sib selection should yield partially Lac^+ cells that are unlikely to have completed the process. During growth limitation, the process is driven to completion by higher rates of exponential growth of each intermediate.

4. The Radman-Taddei-Matic System, a Nonlethal Selection Showing the Accumulation of Rif^R Mutants in "Aging" Colonies.

The formation of mutants resistant to the antibiotic rifampicin (Rif^R) is widely used for measuring mutation rates in growing bacteria. The attractions of the method are the following: (i) it can applied to a variety of bacteria with no need to construct special tester strains; (ii) it measures base substitutions (missense mutations) at a variety of sites within an essential gene (*rpoB*, RNA polymerase β-subunit); (iii) since all Rif^R mutations affect a single gene, they can be easily characterized by DNA sequencing; (iv) at least 69 different base substitution mutations, distributed among 24 coding positions, result in a Rif^R phenotype (21); and (v) Rif resistance arises at a rate that is easy to measure (~10^{-8}/cell/generation in *E. coli* and *Salmonella*).

Unselected mutation rates to Rif^R are measured by a standard laboratory selection (Fig. 6). Cells are grown in rich medium (lacking rifampicin), where it is presumed that resistant and sensitive mutations grow equally well. Mutants form in these cultures and are detected by plated on selective medium containing rifampicin. Unlike the Luria-Delbrück experiment, this selection is not lethal, but it does reliably detect large-effect Rif^R without influencing their frequency. Enumeration of these colonies with allowance for frequency fluctuation allows one to estimate the rate of mutation formation during unselected growth.

Radman, Taddei, and Matic modified this system to study the effects of growth limitation on mutation rates (67, 68). Colonies were allowed to reach a terminal size on rich medium, and the frequency of Rif^R cells was assayed over time as the cells in the colony “aged” without growth. Increases in the frequency of Rif^R cells as a function of time were attributed to a stress-induced increase in mutation rate experienced by a nongrowing population. Colonies were initiated by spotting 100 to 1,000 cells on nitrocellulose filters placed on rich medium. The small starting population ensured that no Rif^R mutants were present at the beginning of colony growth (no founder effects). Bacteria in any colony could be easily retrieved for analysis. On the first day, viable cells (colony forming units, CFU) increased to ~10^9. By the second day, cell number per colony increased further to ~10^{10}. No further increase or decrease in colony population was seen over the following 5 days of incubation. Many artificial colonies were initiated in parallel so that cell number and mutant

frequency could be tested at various times over a period of about a week. It is surprising that the frequency of Rif^R cells increased during the aging period. Laboratory strains show increases up to 100-fold from day 1 to day 7 while the total CFU remained unchanged (67, 68). A set of 787 natural isolates of *E. coli* from diverse ecological niches (6) showed variable responses with a median 7-fold increase in Rif^R mutant frequency between days 1 and 7—a period in which the median CFU increased only 1.2-fold (6).

These results were interpreted as evidence of "stress-induced mutagenesis." It was assumed that stressed cells increased their general mutation rate (6, 67, 68). The phenomenon was referred to as ROSE (resting organisms in a structured environment) and MAC (mutagenesis in aging colonies). As in the Cairns system, there is no apparent growth to serve as the basis for comparing mutation rates in stressed and unstressed cells. That is, the stress of "aging" can not be imposed on growing cells and there is no way to prevent growth without imposing stress. As in the Cairns system, the inferred stress-induced mutagenesis is based on using an increase in mutant frequency (over 7 days of aging) to infer an increase in mutation rate in a nongrowing population. The phenomenon was assumed to reflect mutagenesis and experiments were focused on how this mutagenesis was caused and regulated, not on whether or not the frequency increase reflected mutagenesis.

Tests examined a series of mutant strains, each lacking some candidate function, to determine which function affected accumulation of Rif^R mutants during aging. Accumulation depended on the ability to induce the SOS system for DNA repair and on possession of an active RpoS protein—a stationary-phase sigma factor known to contribute to expression of a variety of genes in growth-limited cells. One particular natural isolate of *E. coli*, C4750, was tested for the predicted genome-wide mutagenesis that the model predicts (6). This isolate showed a 77-fold increase in Rif^R cells between days 1 and 7 but showed no increase in the frequency of mutants resistant to streptomycin or nalidixic acid. The frequency of mutants resistant to 5-fluorouracil, mecillinam, and fosfomycin increased only 3.4-, 1.9-, and 4.8-fold, respectively. While these results were interpreted as support for general mutagenesis, they seem to suggest that another model is needed.

This system violates the rules of laboratory selection which are essential here because an increase in mutant frequency is used to infer an increase in mutation rate. A conclusion requires knowing that the mutant frequency

increase is due to new mutations forming over time under selection and not to faster growth of preexisting mutants. The only evidence for this is that the population as a whole showed no significant growth between the initial growth period (days 0 to 2) and day 7. These measurements could not detect mutations arising in a growing subpopulation. More importantly, the tests did not exclude growth of a Rif^R subpopulation in the vastly larger population of nongrowing Rif^S parental cells.

Recently, Wrande et al. (69) readdressed the question of how Rif^R mutants accumulate in aging colonies. The basic phenomenon was reproduced in both *E. coli* and *Salmonella enterica*, including clinical and natural isolates. As before, accumulation of Rif^R mutants depended on an active *rpoS* gene. However, several striking and significant clues concerning the basis of this phenomenon emerged from new experiments.

1. Accumulation of Rif^R mutant cells continued for up to one month, reaching a frequency of $>10^{-4}$.

2. The frequency increased exponentially (as expected for growth under selection) rather than linearly (as predicted for mutagenesis of a nongrowing population).

3. The Rif^R mutant cells were localized in just one or a few small sectors of an aged colony, as expected if they are clonal subpopulations growing from individual precursor cells (Fig. 11). Mutagenesis of nongrowing cells predicts a normal spatial distribution of new mutants.

4. All Rif^R cells in an individual sector carried the same *rpoB* sequence alteration, demonstrating their clonal relatedness. In contrast, mutant cells from different sectors within the same colony usually carried different sequence alterations.

5. In colonies with many Rif^R mutant cells, most of the mutants were found in a smaller number of localized subclones, consistent with variation in mutant frequency reflecting differences in clone growth rather than differences in the number of clones.

6. The Rif^R mutants showed no significant increase in the number of secondary mutations (the frequency of auxotrophs was tested), suggesting that they had not experienced general mutagenesis.

7. Most of the isolated Rif^R mutants showed a growth advantage over parent cells in colony reconstruction experiments. This growth advantage explains why cells accumulate as colonies age.

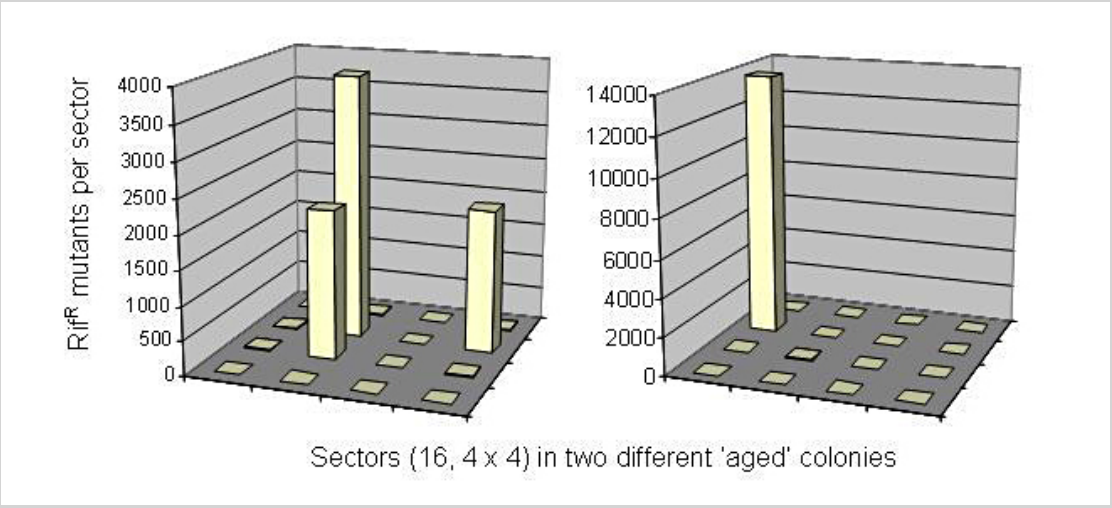

Figure 11The distribution of Rif^R cells in "aged" colonies is nonrandom. Most Rif^R mutants are found to be localized as jackpots in one or a few sectors of the colony, and within each of these jackpots the mutants carry the same sequence alteration, consistent with selective growth of a Rif^R mutant during the period of "aging." Adapted from reference 64 with permission.

Thus, the accumulation of Rif^R mutants in aging colonies is real, but it is explained by selection, not mutagenesis. Apparently Rif^R mutants grow better in "aging" colonies than parent cells even when no rifampicin is present. The detected Rif^R mutants arise during early stages in the experiment and increase in frequency by growing faster than the population at large (69). Mutations appear to arise in nongrowing populations because the total added Rif^R cells are too few to be detected in the total population. This gives the impression that new mutations accumulate in a nongrowing population when, in fact, growth is responsible for the observed increase. Additional experiments will be required to explain exactly why Rif^R mutants grow in aging colonies. It seems likely that some mutant forms of RNA polymerase may allow expression of genes that promote growth under aging conditions. Many Rif^R mutations are known to affect transcription elongation or termination (14, 35, 36, 40, 58, 62,

72), including two of the *rpoB* mutations shown to enhance growth in aging colonies. Originally RpoS was also suggested to regulate the proposed mechanism of "stress-induced" mutagenesis (67) and has also been suggested to control mutagenesis in the Cairns system (20). It seems more likely that RpoS sigma factor and mutant RNA polymerases can act together to facilitate growth within stressed colonies.

SIX OTHER SYSTEMS CITED AS SHOWING STRESS-INDUCED MUTAGENESIS

The Hall System—Rapid Evolution of a New β-Galactosidase (EBG)

In this extensively studied system, an *E. coli lac* deletion mutant acquires the ability to utilize lactose as a carbon and energy source (22, 25, 26, 27, 29). This ability requires two mutations that modify a cryptic operon (*ebg*, for evolved β-galactosidase), whose normal function is unknown but does not include use of lactose. One mutation inactivates the EbgR repressor, thereby allowing constitutive operon expression, and the second mutation alters the EbgAC enzyme, increasing its ability to hydrolyze lactose. Each mutation alone allows very slow growth on lactose (generation times >3 days), but when both mutations are present cells grow rapidly (t_{gen} = 20 min). The *ebgR*-inactivating mutation is expected to be common (10^{-6}/cell/generation) and the *ebgAC* point mutation rare (10^{-9}/cell/generation). Based on these rates, cells with both mutations are expected to arise at a rate of 10^{-15}/cell/generation and (as expected) are not detected in unselected cultures.

In contrast to this expectation, these double mutants arise continuously over a period of several weeks after 10^8 parent cells are plated on selective medium containing lactose. The selection plates also contain a small amount of glycerol (0.01%) that allows some growth, and the lactose-utilizing double mutants are detected as papillae appearing above a sparse bacterial lawn. The lawn population (even after perhaps a 10-fold increase at the expense of glycerol) is still 6 to 7 orders of magnitude too small to ensure coincident occurrence of the two required mutations. The result has been interpreted as evidence that stress induces mutations. Since the recovered lactose-using cells show no increase in the frequency of other mutations, it is concluded that the stress-induced mutagenesis somehow directs mutations to sites that improve growth.

As was true for the Cairns system, this experiment resembles a standard laboratory selection altered so as to estimate the number of mutations occurring in a nongrowing population. This system uses the frequency of mutants (actually double mutants) to draw a conclusion regarding mutation rate. As described above, this procedure requires that the selection conditions adhere to the rules of selection and prevent all growth under selection. It seems likely that selection in this system is not strong enough to prevent growth of small-effect mutations, which can initiate clones and improve their growth ability during growth under selection. If this is true, the system violates the rules and the conclusions regarding mutation rates are unfounded. Below

is one of many possible scenarios by which selection might explain the behavior of this system without recourse to changes in the rate or site specificity of mutation.

Since the frequency of repressor mutants is high (10^{-6}), it is predicted that about 100 null repressor mutants are included in the original population. Many more cells might carry more modest repressor defects. During growth on glycerol, each of these cells could produce a large population in which a duplication of the *ebg* operon arises. This would give multiple copies of a highly expressed operon. Cells growing with an *ebg* amplification could produce many cells, each with multiple copies of the *ebgAC* genes. This increase in target gene number (more cells and more *ebgAC* genes per cell) could allow a rare second mutation to arise without any change in the rate or specificity of the mutation process.

In this experiment, no tests were performed to test for the sequential appearance of the two mutations within the papillae. One might expect some cells within these clones to show unstable amplifications (as seen in the Cairns system). Thus, the system does not eliminate growth of sequential subpopulations and/or gene amplification. While the scenario above is speculative, it suggests that the behavior of this system may be explained by some form of the pathways of genetic adaptation used to explain the Cairns system. This explanation may also explain the *trpAB* revertants described below.

Growth and Selection Explains an Apparent Case of Stress-Induced Mutagenesis—Reversion of Two Separate Mutations (*trpA* and *trpB*) in *Escherichia coli*

In this system, missense mutations in the *trpA* and *trpB* genes of *E. coli* appeared to revert to Trp^+ more frequently when starved for tryptophan than when tryptophan was present (24, 30). In a double mutant carrying both of these mutations, reversion to Trp^+ was expected to be astronomically rare (3.3×10^{-19}) based on the measured reversion rate of the individual mutations tested singly. The observed Trp^+ reversion rate of the double mutant during tryptophan starvation was 4.5×10^{-11} per cell per day. Thus, the *trpA* and *trpB* missense mutations reverted to Trp^+ about 10^8-fold more frequently than would be expected if the two mutations occurred independently of each other. The genome-wide mutation rate (as measured by valine resistance and *lac* reversion) did not increase during Trp starvation and there was no effect on Trp reversion when cells were starved for another amino acid, cysteine. Thus it appeared that stress was inducing mutagenesis and directing mutations preferentially to sites that improve growth.

Hall observed early on that the stress-induced hypermutable state model (which he proposed) was inconsistent with the behavior of the *trp* mutations, because the magnitude of the genome-wide mutagenesis required to account for the reversion would certainly be lethal for any cell. Each base would have a probability of around 0.04 of mutating during the selection period and a vast number of lethal mutations would be expected (as they are expected in the Cairns system). Hall therefore examined the possibility that the two mutations occur sequentially with intervening growth. This analysis was based on the suggestion that the *trpA*, *trpB* double mutant is expected to accumulate the intermediate indole-glycerol-P (due to the *trpA* mutation). This compound breaks down spontaneously to indole, which the TrpB enzyme can convert to tryptophan. Thus, if the *trpB* mutation reverted first, the resulting *trpA*, $trpB^+$ cell could use the spontaneously generated indole and form a growing clone in which the second mutation (to $trpA^+$) could occur. Hall measured the growth rates of single-mutant cells within double-mutant colonies concluded that the most likely explanation of the enhanced reversion of the double mutant was a sequence of two steps, each giving progressively improved growth. That is, *trpA*, *trpB* → indol accumulation →, *trpA* $trpB^+$ → growth on indole → $trpA^+$, $trpB^+$ (28). This illustrates one of the myriad pathways by which selective conditions that incompletely limit growth can give the appearance of

stress-induced mutagenesis. It also makes clear the point that considerable detective work may be required to see how the devious hand of selection can upset even the most careful attempts to foil it.

Stress-Induced Accumulation of Deleterious Mutations in Stationary-Phase Cultures of *E. coli*

Behavior of this system suggests that stressed cells in a nongrowing liquid culture show a stress-induced increase in the formation rate of deleterious mutations (45). Replicate bacterial cultures are grown to full density in liquid medium and then further incubated for up to 100 days with very little additional population increase. Samples were removed from the resting cell suspension at various times and used to inoculate new cultures in fresh medium. These secondary cultures were used to estimate fitness (growth rate). The median decrease in fitness and the increase in variance among the sample lineages were used to infer mutation rates in the resting cell suspension. The genomic mutation rate inferred for cells in prolonged stationary phase was about 0.03/genome/day, about 10-fold higher than values extrapolated from exponential cultures.

While this experiment is not a standard laboratory selection (not done on solid medium), it does share many features with systems used as evidence for stress-induced mutation. That is, a population is held under conditions that allow no net growth and increases in mutant frequency (assessed indirectly) are used to infer increases in mutation rate in the nongrowing population.

To fairly interpret these results requires knowing the amount of growth that might be occurring within the resting population and whether selection for growth or survival in the resting period could affect mutant frequency. As described in Fig. 7 and Fig. 8 above, large cultures that show no net change in population size may support substantial growth of subpopulations in which mutations can arise. The growth may be populations to small to detect or balance growth and death such that no net change in viable cells occurs. One potential explanation for this result that does not involve starvation-induced mutagenesis is undetected growth.

Critics of this experiment have suggested that it involves unappreciated growth where specific subpopulations grow extensively (10) without any change in the population as a whole—the GASP phenomenon (13, 73). This cryptic growth provides added opportunities for random mutations to form at normal rates leading to fitness decreases that are not surprising in view of the large fraction of total mutations expected to be deleterious. A more extreme possibility is that mutations are positively selected during stationary-phase incubation. These mutations may enhance survival in stationary phase but be deleterious under the growth conditions used in the fitness tests.

As in several other systems that involve nongrowing populations, mutation rates cannot be compared with an unstressed control population other than the one growing without restriction. The authors express their apparent mutation rates based on a clock-like view of mutation (mutations/day), whereas their stress-free control rates are based on mutations/cell/generation. It is not obvious if and how clock-like and generation-dependent mutation rates can be directly compared.

Experiments Interpreted as Evidence that Transcription Directs Mutations to Specific Genes in Nongrowing Cells

Yasbin and colleagues made Cairns-type experiments asking whether stress-induced mutagenesis also occurs in *Bacillus subtilis*, a gram-positive species. They measured the accumulation of late-appearing prototrophs from three different auxotrophic mutants (*his* nonsense, *met* nonsense, and *leu* missense) and over 10 days observed increases of ~20-fold for His^+, <5-fold for Met^+, and 2-fold for Leu^+ (54, 65, 66). As in most experiments of this genre the authors made the assumption that the increase in the frequency of prototrophs with time could be interpreted as due to an increase in the rate of mutation in nongrowing cells. No direct tests for increased mutagenesis (for example, by testing for an increase in the frequency of unselected mutations at other locations) were made. Instead, attention was focused on identifying a mechanism to explain the presumed increase in stationary-phase mutation rate. They were intrigued when they found that the accumulation of late-appearing (as well as growth-associated) prototrophs was partly dependent on the gene *mfd* (57). On this basis they proposed that the late accumulation of prototrophs was associated with transcription of the mutant genes. The idea that transcription might "direct" mutations to a particular target, like *lac*, was proposed shortly after publication of the original Cairns experiment (9). The relevance of *mfd* for this class of model is that it codes for TCRF (transcription-coupled repair factor) (59). When RNA polymerase gets stalled in the process of transcription by bulky DNA lesions, the stalled RNAP can be dislodged by TCRF protein, which then recruits Uvr(A)BC to the lesion site, thus promoting nucleotide excision repair. TCRF activity reduces the rate of mutation on the transcribed strand of DNA relative to that on the nontranscribed strand (59). The observation by Yasbin and colleagues that the absence of the TCRF gene decreases the frequency of prototrophs is not readily explained by the known activities of TCRF, and no model has been proposed to explain the phenomenon.

Sequence analysis of the *B. subtilis* prototrophs showed that only a minority of the late-appearing mutants were actually caused by reversion of the auxotrophic alleles. The majority were due to external suppressors (possibly mutant tRNAs), and some of these were late-appearing because they grew slowly (57) and may therefore have been preexisting growth-associated mutations. The leakiness of the three auxotrophic alleles was not tested, but nonsense alleles in other systems are frequently highly leaky and could plausibly support slow growth, especially if the mutant genes were amplified in

a subpopulation of cells, as observed in the *lac* system. Indeed, the two alleles for which the frequency of late-appearing prototrophs was highest are both nonsense mutations. No tests were made for gene duplication or amplification, or for the presence of genetic heterogeneity in prototrophic colonies. TCRF activity was only associated with the infrequent reversion prototrophs and not with the much more frequent external suppressor prototrophs. We do not know why TCRF should associate with a small increase in the frequency of reversions: one possibility we suggest is that the new DNA synthesis following nucleotide excision repair is error prone, fortuitously causing occasional reversion mutations. This has not been tested.

In summary, as in other cases documented here, these experiments are based on the assumption that stress-induced increases in mutation rate were an established fact; the alternative possibility, of selection acting on slow-growing subpopulation, was not tested. The experimental system breaks the rules of laboratory selection by not clearly separating selection from growth. The accumulation of late-appearing prototrophs in this system can plausibly be explained by selection and growth of subpopulations of cells carrying leaky alleles, possibly associated with gene amplifications.

Adaptive Reversion of a *LYS2* Frameshift Mutation in *Saccharomyces cerevisiae*

A Cairns-type experiment was made to test whether the stress of stationary phase caused increased mutation rates also in eukaryotes (63). This experiment measured the accumulation of late-appearing prototrophs from a constructed *lys* frameshift mutation in *S. cerevisiae* and was, in design, methodology, and result, almost identical to the classic Cairns-*lac* reversion experiment (8).

Lys^+ colonies appeared on the selective agar plates after 3 days of incubation, with additional colonies appearing on each of the following 5 days. The numbers of colonies appearing from independent cultures showed a distribution of mutants that fitted well to expectations for random (preexisting) mutations on day 3, calculated according to Lea and Coulson (41). At later times (days 5 to 8) the distribution of mutants fitted well to a Poisson distribution, as expected for mutations occurring after plating and in response to selection. Genetic and physical analysis of the Lys^+ mutants showed that 90% contained a compensating frameshift mutation within the *LYS2* coding sequence, while the remaining 10% were apparently revertants, a conclusion supported by direct sequence analysis in a later study (33). After testing and ruling out cross-feeding and slow-growing mutants as causes of the late-arising Lys^+ mutants, Steele and Jinks-Robertson (63) concluded that at least some of the events leading to the Lys^+ phenotype must have occurred after selective plating.

The experiment showed that there was no significant increase in total cell number as a function of the number of days a plate was incubated. However, these experiments did not test the possibility that subpopulations of cells were growing (those that eventually gave rise to prototrophic colonies) or that within those subpopulations there might have been amplification of the *LYS2* target gene. Either or both of these events would have increased the genetic target for reversion or suppressor mutations without necessarily causing any appreciable increase in the size of the general population of plated cells. By analogy with the Cairns-Foster *lac* system, we suggest that the *LYS2* frameshift mutation might be leaky, and that it could be subject to duplication and amplification under selection for lysine biosynthesis. To our knowledge no one has checked this experimental system for evidence that the *LYS2* mutant allele is leaky, or that it is amplified during the postplating selection period before the visible growth of late-appearing Lys^+ revertants.

Interestingly, the authors define the late-appearing Lys^+ revertants as "adaptive" because they occur specifically in response to lysine starvation. Thus, if cells were starved for other amino acids it did not select for Lys^+ revertants. This showed that starvation did not induce genome-wide mutagenesis. This is in agreement with data that starvation in *Salmonella* does not cause a general increase in mutation rates (34).

Steele and Jinks-Robertson also selected Lys^+ revertants from populations of cells that were UV-irradiated before plating. The numbers of early- and late-appearing Lys^+ revertants were both increased by the irradiation, 5.5-fold and ~10-fold, respectively. Interestingly, the 10-fold increase applied to revertants appearing on days 4, 5, and 6, whereas on day 7 it fell back to a 6-fold increase. But if irradiated cells were allowed to grow before plating, or were held with another starvation before plating, then the number of late-appearing Lys^+ revertants was significantly reduced relative to the numbers found when plating directly after irradiation. These data can easily be explained by the gene amplification model because UV irradiation would increase the number of recombinogenic DNA ends, increasing the probability of duplication and amplification of the *LYS2* allele. This would increase the number of cells in the population that could grow slowly in the absence of lysine, and be substrates for gene amplification and eventual selection of a reversion mutation in the *LYS2* gene. If, however, cells were not plated on the selective medium directly after UV irradiation, then DNA repair systems would have time to repair lesions, unstable and nonselected duplications or amplifications would be lost, and the number of cells in the population with a genome configuration suitable for growth selection to make lysine would be reduced.

Our conclusion is that the late-appearing Lys^+ mutants, selected in the absence of lysine, can best be explained within the parameters of the model for growth advantage associated with gene amplification, essentially as described for the *lac* system. Experiments to test this model have not been made but we would predict that the *LYS2* frameshift allele is leaky and that some late-appearing Lys^+ mutant colonies should be heterogenous and contain cells with duplications and amplifications of the mutant gene.

Mutation under Selection for Recessive Mutations in a Chromosomal Gene

This system resembles that of Cairns and Foster in that one selects for Lac^+ revertants of a Lac^- parent strain (70, 71). The system differs in that it does not involve an F′ plasmid and appears to demand recessive (loss of function) chromosomal mutations, while the Cairns-Foster system demands dominant (gain of function) mutations. This system uses a *lac* operon inserted within a chromosomal purine gene (*purD*) and transcribed from its purine-regulated promoter. Since the *purD* promoter is normally repressed in the presence of excess purines, the strain is phenotypically Lac^- (and requires purine because of the *purD* disruption). When this strain is plated on minimal lactose medium with added purine (adenine), about 100 revertant colonies (Lac^+) arise over the course of 6 days above the lawn of plated cells. The most frequent revertants are expected to be common recessive mutations in the gene for the purine repressor protein (*purR*- 1,000 bp) or rare mutations in the tiny PurR-binding operator sequence (16 bp) near the *purD* promoter. Either type of mutation should activate transcription of *purD* and allow expression of the inserted *lac* genes, resulting in a Lac^+ phenotype.

In the initial description of this system (71), the tester lawn showed very little growth and about 100 Lac^+ revertant colonies accumulated over the course of 6 days of incubation. Results were interpreted as evidence for stress-induced mutagenesis of nongrowing cells. This conclusion was supported by the finding that the ratio of O^c to R^- mutants was much higher than one would expect based on their target sizes. Among day 2 revertants, O^c mutants were 34% of total. Among day 6 revertants O^c mutants were 8% of revertants; the operator target is only 1.6% of the *purR* target. The high relative frequency of O^c mutants is interpreted as evidence that stress directs mutations to sites near those that limit growth (the unexpressed *lac* operon); the higher frequency on day 2 is not explained. In this experiment, the absolute frequency of O^c mutants is also surprising—approximately 10 per 10^8 plated cells—this is approximately 100- to 1,000-fold higher than one would expect for a target of 10 bp.

The conclusion that this system reflects stress-induced mutation rests on the pillars of laboratory selection. That is, growth of the parent strain is must be prevented by selection, and revertant colonies rmust reflect new single large-effect mutations that confer full fitness and are not subject to further

improvement during maturation of the colony. Recent analysis of this system (S. Quinones-Soto and J. R. Roth, unpublished) suggests that substantial lawn growth occurs (about 3 generations) and the process by which Lac^+ colonies arise requires multiple mutations that arise as individual steps in a pathway of genetic adaptation during growth under selection. Growth of the parent on lactose is restricted not only by repression, but also by polar effects of a stem-loop structure present upstream of *lac* in the inserted sequence. Neither a *purR* nor an O^c mutation alone is sufficient to allow a revertant colony to appear under selection. All revertants have lost the stem-loop structure. Thus, the recovered revertants have genotypes that are expected to arise at frequencies of less than 10^{-15}, yet about 100 such revertants arise from 10^8 slow-growing plated cells. Direct tests of these revertants reveal no evidence that they have experienced any global mutagenesis during selection.

Revertant colonies include cells with an unstable Lac^+ phenotype. These cells have an amplification of the *lac* operon due to recombination between the *rrnH* and *rrnE* loci –5 kb direct sequence repeats that flank the *purD*::*lac* region. The yield of revertants in this system is reduced about fivefold by a *recA* mutation. While many details of the reversion process remain to be elucidated, it is clear that the excess of O^c mutations and frequency of stem-loop deletions can be explained by amplification of the *purD*::*lac* region under selection during growth of the developing revertant colonies. This amplification allows growth and provides additional copies of the growth-rate-limiting *lac* region, thereby enhancing the likelihood of mutations that improve growth under selection. This system seems to exemplify the amplification selection process described for the Cairns-Foster system and involves no increase in mutation rate.

CONCLUDING REMARKS

Publication of the Cairns system in 1988 (8) sparked renewed interest in the process by which mutations arise. Work following up on this initial report has revealed methodological pitfalls associated with measuring mutation rates and the difficulty of unambiguously separating the effects of mutation and selection. Thus, as outlined in this chapter, the inference that stress induces mutagenesis can generally be explained by an inability in the different experimental setups used to completely prevent the effects of growth when applying nonlethal selections; i.e., the claims of stress-induced mutagenesis have not strictly excluded the influence of growth and selection in increasing mutant frequencies. Instead many of these observations can be explained by the ability of selection to detect and amplify small differences in growth rate caused by common small-effect mutations. One very common class of mutations that will be important during any adaptive response in which increased activity of some gene can enhance growth is gene amplification. In the widely studied *lac* system of Cairns, amplification of *lac* and occasional coamplification of the nearby *dinB* (coding for the error-prone DNA polymerase, PolIV), can account for the apparent increase in number of Lac^+ revertants without any need to invoke an increased mutation rate.

From a more general perspective, gene amplifications are expected to be a major immediate response whenever growth is limited by an external factor (e.g., low nutrient levels or the presence of a toxic factor) or an internal factor (e.g., a deleterious mutation) and when increased levels of a gene product might mitigate the problem. Furthermore, gene amplification could facilitate the process of acquiring stable adaptive mutations by increasing the number of selected target genes in the population. These stable changes could occur outside of the amplified region and increase in frequency by population expansion alone, or they could affect the amplified genes, in which case target number is increased by both cell growth and added copies per cell. Since the amplification may be dispensable after a stable mutational improvement occurs, it is conceivable that the intermediate amplifications leave no trace in the genome. Thus, we suggest that many adaptive phenomena in fact occur with amplifications being transient intermediates that, because of their intrinsic instability, are rapidly lost and therefore easily underestimated during studies of adaptive evolution. Apart from the *lac* system, recently described examples of such amplification processes of medical significance are provided by the sequential evolution of cephalosporin resistance by TEM β-lactamases (64), resistance to the tRNA synthetase inhibitor mupirocin (53), and

resistance of tumor cells to cytostatic drugs (15). Similar processes of sequential selection of common mutations of small effect (including gene amplification) are likely to apply also for malignant transformations, where cells successively escape the various spatial and temporal control mechanisms that keep growth of individual cells in check (2).

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