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Amplification–mutagenesis—how growth under selection contributes to the origin of genetic diversity and explains the phenomenon of adaptive mutation

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Abstract

The behavior of a particular bacterial genetic system has been interpreted as evidence that selective stress induces general mutagenesis or even preferentially directs mutations to sites that improve growth (adaptive mutation). It has been proposed that changes in mutability are a programmed response to stress in non-growing cells. In contrast, the amplification–mutagenesis model suggests that stress has no direct effect on the mutation rate and that mutations arise in cells growing under strong selection. In this model, stress serves only as a selective pressure that favors cells with multiple copies of a growth-limiting gene. Mutations are made more probable because more target copies are added to the selection plate—more cells with more mutational targets per cell. The amplification–mutagenesis process involves standard genetic events and therefore should apply to all biological systems. Idiosyncrasies of the particular system described here accelerate this process, allowing an evolutionary series of events to be completed in only a few days.

Keywords: Amplification–mutagenesis; Adaptive mutation; Stress; Selection

1. Introduction

1.1. The basic issues

There is general agreement that genetic adaptation relies on two things—mutation and natural selection (see Fig. 1). During growth under selection, mutations arise and influence the reproductive success of the organisms that carry them. Accordingly, some alleles rise in frequency in the population while others are removed, resulting in a change in the predominant genotype. Selection is generally thought to play no role in the origin of mutations, which arise as errors in replication and repair. Although the mutation rate may be evolutionarily set by life history constraints (population size, genome size, recombination rate), we suggest it is not likely to be regulated in response to environmental stimuli. However, the idea that cells might vary their mutability in response to short-term fluctuations in stress has had adherents since the time of Darwin [32]. Some recurring basic questions are the following.

(a) Does stress affect the frequency or target specificity of mutation? More specifically, do cells possess mechanisms to regulate their mutability in response to stress? This specification is needed because of the trivial expectation that some environments would be mutagenic without help from a cellular mechanism (e.g., chemical or physical mutagens may overwhelm the cells mechanisms for preventing genetic change).
(b) Do mutations occur in non-growing cells? If mutations are simply errors in DNA replication or failures of mechanisms that defend against such errors, one might expect that growth (chromosome replication) would be required for mutation. However, one can imagine processes that could induce genetic changes without necessitating full genome replication.

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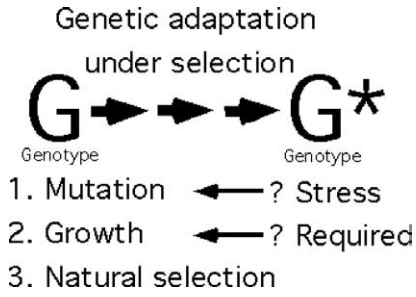

Fig. 1. Persistent questions regarding genetic adaptation. While there may be general agreement that mutation and selection combine to allow adaptation, the open questions are whether mutations are created in response to stress and whether they arise in non-growing cells.

1.2. Classic experiments

Experiments done more than 50 years ago by Luria, Delbrück and Lederberg [27,31] have been widely accepted as demonstrating that mutations arise without the influence of selection. Luria and Delbrück found that the distribution of mutant frequency among independent cultures was much wider (i.e., the mean is much larger than the median) than a Poisson distribution, suggesting that the mutations formed at random during growth of the cultures before the selection was applied. Lederberg used a sib selection to directly demonstrate that colonies arising under selection are initiated by mutant cells that were present prior to imposition of the selective regimen. A criticism of these experiments has been that they employed lethal selections and thus could only have detected pre-existing mutations. To fairly assess the possibility of selection-induced mutations would require a non-lethal selection.

1.3. Adaptive mutation and the genetic system of Cairns and Foster

The caveats inherent in previous work were pointed out by Shapiro [43] and Cairns and co-workers [6], who presented experimental evidence suggesting that some mutations might arise in response to non-lethal selection. The behavior of the Cairns experimental system, as later modified by Cairns and Foster [7], suggested that: (1) stress affects the mutational behavior of bacteria, and (2) stress-induced mutations arise in non-growing cells. We propose that neither conclusion is warranted.

The Cairns–Foster system is described in Fig. 2. The phenotypically Lac$^-$ tester strain carries a *lac* deletion in the chromosome and a leaky *lac* frameshift mutation (+1) on plasmid F'_{128}. During non-selective growth, the *lac* frameshift mutation reverts at a frequency of 10^{-8}/cell/division. Under selective conditions (lactose as sole carbon source), the leakiness of the *lac* frameshift mutation allows slow growth on lactose, which is prevented by adding a 10-fold excess of cells carrying a *lac* deletion mutation; these scavenger cells remove contaminating carbon sources

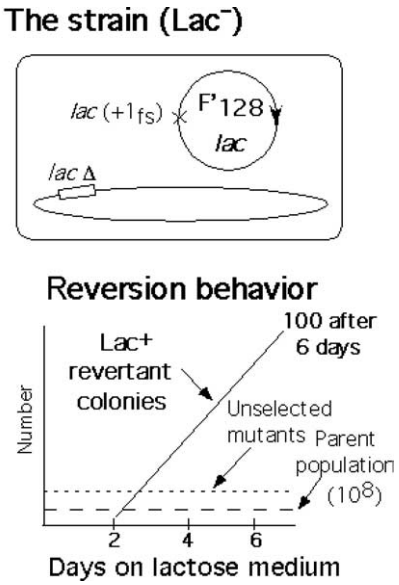

Fig. 2. The Cairns–Foster system. The tester strain used (top of figure) carries its revertible *lac* allele on plasmid F'_{128}. This cell is plated with an excess of scavenger cells with a non-revertible *lac* mutation. Reversion behavior (bottom of figure) includes accumulation of Lac$^+$ revertant clones over a period of days. During this period the plated tester population (between visible colonies) shows little growth or death and accumulates very few unselected mutations.

other than lactose and they serve as competitors that prevent growth of the tester strain.

Over 6 days following plating on selective medium, 10^8 tester cells give rise to about 100 revertant colonies. The late-appearing colonies appear to reflect reversion events that occurred after plating. They are not due to slower growth of pre-existing mutants and the distribution of revertant number among independent plated cultures shows a Poisson distribution rather than the Luria–Delbrück distribution as expected if reversion occurred after plating rather than during non-selective pre-growth [7]. Over the course of the experiment, the plated Lac$^-$ tester population does not grow significantly as judged by samples taken from the surface of the selection plate (between visible colonies). Residual growth of the parent strain is certainly not sufficient to explain the observed 100 revertants using the unselected mutation rate (10^{-8}/cell/division). These results suggested that starvation in the presence of lactose induces the Lac$^+$ reversion events [14,15].

If stress caused a general mutagenesis of the whole tester population (and one treats the 6 day starvation period as essentially one generation), then a 100-fold increase in mutation rate would be required to explain 100 revertants and the non-revertant parent population should show a 100-fold increase in unselected mutant types. This is not seen; the bulk of the population exposed to starvation shows very little increase in unselected mutation frequency [4,23]. When lactose is left out of the selective medium, starvation does not lead to accumulation of revertant cells in the non-growing lawn. Thus it appeared that non-growing cells sense lactose

and respond by causing *lac* reversion without an increase in the general mutation rate [11]. This phenomenon has been called "adaptive mutation". We define this as "any process whereby imposition of stress enhances the yield of mutants with improved fitness". This definition does not stipulate whether fitter mutants arise in growing or non-growing cells or whether actual mutation rates change under selection.

1.4. The question of growth (stationary phase mutagenesis)

The effect of stress on mutation rate would be easily shown if growth were eliminated (see Fig. 1). Without growth, there could be no natural selection and an increase in mutant frequency could confidently be attributed to stress-induced mutagenesis. The appearance of revertant colonies on a plate seeded with a non-growing cell population made it seem likely that such conditions had been achieved and that revertants arose in this population by a first-order process at a rate that was increased by starvation in the presence of lactose. Accordingly, two models were suggested, both of which assume that Lac^+ revertants arise in the stressed non-growing population.

1.5. Directed mutation

This model proposes a mechanism that senses stress and responds by preferentially mutagenizing target sites (in non-growing cells) that are likely to restore ability to grow [10]. Although it is difficult to imagine how this feat might be achieved mechanistically, several models were suggested [8,48,49] and eliminated as explanations of the Cairns system [10]. However improbable mechanistically, this model is theoretically reasonable; it could, in principle, speed up adaptation at little or no cost (i.e., without unselected deleterious mutations).

1.6. Hypermutable state

This model suggests that stress induces intense general (genome wide) mutagenesis in a designated subset (10^5) of the non-growing parent population (10^8) [18]. This model explains the apparent direction of mutation by suggesting that most cells remain quiescent without growth or mutation. Cells in the mutagenized subset survive only if they acquire a Lac^+ reversion prior to any lethal mutation. This model predicts that Lac^+ revertants (but not the parent population at large) should show evidence of mutagenesis (i.e., Lac^+ revertants should carry associated unselected mutations). The predicted mutations were found [52]. However, the measured intensity of mutagenesis was 1000-fold too low to have been responsible for creating 100 revertants from the posited subpopulation of 10^5 cells [41]. Mutagenesis of the intensity required by the model would inflict an unacceptable cost in associated mutations (see below).

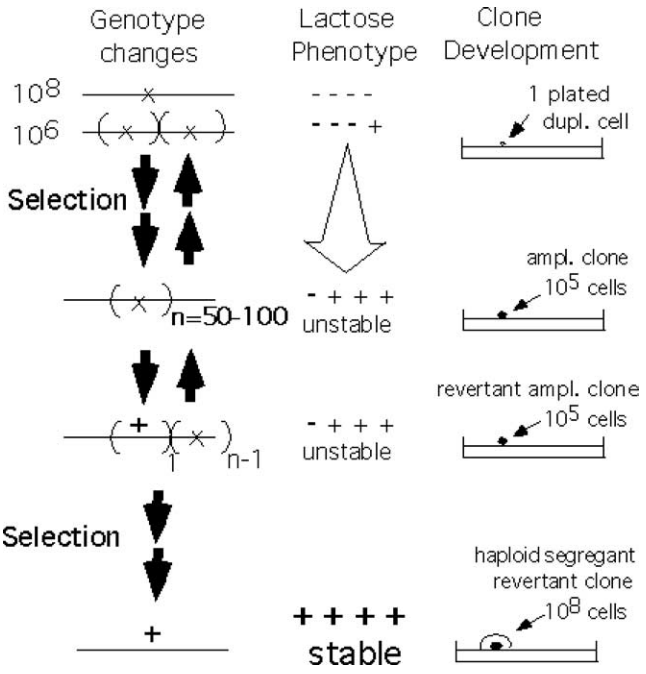

Fig. 3. The amplification–mutagenesis model. This model involves no change in the rate or target specificity of mutation. Rather, the plated population (10^8) includes some pre-existing cells (about 10^6) with a *lac* duplication. The latter cells initiate growing clones, within which further amplification accelerates growth. In each clone, reversion is made more probable by an increase in target *lac* copies. Revertant colonies (left) develop slowly while dependent on an amplified mutant *lac* allele and are ultimately overgrown by haploid revertant lac^+ cells that have lost the mutant copies of the array.

2. Results

2.1. Amplification–mutagenesis and reversion in growing cells

If one abandons the idea that revertants arise in the non-growing parent population, a model is allowed that explains the basic behavior of this system without invoking any change in mutation behavior [3,22]. In this model, stress serves only as an agent of natural selection acting on growing cells and has no effect on the process of mutation. The main points of this model are listed below and diagrammed in Fig. 3.

(a) Although most of the parent population cannot grow on lactose, rare pre-existing cells carrying a duplication of the leaky mutant *lac* allele can initiate slow-growing clones. Of the plated 10^8 cells, about 10^6 carry such a duplication [46]. Only about 100 of these cells initiate clones that are successful.
(b) Selection acts on cells in these developing clones, favoring those with further *lac* amplification. Within each clone, growth of cells with multiple *lac* alleles adds mutational targets and thereby increases the likelihood of a reversion event.

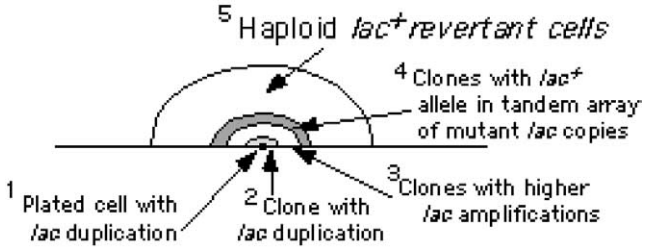

Fig. 4. Diagram of a revertant colony. The model proposes that each revertant arising under selection is initiated by a plated cell with a *lac* duplication which grows under selection and produces faster-growing derivatives with higher *lac* amplification. After reversion, selection holds the lac^+ allele and favors loss of the mutant copies until lac^+ segregants arise and overgrow the colony.

(c) When the number of replicating *lac* copies reaches a sufficient level, the reversion event (−1 frameshift mutation) can occur and further improve growth. This requires no change in mutation rate only more target copies. The weak general mutagenesis that accompanies this process contributes very little to reversion (see below).
(d) A reversion event produces a lac^+ allele in a series of tandemly-repeated mutant copies. A cell of this genotype grows poorly because it frequently loses the revertant allele by segregation events and because the tandemly repeated mutant copies confer a considerable physiological cost. About 0.5–2 Mbp of extra DNA is added to the plasmid carrying the *lac* region [3,22].
(e) Selection favors cells that retain the revertant allele and lose the destabilizing mutant alleles by segregation. Finally a haploid lac^+ cell arises and overgrows the small amplification clone to dominate the final revertant colony (see Fig. 4).

If one is not aware of the growth occurring in the reversion process, it seems as if a non-growing haploid lac^- cell (in the parent population) gave rise to a haploid lac^+ cell that grew up as a colony at a frequency that is elevated by selective stress. If one considers the growth within each developing colony, then about 1000 clones achieve a population of 10^5 cells each with 100 copies of *lac* accumulating 10^{10} replicating *lac* alleles—enough to produce 100 revertants at the normal mutation rate of 10^{-8}/cell/division. The increased number of revertants is explained by an increase in target copy number rather than an increase in mutation rate (per target). The growth that makes this possible is not detected if one samples the lawn of parent cells between colonies; by systematically avoiding colonies, one fails to assess the many cell divisions that occur during development of each visible colony from a single cell. Within each developing colony a series of clones expand as diagrammed in Fig. 4.

2.2. Testing predictions of amplification–mutagenesis

Below is a list of tested predictions made by the model.

(a) The parent mutant allele has residual function (1–2% of revertant activity) that is important to reversion under selection [3]. Cells must starve in the presence of lactose [7].
(b) The parent population includes cells (1%) with a *lac* duplication [46,47].
(c) Each revertant colony appearing under selection contains some cells with a *lac* amplification (unstably Lac^+) and others with a stable (haploid) revertant allele [3,22]. Diagrammed in Fig. 4.
(d) Unstable Lac^+ cells in revertant colonies have 20–100 copies of the *lac* gene [3,21,46].
(e) Stable and unstable Lac^+ cells in a single colony are clonally related, as predicted if they are derived from a single plated cell with a *lac* duplication [3,22].
(f) Time required for development of a clone (from tiny to large) varies widely, as expected if it relies on a series of stochastic events [22].
(g) Revertant number is sharply reduced if *lac* amplification is made deleterious [22].
(h) A constructed *lac* duplication placed in the parent strain stimulates the number of revertants about 100-fold [46].

This model accounts for *lac* reversion under selection without invoking any stress-induced change in mutation rate. However the observed genome-wide mutagenesis must be explained.

2.3. Some Lac^+ revertants experience temporary mutagenesis during a reversion experiment

The amplification–mutagenesis model does not involve any general or directed increase in mutation rate. However, in the course of testing a prediction of the hypermutable state model (described in more detail below), it was observed that Lac^+ revertants arising under selection (but not the Lac^- parent cell population) had a 20–50-fold increased likelihood of carrying an unselected mutation [39,45,52]. This mutagenesis depends on SOS induction of the *dinB* gene (which encodes the error-prone DNA polymerase IV) in the course of the reversion experiment [33,34]. Thus selection appears to do two things—it stimulates the number of Lac^+ revertants and it causes general mutagenesis of those revertants. Below we will argue that this mutagenesis is a side-effect peculiar to this system and is neither necessary nor sufficient to explain the observed *lac* reversion. It makes a negligible contribution to reversion under selection. A model for the source of mutagenesis is described below with some supporting evidence.

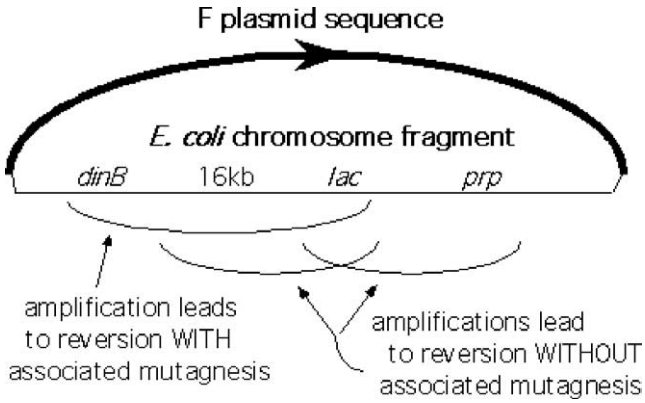

Fig. 5. The arrangement of relevant genes on the F'_{128} *lac* plasmid. In this system, the mutant *lac* gene that is under selection is located on a conjugative plasmid near the *dinB* gene, which encodes an SOS-inducible, error-prone polymerase. We suggest that some (10–20%) developing clones carry an amplification that includes *dinB* as well as *lac*. Amplification of *lac* under selection leads to an increase in the *dinB* copy number and (following SOS induction) to overproduction of DinB and mutagenesis.

2.4. Model for general mutagenesis during growth with a lac *amplification*

The features of the amplification–mutagenesis process thought to be responsible for general mutagenesis are listed below. The structure of the F'_{128} plasmid, which carries the *lac* mutation in this system, is diagrammed in Fig. 5.

(a) On plasmid F'_{128}, the mutant *lac* allele lies about 16 kb away from the *dinB* gene [26], which encodes the SOS-inducible, error-prone DNA polymerase that is essential for general mutagenesis in the Cairns system [33].
(b) Each revertant clone is initiated by a duplication cell whose repeated unit varies in size from 10 to 40 kb [3,22,46].
(c) In 10–20% of clones the *dinB* gene is included with *lac* in the amplified unit [46]. These cells experience an increase in mutation rate.
(d) Growth with an amplification induces the SOS repair system. This induction is due, at least in part to single DNA strands produced from the plasmid transfer origin [5]. Additional induction may be caused by single stranded DNA produced when amplifications undergo spontaneous segregation [22].
(e) Clones whose amplification includes *dinB* produce more DinB protein in response to SOS induction. When this elevated DinB level causes sufficient DNA mismatches to saturate the methyl-directed mismatch repair (MMR) system, mutagenesis results [19,20]. The average mutation rate increases about 20–50-fold [39,45]. The model predicts that the rate increases several hundredfold in the colonies with an amplified *dinB* gene; we have demonstrated that at least a 60-fold increase occurs in these colonies [46].
(f) When reversion occurs, the amplification segregates under selection leaving a revertant lac^+ allele and a single *dinB* allele, which is insufficient for mutagenesis [24,25]. Because of this, mutagenesis stops when selection is relaxed.

2.5. Hypermutable state model—an alternative explanation of general mutagenesis

The hypermutable state model of B. Hall [18] explains behavior of the Cairns–Foster system by suggesting that bacteria possess a mechanism to increase the general mutation rate in response to stress and mutagenesis occurs in non-growing cells. This mechanism is said to have evolved under selection because it increases the speed of genetic adaptation and is therefore beneficial for long-term survival. The general mutagenesis associated with Lac reversion in the Cairns–Foster system was interpreted as supporting this model [52]. The model is described below.

(a) Stress causes a subset of the plated population (10^5 of 10^8 cells) to enter a temporary hypermutable state by inducing SOS (and the error-prone DinB polymerase) and reducing expression of MMR (methyl directed mismatch repair).
(b) Mutagenesis of this subset continues until all cells either die of a lethal mutation or become Lac^+ by reversion of the mutant *lac* allele.
(c) The observed Lac^+ revertants are caused by this genome-wide mutagenesis.
(d) Stress causes this mutagenesis by inducing SOS (and the DinB error-prone polymerase) and by inhibiting methyl-directed mismatch repair.
(e) Lac^+ revertants (but not unreverted parent cells) are predicted to carry associated unselected mutations resulting from the mutagenesis.
(f) Reversion relieves stress and therefore shuts off the mutagenic mechanism.

2.6. Support for the hypermutable state model

Support includes the following:

(a) Associated mutations are more probable in revertants than in the parent population [4,52].
(b) Preventing SOS induction or eliminating *dinB* reduces revertant number about 4-fold [33,34] and eliminates associated mutagenesis [45,46,51].
(c) Overexpression of MutL reduces the number of revertants about 4-fold [19,20].

2.7. The hypermutability state model is implausible

Despite some supportive data, mathematical evaluation suggests that this model is theoretically impossible. The model requires implausibly intense mutagenesis to generate 100 revertants from 10^5 cells—a rate of 10^{-3}/cell. This rate requires a 10^5-fold increase in mutation rate over

the 10^{-8} revertants/cell/division seen during non-selective growth. We know of no laboratory mutagenesis that has achieved this intensity of mutagenesis in non-growing cells. If the required intensity of mutagenesis were somehow obtained, it would produce too many lethals (an average of 8 per cell) to generate 100 Lac$^+$ revertants and those that survived lethals by chance would carry an average of 5 non-lethal null mutations per cell [41]. These mutations would reduce the fitness of revertants (and render unlikely the idea that such a mechanism is beneficial in the long term).

Experimental estimates of the actual mutation rate experienced by revertants suggest a more modest 20–50-fold increase in average mutation rate [39,45]—an intensity that cannot produce even one *lac* revertant if applied to 10^5 cells as required by the model [41]. Furthermore data of Rosche and Foster suggest that mutagenesis is unevenly distributed among revertant clones. Only about 10% of Lac$^+$ revertants show evidence of mutagenesis (a 200-fold increase), while 90% arise with little or no general mutagenesis. Deletion of *dinB* or prevention of SOS induction eliminates associated mutation, but causes only a modest reduction in revertant number [33,45,46,51]. In addition, the hypermutable state model does not explain any of the observations listed above in support of the amplification–mutagenesis model.

2.8. The amplification model is consistent with all published data describing the Cairns–Foster system

(a) Mutation appears directed to *lac* or to F′ plasmid, because only the *lac* region amplifies under selection and only the copy with the *lac*$^+$ allele is retained in the final revertant.
(b) Associated weak general mutagenesis is explained by co-amplification of *lac* and *dinB* in some (about 10%) of the developing revertant clones.
(c) The uneven intensity of mutagenesis is explained by the fact that most revertants arise in clones whose *lac* amplification does not include *dinB* (no mutagenesis), while a few arise in clones that have both *lac* and *dinB* amplified (and are mutagenized).
(d) The two classes of Lac$^+$ revertants observed by Hastings et al. [21] are explained by the fact that clones without mutagenesis have to grow larger with their amplification before realizing a reversion event. In these clones, reversion and segregation occur late and haploid revertant types are unable to dominate the population of the colony. Hastings et al. used inefficient conditions and tested about 100-fold too few cells from each clone and were therefore unable to see that every colony includes both stable and unstable Lac$^+$ types [22].
(e) Eliminating mutagenesis by a *dinB* null mutation or a *lexA*(Ind$^-$) mutation reduces revertant number about 4-fold [33,45,51]. While this was interpreted as evidence that mutagenesis causes 75% of the revertants observed, the minor importance of mutagenesis is apparent when one considers the role of amplification and growth. Two synergistic factors are at work—target amplification and growth on one hand and general mutagenesis on the other. These factors must be multiplied since mutagenesis affects all copies. If one considers that 100 Lac$^+$ revertants arise from 10^6 plated duplication-bearing cells, then the revertant frequency is 10^{-4}—4 orders of magnitude higher than the reversion rate during non-selective growth (10^{-8}). Of this selection-dependent 10^4-fold increase, mutagenesis contributes a 4-fold factor.
(f) Early in the history of the Cairns–Foster system, it was noted that frameshift reversion events arising under selection tend to be -1 mutations in monotonous base runs while reversions occurring during non-selective growth are of a wide variety of types [12,40]. This is explained if selected events tend to be caused by DinB replication while unselected events are due to spontaneous errors of the standard replication machinery. We suggest that in clones without *dinB* amplification, SOS induction causes perhaps a 5-fold increase in DinB-dependent mutagenesis; this would not be detectable as associated mutagenesis by the methods used, but would allow DinB to cause 80% of the *lac* reversion events. In clones that amplify *dinB*, a mutation rate increases several hundred-fold and an even higher fraction of lac reversion events would be caused by DinB.
(g) The number of revertants arising on a series of selection plates inoculated by independent cultures are Poisson-distributed [7]. This was taken as evidence that reversion occurred on the selective medium rather than during non-selective pre-growth. Since the amplification–mutagenesis model posits that revertants arise in clones initiated by duplication cells that form prior to selection, one might expect a Luria–Delbrück distribution of revertant numbers. However, the Luria–Delbrück distribution applies only to situations in which mutations are stable and accumulate in the cultures during growth. In contrast, duplications are highly reversible and are expected, during non-selective pre-growth, to reach an equilibrium frequency dictated by the relative rates of formation and segregation. This predicts a relatively constant duplication frequency in all cultures and a Poisson distribution of revertant numbers, as observed.

3. Discussion

3.1. The amplification–mutagenesis model involves standard genetic events accelerated by idiosyncrasies of the system

The many special features of the Cairns–Foster system that accelerate the amplification–mutagenesis process might lead one to regard its behavior as reflecting impacted artifacts and discount its general relevance. We would disagree. The basic genetic events that underlie the reversion process

are expected for virtually all genetic systems—duplication, amplification, reversion, segregation. The idiosyncrasies of the Cairns system accelerate every aspect of this process and allow it to go to completion within a week. We would expect the same process to operate in all systems, given sufficient time and selection. Below are the contributing idiosyncrasies that have come to light so far.

Cells are poised on the brink of growth The *lac* mutant used is sufficiently leaky that it can form a colony on lactose medium. Growth of this mutant is just barely prevented by addition of competing (Lac$^-$) scavenger cells that assimilate any carbon source (e.g., galactose, acetate or lactate) that escapes from the tester cells. Preventing growth in this way poises the tester cells such that even a two-fold increase in *lac* copy number can allow growth.

The allele under selection is on an F′ *plasmid* The duplication, amplification and segregation events that are at the heart of the amplification–mutagenesis model are all stimulated by DNA ends generated from the plasmid conjugative transfer origin. The transfer (*tra*) genes are thus important to reversion behavior whether or not actual transfer turns out to be important [13,16]. The conjugation (*tra*) system has previously been shown to stimulate intense recombination between F′plasmids and the bacterial chromosome [42,50]. We suggest that the F plasmid contributes to reversion by stimulating duplication, amplification and segregation [44], by helping induce SOS (Kim Bunny, unpublished) and by providing recombination intermediates that serve as sites at which the DinB protein can initiate replication tracts.

The lac *mutation is near the* dinB *gene* This allows some clones to co-amplify *lac* and *dinB* and thereby suffer mutagenesis following SOS-induction. Although this mutagenesis is unnecessary and insufficient, it does contribute slightly to the revertant yield.

3.2. How selection serves to make mutations more probable—the essence of the amplification–mutagenesis model

Selection changes the reversion process radically because it detects mutants with minor growth improvements and allows them to grow into clones that are sufficiently large to permit the next improving mutation. In this way, selection allows a major phenotypic change (e.g., Lac$^-$ to full Lac$^+$) to be achieved by a series of minor improvements—each due to a mutation that is made more likely by the expanded clone of cells of its predecessor. See top of Fig. 6. In the absence of selection (bottom of Fig. 6), mutants with the final phenotype are unlikely because there is no selection for the intermediate clone expansion. Therefore the end phenotype can be attained only by an extremely rare event that provides the final phenotype in one discontinuous step. While mutations may arise that make small improvements in the phenotype, without selection, these clones do not expand and support secondary mutations in the absence of selection.

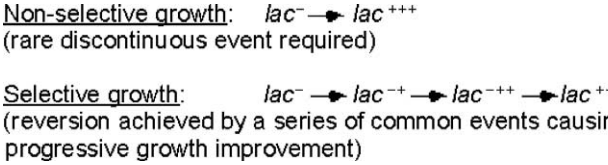

Fig. 6. Effect of selection on the reversion process. Selection breaks up the process of obtaining a reversion event into a series of events. Without selection, a single rare event is required (bottom); with selection, multiple common steps (with intermediate growth) achieve the same goal (top).

While these event series may be achieved in many ways, the *lac* system used here improves growth by adding copies of a growth-limiting mutant gene. Addition of each *lac* copy could be considered an event that initiates a new clone within which the next addition can occur. In this system, the same end product (a haploid revertant *lac*$^+$ allele) is achieved by a rare mutation in the absence of selection and by a series of common progressive growth-improving steps in the presence of selection (see Fig. 3). The stepwise process is accelerated by nested clonal expansions as diagrammed in Fig. 7. Each step leads to production of an expanding population that makes the next event more probable by providing an increased number of targets. This basic procedure can apply to any population under selection.

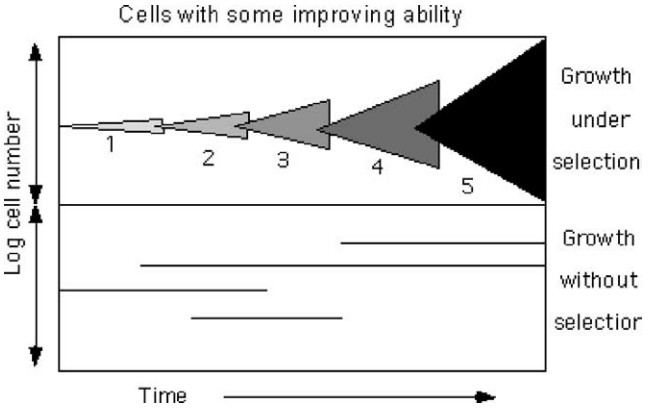

Fig. 7. Role of nested clone expansion in reversion. During selective growth (top) any genetic event that contributes (even modestly) to growth improvement initiates a clone of cells that expands under it reaches sufficient size to realize another event and a further growth improvement. Ultimately (by the process outlined in Fig. 3) a secondary improving event occurs and is made more likely by the large number of cells with multiple *lac* copies that were added to the culture. Each growth-improving event makes the next more likely. During non-selective growth (bottom), events that improve growth may occur but do not lead to clones that expand faster than the overall population. Thus one event does not contribute to the occurrence of a second. Duplication mutations do not improve growth and are subject to segregation. Only rare discontinuous events (*lac* to *lac*$^+$) give a full Lac$^+$ phenotype and these are detected at low frequency.

3.3. The amplification–mutagenesis model is likely to apply directly to important biological processes

Since standard genetic events underlie this model, it should apply to many natural situations. However, the process may be slower in situations that lack the inherent idiosyncrasies of the Cairns–Foster system. Below we list several situations in which the general process may be expected.

Evolution of new genes Since the original suggestions of Ohno [36], it has been widely accepted that genes arise primarily by duplication and divergence of preexisting genes. While this general view seems inescapable in the light of sequence information, the precise process raises some questions. After an act of duplication, how are both alleles raised to high frequency in the population and maintained in a functional state long enough for one to acquire a new function before being eliminated by mutation or segregation? The amplification–mutagenesis model suggests a reasonable possibility.

The initial event might occur before duplication when the parent gene acquires a miniscule level of some secondary function. (It is clear that this occurs, since it is the basis of the generally used procedure of high copy suppression, in which a mutation in one gene is corrected by overexpressing a second gene. The secondary activity of one gene (if amplified) can demonstrably substitute for lack of a different gene.) In a natural context, this would occur when conditions arise under which the second activity provides a fitness advantage. Under these circumstances the original allele (with a modest side activity) is amplified under selection. The extra copies rise to high levels in the population and are maintained by purifying selection acting on the secondary activity. With time, additional divergence can occur that improves the second activity (in some copies) while maintaining the original activity (in at least one other). As gene copies improve their level of the new activity, selection for amplification is relaxed leaving the cell with two genes of different function. This process of improving the second function can be accelerated by recombination between partially improved alleles. According to this model, new genes are formed by a sequence of events—function acquisition, duplication or amplification, copy divergence. Since every step of the process can occur under potentially very strong selection, creation of new genes can be rapid.

Adaptation of bacteria to new situations Natural populations of bacteria are repeatedly exposed to stressful conditions on the fringes of their normal habitat and are placed under strong selective pressure. Many of these situations are medically relevant, such as adaptation of a pathogen to its host (evasion of its defenses) and acquisition of resistance to antibiotics. In these situations, short-term bacterial survival may require genetic adaptation. The rarity of beneficial mutations made it seem reasonable to imagine that bacteria could profitably raise their general mutation rate in response to stress.

We suggest that this mutagenesis might hasten development of the disease, but is unlikely to be of long-term advantage to the pathogen, due to the associated mutational cost [28]. The enthusiasm of some for the benefits of a suddenly increased mutation rate may be conditioned by experience with laboratory experiments, in which strong or lethal selections make slow adaptation unlikely. In such experiments, rare mutations are required and, because genetic variability is limiting, mutagenesis greatly increases the number of revertants. This experience may encourage a belief that variability is also limiting under natural conditions. However, we submit that, unlike the laboratory experiments, natural populations are generally large and usually experience less stringent selections.

The Cairns system allows one to estimate the cost of general mutagenesis [41] and the unlikelihood of its long-term selective advantage. When viewed retrospectively, the Cairns system may resemble many natural situations in that growing cells are placed under strong but non-lethal selection. This has made it possible to observe how fast genetic adaptation can occur when small-effect mutations initiate successive subclones with small growth improvements. The full adaptation is achieved without the costs of associated deleterious mutations.

Duplications are extremely common in bacteria and provide a rich source of variants that could initiate a process of adaptation like that seen in the Cairns system [1,2]. True, reversion in the Cairns system is much less striking when the *lac* allele is in the chromosome instead of on a conjugative plasmid [17,37,39]. However a chromosomal copy of the mutant *lac* allele used in the Cairns system does amplify under selection [44]. Under appropriate selective conditions chromosomal amplifications can reach very high copy numbers [9,35]. We presume that the plasmid location only serves to accelerate a process that could occur more slowly in a standard genetic context. An excellent recent example of the initial stages of this process is the work of Reams and Neidle [38].

Origins of cancer The numerical problem posed by the probability of cancer development shares features with the problem posed by behavior of the Cairns system. In both situations, an apparently non-growing cell population gives rise to variants whose frequency is incomprehensible if one assumes no growth and a reasonable mutation rate. For cancer, it is well established that the progression to malignancy requires the accumulation of several mutations (>5), each of which might occur with rates of $<10^{-8}$/cell/generation. The probability of any cell acquiring all the needed changes is prohibitively low. However the problem is solvable if one considers metazoan cells as individuals whose growth is limited by cell cycle control and host conditions (e.g., low oxygen levels, immune surveillance, apoptosis, need for growth

factors and confinement by adjacent tissues). During growth under selection, a cell can solve these problems serially by a sequence of mutations, each providing a minor growth improvement. Each mutant clone need expand only to a size sufficient to allow the next improving mutation to arise at the ambient mutation rate. The small size of each expanding clone makes variability a limiting factor and explains the positive selection for mutators [29,30].

The parallel between cancer and the *lac* system becomes even closer if one considers the gene amplifications associated with many malignancies. These may serve as important intermediates in cancer development by providing growth improvement simply through increasing the level of a positive regulatory protein or of a gene that is the target of repression. Ultimately sufficient gene copies will be generated to realize a stable mutation with a major effect on growth as described in the amplification–mutagenesis model.

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