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Origin of mutations under selection
(the adaptive mutation puzzle)

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Synopsis

Classic experiments demonstrate that mutations arise at random with respect to their phenotypic consequences or selective value. More recent experiments has been interpreted as evidence that growth-limited bacteria up-regulate their genome-wide mutation rate and direct mutations preferentially to sites that improve growth (adaptive mutation), a challenge to the accepted dogma. However, natural selection can explain these striking results with no change in mutation rates or specificity if one considers the normal mutation spectrum. Common near-neutral mutant alleles are subject frequent duplication and selective amplification, which improves growth. These two very common mutation types, acting in concert, drive rapid adaptation and give the appearance of mutagenesis.

Keywords (10-15)

Adaptive mutation
Directed mutation
Copy number variant
Error-prone polymerase (DinB)
Escherichia coli
Gene amplification
Gene duplication
Hypermutable state
Lactose operon
Local over-replication
Mutagenesis
Plasmid
Recombination
Selective amplification
Stress-induced mutagenesis

Glossary

Adaptive mutation: In standard usage term describes any genetic change that improves fitness. In recent popular usage, the term refers to a hypothetical mechanism said to vary the specificity and increase the intensity of mutagenesis in response to growth limitation. This proposal challenges a widely accepted tenet in evolutionary biology that mutations form as replication or repair errors, independent of need or consequences.

F' *lac*: The F-plasmid of *E. coli* is capable of transferring copies of itself to recipient cells (conjugation). This circular plasmid can exist free or can insert into the circular bacterial

chromosome. Incorrect excision of an inserted plasmid can produce a plasmid derivative (F') that includes bacterial DNA adjacent to the attachment site. The F'*lac*$_{128}$ plasmid carries the chromosomal *lac*, *proAB* and *dinB* genes. The genes encoding the transfer or conjugation functions of F'$_{128}$ are expressed at a high level due to an IS*3* element inserted in the plasmid *finO* gene, which encodes a regulatory function.

Hypermutable state: A hypothetical condition proposed to explain the results of some selection experiments. Non-growing cells are said to induce a mechanism to create mutations (adaptive mutation). The mutable state is attributed to a mechanism that evolved to accelerate adaptation.

Directed mutation: A process proposed to explain results of some selection experiments. A hypothetical mechanism that senses growth arrest, identifies the genomic sites that can restore growth, and preferentially generates mutations at those sites.

Nomenclature:

F'plasmid
F'*lac*
Tet, Tet^R
tetA
Tn*10*

Abstract

Selection and mutation are central contributors to evolutionary change. Mutation produces genetic variability upon which selection acts. The process of natural selection depends on the formation rate of multiple mutation types with different phenotypic consequences. Widely accepted classical experiments provide evidence that mutation and selection are independent processes. That is, mutations are errors in DNA replication or repair that occur at random with respect to their phenotypic consequences or selective value. This pillar of evolutionary theory has been challenged by bacterial experiments interpreted as evidence that bacteria purposefully vary the rate and target specificity of mutation when growth is limited (adaptive mutation). While the idea of environment-induced (adaptive) mutability has many supporters, we suggest here that available evidence is best explained by selection acting alone on amplified near-neutral alleles that arise at a constant rate and site specificity. The mechanisms by which selection imitates mutagenesis may have broad relevance for evolutionary biology.

Introduction

Defining "adaptive mutation" Conventionally, this term describes any genetic change that improves reproductive success. Recently the same term has been used to describe a process by which a proposed mechanism might create beneficial mutations in non-growing cells. Equivalent terms are "stress-induced mutation", "directed mutation" and "stationary phase mutagenesis". It is proposed that this mechanism evolved to accelerate genetic adaptation by increasing the genome-wide mutation rates or by directing mutations preferentially to sites that improve growth. Used in this way, "adaptive mutation" challenges an accepted tenet of evolutionary biology.

The classical view of mutation and selection Experimental genetics of bacteria depends on use of positive selection to identify rare cells in astronomically large populations. Selections serve to detect new mutants, assess mutation rates and demonstrate genetic recombination. This field was put on a sound theoretical footing by demonstrations that positive selection could in fact detect mutants without causing their formation. These classic experiments showed that the mutants detected by stringent, often lethal, conditions actually arose during the non-selective pre-growth period before exposure to selection and therefore could not have been formed in response to selection (Cavalli-Sforza and Lederberg, 1956, Lederberg and Lederberg, 1952, Luria and Delbrück, 1943, Newcombe, 1949, Novick and Szilard, 1950).

The "fluctuation test" devised by Luria and Delbrück showed that mutations form with a constant probability at each cell division. During unselected growth of a culture, cell number increases exponentially and new mutants are added at an exponential rate - more cell divisions, more new mutants (Luria and Delbrück, 1943). Mutants that arise early in the history of a culture appear in a small population and are thus present at a high frequency that remains constant or is enhanced by new mutation events during subsequent growth. Mutant lineages arising at progressively earlier times in the history of the culture are present at exponentially higher frequencies. This is diagrammed in Figure 1.

When multiple replicate cultures are compared, the variance in their mutant frequencies reflects this exponential growth and the times at which mutations happened to occur. This frequency variance exceeds the mean and deviates from a Poisson distribution. Methods were

developed to use the distribution of mutants between parallel cultures to identify a "Luria-Delbrück distribution" and calculate the mutation rate per cell per division. These methods have recently been reviewed in detail (Foster, 2006, Rosche and Foster, 2000). In fluctuation experiments, cultures are grown in non-selective liquid medium and mutants are detected as rare colonies formed when these cultures were plated on strongly selective solid medium where only these mutants can grow. Observation of a Luria-Delbruck distribution of mutant frequencies between replica cultures (left graph in Figure 1) demonstrates that the mutants detected by selection actually form prior to plating and can be used to calculate mutation rate.

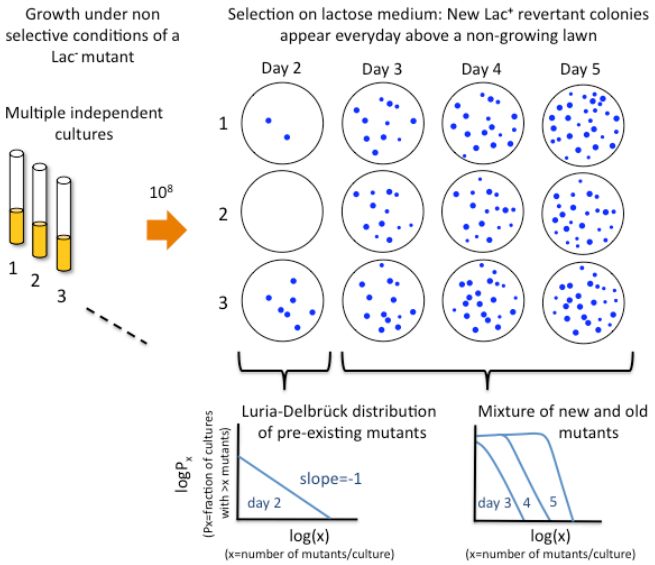

Figure 1. The Luria-Delbrück fluctuation test. Multiple independent cultures are grown non-selectively (left top) and then plated on selective medium to identify mutants that appear over several days (only three are shown). The variance (or fluctuation) in colony number between cultures is used to measure mutation rate (Rosche and Foster, 2000) and also to demonstrate whether the detected mutants arose randomly during non-selective growth prior to plating. Mutants showing a Luria-Delbrück distribution are those that arose during non-selective growth prior to plating and cannot have formed in response to selection. To identify a Luria-Delbrück distribution, the number of mutants (x) in various tubes is plotted as shown at the bottom of the figure above. The horizontal axis displays (log x) for various values of x and the vertical axis shows $\log P_x$, the fraction of tubes with a number of mutants equal or greater than x. For a Luria-Delbrück distribution, this plot shows a slope of -1 (as seen in the graph at left). If mutants form only after cells are plated on the selective medium, a Poisson distribution is expected.

In the *lac* experiment described below, the few revertants appearing on Day 2, show a Luria-Delbrück distribution and reflect pre-existing mutants (Cairns and Foster, 1991). As colony numbers increase with time at a constant rate, the number of mutants on the selection plate shows less variation and deviates from a Luria-Delbrück distribution. (See plots for later days in the graph at left). By Day 5, the bulk of the mutant colonies seem to have arisen on the plate, consistent with being caused by selection. This conclusion may not be warranted for copy

number variants, whose steady state frequency obscures the Luria-Delbrück distribution. (See text.)

With little additional evidence, the conclusion drawn from these beautiful bacterial experiments was broadened and applied to all organisms and conditions regardless of selection stringency. It was accepted that all mutations reflect random errors in DNA replication or repair, regardless of growth rate or phenotypes. Selection thus was inferred to act later to favor or disfavor pre-existing mutations, depending on how their phenotypes influence reproductive success. While this conclusion may well be correct, the classical bacterial evidence does not eliminate the possibility that less stringent growth limitation might change the rate or specificity of mutation. This open possibility was addressed by the "adaptive mutation" challenge described here.

Challenges to the idea of random mutation In standard bacterial genetic experiments, cell populations are plated on solid selective medium that limits growth. Pre-existing mutant cells are detected by the visible colonies they form above the non-growing lawn of parental bacteria. The procedure reliably detects only pre-existing mutants as long as selection is stringent and blocks all growth of non-mutant cells (as was true in the classic experiments). However in practice, this caveat was often forgotten.

In some genetic systems, mutant colonies continue to accumulate for several days after plating, suggesting that selection detects not only pre-existing mutants (as did the classic experiments) but also new mutants that arise after exposure to selection. Some authors interpreted this phenomenon as evidence that growth limitation causes the new mutations (Cairns and Foster, 1991, Cairns et al., 1988, Hall, 1988, Hall, 1990, Hall, 1991, Maenhaut-Michel and Shapiro, 1994, Prival and Cebula, 1992, Pybus et al., 2010, Shapiro, 1984, Sung and Yasbin, 2002, Thomas et al., 1992, Yang et al., 2001).

This conclusion is not warranted when selection conditions are not sufficiently stringent. That is, the new mutants could arise under selection during residual growth of the plated population. In addition partially revertant cells might arise during pre-growth and initiate the late-appearing colonies under selection. These slow-growing clones might improve (evolve) during colony development. Each act of DNA replication provides an opportunity for mutation

and many opportunities are provided during colony growth. This improvement could occur using a constant unenhanced mutation rate.

For most of the exceptional systems used to claim selection-induced mutagenesis, residual growth was not assessed. In some cases, growth and DNA replication proved to be responsible for generating the extra mutants (Gizatullin and Babynin, 1996, Jin et al., 2002, Mittler and Lenski, 1990, Mittler and Lenski, 1992, Prival and Cebula, 1996, Quinones-Soto and Roth, 2011). In one system, however, close attention was paid to population dynamics and evidence of associated general mutagenesis. In this system, a *lac* mutant of *Escherichia coli* did not grow on lactose but did give rise to Lac$^+$ revertant colonies on selective plates (Cairns and Foster, 1991, Foster, 1994).

The *lac* system. To explain the high frequency of cancer, which reflects somatic mutations, John Cairns proposed that growth-limited cells – such a mammalian somatic cells – might increase their mutation rate using a mechanism evolved to enhance genetic adaptation (Cairns, 1978, Cairns, 1998, Cairns et al., 1988). To test the idea of regulated mutagenesis, Cairns and Foster developed a bacterial system in which a cell population is held under selective conditions that prevent growth. Mutants arising in the non-growing population initiate growth and form colonies that appear and accumulate over several days under selection. The general situation resembles non-growing somatic cells and derived malignancies.

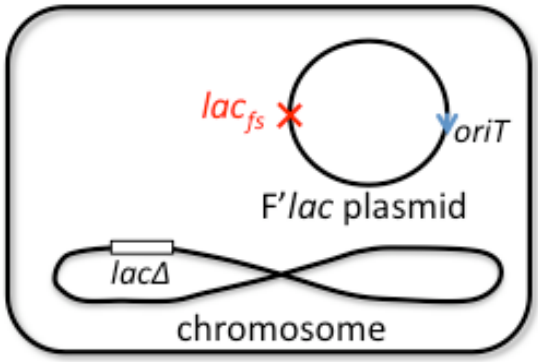

Figure 2 A strain used to seek evidence for selection-induced mutagenesis. The strain FC40 used in the *lac* system carries a leaky (partially functional) mutant *lac* allele on a F'*lac pro* (F'$_{128}$) conjugative plasmid and a deletion of its chromosomal *lac* region. The residual function of the plasmid *lac* allele supports slow growth on lactose, which is prevented when cells are plated with a 10-fold excess of scavenger cells. The Lac- scavenger cells consume any nutrients other than lactose that might contaminate the medium or be excreted by other cells on the plate. The tester cells are poised on the brink of growth.

The parent *E. coli* mutant carries a *lac* mutation on a conjugative low-copy F' plasmid and has a deletion of the normal chromosomal *lac* region. See Figure 2. A lawn of 10^8 mutant cells is plated on solid lactose medium, where it cannot grow but gives rise to about 100 Lac$^+$ revertant colonies, which accumulate over the course of 5-6 days. The reversion rate of the mutant *lac* allele during non-selective growth is 10^{-8}/cell/per division (Foster and Trimarchi, 1994). Under selection, the population produces 100 Lac$^+$ revertant colonies over 6 days. The colonies are of two types. The majority (90%) includes cells that have acquired a compensating frameshift mutation and thereby a fully functional *lac*$^+$ allele. These cells form stable Lac$^+$ colonies when streaked on non-selective medium. The minority type (10%) have an unstable Lac$^+$ phenotype and form sectored (Lac+/Lac$^-$) colonies when streaked on non-selective rich medium (Andersson et al., 1998). These cells have a tandem *lac* amplification with multiple copies of the leaky mutant *lac* allele. (See Figure 3).

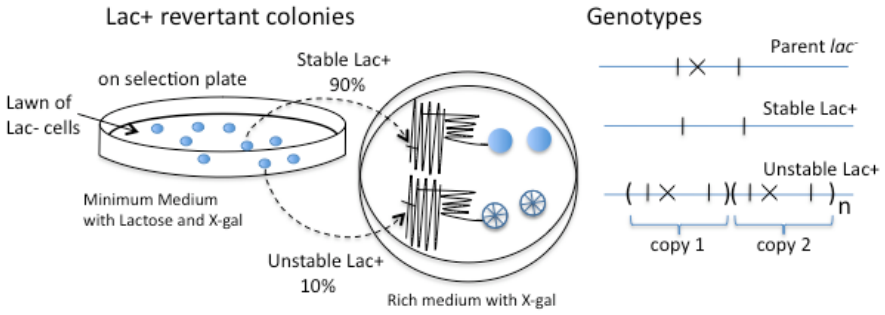

Figure 3 Two types of Lac$^+$ revertant colonies The majority of the revertant colonies that appear on the selection plate include cells that are stably Lac$^+$ when streaked on non-selective medium with X-Gal, a chromogenic β-galactosidase substrate. These cells have acquired a compensating frameshift mutation and carry a fully functional *lac*$^+$ allele. On day 5, about 10% of revertant colonies are unstably Lac$^+$ and form sectored (blue/white, Lac$^+$/Lac$^-$) on non-selective medium. These cells carry a tandem array of 10-100 (n) copies of the mutant *lac* region and grow under selection by virtue of to their multiple partially-functional *lac* copies. On non-selective medium, the amplification is frequently lost, leading to a Lac$^-$ (white) colony sector.

The course of a reversion experiment is shown in Figure 5. The two colony types accumulate on the selection plate with different trajectories. Stable Lac$^+$ colonies accumulate linearly over 5 days, consistent with their arising in a non-growing population. Unstably Lac$^+$ colonies (10% of total) carry a tandem amplification (10-100 copies) of the original mutant *lac*

allele. These colonies accumulate exponentially with time. This suggests that they might develop from a growing population. The parent lawn population as a whole shows very little growth over the course of the experiment (See Figure 5). The question is, "How does a non-growing parent population give rise to these two types of Lac⁺ revertant colonies?" Some say that growth limitation is mutagenic (perhaps without DNA replication). Others say that selection acts on pre-existing partially-functional variants allowing them to develop a fully Lac⁺ phenotype without mutagenesis (perhaps without cell division).

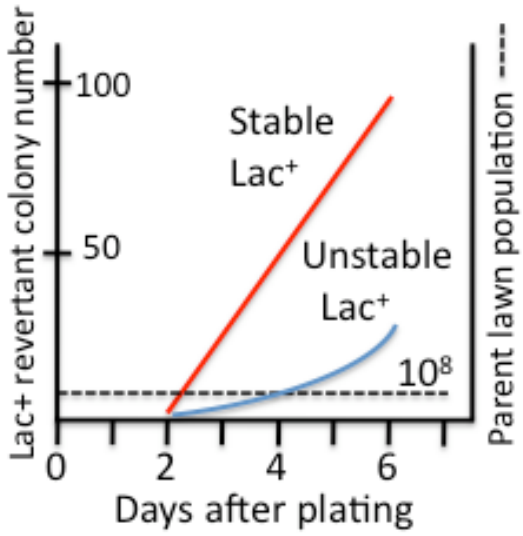

Figure 4 Accumulation of the two revertant types above non-growing lawn population. After 10^8 cells are plated on day 0, the lawn shows little or no growth, but revertant colonies accumulate over several days. Revertants are tested by streaking on non-selective medium to test the stability of their Lac⁺ phenotype. Colonies of stably Lac⁺ revertants accumulate linearly, suggesting formation from a non-growing cell population. In contrast, unstably Lac⁺ colonies accumulate exponentially, suggesting that they form in a growing population. This behavior is explained by a new model described below.

A clue for solving this puzzle may be the nature of the parent mutant *lac* allele. The *lac* allele carries a leaky frameshift mutation and produces about 2% of the β-galactosidase (LacZ) level found in a revertant. The ability of the strain to grow on lactose using its residual LacZ level is prevented by adding a 10-fold excess of scavenger cells that carry a *lac* deletion mutation. Scavengers are titrated so they just barely prevent growth of the tester by consuming carbon sources other than lactose that might contaminate the medium or be excreted by cells on the plate. Thus selection conditions are not stringent -- they prevent cell division, but leave cells poised on the brink of growth.

The adaptive mutation paradox – The paradox of this system is that selection appears to cause a 100-fold increase in the number of Lac⁺ revertants, but does so with very little evidence of general mutagenesis. Without selection, the parent mutation reverts at 10^{-8}/cell/division.

Under selection, 10^8 plated cells produce 100 revertant colonies over 6 days. This represents a 100-fold rate increase, if one assumes one division under selection. However, analysis of this system has revealed little evidence of an increase in general mutation rate. That is, the starved lawn population shows no increase in the frequency of unselected mutations on the chromosome (Rosche and Foster, 1999, Slechta et al., 2002b, Torkelson et al., 1997). The revertant Lac^+ colonies show a 10-fold increase in the likelihood of secondary unselected mutation, but that increase is unevenly distributed. About 90% of Lac^+ revertants show no evidence of general mutagenesis, while 10% have experienced a 200-fold increase in mutation rate (Rosche and Foster, 1999). How can the number of Lac^+ revertants increase without exposing the genome at large to mutagenesis?

How do Lac^+ mutants arise under selection? --- Three models and their problems

a) **Directed mutation –** In the initial model, an evolved mechanism senses the physiological situation and directs mutations to genomic sites whose alteration will restore growth (Foster, 1999). Directed mutation would help explain the lack of evidence for genome-wide mutagenesis. The difficulty lies in defining a process that can sense the cause of growth limitation and direct mutations to rare genomic sites that solve the problem. This would seem to require clairvoyance, but there is a precedent in the mammalian immune system, which mutagenizes local genomic regions in response to infection (Teng and Papavasiliou, 2007). Several clever ways to accomplish this in starving bacteria were suggested (Stahl, 1988), but the possibility of an increase in transcriptional errors or defects in post-replicative mismatch correction system later proved incorrect (Foster and Cairns, 1992, Stahl, 1992). Formation of Lac^+ colonies on selective medium relies on a functional recombinase (RecA) and some DNA replication. The selective amplification model (below) has the effect of directing mutation to valuable sites by amplifying the target gene and selectively maintaining only the revertant copy (Roth et al., 1996).

b) **Selection-induced hypermutability** This model proposes that growth limitation induces an evolved mechanism for genome-wide (undirected) mutagenesis in a subset of the growth-limited population. This model was initially suggested by Hall (Hall, 1990) and supported by Torkelson at al (Torkelson et al., 1997), who found that Lac^+ revertants arising in the Cairns-Foster system were about 20-fold more likely to carry associated, unselected mutations. In

this model, mutagenesis of the starved population is not detected because so few starved cells are actually mutagenized. This model, in its simplest form, is not feasible because it requires generation of 100 mutants from a sub-population 10^5 cells – a rate of 10^{-3}/cell/generation. This rate is a 10^5-fold increase of the *lac* reversion rate measured during non-selective growth and is substantially more intense than mutagenesis by any chemical or physical mutagen acting on non-growing cells (Adelberg et al., 1965, Miller, 1992, Wechsler et al., 1973). This model may explain the modest increase in unselected mutations in revertants, but it cannot alone explain the origin of mutants in the *lac* system.

c) **Selective amplification of the mutant gene.** This model proposes that mutants with a partially restored LacZ function arise during prior non-selective growth. When placed on selective medium, these cells grow slowly and improve with no increase in mutation rate, leading ultimately to a late-appearing visible colony. It was first suggested that the pre-existing cells carry a duplication of the leaky mutant *lac* allele, which supports slow growth on lactose. Growth improves as the amplification expands under selection. Ultimately some copy the *lac* region acquires a mutation that restores a functional *lac*$^+$ allele. The likelihood of such a mutation increases with the number of target sequences – more *lac* copies per cell and more cells in each slowly growing colony. No mutagenesis is required.

The selective amplification model has been shown to operate in many biological systems (Andersson and Hughes, 2009, Elliott et al., 2013, Kondrashov, 2012), including adaptation by which poxvirus evades host defenses (Elde et al., 2012), and bacterial acquisition of antibiotic resistance (Nilsson et al., 2006, Paulander et al., 2010, Pranting and Andersson, 2011, Sun et al., 2009). Most notably, this model explains evolution of new genetic functions under continuous selection (Bergthorsson et al., 2007) and has been shown experimentally to underlie evolution of a gene with a novel function within 3000 generations of growth (Näsvall et al., 2012). The model probably explains the *Salmonella* version of the Cairns-Foster system, in which plated cells grow about one division per day (Hendrickson et al., 2002, Kugelberg et al., 2006, Slechta et al., 2002b).

However, despite these successes, the original selective amplification model does not explain the original *E. coli* version of system, where plated cells show very little growth. The lack of growth makes it hard to imagine how plated cells with a duplication achieve the amplification and cell number increase needed to explain reversion without mutagenesis.

The philosophical problem of the adaptive mutation. --- Why has this question been so hard to resolve? Since its first description (Cairns and Foster, 1991, Cairns et al., 1988), this system has been extensively investigated by multiple groups (Foster and Trimarchi, 1994, Galitski and Roth, 1995, Harris et al., 1994, Peters et al., 1996, Powell and Wartell, 2001, Prival and Cebula, 1996, Radicella et al., 1995) from multiple points of view. The accumulated body of data is of high quality and is generally accepted by all participants. However results have been interpretable in different ways and viewpoints have not converged with time, despite occasional declarations of victory. The subject has been reviewed repeatedly from multiple perspectives (Andersson et al., 2011, Foster, 2004, Foster, 2007, Galhardo et al., 2007, Rosenberg, 2001, Roth et al., 2006). The difficulty in sorting this out may be that many experiments were designed to verify one particular model rather than to decide between models. The few data conflicts promise to decide the issue.

The generally accepted body of evidence Analysis of the *lac* system has revealed several bits of data that appear central to the mechanism but remain controversial. First, the yield of revertants depends heavily on homologous recombination. Virtually no revertant colonies appear in strains lacking RecA or RecBCD proteins, which are central to recombination initiated at double strand breaks (Cairns and Foster, 1991, Harris et al., 1994). However, the opposite effect is seen in strains lacking only the nuclease RecD; the number of Lac+ revertants increases in proportion to the increase in F' *lac* plasmid copy number caused by the RecD deficiency (Foster and Rosche, 1999). Second, revertant yield depends heavily on ability of the F'*lac* plasmid to transfer conjugatively to recipient strains (Foster and Trimarchi, 1995, Peters et al., 1996, Radicella et al., 1995). Virtually no revertants are seen when the transfer (*tra*) functions of F'plasmid are inactive, especially in absence of TraI the endonuclease that initiates transfer replication. Third, the total revertant colony number is reduced about 5-fold in strains that lack the error prone DNA repair polymerase, DinB (Foster, 2000, McKenzie et al., 2001, Slechta et al., 2003). Lack of DinB does not affect unstable revertant number, but reduces the number of stable revertants about 10-fold. Thus without DinB, mutants still arise under selection, but are evenly divided between stable and unstable revertant types. It is interesting that the reversion rate of the same *lac* mutation during non-selective growth is not affected by RecA (Bull et al., 2001) or by

the chromosomal DinB allele (Gawel et al., 2008, Kim et al., 2001, Kuban et al., 2004, Strauss et al., 2000, Wolff et al., 2004). The same studies show however a two- to four-fold reduction in mutagenesis on F' *lac* when the *dinB* copy on that plasmid is deleted. The three models outlined above explain the behavior under selection in different ways. The key to understanding this system may lie in the conflicting data bits that seem to decide between models.

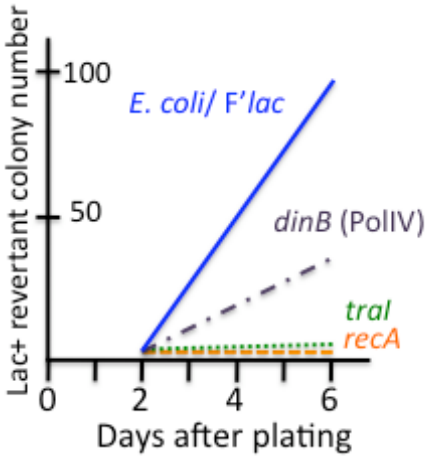

Figure 5 Three requirements for revertant yield in the *lac* system. The revertant yield (blue line) seen in the parent strain is reduced more than 25-fold in strains carryng a *recA* mutation, which blocks homologous recombination (dotted green line). A similar reduction is caused by a *traI* mutation on the plasmid, which eliminates a single-strand endonuclease needed to initiate conjugational transfer (dotted orange line). A functional transfer origin is needed for reversion (Galitski and Roth, 1995, Peters et al., 1996, Radicella et al., 1995), but the role of actual transfer is still unclear. Direct tests show a low frequency of actual plasmid transfer associated with reversion (Foster and Trimarchi, 1995, Maisnier-Patin and Roth). However, in support of mating, Tn*10* is lost during reversion, apparently stimulated by single-strandedness associated with conjugation(Godoy and Fox, 2000). In addition, most Tra proteins are required are required for reversion, including those needed for mating pair stabilization (Maisnier-Patin and Roth). In the absence of DinB, total revertant yield drops 5-fold; unstable revertants are unaffected while stable revertants drop 10-fold. Without DinB, residual revertant number is evenly divided between stable and unstable types.

Resolving conflicts and deciding between models.

a) Do the cells that initiate stable revertant colonies arise before or after selection?

Mutagenesis models predict that revertant colonies are initiated on the selection plate by new mutant cells formed in response to the selective environment. In contrast, selection models predict that initiating cells carry a *lac* amplification that forms during non-selective pre-

growth. On selective plates, these cells grow slowly and acquire a mutation that generates a *lac*$^+$ allele and allows rapid exponential growth.

The first attempt to decide the "before or after" question was a classic Luria-Delbrück fluctuation test (Cairns and Foster, 1991). Tests of this type demonstrated long ago that stringent selections detect mutants whose frequency fluctuates because they arose at different times prior to plating (see above). In the *lac* system, the number of revertant colonies formed over several days does not show fluctuation from one parallel culture to the next (Cairns and Foster, 1991). This was taken as evidence that Lac$^+$ mutants were initiated on the selection plate, consistent with either model of "stress-induced" mutagenesis. Recent results suggest that this conclusion is not warranted for copy number variants whose frequency does not fluctuate.

Unlike point mutants, duplications are not subject to Luria-Delbrück fluctuation. During non-selective growth, cells with duplication or amplification of any gene come to a steady-state frequency maintained by a balance between a high formation rate (typically 10^{-5}/cell/division) on one hand and a higher loss rate (10^{-3}/cell/division) plus fitness cost of the other (Reams et al., 2010). The forces that maintain this steady state obscure fluctuations due to the timing of duplication formation events. If revertants are initiated by copy-number variants (as proposed by the selection model), fluctuation tests cannot decide whether they form before or after plating. A different sort of evidence is required.

A new test recently showed that revertant colonies are initiated by pre-existing cells with multiple *lac* copies (Sano et al., 2014). These experiments use a *tetA* (tetracycline resistance) gene inserted at various points on the F'*lac* plasmid. The tetracycline analogue anhydro-tetracycline (AnTc) induces expression of *tetA*. This induction inhibits growth of cells with multiple copies of *tetA*, but has no effect on cells with a single copy.

The *tetA* gene is located on the same plasmid as *lac* and the two genes are expected to co-amplify. The selection model described above predicts that cells with multiple copies of mutant *lac* allele initiate Lac$^+$ revertant colonies. Pre-growth with AnTc counter-selects rare cells with multiple copies of *tet* (and often *lac*). This reduces then the number of revertant colonies appearing on selective medium. This result was seen regardless of the position of the inserted *tetA* gene on the F'*lac* plasmid, suggesting that the critical cells have multiple copies of the whole F'*lac* plasmid (which includes both *tetA* and *lac*) rather than an internal tandem

amplification of the *lac* region. These experiments led to the conclusion that cells with multiple copies of the F'*lac* plasmid initiate stable Lac$^+$ revertant colonies.

Unstable Lac$^+$ revertant colonies are also initiated by cells with multiple copies of F'*lac* (Sano et al., 2014), but the plasmids in these cells appear to carry a tandem amplification of the *lac* region (see Figures 3 and 4). Earlier evidence suggested that these unstable revertants are initiated by pre-existing cells with a short *lac* duplication (Kugelberg et al., 2006). We suggest that both findings are true --- unstable revertants are initiated by cells whose high copy F'*lac* plasmid includes a short *lac* duplication (Kugelberg et al., 2006). In the model below, we will assume that the extra number of *lac* copies supports growth and allows these cells to initiate unstable revertant colonies. Since both stable and unstable revertant colonies are initiated prior to selection, neither revertant type can be induced by selection.

b) Does the plated population grow before mutation? The lawn population grows very little over the course of the experiment. This has been interpreted as evidence that mutants arise in non-growing starved cells. However it is hard to eliminate the possibility that a subset of lawn population grows or that mutations arise within slowly-growing colonies (as suggested by the selective amplification model).

Support for mutagenesis within growing colonies was the presence of rare unstable Lac$^+$ cells within colonies of otherwise stably Lac$^+$ colonies. These cells were taken as evidence for unstable precursors of stably revertant cells (Hendrickson et al., 2002). Recently, we confirmed earlier evidence (Hastings et al., 2004) that the unstable Lac$^+$ phenotype of these rare cells is not heritable -- all descendent cells are either Lac$^+$ or Lac$^-$ (Roush et al.). This is inconsistent with tandem amplifications of *lac*, which are heritable and suggests that the rare unstable Lac$^+$ cells have multiple copies of the F'*lac* plasmid – some *lac*$^+$ and some *lac*$^-$. Thus the preponderance of evidence implies that plated cells grow very little under selection before the reversion event. This explains the linear accumulation of stable revertants. The new model below explains the mixed plasmid types seen in rare unstable Lac$^+$ cells within otherwise stable Lac$^+$ colonies.

c.) What activates the DinB polymerase -- induction or amplification? Strains lacking the error-prone DinB polymerase show 10-fold fewer stable revertants, but the same number of

unstable revertants (McKenzie et al., 2001). The DinB (polIV) enzyme, which belongs to the Y-family of DNA polymerases, can accommodate some bulky lesions and catalyze translesion DNA synthesis of damaged template strands (Fuchs and Fujii, 2013, Sale et al., 2012). When overproduced, DinB makes frequent mistakes including many frameshift mutations, during replication of undamaged DNA (Kim et al., 2001). Expression of the *dinB* gene is induced by DNA damage as part of the SOS response (Wagner et al., 1999) and by cessation of growth (RpoS) (Layton and Foster, 2003). Mutagenesis models propose that growth limitation induces *dinB* gene expression and consequently causes mutagenesis of the genome (Layton and Foster, 2003, Lombardo et al., 2004). This does not explain the uneven genomic distribution of mutagenesis (Rosche and Foster, 1999). Selection models suggest that the significant increase in DinB levels occurs because the *dinB* gene happens to be located close to *lac* on the F' plasmid (16kb away) (Kofoid et al., 2003). This position allows the two genes to be co-amplified during selection for more copies of the mutant *lac* gene.

Determining the effect of *dinB* gene position should be decisive, since mutagenesis models predict no role of position and selection models expect a critical role. Our lab found that revertant yield depended heavily on presence of a functional *dinB*$^{+}$ allele on the F'*lac* plasmid and not at all on the state of the chromosomal *dinB* gene (Slechta et al., 2002a). Another lab found the position of *dinB*$^{+}$ to be irrelevant to revertant yield (McKenzie et al., 2001). We have repeated these experiments and are extending the tests, but all current evidence supports the importance of position (Roush et al.). In the model described below, we assume that this conflict will be resolved in favor of a *dinB* gene position effect.

A new model accounts for all available data. Resolution of these conflicts listed above was helpful, but in fact none of the individual models withstood the tests. Here we presented a new model that incorporates features of all three models and seems consistent with all available information. The new model is described below. (With the relationship of each point to the previous models.

a) All revertant colonies are initiated by pre-existing cells with multiple copies of the F'*lac* plasmid. Sufficient energy for reversion may be supplied by a single copy of the leaky mutant *lac* allele (Stumpf et al., 2007). The extra plasmid copies allow over-replication of *lac* and

over-production of DinB. (Pre-existing cells with extra *lac* copies initiate revertant colonies, as in the selection model.)

b) The cells that initiate the stable revertants have insufficient energy to replicate their chromosome from its regulated origin. These cells cannot divide until they acquire a *lac*+ allele by mutation. (Growth is blocked, as in mutagenesis models.)

c) Initiator cells have sufficient energy to support continued F'*lac* replication from existing replication forks, initiated by the plasmid transfer origin or at intermediates in recombination between plasmid copies. These forks replicate the entire plasmid repeatedly over a period of many days with little or no cell division. (Mutations are made more likely by repeated replication, as in the selection model.)

d) The F'*lac* plasmid encodes the *dinB* gene (error-prone polymerase) so cells with multiple plasmid copies have more DinB protein and a higher mutation rate. This mutagenesis affects only the plasmid, since the chromosome is not replicating. This mutagenesis is not due to an evolved mechanism, but is an artifact resulting from the chance location of *dinB* on the F'*lac* plasmid and leakiness of the *lac* mutation. (Mutagenesis is directed to the plasmid.)

e) As soon as a mutation event produces a lac^+ allele in any of the multiple *lac* copies, energy is supplied and cell division starts. Revertant cells initiate exponential growth under selection to maintain their new lac^+ allele and lose any non-revertant plasmid copies. The rare unstable Lac^+ cells in a stable revertant colony have a mixture of plasmid types that have not yet segregated. The selective plasmid retention has the effect of directing mutation to DNA sites that limit growth. Plasmid copy number and over-replication increases the formation rate of any mutation on F'*lac*, but only enhances retention of selected mutations. Recovery of unselected mutations is not enhanced. This is true because only a selected plasmid copy is retained. (Mutagenesis appears directed to sites that limit growth).

f) Unstable Lac^+ revertant colonies are initiated by cells with a small tandem duplication of *lac* in each of their multiple plasmid copies. Multiple copies of an F'*lac* with a duplicated *lac* region provide sufficient *lac* copies (and energy) to support growth on lactose. Cells grow slowly under selection and expand their duplication into a long amplified array as their plasmid copy number drops. Cells within these clones grow exponentially and replicate their chromosome, while selectively holding multiple tandem copies of *lac.* Cells whose amplified *lac* region

includes *dinB* show increased mutagenesis of their entire chromosome. (This explains the observed modest level of general (undirected) mutagenesis.)

Explaining behavior of the *lac* system in terms of standard evolutionary ideas.

Basic evolutionary theory predicts that imposed selection will increase the rate of sequence change without increasing mutation rates. This is diagrammed in Figure 6. Assume that mutations arise as random errors whose frequency and spectrum of types is unaffected by growth limitation. Consider first a well-adapted population growing under optimum conditions. A large fraction of new mutations have phenotypes that are near-neutral (synonymous, silent or having tiny positive or negative effects on growth). These mutations tend to remain in the population and are subject to random loss by drift. Perhaps a similar fraction of mutations causes a palpable loss-of-function phenotype that impairs growth (deleterious). These mutations are continuously removed by purifying selection. Very few new mutations improve growth if the population that is well-adapted to its circumstances. Figure 6 (center).

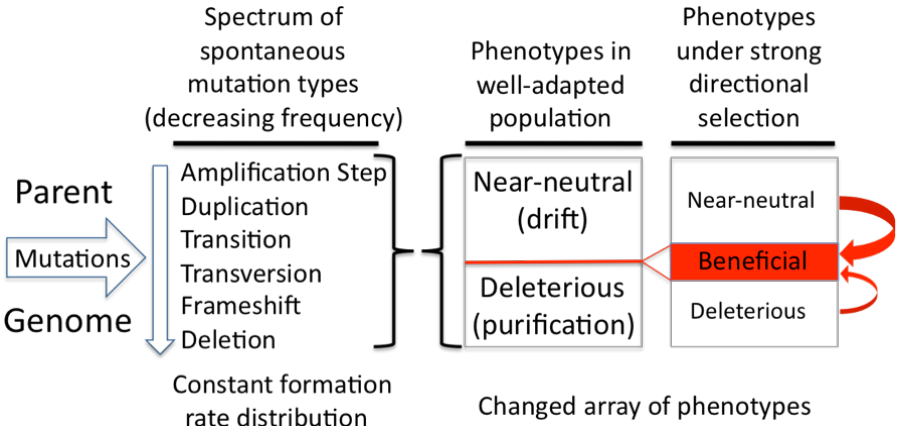

Figure 6 The conventional wisdom and how it can imitate mutagenesis. The primary event is formation of the several mutation types. These arise a vast array of frequencies and can have phenotypic effects of vastly different magnitude. In *Salmonella*, gain-of-function mutation types arise at rates that vary over a million-fold range . Substitution mutations that cause qualitative changes in a particular protein arise at less than 10^{-8}/cell/generation. Duplications of a gene form at 10^{-5}/cell/generation and further copy number increases at about 10^{-2}. Base substitutions in a particular gene arise at about 10^{-7}/cell/division and most cause a loss-of-function phenotype. The magnitude of their effects range from synonymous and near-neutral conservative missense substitutions that are about third of substitutions to the completely null mutations (nonsense), which, together with frameshift and deletion types make up about 1% of

genetic changes. The selective value (or costs) of mutations also varies over a wide range, but beneficial mutations are extremely rare in a well-adapted population. When a population is placed under strong directional selection, a larger fraction of available variability contributes to improved growth. Thus the predominant genotype in a population changes more rapidly under selective, even when the source of variability remains constant.

Now shift the population to conditions that impair growth but leave open many mutational ways to adapt and improve. A larger fraction of new mutations are beneficial and are brought to high frequency in the population by positive selection. Some were previously near neutral or even deleterious. Due to selection, the preponderant genotypes in the population change progressively. More mutations are being fixed in the genome even though the mutation rate and spectrum of mutation types is unchanged. New mutations form (at an unchanged rate) but rise in frequency and are held in the genome by selection. This looks like mutagenesis, when in fact selection has just detected beneficial alleles in the pool of near-neutral and deleterious types. The rate of adding new alleles to the preponderant genome increases due to selection alone. In the adaptive mutation models discussed here, these new mutations are attributed to an evolved mutagenic mechanism induced by stress. In the revised final model, polymorphic near-neutral alleles gain a significant phenotype when amplification under selection. Adaptation occurs without mutagenesis.

Evolutionary insights provided by the *lac* system Several principles of broad relevance to evolutionary biology have emerged from study of the *lac* system. This is true despite the fact that behavior of the system relies on specialized features that are peculiar to the strain used. That is, selection is imposed on a *lac* allele that happens to lie near the *dinB* gene, which encodes an error-prone polymerase. Both *lac* and *dinB* genes are carried by a conjugative plasmid with a replication origin that normally supports transfer from one cell to another. Scavenger cells prevent growth under selection but poise cells on the brink of growth. Below is a list of generally applicable principles that have emerged from analysis of this peculiar system.

a) Near-neutral beneficial alleles can gain a significant phenotype when amplified under selection. The minimal activity of the mutant *lac* gene can support growth in this system only when present in multiple copies, as seen during formation of unstable revertant colonies.
b) The two mutation types contributing to revertant formation are extremely common. Duplications have recently found to be the most common of all spontaneous mutations in

many organisms (10^{-5}/cell/division in *Salmonella*) and steps in higher amplification are even more frequent (10^{-2}/cell/division in *Salmonella*) (Reams and Roth, 2014 In press). The frequency of these copy number variants comes to a high steady state frequency in the population before any selection is imposed (10^{-3} for any gene in *Salmonella*) (Reams et al., 2010). Thus these copy-number variants are essentially common stable polymorphisms that form and are lost at high frequency.

Near neutral mutations have small phenotypes and thus are not selectively increased or removed from the population but are carried as neutral polymorphisms subject only to drift. These polymorphic alleles are available when some new selection pressure is imposed. Individuals with multiple copies of a near-neutral allele are frequent and selective response can be rapid.

c.) Amplification under selection is rapid and supplies multiple copies of a growth-limiting allele, all of which are subject to mutational improvement. Thus amplification increases the likelihood of sequence improvement by providing more targets (not by mutagenesis). Amplification is reversible such that the extra copies are lost once selection is relaxed by a beneficial mutation in some copy. This process of selective amplification and later loss has the effect of directing new mutations to beneficial sites.

The apparent direction of mutations to valuable sites by amplification is diagrammed in Figure 7. A region including the gene under selection amplifies under selection. This enhances growth and provides more targets for mutation. The extra copies make any mutations in this region more likely, regardless of effect on phenotype. If a beneficial mutation occurs in some copy, selection for amplification is relaxed and the improved allele is held selectively. This allows all other copies and any non-selected mutations to segregate and be lost, but retains the copy that has a beneficial mutation. The net effect of this process is to increase the frequency of beneficial mutations in the local region with no effect on non-selected mutations. The chance of retaining an unselected mutation in the amplified region is the same as the likelihood of this mutation without amplification.

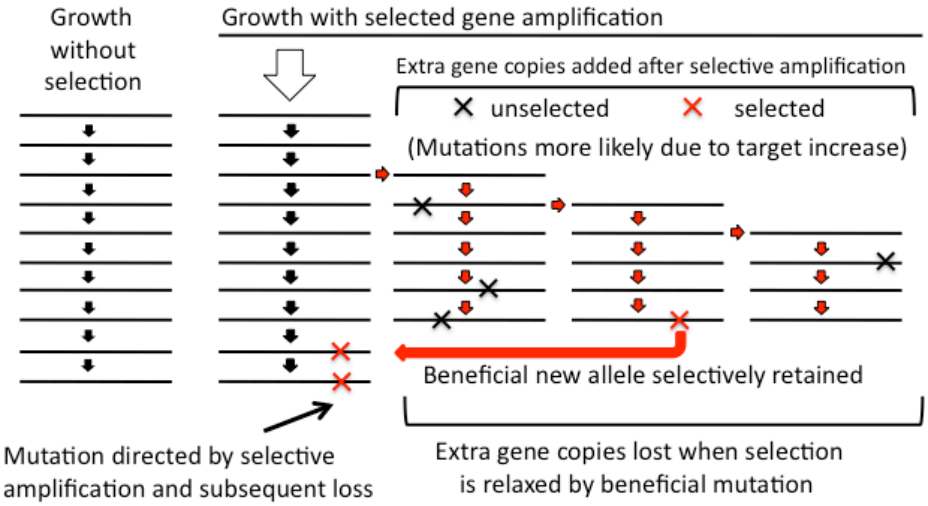

Figure 7 Selective amplification directs new mutations to sites that improve growth. The left column shows a genome region that replicates serially with no selection on copy number. The right column shows the same region that is amplifying under selection for increases in some encoded function. Amplification provides more copies of the entire region and increases the likelihood that a cell with the amplification will experience some mutation in this region. If the mutation is beneficial, selection to hold the amplification is relaxed and all copies are lost except the improved version, which is selectively maintained. Any copy with an unselected mutation is lost. The end result is a genome with an improved allele whose probability has been increased without increasing the likelihood of any other mutation in the genome.

d.) Selection can operate in non-growing cells if a local region is over-replicated and beneficial mutations are held selectively. This can be seen in Figure 7, where the extra copies are added by repeated replication in non-growing cells. Each act of replication presents an opportunity for mutation. Selection holds only a copy with a beneficial mutation. In essence, the multiple copies of the sub-genomic region serve as a population upon which selection can act in non-dividing cells. This process may prove relevant to origins of cancer whose progression is favored by fragile sites in the metazoan chromosome (Albertson, 2006). Repeated breakage and replicative repair of such sites can occur during one generation of a single cell and the lifetime of a single metazoan individual.

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