

Review for Howard Ochman ---

CSH book **Microbial Evolution**

The origin of mutants under selection: How natural selection mimics
mutability (adaptive mutation)

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Short title: Origin of mutations under selection

Abstract: (100-140 words)

Selection detects mutants but does not cause mutations. Contrary to this dictum, Cairns and Foster plated a leaky *lac* mutant of *E. coli* on lactose medium and saw revertant (Lac^+) colonies accumulate with time above the non-growing lawn. This result suggested that bacteria mutagenize their own genome when growth is blocked. However, the conclusion is suspect in the light of recent evidence that revertant colonies are initiated by pre-existing cells with multiple copies the conjugative *F'*/*lac* plasmid that carries the *lac* mutation. In some plated cells, the plasmid includes a tandem *lac* duplication and provides sufficient LacZ to support slow growth leading to an unstable Lac^+ colony. Other plated cells have multiple copies of a simple *F'*/*lac* and sufficient LacZ activity for plasmid replication but not cell division. Repeated plasmid replication in non-growing cells increases the likelihood of a reversion event. Reversion to *lac*⁺ triggers exponential cell growth leading to a stable Lac^+ revertant colony. Cells with multiple copies of the *F'*/*lac* plasmid have an increased mutation rate, because the plasmid encodes the error-prone (mutagenic) DNA polymerase, DinB. Without DinB, unstable and stable Lac^+ revertant types form in equal numbers and arise with no mutagenesis. Amplification and selection are central to behavior of the Cairns-Foster system, while mutagenesis is a system-specific side-effect of *dinB-lac* co-amplification. Study this system has revealed several general principles: a) In all populations, gene duplications are frequent stable genetic polymorphisms. b) Common near-neutral mutant alleles can gain a positive phenotype when amplified and c) Natural selection can operate without cell division on variability generated by over-replication of local genome sub-regions.

INTRODUCTION

An initial point to consider In considering the possibility of regulated mutagenesis, it is useful to consider a basic, but often forgotten truism. Most new mutations are near-neutral or deleterious types that are easily missed or ignored. Similarly, copy number changes of any particular gene are extremely common (10^{-5} /cell/division in *Salmonella*) but hard to detect. In contrast, rare beneficial mutations with big effects are obvious because selection allows them to increase in frequency. Genetic analysis has misdirected our attention toward these rare large-effect beneficial changes. It's easy to forget the "silent majority" -- the near-neutral and deleterious changes, but it's important to remember that natural selection sees almost everything and is always watching.

The major question under consideration Bacterial genetics uses stringent selection conditions to detect rare mutants. Genetic analysis of bacteria was made possible by the demonstration that strong selection could reliably detect and enumerate pre-existing mutants without contributing to their formation. Classical experiments by Luria and Delbrück (Luria and Delbrück 1943), the Lederbergs (Lederberg and Lederberg 1952) and Newcombe (Newcombe 1949) used lethal selections to show that mutants arise prior to selection and do not require growth limitation for their formation. These selections could only detect pre-existing mutants and therefore could not demonstrate whether or not some adaptive mutations form at a higher rate in response to selection. However the classic experiments were interpreted as evidence that selection does not affect mutation rates and led to the ultimate conclusion that all mutants arise as replication errors that are random with respect to functional or selective consequences.

Later experiments seemed to contradict this conclusion. In several systems, microbial growth was blocked by non-lethal conditions and mutant colonies accumulated over time above the lawn of plated cells (Shapiro 1984; Cairns et al. 1988; Hall 1988; Steele and Jinks-Robertson 1992; Maenhaut-Michel and Shapiro 1994; Sung and Yasbin 2002). The behavior of these systems was interpreted as evidence that non-growing cells increased their own mutation rates to help them "get off the dime". The cost of mutagenesis (n deleterious mutations) was not evaluated and the possibility that cells grow under selection prior to mutation was not eliminated. If cells grow under selection, any observed mutants could result from unappreciated parent cell divisions or from proliferation of pre-existing small-effect mutants rather than an increased mutation rate. When

selection is imposed on a growing population, the effect of mutagenesis on mutant frequency is hard to quantify in the face of the enormous exponential effects of positive selection.

To avoid interference from selection, mutation rates are classically measured by focusing attention on mutant types that can grow well under some permissive growth conditions and can be detected later by stringent selection (reviewed by Rosche and Foster 2006). Genome-wide mutation rates have also been estimated by examining the whole genome sequence of populations that have been passed through repeated small bottlenecks to minimize accumulation of fitter mutants and loss of mutants that impair growth (Lee et al. 2012). These devices solve the rate measurement problem, but avoid the real issue – “Do adaptive (beneficial) mutations occur at a higher rate under selection?”

The Cairns-Foster system (Cairns et al. 1988; Cairns and Foster 1991) was designed to solve these problems. The goal was to assess the formation rate of adaptive or beneficial mutations under selective conditions that prevent growth, but are not lethal. In interpreting the Cairns-Foster experiment, it is assumed that, if cells aren't dividing and chromosomes are not replicating, then any increase in mutant frequency must be due to mutagenesis and not selection. To eliminate growth, conditions were carefully adjusted (using scavenger cells as described below). Selection was deliberately set at a stringency that is just enough to prevent growth (Cairns and Foster 1991). The parental cells do not grow, but are poised such that very minor improvements may allow growth to start. The system has several problems. There is no easy way to compare mutation rates in growing and non-growing populations. During growth, one measures mutations/cell per generation. Without growth, one measures mutations/cell per unit time. Because selection stringency is low, it is hard to eliminate cryptic growth of a sub-set of the plated population or within slowly developing, partially revertant colonies. Selection never sleeps. We'll suggest later that the problem is even worse --- selection may even operate even when cell division is blocked.

Phenomenology of the Cairns-Foster system – how the experiment is done. In the basic *Escherichia coli* tester strain, the *lac* operon is located on the low-copy *F'**lac* plasmid (Cairns and Foster 1991) (See Figure 1). The *lacZ* gene (β -galactosidase) is fused to *lacI* (repressor) and transcribed from a constitutive (i^Q) promoter. Expression of LacZ activity is prevented by a leaky +1 frameshift mutation within the *lacI* portion of the hybrid gene, which reduces the level of β -galactosidase (LacZ activity) to 2% of that seen in revertants (Foster 1994). Cells are pre-grown in minimal glycerol medium, washed and plated (10^8) on lactose medium, where any residual growth is prevented by a 10-fold excess of scavenger cells with a *lac* deletion mutation. Scavengers can't use lactose or revert, but can consume any residual nutrients in the agar or compounds excreted from

revertant colonies (notably galactose). The number of scavenger cells is set to just barely prevent growth of the tester, which is poised on the brink of growth (Cairns and Foster 1991).

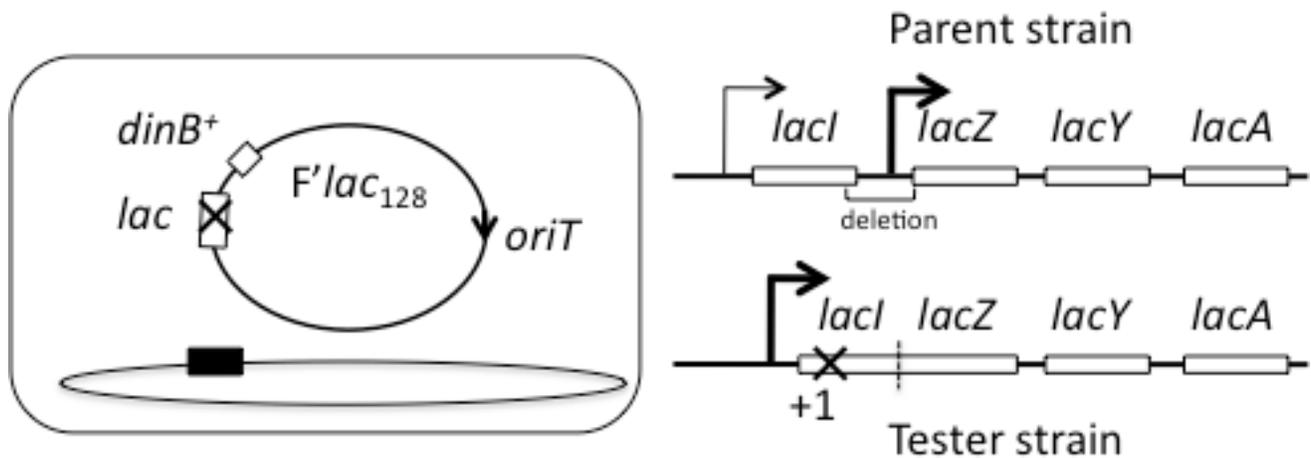


Figure 1 The strains used in the Cairns-Foster system. The *Escherichia coli* tester strain carries an *F'**lac* plasmid with a mutant *lac* allele; the chromosomal *lac* region has been deleted. On the *F'**lac* plasmid, the *lacI* and *lacZ* genes have been fused so they encode a single protein with β -galactosidase (LacZ) activity. This hybrid gene is transcribed from a constitutively expressed *lacI*^o promoter. The ability of the tester to grow on lactose is blocked by a +1 frameshift mutation within the *lacI* portion of the hybrid gene. This mutation is leaky and allows production of about 2% of the revertant enzyme activity (Cairns and Foster 1991). The *F'**lac* plasmid also carries the *dinB* gene, which encodes an SOS-inducible error-prone (mutagenic) DNA repair polymerase. The transfer (*tra*) functions of plasmid *F'**lac*₁₂₈ that allow conjugation are constitutively expressed. Conjugal replication and transfer processes are initiated at a transfer origin (*oriT*) by the single strand endonuclease TraI.

The course of an experiment is described in Figures 2 and 3. Tester cells (10^8) are grown non-selectively, washed and plated at day 0. The Lac⁺ revertant colonies start appearing by day 2 and accumulate linearly for about 5-6 days thereafter. During this time, the plated lawn shows little growth and this stasis is not a balance of death and growth (Foster 1994). The colonies appearing first -- before day 2 -- are initiated by pre-existing full *lac*⁺ revertants (with a compensating -1 frameshift mutation). Colonies that accumulate later, above a non-growing lawn, are the critical revertants to explain. The yield of these late revertants depends on residual LacZ expression from the leaky mutant gene. This residual expression could in principle support very slow lawn growth or could simply supply energy needed for the reversion process, a point that will become critical. There are two types of late revertant colonies. Ninety percent of colonies appearing on day 5 contain cells with a stable *lac*⁺ mutation (a compensating -1 frameshift). The remaining colonies (10%) contain

cells with a tandem amplification (10-100 copies) of the original mutant *lac* allele. Most of these are standard direct-order repeats with short (10bp) junction sequences (SJ or short junction types). About 20% of unstables have tandem repeats arranged in alternating orientation (TID or tandem inversion duplication). Stable types accumulate linearly, unstable types exponentially (Figure 3).

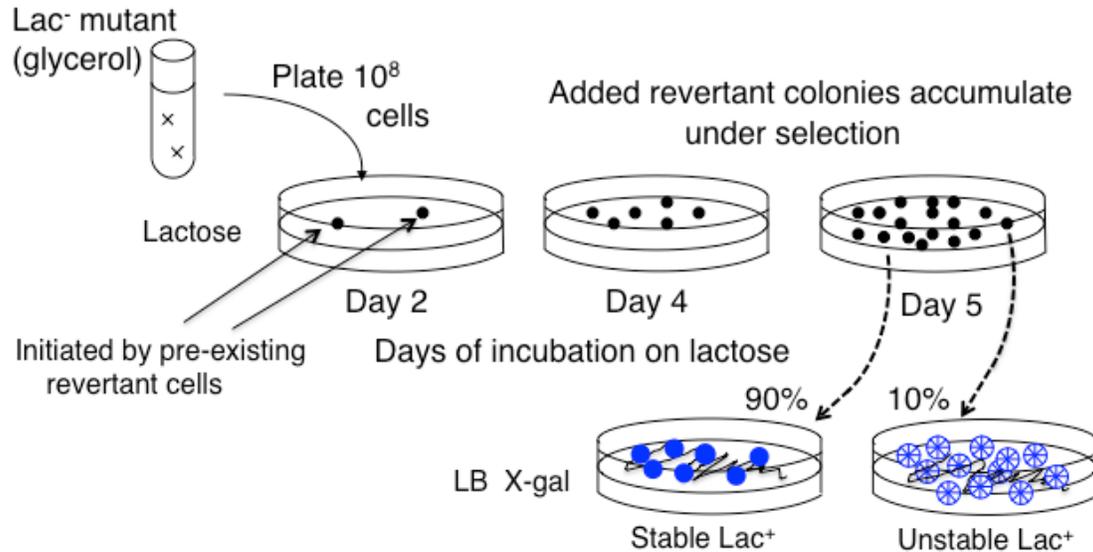


Figure 2 The Cairns-Foster reversion experiment. Cells of the strain described above are pre-grown on glycerol, washed and plated on lactose medium. The few revertant colonies that appear on lactose within 2 days are initiated by fully *lac*⁺ revertant cells that arose during prior non-selective pre-growth. More *Lac*⁺ colonies accumulate on the plate over the next 4 days. On day 5, ninety percent of new late colonies are made up of stable *lac*⁺ cells that have acquired a compensating frameshift mutation and therefore a functional *lac*⁺ allele. The other 10% of revertant colonies are made up of cells with an amplified array of the original mutant *lac* allele. Amplification-bearing cells are unstable and frequently lose their *Lac*⁺ phenotype. When these revertants are streaked on rich non-selective medium containing the chromogenic LacZ substrate X-gal, they form sectorized (blue/white) colonies that reveal their frequent loss of ability to use lactose. (See Figure 3)

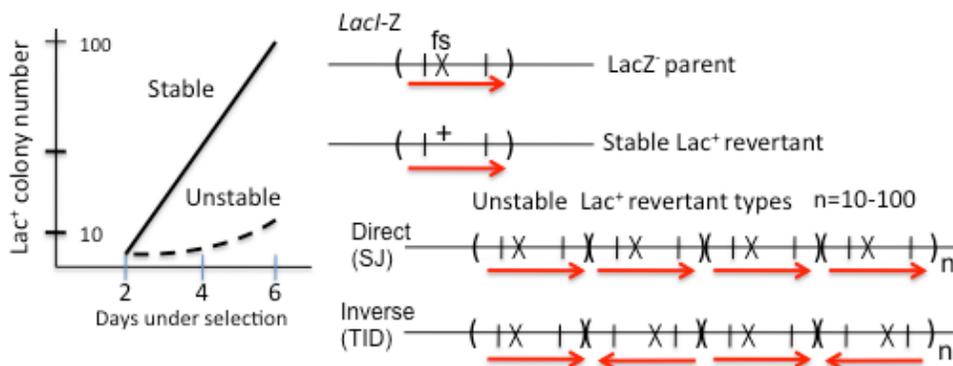


Figure 3 The time course of accumulating stable and unstable revertants in the Cairns-Foster experiment. After the tester strain is plated on lactose medium (on day 0), revertant colonies accumulate over several days. Stable *lac*⁺ colonies accumulate linearly with time, while unstable *Lac*⁺ colonies accumulate exponentially with time. The genotype of the parent tester strain is diagrammed at top right. Stable *Lac*⁺ colonies have acquired a compensating (-1) frameshift mutation and thereby a *lac*⁺ allele. Cells in unstable *Lac*⁺ revertant colonies have multiple copies (n) of the original leaky mutant *lac* allele, arranged either as tandem direct-order repeats (SJ) or

expanded tandem inversion duplications (TID) with copies in alternating orientation. Cells with sufficient copies of the partially functional mutant *lac* allele can grow on lactose and form a sectored blue/white colony on rich X-gal medium due to loss of *lac* copies.

Numerology of the Cairns-Foster system – the mutation problem to be solved. The curious thing about the Cairns-Foster system is the abundance of revertant colonies and the paucity of evidence for any general mutagenesis that might cause their appearance. The *lac* → *lac*⁺ reversion rate is 10⁻⁸/cell/division during non-selective growth in liquid medium (Rosche and Foster 2006). A lawn of 10⁸ plated cells produces 100 colonies over 5-6 days (about 100-fold more than expected) and does so with no apparent lawn growth and very little general mutagenesis. If every plated cell divided once, the number of revertants could be explained by a 100-fold increase in general mutation rate. However the lawn of parent cells shows no increase in the frequency of unselected mutations (Torkelson et al. 1997; Rosche and Foster 1999; Slechta et al. 2002). The Lac⁺ revertant colonies show a 20-fold increase in the frequency of unselected associated mutations, which is unevenly distributed. Ninety percent of Lac⁺ revertants show no evidence of mutagenesis, while 10% have experienced about a 100-fold increase in genome-wide mutation frequency (Rosche and Foster 1999). It seems clear that some mutagenesis occurs that is insufficient to explain the revertants and this mutagenesis is being distributed in interesting ways.

Three suggestions to explain the uneven distribution of mutagenesis Several ideas have been proposed to explain how Lac⁺ revertant number can increase 100-fold with very little general genome-wide mutagenesis. The first two ideas below have been used, individually or together, as part of models that explain the Lac⁺ mutants by stress-induced mutagenesis. The third idea (our favorite) suggests that stress does not induce mutagenesis at all. It attributes the modest observed increases in mutation rate to a non-essential artifactual side effect of selection in this system.

1) Directed mutagenesis – Stress induces predominantly beneficial mutations in all cells of the population. This model proposes a regulatory mechanism that senses the physiological problem and directs mutagenesis to sites that improve growth (Foster and Cairns 1992; Foster 1993). Direction to useful sites explains how the number of Lac⁺ revertants might increase more than the number of mutants at large. That is, the mechanism makes predominantly valuable changes. While a mechanism to direct mutagenesis may seem difficult to imagine, clever ways of achieving this have been suggested (Stahl 1988). Those include an increase in transcription errors, or a failure in the post-replicative mismatch correction system. These possibilities were later rejected (Foster and Cairns 1992; Stahl 1992). One important early observation was that the recombinase RecA and some DNA synthesis is required for appearance of Lac⁺ revertants under selection (Cairns and Foster 1991; Foster and Cairns 1992). The selective-amplification model described

below relies on RecA and has the effect of directing mutagenesis to the precise positions that limit growth (Roth et al. 1996).

Later experiments showed that mutagenesis is not directed in the strictest sense. Starvation of *lac* mutants on lactose stimulates selective reversion of mutations in other genes on the F' plasmid (+1 frameshifts in *tetA*), not just in *lac* genes (Foster 1997). During starvation on lactose, tetracycline-resistant mutants (TetA⁺) accumulated at the same linear rate as did Lac⁺ revertants. This was evidence that limitation of growth on lactose does not direct mutations specifically to the *lac* operon, but also affects other sites close to *lac* on F'/*lac*. The formation rate of both types of apparently directed mutations can be enhanced by DinB if the *dinB*⁺ gene is also present on the plasmid that carries *tetA* and *lac* (see model below).

- 2) Hypermutable states – Stress induces genome-wide mutagenesis in a subset (10^{-3}) of the plated population. This model was suggested by Barry Hall (Hall 1990) and was supported by the discovery that Lac⁺ revertants have an increased probability of carrying associated unselected mutations (Torkelson et al. 1997; Rosche and Foster 1999; Slechta et al. 2002). The Lac⁺ revertant cells with associated mutations do not arise in cells with a heritable mutator mutation (Rosche and Foster 1999). The frequency of unselected mutants in the starved population is low, and the small fraction of those cells that were mutagenized has been estimated at about 1 cell in a 1000 (Rosche and Foster 1999; Bull et al. 2000). If the accumulated 100 Lac⁺ revertants were initiated by cells that arose in the mutagenized subset of cells, they would have to arise from 10^5 instead 10^8 plated cells. Generating 100 revertants from 10^5 cells (a rate of 10^{-3}) would require a 10^5 -fold increase in mutation rate over that measured in unselected cells. When inflicted on non-growing cells, this intense mutagenesis is higher than can be achieved by any chemical mutagen and is expected to add about 8 null mutations to each Lac⁺ genome (Roth et al. 2006a). Thus general mutagenesis alone can't explain the revertants appearing in the Cairns-Foster system, but might contribute if combined with the directed mutagenesis described above.
- 3) Selective improvement of pre-existing small-effect mutants – Stress is not mutagenic -- only an agent of selection. This model proposes that cells with multiple copies of the leaky mutant *lac* allele arise prior to selection and initiate the colonies that appear later on lactose plates. Due to the residual activity of the mutant *lac* allele, multiple copies of the mutant gene might provide sufficient energy to support growth or at least allow repeated plasmid replication under selection. Repeated replication of multiple *lac* target sites might enhance the likelihood of an improving mutation using only the basal mutation rate. This *lac* amplification may occur in slowly-growing cells or maintained non-growing cells that continue to replicate just their F'/*lac* plasmid.

The enhanced yield of Lac⁺ revertants under selection and the associated mutagenesis seen in 10% of Lac revertants is attributed in part to activity of the DinB polymerase. This polymerase is encoded by a gene located near *lac* on the F'*lac* plasmid and is thus subject to increased expression in strains with multiple F'*lac* copies or tandem duplications large enough to include both the *lac* and *dinB* genes.

The philosophical conundrum posed by the Cairns-Foster system. Work on the Cairns-Foster system has generated a body of high quality data. Parties to the debate accept most of these results. The few remaining conflicts will be discussed below. Despite their general acceptance, these results have been interpreted in diametrically opposite ways. One side concludes that cells have mechanisms to create mutations whenever growth is blocked (“adaptive mutation”, “stress-induced mutagenesis”, “directed mutation”, “stationary phase mutagenesis” or “hypermutable states”). The other side (that’s us) interprets the same results as evidence that growth limitation does not change the mutation rate, but serves as an agent of selection that favors growth and improvement of pre-existing partial revertants. The failure to resolve this issue may result from a tendency of both sides to test predictions that verify their point of view rather than doing experiments that decide between the two general possibilities. Experiments discussed below may prove decisive.

Factors that affect the yield of stable and unstable revertants – the major facts. The accumulated body of information has been reviewed repeatedly, both from the viewpoint of stress-induced mutagenesis (Foster 1993; Foster 1999; Rosenberg 2001; Hersh et al. 2004; Foster 2005; Foster 2007; Galhardo et al. 2007; Rosenberg et al. 2012) and from the position of selection alone (Roth and Andersson 2004; Roth et al. 2006b; Andersson et al. 2011). Below we described the most striking findings. These results are agreed upon but interpreted differently. Later we’ll combine these results, resolve some data conflicts and offer a comprehensive view of reversion in the Cairns-Foster system that has broader implications for evolutionary processes.

1) F' plasmid transfer replication The yield of Lac⁺ revertants is essentially eliminated by mutations that inactivate the ability of the F'*lac* plasmid’s ability to transfer conjugatively to a recipient cell (Foster and Trimarchi 1995a; Galitski and Roth 1995; Radicella et al. 1995; Peters et al. 1996). This is most striking for strains lacking *Tral* – a plasmid protein with DNA nickase and helicase activities, both of which contribute to transfer replication and to reversion (Traxler and Minkley 1988; Foster and Trimarchi 1995a). During the reversion process, a *Tn10* inserted on the F' plasmid is subject to frequent loss which is known to be stimulated by single-strandedness (Syvanen et al. 1986). Since conjugal replication origin produces a single-strand product, frequent *Tn10*

loss suggested that reversion involves plasmid transfer or transfer-associated replication from the *oriT* transfer origin (Godoy and Fox 2000). However, transfer of the whole plasmid is seldom associated with reversion (Maisnier-Patin and Roth ; Foster and Trimarchi 1995a; Foster and Trimarchi 1995b). These results do not eliminate the possibility that *Tral* initiates internal *F'/lac* replication from the transfer origin (*oriT*) or contributes to transfer within a single cell or between daughter cells. The F-plasmid conjugation functions are needed for both stable and unstable Lac^+ revertants (Ponder et al. 2005). Consistent with the above points, the effect of growth limitation on *lac* reversion depends on the leaky *lac* allele being located on the F' plasmid (Slechts et al. 2003).

2) Recombination proficiency. Lack of the RecA (DNA strand exchange) protein or the RecBC/RuvABC proteins (double strand break repair) reduces revertant number severely (Cairns and Foster 1991; Harris et al. 1994). Other recombination defects cause a generally smaller reduction (He et al. 2006). The several schools of thought suggest that recombination either plays a role in initiating mutagenic DNA replication or contributes to the process of gene amplification. Ability to recombine, like ability to transfer, is required for both stable and unstable Lac^+ revertant colonies.

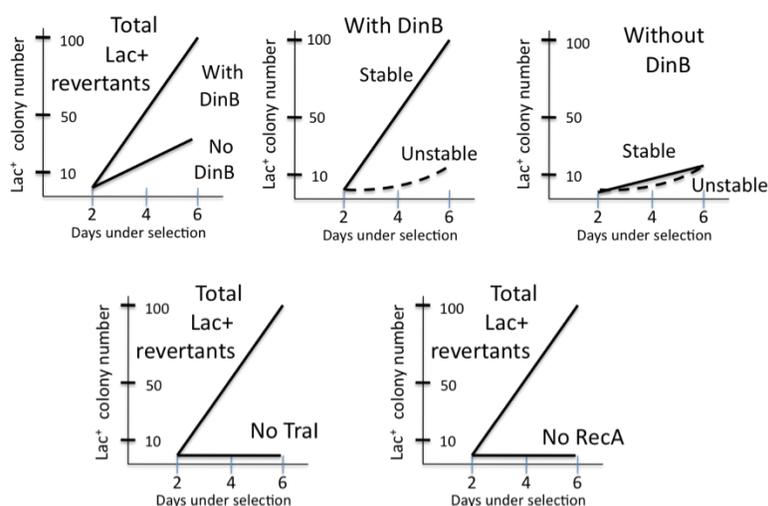


Figure 4 Major contributors to revertant yield A considerable body of data has been accumulated to test the effect of various chromosome- and plasmid-encoded functions on revertant yield. Top left shows dependence of revertant yield on the error-prone repair polymerase DinB (4-5 fold). Top center shows that in a $DinB^+$ strain, stable revertants are about 90% of total and unstable revertants are 10%. Top right, shows that without DinB, the number of stable revertants is reduced about 10-fold and becomes roughly equal to the number of unstable revertants (Foster 2000; McKenzie et al. 2001). That is, even without the mutagenic DinB polymerase, the number of stable revertants increases with time under selection. Two of the most important functions for reversion are *Tral* (bottom left), which nicks and unwinds plasmid DNA during conjugative

transfer, and RecA, a strand-exchange protein that is central to homologous recombination. Mutants in *recBC* and *ruvABC* genes have similarly strong effects on revertant yield.

3) The DinB error-prone DNA repair polymerase. This polymerase (also called Pol IV) belongs to the Y-family of translesion DNA polymerases and is part of the SOS response to DNA damage (Friedberg 2005). DinB can efficiently replicate a template containing damaged bases that block normal replication, especially bulky lesions formed at the N2-position of deoxyguanosine (Jarosz et al. 2007; Walsh et al. 2011). DinB can also elongate misaligned primers and incorporate modified nucleotides (Nohmi 2006). Overexpression of DinB leads to spontaneous mutagenesis of undamaged DNA. In particular, a high level of DinB makes -1 mutations by template-slippage in repetitive sequences (Kim et al. 2001; Nohmi 2006) --- the type of sequence found at the site of the *lac* mutation in the Cairns system.

In the absence of DinB, the number of stable Lac⁺ revertants drops about 10-fold but the yield of unstable revertants is unaffected (McKenzie et al. 2001). Without DinB, stable and unstable revertants appear in equal numbers (1:1 instead of a 9:1 ratio) and total revertant yield is reduced about 5-fold. (See Figure 4). Thus DinB contributes substantially, but is not absolutely required for the accumulation of revertants under selection.

4) Duplication and amplification of the *lac* operon. Gene duplications form at a high rate and come to a high steady-state frequency during non-selective growth (Reams et al. 2010). This steady state is maintained by the balance between high formation rates on one hand and loss rates plus fitness cost on the other. Cells with higher amplification are expected to come to lower steady-state frequencies. Cells whose F'*lac* carries a large duplication (100kb) of the plasmid *lac* region are carried at a high steady-state frequency of 1/500. These common *lac* duplications form on F'*lac* between flanking copies of IS3 (1.2kb). Unstable Lac⁺ revertant colonies consist of cells with an amplification of a smaller *lac* region (20-30kb). The duplication underlying these amplifications often form between palindromic REP elements (Bachellier et al. 1999; Kofoid et al. 2003; Slack et al. 2006; Kugelberg et al. 2010). The cells in unstable Lac⁺ revertant colonies grow under selection because they have 10-100 copies a small region (lower fitness cost) that includes the leaky mutant *lac* allele.

The amplifications found in unstable Lac⁺ revertants are of two types. Some have tandem direct-order repeats with a short junction sequence (SJ) and appear to arise by an exchange between very short repeats (10bp). Others have an array of tandem copies in alternating

orientation (head-to-head and tail-to-tail) (Kugelberg et al. 2006; Slack et al. 2006; Kugelberg et al. 2010). These are known as tandem-inversion-duplications (TID). (See Figure 3.) Both duplication types allow slow growth on lactose and expand into higher amplifications by unequal recombination between the directly repeated sequences (Kugelberg et al. 2010) (Reams et al. unpublished results). Copy number expansion is expected whenever the growth improvement provided by additional *lac* copies exceeds the fitness cost of the duplication.

Two diametrically contradictory models – and how they grew. The two models described below use the same basic facts outlined above, but explain reversion in the Cairns-Foster system in different ways. In the first, colonies are initiated on the selection plate when an evolved stress-induced cellular mechanism causes a discontinuous event in a non-growing cell. This event either creates a new large-effect frameshift mutation in *lacI-lacZ* gene or a sudden amplification of *lac* copy number. In the second model, colonies are initiated by pre-existing mutants with a *lac* duplication. These cells grow slowly at first and improve under selection. We will first describe one simple versions of these two opposite models and then will consider some data conflicts and present a unified model that we think accounts for all of the available information.

1) Stress-induced mutagenesis. In this model, none of the plated tester cells grow under selection but all remain alive and respond to starvation by inducing a set of genes that lead to DinB-dependent mutagenesis. Increased expression of DinB is mediated by LexA, repressor of the SOS DNA repair system, and by RpoS, activator of genes during slow growth (Layton and Foster 2003; Lombardo et al. 2004; Galhardo et al. 2009). Although the tester cells are not dividing, the leakiness of the *lac* allele is thought to give enough energy for occasional firing of the vegetative plasmid origin on F' and DNA synthesis. If a replication fork encounters a nick produced at the conjugational replication origin *oriT* by the endonuclease Tral, the fork collapses creating a double strand break. Normally such ends are processed in an error-free manner by RecA/BC-dependent recombination. However during growth limitation, the model proposes that repair becomes mutagenic because RpoS and DinB proteins are up-regulated. Both these proteins are crucial to the formation of the point mutations (-1 frameshifts) that produce a *lac*⁺ allele and a stable revertant colony. In other tester cells, starvation causes a sudden *lac* amplification and the multiple mutant *lac* alleles allow growth and formation of an unstable revertant colony. In this model, both stable and unstable revertant types are independently initiated by discrete events that are formed under selection by a stress-induced mechanism. These events all occur in non-growing cells.

2) Amplification under selection. In the initial form of this model, the reversion process starts with a duplication-bearing cell formed during non-selective pre-growth prior to plating. Such cells are extremely common (1/500) and grow very slowly under selection. Their growth rate increases progressively by expansion of the tandem array of *lac* alleles. Ultimately the mutant *lac* allele is replicated a sufficient number of times that one copy acquires a reversion event to *lac*⁺. This can occur at the standard unenhanced mutation rate. As soon as a frameshift mutation generates a revertant *lac*⁺ allele, growth accelerates and a stable revertant colony forms. If a deletion removes a junction of the initial duplication the repeat size is reduced. This lowers the fitness cost of amplification, allowing higher amplification and formation of an unstable revertant colony.

This model requires no mutagenesis, but does benefit from multiple copies of the *dinB*⁺ gene. Mutation rates increase in colonies whose *lac* amplification includes the nearby *dinB* gene, which just happens to lie 16kb away from *lac* on the F'*lac* plasmid. This model was developed based on experiments with the Salmonella version of this system in which the *lac* mutation supports higher lawn growth. The model seems to explain the behavior of Salmonella, but does not account for behavior of the *E. coli* system in which much less growth occurs.

Four data conflicts that have delayed a final decision. Below are four questions whose answers should help decide between “stress-induced mutagenesis” and “amplification under selection.” We think that these answers are near.

1) Are revertants initiated before or after selection? A decisive difference between the models above is whether revertant colonies are initiated by cells that arise on the selection plate, possibly induced by stress. Alternatively colonies could be initiated by weakly Lac⁺ cells that form before plating as proposed by the selection models. One of the strongest arguments in favor of “stress-induced mutagenesis” is the outcome of a Luria-Delbrück fluctuation test applied to reversion in the Cairns-Foster system (Cairns and Foster 1991). Multiple parallel cultures were plated independently on selective medium and day 5 revertant yields were found to show a Poisson distribution. The absence of “jack-pots” characteristic of a Luria-Delbrück distribution was interpreted as evidence that Lac⁺ colonies cannot be initiated prior to selection but must arise on the selection plate. This result is opposite to that of classical experiments showing that mutants detected by lab selections arise prior to selection (Luria and Delbrück 1943; Newcombe 1949; Lederberg and Lederberg 1952). The results were taken as evidence that the Lac⁺ colonies arising in the Cairns-Foster system were initiated on the plate by mutations formed in response to selection or “stress”.

Since those fluctuation tests were done, it has become clear that duplication mutations are immune to Luria-Delbrück fluctuation tests (Reams et al. 2010). The frequency of duplications and higher amplifications in unselected cultures comes to a steady-state frequency that is maintained by a balance between the high rate of gene duplication formation (10^{-5} /cell/generation) on the one hand, and the even higher loss rate (typically 10^{-2} /cell/generation) plus the fitness cost of the duplication on the other hand. The forces that drive the frequency of cells with a gene duplication (or amplification) toward steady state act on any aberrant frequency due to timing of formation events. This minimizes the occurrence of jackpots. Because these steady-states obscure fluctuation, a different test was required to determine whether pre-existing duplications initiate revertant colonies that appear under selection.

Recent evidence suggests that revertant colonies are initiated by cells that form prior to selection and thus cannot be stress-induced (Sano et al. 2014). This was determined by placing the *tetA* gene near *lac* on the *F'*/*lac* plasmid. Multiple copies of *tetA* are toxic when induced by the analogue anhydro-tetracycline, AnTc. If cells with multiple copies of *lac* initiate revertants, many of those cells should also have multiple copies of the nearby *tetA* gene and be sensitive to inhibition by AnTc. Reducing the frequency of high-copy variant cells (lowering their steady state frequency) should reduce the number of revertant colonies seen later under selection. When cultures of the tester strain with *tetA* on the *F'*/*lac* plasmid were pre-grown in the presence of AnTc, the number of Lac⁺ revertants was reduced sharply. The revertant number is restored by a few additional generations of growth in the absence of AnTc. Unexpectedly the reduction in revertant yield was seen regardless of the position of *tetA* on the *F'*/*lac* plasmid. This suggested that the critical cells have multiple copies of the whole *F'*/*lac* plasmid, rather than an amplification of a small *lac* region. This finding is parallel with the “amplification under selection” model, except that amplification occurs at the level of plasmid copy number instead of by tandem repeats of only the *lac* region. Cells with multiple copies of the *F'*/*lac* plasmid initiate both the stable and unstable Lac⁺ revertants. If all revertants are initiated prior to selection, we conclude they cannot be “stress-induced”.

- 2) Must *dinB* be located on the *F'*/*lac* plasmid? In the original “amplification under selection” model, expression of DinB is increased by selective co-amplification of the *dinB* and *lac* genes. This requires that the two genes be located close together, as they happen to be in the original tester strain. In contrast, “mutagenesis” models propose that stress increases the level of DinB protein by transcriptional gene regulatory mechanisms (e.g. RpoS, LexA) and gene position should be irrelevant. We found a clear effect of *dinB* gene position on revertant yield -- Lac⁺ revertant

number was severely decreased by removing the *dinB*⁺ allele from its position cis to *lac* on the F'*lac* plasmid (Slecht et al. 2003). Functionality of the chromosomal *dinB* allele was not required. The conflict arose when the same experiment (done in another lab) showed no effect of position (McKenzie et al. 2001). We have reconstructed all of the critical strains and still get our original result – a full yield of stable revertants requires a functional *dinB*⁺ allele located on the same plasmid as *lac*. Resolving this “he said, she said” stand-off will require more testing, but we’re convinced that *dinB* gene position is critical and observed mutagenesis requires co-amplification of *dinB* with *lac*.

- 3) Is there growth before mutation under selection? Mutagenesis models depend on mutants arising in non-growing cells. In contrast, the original amplification-selection model relies on growth under selection. Most other systems used to support stress-induced mutagenesis have not eliminated growth under selection, making it hard to decide what is happening (Mittelman 2013). In the Cairns-Foster system, supporters of mutagenesis have taken pains to show that cells are not growing (see below). Supporters of selective amplification argue that these tests have missed important growth within developing colonies, which is systematically missed by assays of the plate population. The question of growth could be decisive.

A considerable body of evidence supports the belief that mutations arise in non-growing cells. This evidence must be accounted for in any final model. Notice that the preponderance of evidence below argues against growth.

- a. The total number of parental cells in the lawn (outside of colonies) does not increase for 4 days. The constant lawn population is not a balance between growth and death (Foster 1994). However a large population (10^8) is being tested so one doubling would be hard to detect and yet would provide 10^{-8} opportunities for mutation. In addition these tests don't assess growth within the developing colony.
- b. If growth occurs within colonies, as suggested by the original amplification model, then the number of visible colonies is expected to increase exponentially since an exponentially increasing number of precursor cells is present in each colony. The graphs describing revertant accumulation show an upward flex in some experiments but in general this accumulation is linear with time, consistent with very little general population growth over 4 days. This argues against growth within the developing colony prior to the reversion event.
- c. Newcomb spreading experiments did not show evidence that *lac*⁺ revertants arise from cells growing under selection prior to mutation (Newcombe 1949). This classic experiment is a contemporary of the Luria-Delbrück fluctuation test and the Lederbergs' replica-printing

experiment, which originally demonstrated that mutations are not caused by the selection. Newcombe plated bacteria cells on non-selective medium and allowed time for a few divisions. He then spread the population of the plate before imposing a selection (phage resistance). If resistant mutants were present in the plated population and grew prior to selection (which they did), the act of spreading multiple cells within a developing colony should seed multiple colonies that could be detected after selection was imposed. Spreading should not increase selected mutant number if mutations are induced by exposure to selection.

If colonies are initiated by rare pre-existing cells that grow prior to selection, then spreading plate shortly after plating should increase the number of detected after selection is imposed. This happened in Newcomb's experiment, indicating that the revertant clones arose and grew before selection. In the Cairns-Foster experiment, this experiment would test whether pre-existing slow-growing cells grow before acquiring a full *lac*⁺ reversion mutation, as suggested by the amplification model. This experiment is difficult because spreading must be done after some growth has occurred but before any developing clone has acquired a full *lac*⁺ allele. It's also crucial to avoid pre-existing Lac⁺ cells, which will certainly grow and produce many colonies after spreading. The experiment was tried by Foster (Foster 1994) and by us (Quiñones-Soto and Toofan unpublished results). Both attempts failed to provide evidence of pre-existing cells that grow prior to reversion.

- 4) Are amplifications remodeled under selection? The original "amplification selection" model proposed that revertants were initiated by cells with a duplication or amplification of the large 100kb *lac* amplifications between IS3 copies. These cells were suggested to grow and amplify a bit under selection and then acquire either a point mutation to *lac*⁺ (aided by their multiple copies of DinB) or a join point deletion. The join point deletion reduces the size of the repeated unit and thereby the fitness cost so as to allow high amplification under selection. Without growth, this remodeling process seems unlikely.

Since then we've found evidence that the common large IS3-mediated duplications make no contribution to reversion and the shorter duplications (see Figure 3) that underlie the amplifications in unstable revertants actually arise during non-selective pre-growth (Kugelberg et al. 2010). These short duplications are sufficiently frequent in the plated population to explain all of the unstable Lac⁺ revertants (Reams et al. 2012). If SJ and TID duplications arise by remodeling of a pre-existing duplication, this must all occur during growth prior to selection. Because unstable revertants are initiated by cells that arise prior to plating, they cannot be

“stress-induced” and are essentially already remodeled prior to selection. We will propose below that the precursors of unstable revertants grow immediately after plating and improve under selection by further expansion of their amplified array. As expected for exponentially growing clones that become visible with time, the number of unstable revertants increases exponentially over a series of days (Figure 4). It seems clear that growth is central to development these colonies and they are initiated by cells that arise prior to selection. The question of growth involves primarily the origin stable Lac⁺ revertants.

The deeper meaning of growth and mutagenesis The question of growth under selection is critical because growth and mutagenesis are essentially trade-offs in trying to account for the 100 revertants that arise in this system. If growth is truly eliminated by selection, as seems likely, mutagenesis of some kind seems essential to explain the observed mutations. If growth occurs, then the exponential expansion may provide sufficient acts of replication to produce the revertants using the standard unenhanced basal mutation rate. We have resisted the idea of mutagenesis because the idea of an evolved mechanism to increase the genome-wide mutation rate temporarily seemed numerically unsanitary. Mathematical modeling of the “hypermutable state” model suggested that temporary up-regulation of genome-wide mutagenesis rate in a subset of the population is unlikely to be useful long-term due to the high cost of deleterious mutations and the persistence of deleterious mutations after any episode of hypermutability (Roth et al. 2006a). This modeling does not eliminate the mechanistically problematic possibility of “directed mutation”.

Our persistent confidence that growth must occur but somehow escape the tests listed above was based on the finding that stable Lac⁺ revertant colonies include rare cells that are unstably Lac⁺ (Hendrickson et al. 2002). The growth and amplification model predicts that stable revertant colonies should contain a few unstable precursor cells with an amplified mutant *lac* allele. Such ancestral cells were identified by their formation of sectorized (blue/white) colonies when streaked on rich X-gal medium and were taken as evidence for amplification and growth within the developing colony prior to reversion. These unstably Lac⁺ cells represented about one cell in 1000 stably Lac⁺ cells (Hendrickson et al. 2002). These sectorized colonies are visually indistinguishable from the extensively characterized cells in unstable Lac⁺ colonies, which carry an extensive *lac* amplification. However Hastings et al reported that the rare unstable cells within otherwise stably Lac⁺ colonies showed an unstable Lac⁺ phenotype that is not heritable (Hastings et al. 2004). We have confirmed their observation and found that the rare unstable cells do not have *lac* amplifications (which are heritable) but seem to have multiple copies of the F'*lac* plasmid, some Lac⁺ and some Lac⁻. This is an extremely unstable (poorly heritable) situation due to the mechanisms that control plasmid

copy number and distribution. This result suggested that *lac*⁺ revertant alleles might not arise in cells growing with amplification, but rather in cells with multiple copies of the whole F'*lac* plasmid. This is consistent with the evidence above that revertants are initiated by cells with multiple plasmid copies. This results re-opened the question of growth, since cells with multiple plasmid copies seem too unstable to form extensive clones under selection but might persist when division was blocked. Without growth it is difficult to explain the revertant number without some mutagenesis.

A new model resolves the conflicts. This model is consistent with the entire body of information accumulated by both sides of the Cairns debate and outlined above. In this model, reversion starts before selection (with a plasmid copy number increase) and the critical frameshift mutations occur under selection without prior growth and with no programmed increase in mutation rate.

- 1) The major feature of this model is that both stable and unstable Lac⁺ revertants are initiated by cells with an increased F'*lac* plasmid copy number. These cells arise before selection so the revertant colonies they initiate cannot be stress-induced.
- 2) While the initiator cells with multiple copies of F'*lac* arise before selection, their precise structure is unclear. These cells seem to have evaded standard plasmid copy-number controls. These plasmids may have transfer replication forks that have switched to rolling circle replication and produce double-stranded linear products that may be degraded as they are made. Alternatively actual transfer might be occurring as suggested in earlier studies (Galitski and Roth 1995; Radicella et al. 1995; Peters et al. 1996; Godoy and Fox 2000). Transfer could inject a single strand end that becomes double stranded, recombines and initiates a rolling circle replication fork using the recipient plasmid as template. Additional replication of *lac* in these non-growing cells may result from nicks introduced at *oriT*, which become double strand breaks when hit by a replication fork as suggested previously (Foster et al. 1996; Harris et al. 1996). Regardless of the nature of these cells, they replicate *lac* repeatedly over the course of several days using energy provided by the leaky *lac* allele. Each act of *lac* replication provides an opportunity for reversion. The DNA single strands exposed during this process induce an SOS response. The absence of cell growth induces the RpoS regulon. These two features have been suggested previously (Foster 2007; Galhardo et al. 2007).
- 3) After plating, these initiator cells do not grow exponentially, because they lack sufficient energy to fire their chromosomal replication forks. They use the energy provided by multiple *lac* copies to repeatedly replicate and repair their plasmids. The plasmid replication (from an unregulated origin) exploits available energy and allows continued copying of the plasmid in non-dividing cells. The

failure of cells to divide leaves them unable to easily reduce their plasmid copy number until growth resumes.

- 4) Formation of stable revertants (-1 frameshifts) is enhanced about 10-fold by the presence of a *dinB*⁺ gene anywhere on the F'*lac* plasmid. The increase in DinB protein results from the repeated *dinB* gene on the several plasmid copies. Mutations to *lac*⁺ form during localized over-replication of *lac* on the multiple plasmid copies. This replication alone is sufficient for some reversion, but the revertant yield is enhanced about 10-fold if DinB is amplified as part of the plasmid. Since cells are not dividing, the main chromosome is not replicating and is not subject to DinB mutagenesis.
- 5) The events described above do not reflect a process that is stress-induced in a standard sense. Selective conditions prevent cell division but allow repeated local over-replication of the F'*lac* plasmid. This over-replication is excessive only because chromosome replication and cell division is blocked.
- 6) When any one of the many copies of the mutant *lac* allele acquires a *lac*⁺ reversion event (-1 frameshift), energy is immediately supplied, allowing resumption of cell division and chromosome replication. All plasmids with non-revertant *lac* copies are lost by segregation as the cell divides and copy number controls come into play. The revertant F'*lac*⁺ copy is selectively maintained in cells with a normalized copy number. This stably *lac*⁺ colony contains no cells with a tandem *lac*⁺ amplification, but may include a few cells that have not yet diluted out all of their unreverted F'*lac* copies.
- 7) The unstable Lac⁺ revertants are also initiated by pre-existing cells with multiple copies of an F'*lac* plasmid, but the plasmid in these cells carry an internal *lac* duplication (SJ or TID). Cells with multiple copies of a duplication-bearing F'*lac* plasmid have enough energy to fire their replication origins and divide. Their plasmid copy number can be maintained in part by continued replication (requiring Tra functions), but drops progressively by unequal plasmid partitioning during growth. As plasmid copy number returns toward normal, the *lac* amplification expands and progressively improves growth. These cells ultimately produce a colony of unstable Lac⁺ colonies.
- 9) In principle, this model is identical to the initial selected tandem amplification model described above except that a) Plasmid copy number increases before selection and is maintained without

growth. Stable elevated plasmids copy numbers in non-growing cells take the place of tandem copy amplification during growth under selection. b) Local over-replication of the whole plasmid in non-growing cells replaces cell growth to provide opportunities for reversion.

10) Pre-existing cells with multiple copies of an F'/lac plasmid may be the equivalent to the rare “hypermutable” cells in mutagenesis models. Under selection, cells in this state replicate their F' plasmid and lac region in the presence of high levels of DinB, but do not replicate their chromosome are not generally mutagenized.

11) Mutagenesis in this model is “directed” in two senses. Only the F'/lac replicates in the presence of elevated DinB levels. Following a rare act of reversion, the population of unreverted lac alleles that allowed this event is lost and only the rare lac^+ allele is retained selectively. This has the effect of directing mutagenesis to the precise base pairs that limit ability to grow on lactose (Roth et al. 1996). This point will be discussed more below.

Relevance of the Cairns-Foster system to evolution under selection. Since the advent of the Cairns-Foster system, it has become clear that copy number variations are the most common genetic changes in natural populations and in populations of metazoan somatic cells (reviewed by Reams and Roth 2014 (in press)). Selective gene amplification underlies progression of many malignancies (Albertson 2006). The original selection-amplification model for the Cairns system involved growth and improvement under selection. This first model seems to explain the Salmonella version of the Cairns-Foster system and has been experimentally verified for a variety of other biological situations, including evolution of bacterial antibiotic resistance (Nilsson et al. 2006; Sandegren and Andersson 2009; Sun et al. 2009; Paulander et al. 2010; Pranting and Andersson 2011) and adaptation of poxvirus to host defenses (Elde et al. 2012). Most generally, the selective amplification model offers an explanation for evolution of novel genes under selection (Bergthorsson et al. 2007) and has been experimentally shown to produce a new gene within 3000 generations of growth under selection (Näsvalld et al. 2012). Thus the Cairns-Foster system has suggested a broadly relevant biological process.

Despite these successes, it now appears that this original model does not quite explain the *E. coli* version of the Cairns-Foster system in which very little growth occurs. Resolution of several conflicts led to the new model described above which does explain the *E. coli* system. This new model may provide answers to questions that have broad evolutionary applicability.

- 1) How does amplification mimic directed mutagenesis? Despite a considerable fitness cost, duplications and amplifications are held as stable polymorphisms in unselected populations (Reams et al. 2010). These steady state frequencies are high because duplications and amplifications form at a high rate. Steady-state frequencies increase when selection favors cells with multiple copies of some near-neutral allele. Amplification of such an allele can provide a selective value that exceeds the cost of the duplication itself. This allows lineages with amplifications to expand and add copies of a growth-limiting allele to the genome. These cells grow faster and replicate more copies of the rate-limiting allele at each division. Each act of replication provides an opportunity for further mutations that improve the functionality of the limiting allele. In the Cairns-Foster system, the mutant *lac* allele has a very low ability to support growth on lactose and is, in a sense, a near-neutral allele that can provide a beneficial phenotype when amplified. Thus selection gives the appearance of mutagenesis that actually results from replicating more copies of the target allele. (See Figure 5)
- 2) How can selection direct mutation to critical sites sites? Direction of mutation to selectively valuable sites is a side-effect of amplification under selection. As soon as an improving mutation occurs in any one of the multiple *lac* copies, selection holds that improved allele and allows loss of other copies of that gene. Deleterious alleles of the gene in question are selectively lost from the lineage. The apparent directedness is due to the dynamic control of gene copy number --- loss and reamplification occurs continuously and affects genes carried in either tandem arrays or on multi-copy plasmids. In essence, the multiple gene copies in an amplified array (or multiple plasmid copies in a cell) constitute a population that is under selection to improve. The probability that a final revertant plasmid has an associated unselected mutation is the same as the probability of that associated mutation arising in any single copy of the gene with no selection or amplification. Thus amplification enhances the likelihood that some *lac*⁺ mutation will occur, but does not enhance the likelihood of an associated unselected mutation, because selection fixes only the revertant allele. Mutations are in-effect directed to the exact base pairs that provide the selectable phenotype and are not made more likely at other sites.

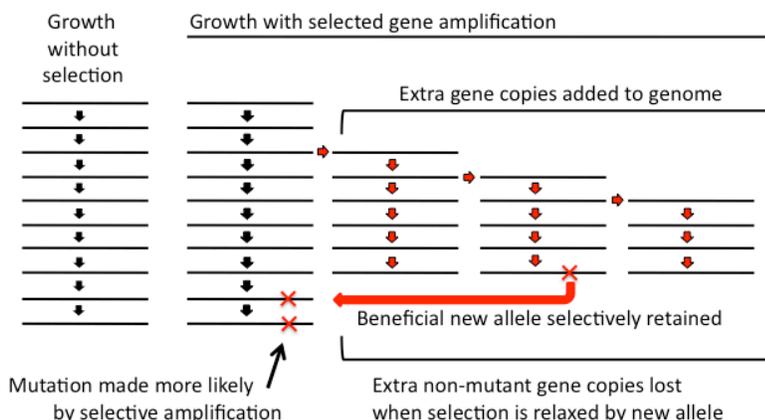


Figure 5 How *lac* amplification enhances revertant yield without mutagenesis Each act of *lac* replication provides an opportunity for a reversion event (frameshift). A cell with only one *lac* copy has one opportunity to revert for each cell division or plasmid replication (see left column). As the *lac* allele amplifies, each cell gains additional chances for a reversion event with no increase in mutation rate (right column). If the amplified array improves growth on lactose, then the lineage on the right expands faster than that on the left (without an amplification) and this growth also adds to the likelihood of a reversion event. In effect, amplification directs mutations to the exact base pairs that limit growth, because once a revertant allele forms, selection holds only that allele while the non-revertant alleles are no longer selectively retained. The likelihood of an unselected mutation near *lac* is the same with and without selection, since only the revertant allele is kept in the genome.

3) Natural selection without reproduction. It is generally assumed that natural selection requires cell division since improvement (or purification) is attributed to differential reproductive success of cells with different genotypes. In the Cairns-Foster system, the plated cell population is subjected to selection that prevents growth (just barely). The growth-limiting gene (on the F'/lac plasmid) is repeatedly copied until an improving mutation occurs. A revertant allele triggers exponential growth and is selectively held, while non-revertant alleles are lost as soon cell growth starts and as the population expands.

The increase in *lac* copy number occurs prior to selection, by some loss of plasmid copy number control. On lactose medium, cells cannot divide and have no way to reduce their copy number. They can however replicate their plasmids and use DNA repair mechanisms to heal plasmid breaks introduced by the *Tral* protein and/or recombine DNA fragments with recipient

plasmids during redundant conjugation. Thus the plasmid is a subset of the genome that is over-replicated in non-dividing cells. Cells are trapped in this state until a reversion event occurs.

Mutagenesis by over-replication may be relevant to progression of some malignancies (Albertson 2006). Somatic cells that grow very little may repeatedly repair regions of their genome surrounding fragile sites, which are subject to frequent breakage. In this situation, non-growing cells are under strong selection to grow and may repeatedly replicate specific regions of their genome during break repair. This may be mimicked in the Cairns-Foster system where breaks induced at *oriT* stimulate mutagenesis of the *F'**lac* plasmid. We submit that the phenomenon of break-induced mutagenesis (Shee et al. 2011; Rosenberg et al. 2012) may reflect repeated repair and local over-replication in non-growing cells and involve no increase in mutation rate.

Concluding remarks At first glance, the Cairns-Foster system appears simple and seems to demonstrate stress-induced mutagenesis of non-growing cells. This conflicts with the classical conclusion that selection and mutation are independent. Deeper inspection reveals that the behavior of this system does not require mutagenesis but relies on several aspects of selection. The apparent direction of mutations to valuable sites results from selective amplification of the growth-limiting gene and selective retention of the improved allele (a form of selection). The modest increase in general mutation rate caused by *DinB* is an artifact due to the chance proximity of the *dinB* and *lac* genes, which enables selective co-amplification. Cells in a hypermutable state are those with multiple copies of the *F'**lac* plasmid that includes *lac* and *dinB*. These cells arise prior to selection and are not stress-induced.

The response of populations to selection can be rapid when it exploits two of the most common mutation types known. Duplications and amplifications form frequently and are held in populations as stable genetic polymorphisms. Near-neutral beneficial alleles are extremely common and gain significant value when amplified. Selection thus gives the appearance of mutagenesis by driving rapid progressive genetic adaptation with no programmed change in mutation rate.

ACKNOWLEDGEMENTS

We thank lab mates Emiko Sano, John Paul Aboubechara, Semarhy Quiñones-Soto, Andrew Reams, Eric Kofoid, Douglas Huseby for ideas and critique. We thank Ivy Roush for allowing us to cite her unpublished work. We thank colleagues Wolf Heyer, Neil Hunter and Stephen Kowalczykowski for suggestions. We thank collaborators Dan Andersson and Diarmaid Hughes of Uppsala University for constant encouragement. Most of all we thank John Cairns and our loyal

opposition – Susan Rosenberg and Pat Foster -- and for their persistent and incisive defense of stress-induced mutagenesis. Their models have provided constant stimulation even though we're confident that they are incorrect.

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