Evidence That Selected Amplification of a Bacterial *lac* Frameshift Allele Stimulates Lac$^+$ Reversion (Adaptive Mutation) With or Without General Hypermutability

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ABSTRACT

In the genetic system of Cairns and Foster, a nongrowing population of an *E. coli lac* frameshift mutant appears to specifically accumulate Lac$^+$ revertants when starved on medium including lactose (adaptive mutation). This behavior has been attributed to stress-induced general mutagenesis in a subpopulation of starved cells (the hypermutable state model). We have suggested that, on the contrary, stress has no direct effect on mutability but favors only growth of cells that amplify their leaky mutant *lac* region (the amplification mutagenesis model). Selection enhances reversion primarily by increasing the mutant *lac* copy number within each developing clone on the selection plate. The observed general mutagenesis is attributed to a side effect of growth with an amplification—induction of SOS by DNA fragments released from a tandem array of *lac* copies. Here we show that the *S. enterica* version of the Cairns system shows SOS-dependent general mutagenesis and behaves in every way like the original *E. coli* system. In both systems, *lac* revertants are mutagenized during selection. Eliminating the 35-fold increase in mutation rate reduces revertant number only 2- to 4-fold. This discrepancy is due to continued growth of amplification cells until some clones manage to revert without mutagenesis solely by increasing their *lac* copy number. Reversion in the absence of mutagenesis is still dependent on RecA function, as expected if it depends on *lac* amplification (a recombination-dependent process). These observations support the amplification mutagenesis model.

ACCORDING to the neo-Darwinian view of evolution, mutations arise at random with respect to their phenotypic consequences. Selection does not stimulate mutation formation, but acts at a population level to eliminate deleterious mutations and favor the rare mutations that improve fitness (Mayr 1982). Classic experiments demonstrate that at least a fraction of total mutations arise independently of selective stress (Luria and Delbruck 1943; Lederberg and Lederberg 1952). However, this evidence does not exclude the possibility that another fraction of total mutations arises in response to selective conditions. Over the past 10 years, experimental observations have suggested that bacteria might be able to alter their mutability purposefully in response to stress (Cairns *et al.* 1988; Hall 1990, 1997; Cairns and Foster 1991; Torkelson *et al.* 1997; Wright 2000).

In an experimental system designed by Cairns and Foster, a population of *Escherichia coli* cells with a particular *lac* frameshift mutation is starved on medium containing lactose as the sole carbon source. The nongrowing population appears to give rise specifically to Lac$^+$ revertants, but very few unselected mutations (Cairns *et al.* 1988; Cairns and Foster 1991; Bull *et al.* 2001). Initially, this behavior was attributed to a mechanism that senses starvation and directs mutations to sites that relieve stress (Cairns *et al.* 1988; Foster and Cairns 1992). Several proposed mechanisms were eliminated for this system (Davis *et al.* 1980; Stahl 1988; Foster 1992). Interest in directed mutation waned when it was found that Lac$^+$ revertant clones (but not the starved parent population) are generally mutagenized in the process of reversion (Torkelson *et al.* 1997; Rosche and Foster 1999).

According to the hypermutable state model (Hall 1992), the behavior of the Cairns system reflects stress-induced mutagenesis. This model proposes a regulatory mechanism (evolved under selection) that responds to selective stress by generally mutagenizing a subset (10^5 cells) of the nongrowing population (10^8 cells). This mechanism evolved because it facilitates genetic adaptation to stress. Mutations appear to be directed to valuable sites, because mutagenesis kills (with lethal mutations) most of the mutagenized cells; only Lac$^+$ revertants escape, because they relieve the stress and shut off muta-

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genesis. The detected Lac$^+$ revertants reflect reversion events that occurred before any lethal mutation. The unselected mutations associated with *lac* reversion are those that occurred prior to *lac* reversion.

In contrast, the amplification mutagenesis model proposes that selection has no direct effect on mutation, but acts only at a population level to favor a succession of progressively faster-growing cell types (Andersson *et al.* 1998; Hendrickson *et al.* 2002). Cells with a *lac* duplication arise during nonselective growth. On selective medium, such cells initiate clones within which a sequence of events occurs—*lac* amplification (tandem repeats), reversion of one allele to *lac*$^+$, and segregation of haploid *lac*$^+$ cells. Each step in the process allows faster growth. This process culminates when haploid *lac*$^+$ revertant cells overgrow the original clone of cells with an amplification (Andersson *et al.* 1998; Hendrickson *et al.* 2002). Outside of these clones, the plated lawn (10^8 cells) may grow very little or not at all (Cairns and Foster 1991; Foster and Cairns 1992; Foster 1994). The process is proposed to be rapid because duplication, amplification, and segregation are all stimulated by the F′ plasmid (Slechta *et al.* 2002) on which *lac* must be located (Galitski and Roth 1995; Radicella *et al.* 1995; Godoy and Fox 2000). This basic model does not include general mutagenesis, but proposes that selection enhances appearance of Lac$^+$ revertants only by adding replicating *lac* copies (mutation targets) to each developing clone (amplification and growth). However, unselected mutations are frequent in *lac*$^+$ revertants (Torkelson *et al.* 1997) and must be explained.

General mutagenesis associated with reversion in the Cairns system is due to induction of the error-prone DinB polymerase, which is part of the SOS repair system (McKenzie *et al.* 2001), possibly in combination with inhibition of the methyl-directed mismatch repair (MMR) system (Harris *et al.* 1997; Foster 1999a). Interpreted according to the hypermutable state model, these results suggest that the evolved mechanism induces SOS and inhibits MMR in response to starvation. This results in general mutagenesis, which must occur during recombinational replication since it is claimed that mutagenized cells are in stationary phase (Foster 1999b). This programmed mutagenesis is said to be central to reversion under selection(Rosenberg 2001).

On the contrary, the amplification mutagenesis model proposes that mutagenesis is not regulated but is rather an unavoidable side effect of growth with a gene amplification—SOS induction. The model suggests that during growth with an amplification, segregation events release from the tandem array DNA fragments that are resected to single strands—the inducer of SOS and its error-prone DinB polymerase. Segregation and reamplification occur repeatedly during growth under selection. The mismatches caused by induced DinB saturate the MMR system and lead to mutations. SOS mutagenesis is thus an unavoidable side effect of growth with an amplification and is not caused by an evolved stress-sensing mechanism. Mutagenesis increases the yield of *lac* revertants (but also adds deleterious associated mutations). The model proposes that mutagenesis is not essential to the primary process (amplification and growth) by which selection stimulates reversion. This proposal is examined here.

A *Salmonella enterica* analog of the Cairns system was used to test several aspects of the amplification mutagenesis model. In *S. enterica*, as in *E. coli*, *lac* revertants that arise under selection experience, respectively, an SOS-dependent 50- and 20-fold increase in general mutation rate. Surprisingly, in both organisms, blocking mutagenesis reduces revertant yield only 2- to 4-fold. The discrepancy in mutagenic effects is explained by the amplification mutagenesis model, which predicts that, without mutagenesis, amplification clones continue growing and some attain sufficient *lac* copies to realize reversion without mutagenesis. Two predictions were verified. First, RecA function is required for reversion even in the absence of mutagenesis, presumably because recombination is needed for amplification. Second, revertant clones arising without mutagenesis contain a higher proportion of cells with an amplification (an unstable Lac$^+$ phenotype), presumably because the clone of amplification cells grew larger before reversion and was less overgrown by haploid revertant types. These results are discussed in terms of the current evidence (and persistent questions) for the amplification mutagenesis model.

MATERIALS AND METHODS

Strains: All strains are derivatives of *S. enterica* (serovar Typhimurium, LT2) (see Table 1). The mutant F′ *lac* plasmid was provided in an *E. coli* strain (FC40) by Pat Foster. A plasmid with the *E. coli lexA3*(Ind$^-$) allele was provided by John Little.

Media and chemicals: The minimal medium was NCE salts (Berkowitz *et al.* 1968), containing the appropriate carbon source (glycerol or lactose) at a concentration of 0.2% (w/v) plus nutritional supplements at the concentrations recommended by Davis *et al.* (1980). The rich medium was nutrient broth (NB; Difco Laboratories, Detroit) supplemented with 5 g/liter NaCl. MacConkey agar base medium was used to identify carbon source utilization mutants (propanediol, maltose, xylose, or fructose added at 1%). E-glucose leucine medium was used to identify auxotrophs. Media were solidified with 1.5% BBL agar. Final concentrations of antibiotics in rich media were 50 μg/ml kanamycin sulfate (Kn), 20 μg/ml tetracycline (Tc), and 10 μg/ml chloramphenicol (Cm). The chromogenic β-galactosidase substrate 5-bromo-4-chloro-3-indolyl-β-d-galactopyranoside (X-Gal; Diagnostic Chemicals, Oxford, CT) was used at either 25 μg/ml in minimal media or 40 μg/ml in NB for identifying sectored unstable *lac*$^+$ colonies. Unless otherwise specified, chemicals were obtained from Sigma Chemical Company (St. Louis).

Construction of the *lexA33*(Ind$^-$) and *recA*(O^c) mutants: The *lexA3* mutant of *E. coli* is strongly deficient in SOS induction (Markham *et al.* 1981). The entire *E. coli lexA3* gene was PCR amplified and introduced in place of the chromosomal *S.*

TABLE 1

Strains

Strain	Genotype	Source
LT2	Wild-type *S. enterica* (serovar Typhimurium, LT2)	(TR10,000) lab collection
TT18302	*leuD21 proB1657*::Tn*10/F*$'_{128}$ *pro*$^+$ *lacI*^{q} *lacI33*(fs) *lacIZ* (Ω)	Plasmid from Pat Foster
TT18306	*leuD21 proB1657*::Tn*10 recA1/F*$'_{128}$ *pro*$^+$ *lacI*^{q} *lacI33*(fs) *lacIZ* (Ω)	
TT21657	*leuD21 proB1661*::Tn*5*/F$'_{128}$ *pro*$^+$ *lacI*^{q} *lacI33*(fs) *lacIZ* (Ω)	
TT22964	*lexA33*::(Cm *lexA3)*(sw)	
TT23152	*leuD21 proB1657*::Tn*10 recA281*(O^c) *zgc-9189*::Cm (sw)/F$'_{128}$ *pro*$^+$ *lac*I^q *lacI33*(fs) *lacIZ* (Ω)	
TT23153	*leuD21 proB1657*::Tn*10 lexA33*::(Cm *lexA3)*(sw)/F$'_{128}$ *pro*$^+$ *lacI*^{q} *lacI33*(fs) *lacIZ* (Ω)	
TT23154	*leuD21 proB1657::Tn10* recA281(O^c) *zgc-9189::Cm(sw) lexA33*::(Cm *lexA3)*(sw)/F$'_{128}$ *pro*$^+$ *lacI*^{q} *lacI33*(fs) *lacIZ* (Ω)	
TT23254	*leuD21 proB1661*::Tn*5 lexA33*::(Cm *lexA3)*(sw) *recA642*::Tn*10*dT-POP/F$'_{128}$ *pro*$^+$ *lacI*^{q} *lacI33*(fs) *lacIZ* (Ω)	
TT23253	*leuD21 proB1661*::Tn*5 lexA33*::(Cm *lexA3)*(sw)/F$'_{128}$ *pro*$^+$ *lacI*^{q} *lacI33*(fs) *lacIZ* (Ω)	

enterica lexA$^+$ gene by linear transformation (Poteete and Fenton 1984; Datsenko and Wanner 2000; Yu *et al.* 2000; K. Bunny, J. Liu and J. R. Roth, unpublished results); a nearby chloramphenicol resistance determinant was used as a selective marker; the resulting allele is designated *lexA33* and is designated below with its phenotype (Ind$^-$) to make clear that it is defective for induction of SOS (not constitutive as expected for a null allele). A *recA*(O^c) mutation characterized in *E. coli* (Ginsburg *et al.* 1982) was synthesized within a primer used to amplify a chloramphenicol resistance determinant and introduced near *recA*$^+$ in the *S. enterica* chromosome by linear transformation. Details of these constructions will be presented elsewhere (K. Bunny, J. Liu and J. R. Roth, unpublished results).

Reversion tests: Strains were pregrown overnight in NCE glycerol medium with amino acid supplements (if needed) at standard concentrations (Galitski and Roth 1995, 1996). Cells were pelleted, washed in NCE, plated (2×10^8) on selective medium (NCE lactose, X-gal, and leucine), and incubated at 37° for 6 days. In addition to the *lac* tester cells, 1×10^9 scavenger cells (*S. enterica* LT2 or derivatives, which do not contain a *lac* operon) were also added to the lactose plates to consume any carbon sources other than lactose that might contaminate the agar. The number of Lac$^+$ revertant colonies was counted each day from day 2 to day 5. Each data point in the numbers is the mean and standard error of at least 20 independent measurements.

Identifying *lac*$^+$ revertants with associated nonselected mutations: The *lexA*$^+$ and *lexA33*(Ind$^-$) tester strains (TT18302 and TT23153) were grown overnight in NCE glycerol medium, diluted into the same medium, and dispensed into 96-well plates at a density of 10^4 cells per 200 μl. The cultures were incubated overnight with shaking at 37° and then pelleted and resuspended in 100 μl of NCE. Cells were plated onto NCE lactose X-gal selection plates with a collection of amino acids, nucleic acid bases, and vitamins that cannot be used as carbon sources by *S. enterica* (Typhimurium, LT2; Gutnick *et al.* 1969). These supplements (histidine, isoleucine, leucine, lysine, methionine, phenylalanine, threonine, tryptophan, tyrosine, valine, adenine, guanine, thymine, uracil, and thiamine) were added at concentrations described previously (Davis *et al.* 1980). The appropriate scavenger cells (LT2 or TT22964) were plated (1×10^9 cells) with the testers and plates incubated for 5 days at 37°. Newly appearing Lac$^+$ revertant colonies were picked on days 2 and 5, purified on selection medium, patched to E-glucose plates containing the above supplements, and incubated overnight at 37°. These patches were replica printed to E-glucose leucine medium to identify auxotrophs and to MacConkey-maltose, MacConkey-fructose, MacConkey-xylose, and MacConkey-propanediol to identify various fermentation mutations. To determine whether the revertant had arisen in the tester (Tc^R) or in the scavenger strain (Tc^S) following F′ *lac* transfer, revertants were printed to NB-tetracycline. All replica plates were incubated overnight at 37°. Propanediol plates were incubated anaerobically to identify mutants deficient in propanediol utilization or cobalamin (vitamin B_{12}) synthesis. (Both mutant types fail to use propanediol under these conditions.) Auxotrophs were identified by inability to grow on E-glucose leucine medium; carbon source utilization mutants were identified as white patches on one of the MacConkey sugar media. Revertants that occurred following transfer of F′ *lac* to a scavenger were identified by their sensitivity to tetracycline.

Identifying unstable Lac$^+$ cells in revertant colonies: The entire revertant colony was suspended in saline, diluted, and plated on NB medium containing the chromogenic β-galactosidase substrate, X-gal. Unstable Lac$^+$ cells form colonies that are blue with many white (Lac$^-$) sectors; it has been shown elsewhere that cells that form colonies with this phenotype carry a tandem array of *lac* copies (Tlsty *et al.* 1984; Whoriskey *et al.* 1987; Andersson *et al.* 1998; Hastings *et al.* 2000; Hendrickson *et al.* 2002).

RESULTS

Experimental design: Lac$^+$ revertants were selected on minimal lactose medium containing a mixture of added nutrients that cannot serve as a carbon source but can satisfy the nutritional requirement of a variety of auxotrophs (Gutnick *et al.* 1969). Each Lac$^+$ revertant was scored to detect an associated unselected mutation in any of 100 genes. All of the experiments described here were performed with cells of *S. enterica* (Typhimurium, LT2) that carry the mutant F′ *lac* plasmid used by Cairns and Foster (1991). These mutant cells (10^8) were plated with 10^9 scavenger cells—nonrevertible Lac$^-$ mutants that carry no F′ plasmid. Scavenger cells are included to consume traces of usable carbon sources contaminating the medium, but they can also act as conjugational recipients and acquire a plasmid by transfer from tester cells. In the course of the reversion experiment, tester cells divide less than once a day, not enough to account for the yield of revertants. The *S. enterica* system used behaves in every way like the *E. coli* system described previously (Foster 1999b). The *S. enterica* and *E. coli* systems are compared later.

Transfer of the F′ plasmid from tester to scavenger cells is not restricted during reversion under selection. A mutant F′ *lac* plasmid can be transferred into a scavenger cell and then revert to Lac$^+$. To test the importance of SOS induction, we used various tester and scavenger pairs that carried either the *lexA*$^+$ allele (allows SOS induction) or the *lexA33*(Ind$^-$) allele (prevents SOS induction; Friedberg *et al.* 1995; McKenzie *et al.* 2000; K. Bunny, J. Liu and J. R. Roth, unpublished results).

According to the hypermutable state model, SOS mutagenesis causes both reversion to Lac$^+$ and general mutability. That is, all Lac$^+$ revertants should appear only in *lexA*$^+$ cells (which can induce SOS). They can therefore arise in a *lexA*$^+$ tester or in a *lexA*$^+$ scavenger following transfer. In contrast, the amplification mutagenesis model predicts that selection can stimulate reversion without mutagenesis simply by allowing growth and increasing the *lac* copy number; the secondary SOS mutagenesis contributes to, but is not essential for, *lac* reversion. According to this model, failure to induce SOS should eliminate associated unselected mutations but cause only a partial defect in *lac* reversion. The latter prediction is fulfilled by the results below.

Effect of a *lexA33*(Ind$^-$) mutation on reversion to Lac$^+$ under selection: Figure 1 shows the Lac$^+$ reversion behavior of the four tester/scavenger cell combinations [*lexA*$^+$/*lexA*$^+$, *lexA*$^+$/*lexA33*(Ind$^-$), *lexA33*(Ind$^-$)/*lexA*$^+$, and *lexA33*(Ind$^-$)/*lexA33*(Ind$^-$)]. The following points should be noted. Preventing SOS induction in both the tester cells and the scavenger cells [*lexA33*(Ind$^-$)/*lexA33*(Ind$^-$)] causes the maximal three- to fourfold decrease in Lac$^+$ revertants under selection, as shown previously (McKenzie *et al.* 2000, 2001). A *lexA33*(Ind$^-$) mutation in the scavenger has very little effect on revertant number when the tester is *lexA*$^+$. A *lexA33*(Ind$^-$) allele in the tester causes only about a twofold reduction in total revertant number when the scavenger is *lexA*$^+$, suggesting that scavenger cells might contribute to some reversion events (confirmed below).

Blocking SOS induction eliminates associated mutations: Regardless of the *lexA* genotypes, most (>70%) of the Lac$^+$ colonies arising on day 5 reflect reversion events on plasmids carried by the original tester strain (Table 2). In the *lexA33*(Ind$^-$)/*lexA*$^+$ (tester/scavenger) combination, the overall frequency of Lac$^+$ revertants dropped only twofold (Figure 1), and 70% of the Lac$^+$ revertants arose in the *lexA33*(Ind$^-$) tester strain (Table 2). However, none of these Lac$^+$ revertants showed associated mutations. Thus considerable *lac* reversion occurs without SOS induction or general mutagenesis. In contrast, the minority of Lac$^+$ (30% = 289) revertants that arose following transfer to the *lexA*$^+$ scavenger cells was frequently associated with unselected mutations (11/289). All of the 42 associated mutations detected in this experiment were found in a *lexA*$^+$ background.

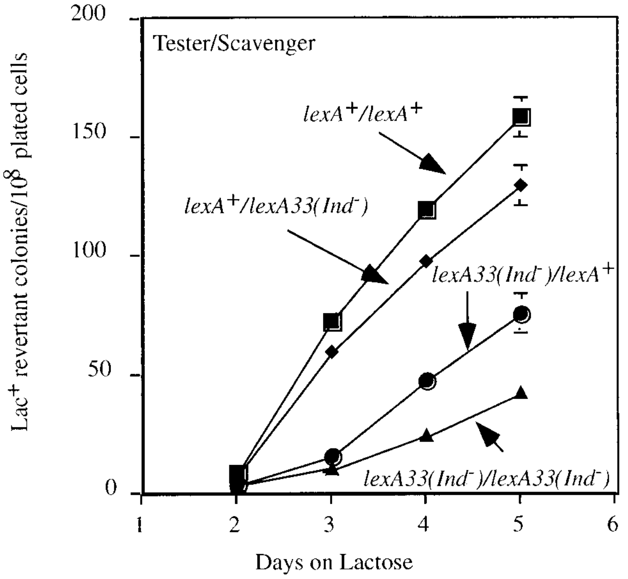

Figure 1.—Effect of *lexA*$^+$ and *lexA33*(Ind$^-$) mutations on frequency of *lac* revertants. Tester strains carried the revertable *lac* frameshift mutation on an F′ *lac* plasmid and, in the chromosome, either a *lexA*$^+$ or a *lexA33*(Ind$^-$) allele (strains TT18302 or TT23153). Scavenger cells had no *lac* region and thus were nonrevertably Lac$^-$; these were either *lexA*$^+$ (LT2) or *lexA33*(Ind$^-$) (TT22964). In the graph, each line is designated with the *lexA* genotype of tester and scavenger. Revertant colonies from one set of plates were counted on each day of the experiment. A larger parallel set of plates was used as the source of colonies picked on day 2 and day 5 that were tested for the frequency of associated unselected mutation.

It is interesting to note that the intensity of mutagenesis appears to be slightly higher in revertants that arose following transfer to a *lexA*$^+$ scavenger. Of 476 *lac* revertants that arose in this way, 12 (2.5%) had an associated mutation. Of the 1856 Lac$^+$ revertants that arose in a *lexA*$^+$ tester (with no required transfer), 30 (1.6%) carried an associated mutation. This suggests that F′ transfer *per se* may help induce SOS and mutagenesis independent of (or in addition to) the induction caused by amplification under selection (R. Kamph and D. I. Andersson, unpublished results). In *lexA33*(Ind$^-$) testers, the number of Lac$^+$ revertants is reduced only ~3-fold (see Figure 1), but the frequency of associated mutations in those revertants is reduced at least 30-fold.

This was determined by taking the number of day 5 Lac$^+$ revertants generated from experiments with a *lexA33*(Ind$^-$) tester (Figure 1) and subtracting the fraction that arose after transfer to a *lexA*$^+$ scavenger (Table 2). This corrected number of Lac$^+$ revertants and their

TABLE 2

Distribution of Lac$^+$ revertants and associated mutations in different *lexA* backgrounds

Genotype of cells[a] (ratio 1:10)		Day 2 revertants			Day 5 revertants		
		Lac$^+$ revertants	Associated auxotrophs		Lac$^+$ revertants	Associated auxotrophs	
Tester scav.[b]	Scavenger	[% in tester (total scored)]	No. in tester[b]	No. in scav.[b]	[% in tester (total scored)]	No. in tester[b]	No. in scav.[b]
lexA$^+$	*lexA*$^+$	99 (1047)	2	0	83 (1113)	16 (926)	1 (187)
lexA$^+$	*lexA33*	98 (1012)	0	0	92 (1009)	14 (930)	0 (79)
lexA33	*lexA*$^+$	98 (931)	0	0	71 (1013)	0 (724)	11 (289)
lexA33	*lexA33*	100 (936)	0	0	96 (985)	0 (947)	0 (38)

[a] The *lexA33* allele confers inability to induce the SOS regulon.

[b] Differences between the aggregate auxotroph frequency on day 5 in *lexA*$^+$ (42/2332) and *lexA33* (0/1788) strains are extremely significant ($P < 0.0001$). Differences between auxotroph frequency on day 2 and day 5, when both tester and scavenger were *lexA*$^+$ (2/1047 and 17/1113), are very significant ($P = 0.022$). The aggregate frequencies of auxotrophs on days 2 and 5 (2/3926 and 42/4120) are extremely significant ($P < 0.0001$). The frequencies of auxotrophs found for various combinations of tester and scavenger on day 2 (2/1047, 0/1012, 0/931, 0/936) are not significantly different ($P > 0.5$).

frequency of associated mutations was compared to the same values seen for revertants that arose in the *lexA*$^+$ tester strain.

We estimate that (in a *lexA*$^+$ strain) selection increased the general mutation frequency in Lac$^+$ revertants ∼50-fold. To make this estimate, the day 2 Lac$^+$ revertants from all experiments (assuming they had not experienced induced mutagenesis) were pooled with the Lac$^+$ revertants that arose in *lexA33*(Ind$^-$) cells (where SOS induction was impossible). The frequency of associated mutations in this unmutagenized pool was compared to the frequency of associated mutations in Lac$^+$ cells that arose under selection in *lexA*$^+$ cells. This gave a basal frequency of associated mutations of 0.035% (2/5714) without SOS and 1.8% (42/2332) with induced SOS mutagenesis—roughly a 50-fold increase. This is in general agreement with the *E. coli* data of Rosche and Foster (1999), who estimated a 20-fold increase. Since both estimates are based on small numbers, we have used the average (35-fold) when discussing increased mutability during selection.

General mutagenesis is not associated with reversion under nonselective conditions: Revertant colonies that appeared on day 2 are presumed to reflect mutational events that occurred during nonselective pregrowth on glycerol (see materials and methods). Almost all (>98%) of these Lac$^+$ revertant colonies carry the revertant F′*lac* plasmid in the tester strain regardless of the *lexA* genotype and only 2 of 4000 Lac$^+$ revertants tested showed an associated mutation (Table 2). Finding two hits on a 100-gene target among 4000 colonies corresponds to a frequency of mutants of $\sim 5 \times 10^{-6}$ per gene, which is reasonable for spontaneous null mutations.

The effect of the *lexA33*(Ind$^-$) mutation is not due to reduced RecA levels: Reversion under selection is strongly reduced by a *recA* mutation (Foster 1999b) and less severely by a *lexA33*(Ind$^-$) mutation (McKenzie *et al.* 2000, 2001). These effects might be related since the *recA* gene is repressed by the LexA repressor protein. Therefore it is possible that a *lexA33*(Ind$^-$) mutation might simply be super-repressing expression of RecA protein. We eliminated this possibility by introducing a *recA* operator constitutive mutation, defined in *E. coli* (Ginsburg *et al.* 1982), into *S. enterica* as described in materials and methods (K. Bunny, J. Liu and J. R. Roth, unpublished results). This operator constitutive mutation restores full recombination ability to *S. enterica* strains with overproduced LexA or LexA33(Ind$^-$) protein (K. Bunny, J. Liu and J. R. Roth, unpublished results). Moreover, the chromosomal *lexA33*(Ind$^-$) mutation causes no reduction in recombination ability as judged by transduction (K. Bunny, J. Liu and J. R. Roth, unpublished results). As seen in Figure 2, the *lexA33*(Ind$^-$) mutation reduced reversion just as much in the *recA*(O^c) strain as in a *recA*$^+$ strain. Thus the *lexA33*(Ind$^-$) mutation seems to reduce reversion by preventing SOS induction and not by reducing recombination ability. This was demonstrated previously for *lexA* mutants of *E. coli* (McKenzie *et al.* 2000).

SOS-independent reversion under selection depends on RecA function: The amplification mutagenesis model proposes that growth and amplification under selection should stimulate reversion during selection even without general mutagenesis. This would occur simply because of the increase in *lac* copy number within the developing clone. If this is true, then any function that contributes to amplification should have an effect on reversion even in strains that lack SOS mutagenesis.

Several observations provide indirect support for this idea. In both *S. enterica* (Figure 1) and *E. coli* (McKenzie *et al.* 2000, 2001) reversion dropped only ∼2- to 4-fold in *lexA33*(Ind$^-$) strains that cannot induce SOS muta-

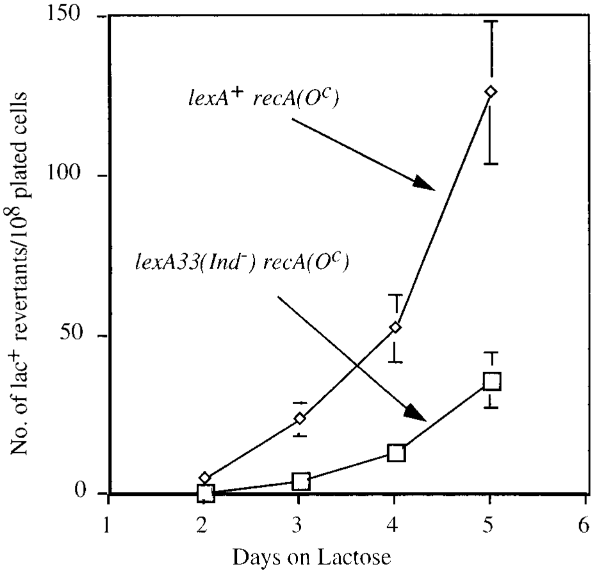

FIGURE 2.—The effect of a *lexA33*(Ind$^-$) mutation is not due to repression of *recA*. Strains tested both carry a *recA*(O^c) operator constitutive mutation and either a *lexA*$^+$ (strain TT23152) or a *lexA33*(Ind$^-$) allele (strain TT23154). In both cases, LT2 cells served as scavengers. The *recA*(O^c) mutation was shown elsewhere to prevent repression of *recA* by overexpressed LexA33(Ind$^-$) protein (K. BUNNY, J. LIU and J. R. ROTH, unpublished results).

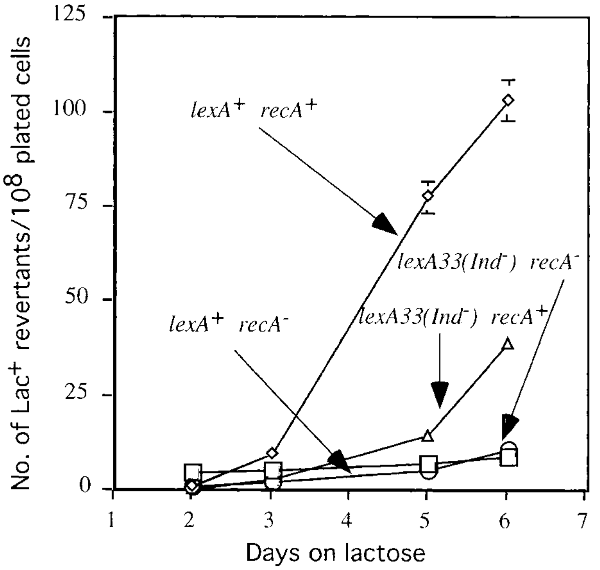

FIGURE 3.—Reversion under selection in a *lexA33*(Ind$^-$) strain (no SOS) depends on RecA function. Tester strains used were the following: *lexA*$^+$, *recA*$^+$ (TT18302), *lexA*$^+$, *recA* (TT18306), *lexA33*(Ind$^-$), *recA*$^+$ (TT23153), *lexA33*(Ind$^-$), *recA* (TT23254). In each case, strain LT2 served as the scavenger cells.

genesis. In contrast, a *recA* mutation reduces reversion >10-fold (GALITSKI and ROTH 1995; FOSTER 1999b; SLECHTA *et al.* 2002). Similarly, blocking growth of cells with an amplification caused at least a 10-fold reduction in reversion (HENDRICKSON *et al.* 2002), while lack of mutagenesis caused only a 3-fold reduction. These results suggest indirectly that recombination (and amplification) contribute to reversion in ways that are independent of SOS induction and general mutagenesis. According to the amplification mutagenesis model, the main role of RecA in the Cairns system is in supporting amplification.

Similarly the F′ plasmid contributes to reversion under selection by some means that do not require increasing general mutability. More revertants accumulate when *lac* is on the F'_{128} plasmid than when it is in the chromosome (RADICELLA *et al.* 1995; GODOY *et al.* 2000; SLECHTA *et al.* 2002). Location of *lac* on an F′ plasmid stimulates reversion in *lexA33*(Ind$^-$) strains, which do not show general mutagenesis (S. SLECHTA and K. BUNNY, unpublished data). This fits with the idea that conjugative plasmids stimulate recombination (duplication and amplification; SLECHTA *et al.* 2002) and this recombination can contribute to reversion with or without general mutagenesis.

A direct test of this idea (Figure 3) shows the RecA dependence of residual reversion in the absence of SOS. We suggest that RecA function contributes to reversion in two ways. It permits amplification and therefore growth (by supporting recombinational exchanges) and it allows SOS induction and mutagenesis (by serving as coprotease for LexA repressor).

Discordant effects of SOS on reversion and associated mutations: There is a discrepancy between the effect of SOS induction on general mutability and its effect on reversion of the *lac* mutation. In both *E. coli* and *S. enterica*, elimination of SOS mutagenesis [*lexA33*(Ind$^-$) or *dinB*] reduces reversion only 2- to 4-fold but eliminates the associated general mutagenesis that has been shown to increase mutation rate ~35-fold (ROSCHE and FOSTER 1999; MCKENZIE *et al.* 2000; for *S. enterica* see Figure 1 and Table 2). How can general SOS mutagenesis stimulate associated mutagenesis so much and reversion so little?

A related problem was identified by Rosche and Foster, who concluded that general mutagenesis could explain only 10% of Lac$^+$ revertants (ROSCHE and FOSTER 1999). They estimated that *lac*$^+$ revertants arising under selection show an overall average of a 20-fold increase in the frequency of unselected mutations, but they demonstrated that the intensity of mutagenesis was highly variable. They concluded that their data would be explained if 10% of the *lac* revertants had experienced a 200-fold increase in associated mutations. While they did not comment on the source of the remaining 90% of the *lac* revertants, their results imply that some other factor is at work in the process of reversion under selection.

The amplification-mutagenesis model offers an explanation for both of the above observations. If simple amplification (more *lac* copies) makes a major contribution to reversion under selection, then amplification will also increase the effect of mutagenesis by providing multiple targets within each mutagenized cell. A lower intensity of mutagenesis can explain all observed revertants if each mutagenized cell has multiple copies of the *lac* operon. The intensity of mutagenesis is expected to vary widely because reversion reflects a succession of stochastic events. Clones will experience less mutagenesis if they happen to acquire a *lac* reversion early in their history and will be more heavily mutagenized if they happen to spend more time growing and being mutagenized prior to reversion. These considerations lead to a testable prediction.

A testable prediction: The amplification-mutagenesis model proposes that clones of cells with an amplified *lac* region grow slowly until they acquire a *lac*$^+$ reversion, at which point selection holds the revertant allele and counterselects the mutant alleles. Ultimately a fast-growing, stable *lac*$^+$ haploid segregant cell arises, overgrows, and becomes the predominant cell type in the revertant colony. In the final mature revertant colony, the fraction of cells with a *lac* amplification will depend on how early in the history of the colony the *lac*$^+$ reversion event occurred. If SOS mutagenesis is induced, the reversion event will (on average) occur early in the process when the clone is small; the final colony will contain few amplification cells because most colony growth will be due to haploid overgrowth (Andersson *et al.* 1998; Hendrickson *et al.* 2002). Under standard LexA$^+$ conditions, ~0.5% (median value) of total cells in a typical revertant colony are found to carry an amplification (Hendrickson *et al.* 2002 and this work). In contrast, if the mutation rate is reduced, as in *lexA33*(Ind$^-$), the amplification clone is expected to continue to grow and accumulate cells and *lac* copies until it reaches sufficient size to realize the reversion event (now rarer because of a lower mutation rate). In this situation, a sizable colony of cells with an amplification will be present before the haploid type arises and overgrows. The amplification model therefore predicts that, under *lexA33* (Ind$^-$) conditions, the mature revertant colonies should, on average, be richer in ancestral cells with a *lac* amplification. We tested this prediction.

Cells with a *lac* amplification are more prevalent in revertants that arise without SOS induction: The frequency of unstable Lac$^+$ cells was tested for revertant colonies that appeared on day 5 (and were not visible on day 4). We tested 10 colonies from a LexA$^+$ tester (plated with a LexA$^+$ scavenger) and 10 from a LexA33(Ind$^-$) tester [plated with a LexA33(Ind$^-$) scavenger]. Roughly 3000 cells were tested from each of these 20 revertant colonies; cells were distributed on medium containing X-gal, where unstable Lac$^+$ cells with an amplification form blue colonies with multiple white (Lac$^-$) sectors and stable Lac$^+$ cells form solid blue colonies. The results are in Table 3 and procedures are described in materials and methods.

As predicted by the model and seen previously (Andersson *et al.* 1998; Hendrickson *et al.* 2002), every revertant colony that arose under selection included some cells with a *lac* amplification (regardless of *lexA* genotype). The frequency of amplification-bearing cells varied widely from one clone to the next, as predicted for a process with multiple stochastic steps and seen previously for *E. coli* (Hendrickson *et al.* 2002). In both *S. enterica* and *E. coli*, evidence has been presented that the unstable Lac$^+$ cells are clonally related precursors of the stable Lac$^+$ cells in the same colony (Andersson *et al.* 1998; Hendrickson *et al.* 2002). In keeping with the prediction of the amplification-mutagenesis model, revertants arising in the *lexA33*(Ind$^-$) strain contained more amplification-bearing cells than those arising in the *lexA*$^+$ cells. In the *lexA33*(Ind$^-$) strain the median frequency of amplification-bearing cells was 82% whereas the *lexA*$^+$ strain had only 0.5%. We suggest that more extensive growth with amplification prior to reversion explains this higher frequency of unstable Lac$^+$ cells in revertants arising without mutagenesis.

These results can also explain why eliminating the 35-fold increase in mutation rate caused by SOS induction results in only a 2- to 4-fold reduction in revertant frequency. When mutagenesis is eliminated, cells continue growing and some of them achieve reversion even with a low mutation rate. Thus more extensive growth prior to reversion adds more *lac* copies to the clone and, in part, compensates for the reduced mutation rate. This predicts a later appearance of revertants in a *lexA33* (Ind$^-$) strain, which is apparent in Figure 3. This also predicts that the difference in revertant number seen in *lexA*$^+$ and *lexA33*(Ind$^-$) strains should be smaller at later times in the reversion experiment. We suggest that the 2- to 4-fold reduction observed on day 6 may reflect clones of amplification cells that would have shown a revertant if mutagenized, but went extinct early and failed to reach a size sufficient to provide spontaneous reversion.

One aspect of the data in Table 3 should be noted. The increase in the frequency of unstable Lac$^+$ cells (160-fold) is greater than one might have predicted. The effect of reducing the mutation rate 35-fold by means of a *lexA33*(Ind$^-$) mutation should have been corrected by about a 35-fold increase in the size of the amplification clone. The mean increase is higher than this. Some of the revertants arising in the *lexA33*(Ind$^-$) background showed no stable Lac$^+$ revertants; that is, all of the 3000 tested cells were unstably Lac$^+$, suggesting that the colony had appeared without a reversion event. (Preliminary analysis of these colonies suggests a new aspect of the Cairns phenomenology that supports the amplification model and will be described elsewhere.) If one ignores these pure amplification clones and considers only colonies that include some stable Lac$^+$ cells,

TABLE 3

Revertant colonies arising in *lexA33(Ind⁻)* strains show more cells with an amplification

Colony no.	Revertants of a *lexA*$^+$ tester plated with a *lexA*$^+$ scavenger[a] Total Lac$^+$ scored (stable + unstable)	No. unstable Lac$^+$	% cells unstable Lac$^+$	Revertants of a *lexA33*(Ind$^-$) tester plated with a *lexA33*(Ind$^-$) scavenger[a] Total Lac$^+$ scored (stable + unstable)	No. unstable Lac$^+$	% cells unstable Lac$^+$
1	3122	16	0.5	2928	427	14.5
2	3002	49	1.6	3012	3012	100
3	2927	126	4.3	3220	3220	100
4	2862	2176	76	2414	426	17
5	3127	4	0.12	2328	1917	82
6	2726	12	0.5	3014	3014	100
7	3412	17	0.5	3116	295	9.4
8	3100	1	0.03	3627	3627	100
9	2628	8	0.3	3445	3445	100
10	3127	3	0.1	3536	1925	54

[a] See MATERIALS AND METHODS for the procedures used.

the increase in frequency of unstable Lac$^+$ cells is closer to that predicted by the model. The observed increase in frequency of unstable Lac$^+$ cells was predicted by the amplification model and is therefore consistent with that model. These results are not predicted by the hypermutable state model.

DISCUSSION

The amplification-mutagenesis model (A-M) proposes that revertants appear during starvation primarily because preexisting duplication cells initiate clones within which natural selection favors growth of a subpopulation with an amplified mutant *lac* region. This adds *lac* copies to each developing clone until reversion occurs. At this point, the mutant alleles are lost by segregation and a haploid revertant clone overgrows the colony. Each final haploid revertant type is derived from one preexisting duplication cell (Andersson *et al.* 1998). According to the A-M model, general mutagenesis occurs within the clone and contributes to the yield of revertants, but is a secondary, nonessential aspect of the overall process (Hendrickson *et al.* 2002).

Comparing *S. enterica* and *E. coli*: The original system used *E. coli* (Cairns and Foster 1991) and experiments in that organism have been used to support the hypermutable state model (Rosenberg 2001). The original form of the A-M model was based on work in the parallel *S. enterica* system (Andersson *et al.* 1998). Arguments have been made that *S. enterica* behaves differently from *E. coli* (Foster 1999b; Hastings *et al.* 2000). The main concern was that the *S. enterica* tester strain grows more under selection than does the *E. coli* analog. We have compared these testers directly and find that both produce ~2 Miller units of β-galactosidase. Both the *S. enterica* and *E. coli* testers are able to grow and form colonies on lactose, but this growth can be inhibited if sufficient Lac$^-$ scavenger cells are added to the plate. For *E. coli*, ~10^9 added scavengers can just prevent growth and leave cells poised on the brink of growth (such that even a duplication of *lac* might initiate growth). When *S. enterica* is placed under the same conditions it divides less than once a day. In our hands, *E. coli* divides once every 2–4 days; the variability may reflect complexities of blocking growth with a competing scavenger. Thus *S. enterica* revertants appearing on day 6 reflect mutations that occurred (2 days previously) when the starved population had undergone about three divisions. This growth is not sufficient to explain the revertant colonies, but is more than that seen for *E. coli.*

For the hypermutable state model, cessation of growth is critical. Only if growth of the plated population is completely blocked is one forced to attribute later reversion to regulated mutability. The residual growth in *S. enterica* might well be expected to affect this process. In the amplification-mutagenesis model, however, the critical growth occurs within each developing clone and growth of the plated population is largely irrelevant (but could add a few duplication cells to the plate). The amplification-mutagenesis model should operate with or without tester population growth, as long as the growth rate is sufficiently limited that selection can detect a progressive improvement of growth caused by duplication, amplification, reversion, and segregation. Thus the two models make very different predictions regarding the effect of residual growth on reversion behavior.

Despite their growth differences, *S. enterica* and *E. coli* behave very similarly. Previous data have revealed the following shared properties.

1. It is critical that the *lac* mutation be slightly leaky (Andersson *et al.* 1998).
2. It is critical that the *lac* mutation be located on a conjugative plasmid with expressed *tra* functions rath-

er than on the chromosome (Foster 1995; Galitski and Roth 1995; Radicella *et al.* 1995; Godoy and Fox 2000; Godoy *et al.* 2000; Slechta *et al.* 2002).

3. Reversion requires recombination functions (Foster and Cairns 1992; Galitski and Roth 1995; Andersson *et al.* 1998; Foster 2000; Slechta *et al.* 2002).
4. Cells with a *lac* amplification are absent from day 2 revertant colonies, prominent in tiny colonies arising under selection, and rare (but always present) in mature revertant colonies (Andersson *et al.* 1998; Hendrickson *et al.* 2002; and this work).

Additional similarities are described here by confirming, for *S. enterica*, observations made previously in *E. coli.*

5. Revertants have an increased probability (∼35-fold) of carrying an unselected mutation (Torkelson *et al.* 1997; Rosche and Foster 1999; Table 2 of this work).
6. Preventing SOS induction eliminates the 35-fold increase in associated mutations but reduces revertant yield only 2- to 4-fold (McKenzie *et al.* 2000, 2001; Figure 1 and Table 2 of this work).

These similarities between the two systems make it clear that the slight growth difference is not critical to the overall process of reversion. The independence of background growth supports amplification-mutagenesis.

Previous evidence for multiple contributions to reversion under selection: Work of others on *E. coli* (confirmed here for *S. enterica*) revealed the surprising fact that preventing SOS mutagenesis reduces general mutability at least 30-fold, but reduces *lac* reversion only 2- to 4-fold (McKenzie *et al.* 2000, 2001). This discrepancy suggested that a second factor contributes to reversion; this factor is shown here to be amplification and growth. Similarly, blocking recombination (*recA*) reduces reversion 10- to 15-fold, while blocking SOS mutagenesis [*dinB* or *lexA33*(Ind$^-$)] reduces reversion only 2- to 4-fold, suggesting that some RecA-dependent mechanism contributes, even in the absence of general mutagenesis. We suggest that this second RecA-dependent contribution is amplification (and consequent growth).

The existence of a second factor was also implied by the demonstration of a variable intensity of mutagenesis (Rosche and Foster 1999). These authors inferred that general mutagenesis could account for only 10% of *lac* revertants, but they did not comment on the source of the bulk (90%) of the observed revertants, which must have been induced by some other process. (These numbers are nicely explained if each mutagenized cell carries 10 copies of *lac.*) In another study, the central role of amplification was missed for technical reasons, but the conclusion was drawn that amplification and induced mutagenesis were parallel independent processes that make additive contributions to reversion (Hastings *et al.* 2000). Thus both of the above observations were interpreted as indicating additive sources of mutations. We suggest that the two factors contributing to reversion are amplification and mutagenesis and their effects should be multiplied rather than added.

The relative contributions of amplification and mutagenesis to reversion under selection: Evidence is presented here that selection can stimulate reversion by amplification alone with no contribution from general mutagenesis. That is, revertant colonies arising without mutagenesis are (1) still RecA dependent and (2) include an increased frequency of cells with a *lac* amplification. Under conditions allowing mutagenesis, both factors contribute to the yield of revertants, raising the question of their relative contributions.

The 2- to 4-fold reduction in revertant number caused by eliminating SOS mutagenesis [with a *lexA*(Ind$^-$) or a *dinB* mutation] immediately suggests that general mutagenesis is responsible for as much as 75% of reversion under selection and leaves only 25% to be caused by amplification of the *lac* gene (Hastings *et al.* 2000; McKenzie *et al.* 2000, 2001). This conclusion depends on believing that mutagenesis and amplification make additive contributions to reversion (Hastings *et al.* 2000). According to the A-M model, on the contrary, the two contributions are factors that should be multiplied to estimate their combined effect on reversion. That is, the added *lac* copies are all subject to mutagenesis. Furthermore these factors should be viewed as acting on the 100 initial cells with duplications that initiate revertant colonies and not on the entire plated population (10^8) or even a suggested hypermutable subpopulation (10^5).

The reversion rate of the *lac* frameshift mutation is $\sim10^{-8}$/cell/division. To obtain a *lac*$^+$ revertant requires accumulating a population of cells in which the reversion event can be realized at the ambient mutation rate. We propose that each duplication cell grows and amplifies *lac* until it reaches a population of $\sim10^5$ cells, each with ∼30 copies of the *lac* region. The probability of a revertant derived from the parent duplication cell is thus increased 3×10^6-fold by growth and amplification. If the reversion rate (10^{-8}) is increased 35-fold by SOS induction, then the product of these factors, (3×10^6 *lac* copies) × (35×10^{-8} revertants/*lac* copy), is ∼1 revertant—assuring a reversion event within the clone. This reversion event was made possible by two multiplied factors—*lac* copy increase and mutagenesis—contributing in a ratio of $\sim10^5$:1. Viewed in this way, general mutagenesis is a minor factor indeed, despite the fact that removing mutagenesis reduces revertant yield ∼2- to 4-fold.

Evidence supporting the amplification-mutagenesis model: Previous supports for the A-M model (Andersson *et al.* 1998; Hendrickson *et al.* 2002) are the following:

1. Cells with a *lac* amplification are found within each clone arising under selection. These amplification-bearing cells are not found in colonies that are initiated under nonselective conditions.
2. Cells with a *lac* amplification are clonally related pre-

decessors of the stable Lac$^+$ revertant cells in the same colony.

3. Reversion is eliminated if one prevents growth of cells that carry an amplification of *lac*.

Here several additional lines of support are presented:

4. Selection enhances appearance of revertants even when general mutagenesis is prevented by blocking SOS induction. According to the A-M model, this is due to amplification and growth under selection, which add copies of the *lac* region (mutation targets) to each clone.
5. The residual reversion seen without general mutagenesis is dependent on RecA function. The model attributes this to the essential role of RecA in gene amplification and segregation.
6. When general mutagenesis is prevented, revertant clones carry a higher frequency of cells with a *lac* amplification. Delayed reversion allows continued growth of the original amplification clone, which is less extensively overgrown by haploid *lac*$^+$ cells after reversion and segregation.

Questions regarding the amplification-mutagenesis model: Several key aspects of the A-M model have not yet been supported by direct evidence (although several have circumstantial support):

1. Are revertants all initiated by duplication-bearing cells that arose prior to selection? This part of the A-M model has not been directly addressed, but would make reversion under selection appear to fit well with classic work demonstrating that mutations preexist selection (Luria and Delbruck 1943; Lederberg and Lederberg 1952).
2. How is the SOS regulon induced during the Cairns experiment? That this induction does occur is demonstrated by the fact that mutagenesis requires the SOS-induced DinB polymerase and is prevented by a *lexA*(Ind$^-$) mutation that blocks SOS induction (McKenzie *et al.* 2001). The SOS regulon is normally induced by single-stranded DNA (Walker 1996). The A-M model proposes that degradable ends or linear DNA molecules are released during segregation of the amplified array, but this has not been directly demonstrated.
3. How is the error-prone DinB polymerase activated during a Cairns experiment? In otherwise wild-type strains, simple induction of SOS is not mutagenic; *lexA* null mutants (constitutive for SOS) are not mutators (Friedberg *et al.* 1995; K. Bunny, J. Liu and J. R. Roth, unpublished results). The UmuCD polymerase requires RecA-dependent processing for its activation(Walker 1996). As of yet, no such process is known for DinB, but some clues are beginning to emerge (Wagner and Nohmi 2000; Kim *et al.* 2001).
4. Why does the Cairns experiment require that the *lac* gene be located on a conjugative plasmid? The exact role of the F′ has not been defined, but it seems clear that transfer (*tra*) functions of F are important for reversion (Foster and Trimarchi 1995; Galitski and Roth 1995). Tests of the effect of genomic position on reversion suggest that both duplication and amplification are stimulated for genes on the F′ or pSLT plasmids (Slechta *et al.* 2002). Loss of a Tn*10* insertion near *lac* is stimulated by conjugation and by selection for *lac* reversion, suggesting that transfer replication may be involved in reversion (Godoy and Fox 2000). The A-M model proposes that DNA ends are created by firing of the plasmid transfer origin (internally or during mating). While it seems likely that such ends would stimulate duplication, amplification, and segregation on the plasmid, this has not been directly demonstrated.
5. How is amplification achieved? Extensive evidence has been presented that arrays of tandemly repeated *lac* regions are generated on the F′ plasmid in the course of reversion in this system (Andersson *et al.* 1998; Hastings *et al.* 2000; Hendrickson *et al.* 2002). Reversion of *lac* mutations by amplification was first seen in closely related strains that also carry a leaky *lac* mutation on an F′ plasmid (Tlsty *et al.* 1984; Whoriskey *et al.* 1987). Presence of these amplified tandem arrays does not eliminate the possibility that plasmid copy number increases during growth limitation, as has been seen for the F plasmid under some conditions (Foster and Rosche 1999a). Similarly, some amplified arrays may excise as circles and be transmitted linearly at cell division. Amplification may occur by rolling-circle replication following recombinational repair of an end in cells carrying a *lac* duplication (Roth *et al.* 1996; Slechta *et al.* 2002). Such secondary contributions to *lac* amplification have not yet been investigated.
6. Can one cell really produce a haploid revertant within 20 generations of growth? This is the ultimate test of the model. The A-M model suggests that, within 20 generations of growth under selection, a single *lac* cell with a duplication can generate descendants with ~30 *lac* copies/cell while the clone reaches a total size of ~10^5 cells. This is a tall order. To test this central aspect of the model will require mathematical modeling of the process and determining the several rates (duplication, recombination, mutation, and segregation) to see if it is feasible. We suggest that the unique features of the Cairns system act together and can indeed allow cells to complete this process in so few generations.

The good and the bad news regarding the Cairns system: The Cairns system first appeared to demonstrate directed mutation (Cairns *et al.* 1988; Cairns and Foster 1991) and later stress-induced mutation (Hall 1992; Torkelson *et al.* 1997). Behavior of this system was extrapolated to suggest that living things regulate their mutational behavior (Foster 2000; Rosenberg 2001). On the basis of the behavior of this system, stationary phase automutagenesis was inferred (Foster and Rosche 1999b; Lombardo *et al.* 1999) and mutagenic recombination (Foster *et al.* 1996; Harris *et al.* 1996) was suggested. The A-M model argues that none of these interpretations is likely to be correct.

Over the past 12 years, analysis of the Cairns system has revealed a set of features that underlie its behavior. These include placement of an unusually leaky +1 frameshift mutation on a conjugative plasmid that constitutively expresses its transfer functions. Another feature is its use of scavenger cells to prevent multiplication and poise cells near the point of growth. Additional special features soon will be added to this list (S. Slechta and K. Bunny, unpublished data). A skeptic might reasonably conclude that the system is a mass of impacted, interacting artifacts and is therefore unsuitable for the study of genetic adaptation. We disagree.

The A-M model proposes that the peculiarities of the Cairns system allow it to complete a broadly relevant evolutionary process within a few days. A simple duplication can initiate growth; the F′ plasmid stimulates duplication, amplification, and segregation and thereby generates frequent variants upon which selection can act. These features accelerate the underlying process—amplification under selection allows growth and also increases the target size for valuable mutations. The same process may occur slowly in many natural situations such as the evolution of new genes and origins of some cancers. Thus while the bad news is that the Cairns system is extremely atypical, the good news is that its idiosyncrasies accelerate and therefore make experimentally accessible a genetic process that is of very general importance.

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