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MECHANISMS OF STATIONARY PHASE MUTATION: A Decade of Adaptive Mutation

P. L. Foster

Department of Biology, Indiana University, Bloomington, Indiana 47405

P. L. Foster: pfoster@bio.indiana.edu

Abstract

A decade of research on adaptive mutation has revealed a plethora of mutagenic mechanisms that may be important in evolution. The DNA synthesis associated with recombination could be an important source of spontaneous mutation in cells that are not proliferating. The movement of insertion elements can be responsive to environmental conditions. Insertion elements not only activate and inactivate genes, they also provide sequence homology that allows large-scale genomic rearrangements. Some conjugative plasmids can recombine with their host's chromosome, and may acquire chromosomal genes that could then spread through the population and even to other species. Finally, a subpopulation of transient hypermutators could be a source of multiple variant alleles, providing a mechanism for rapid evolution under adverse conditions.

Keywords

spontaneous mutation; evolution; selection; hypermutation; directed mutation

INTRODUCTION

For over 40 years it was dogma that spontaneous mutations arise as random errors during genomic replication. In 1988, Cairns and his collaborators published a paper challenging that dogma (27). They confirmed and extended previous studies by Ryan (137–139) and Shapiro (141) showing that mutations can arise in apparently static bacterial populations when subjected to nonlethal selective pressure. In addition, Cairns et al presented evidence that only selected mutations, not deleterious or neutral mutations, appeared in a population during selection. This phenomenon has come to be called adaptive mutation.

The adaptive mutation controversy has generated a wealth of research. The history behind the controversy, the early work contradicting the random mutation dogma, and research prior to 1993 have been previously reviewed (48). This review covers prokaryotes only and focuses on developments since 1993. For different perspectives readers should consult other recent reviews (20,25,82).

Although it was clear by 1993 that adaptive mutation is not a single phenomenon, it was thought that some basic principles would emerge. Few, if any, have. Indeed, at this point it seems that nearly every case of mutation in cells under selection may have a different underlying mechanism. This review is therefore organized by mutational system, and only at the end are some general issues discussed.

What's in a Name?

Various names have been given to the phenomenon here called “adaptive mutation” but they all tend to have some unwanted connotations. The original name, “directed mutation” (attributable to the editors of *Nature*), implied a transfer of information from the environment back to the genome, an idea that was rejected early on (53). However, that name did promote the useful (and first) meaning of “mutation” as the process that generates DNA sequence changes in addition to the sequence change itself. “Mutagenesis” would convey the same meaning, but is usually used for the process by which mutations are induced by exogenous mutagens. “Mutability” means “the ability to be mutated”; thus, “adaptive mutability” (68) means “the ability to be mutated adaptively.” Some acronyms have been suggested. SLAM, stressful-lifestyle associated mutation (134), conjures up aggression and violent behavior (or at least a loud noise). SAM, starvation-associated mutation (17), has already been designated for S-adenosylmethionine. Stationary-phase mutation (SPM) was the name given to Ryan’s work, although not by him (71). However, are the cells that mutate really in stationary phase or starving? In most cases, it is not known if the mutating cells are expressing the gene products associated with stationary phase, and a slow input of energy may be required for mutation. My nickname for the process, FRED, means absolutely nothing.

The term “adaptive mutations,” introduced in 1991 (26), was defined as a process that produces mutations specific to the selective pressure but does not produce mutations that are useless or deleterious (48). As discussed below, in the case of the best-studied example of adaptive mutation, *Escherichia coli* strain FC40, nonselected mutations arise and persist during selection. Nonetheless, mutations giving an adaptive phenotype appear by a process distinct from the process that produces random mutations during nonselective growth. Therefore, in the absence of a better term, adaptive mutation is retained here to mean the process that during selection produces mutations that relieve the selective pressure, whether or not nonselected mutations are also occurring. “Adaptive mutations,” meaning the sequence changes themselves, is used in the same sense as it is used by evolutionists to distinguish beneficial from neutral or deleterious mutations.

ADAPTIVE MUTATION IN *ESCHERICHIA COLI* STRAIN FC40

E. coli strain FC40 cannot utilize lactose (Lac^-) but readily reverts to lactose utilization (Lac^+) when lactose is its sole carbon and energy source. Since its description in 1991 (26), FC40 has become the most popular *E. coli* strain for the study of adaptive mutation. The reason is clear—the abundance of adaptive Lac^+ mutations that appear makes the phenomenon easy to study and obvious artifacts easy to eliminate. Two days after plating FC40 cells on minimal lactose plates, Lac^+ colonies start appearing at a constant rate of nearly 1 per 10^7 cells per day. Of the Lac^+ colonies that appear during a week of incubation on lactose, 95% are due to mutations that occurred after plating. Were these normal growth-dependent mutations (i.e. the mutations that occur during nonselected growth), every cell would have to be replicating once an hour during lactose selection, about the same rate of replication achieved by cells during nonselective growth in minimal medium (26,49).

FC40 has been so extensively studied that it has become a paradigm of adaptive mutation. This is unfortunate and unintended, as it was clear early on that not all aspects of adaptive mutation in FC40 are generalizable to other systems (47). Nonetheless, studies of FC40 have revealed in detail one mechanism by which mutations can arise in cells during selection. Adaptive mutation in FC40 has been recently reviewed (51,59), so only a summary is given here.

Properties of the Lac⁻ Allele

The Lac⁻ allele in FC40 is derived from a fusion of the *lacI* gene to the *lacZ* gene that eliminates the coding sequence for the last four residues of *lacI*, all of *lacP* and *lacO*, and the first 23 residues of *lacZ*. Constitutive transcription is initiated from the *lacI^q* promoter (120). Mutant versions of this fusion constructed by J.H. Miller and coworkers have been used for a variety of mutational studies [for an example, see (113)].

The Lac⁻ allele carried by FC40, $\Phi(lacI33-lacZ)$, has an ICR191-induced +1 frameshift at the 320th codon of *lacI*, changing CCC to CCCC (28). The allele is slightly leaky, producing about 2 Miller units of β -galactosidase, which is not sufficient to allow FC40 to grow on lactose (49). The frameshift is polar on *lacY*; thus, FC40 is also lactose permease-defective (PL Foster, unpublished observations). However, stationary-phase cells of FC40 do not revert to Lac⁺ unless lactose is present (26), so the energy provided by the residual amount of β -galactosidase and permease activity might be required for the mutational process. Indeed, Andersson et al (2) presented evidence that the amount of energy produced by the $\Phi(lacI33-lacZ)$ allele determines the adaptive mutation rate, although it is not clear how they distinguished mutation rate from the rate at which Lac⁺ mutants grew.

Characteristics of Adaptive Mutation in FC40

In FC40, adaptive mutation to Lac⁺ has the following characteristics. (a) Whereas growth-dependent Lac⁺ mutations include deletions, duplications, and frameshifts, adaptive Lac⁺ mutations consist almost exclusively of -1 base-pair (bp) frame-shifts in runs of iterated bases (60,133). Over half of the adaptive mutations occur at one hotspot, which was the site of the original +1 frameshift (56,59). (b) Recombination functions, specifically the activities of the RecABCD pathway for double-strand end repair, are required for adaptive but not growth-dependent Lac⁺ mutations (26,48,90).¹ (c) *E. coli*'s two enzyme systems for the branch migration of recombination intermediates have opposite roles in adaptive Lac⁺ mutation (63,91), whereas both contribute to normal recombination (162). (d) In *E. coli* and related bacteria, DNA damage induces the "SOS response" (65). SOS mutagenesis functions, whether encoded by the *umuDC* operon or the *dinB* gene, are not required for adaptive mutation (26). But if induced, SOS mutagenesis will increase adaptive mutation (PL Foster, unpublished observations). (e) Both the high rate of adaptive reversion to Lac⁺ and its *recA*-dependency requires that the Lac⁻ allele be on the F' episome (61,126,129). (f) When on the episome, the high rate of adaptive reversion of the Lac⁻ allele, but not its *recA*-dependency, requires that one or more conjugal functions be expressed (61,67,129); however, actual conjugation is not required (61,62).

Adaptive mutation to Lac⁺ resembles growth-dependent mutation in two respects. (a) Adaptive Lac⁺ mutations are produced by *E. coli*'s replicative polymerase, DNA polymerase III, (Pol III) (56,88).² DNA polymerase II (Pol II) also replicates DNA, particularly in stationary-phase cells, but it produces few errors (42,56,127). (b) Adaptive mutations are corrected by the methyl-directed mismatch repair (MMR) pathway (53,63,129), which corrects mismatches in hemimethylated DNA in favor of the methylated strand (119).

¹*recD* mutants are nuclease minus but retain the helicase activity of the RecBCD enzyme. Although adaptive mutation in FC40 is increased in *recD* mutant cells (90), the mechanism of mutation is different from that in wild-type cells. In *recD* mutant cells episomal replication increases dramatically, probably as a result of aberrant rolling-circle replication, accounting for the increase Lac⁺ mutation (58).

²The polymerase subunit of Pol III is encoded by *dnaE*. Adaptive mutations in FC40 are decreased by an antimutator allele of *dnaE* (56,88), and the simplest interpretation of this result is that the mutations are due to errors made by Pol III. However, it was recently shown that incubation of a temperature-sensitive *dnaE* mutant at the nonpermissive temperature inhibits recombination in *recD* mutant cells (100). Thus, if the antimutator *dnaE* allele used, *dnaE915* (44), is also partially defective for replication, the antimutator effect seen in FC40 could be due solely to inhibition of the recombination required for adaptive mutation. However, *dnaE915* did not affect adaptive mutation in a strain carrying an exonuclease-defective allele of Pol II, yet these mutations were *recA*-dependent (56).

Overproduction of MMR enzymes can additionally reduce adaptive mutations two- to fivefold (56,89). Although it has been argued that this latter result means that MMR is limiting for adaptive mutation (89), a similar reduction in growth-dependent mutations can be demonstrated (52,56) (further discussed below).

A Model for the Mechanism of Adaptive Mutation in FC40

Nicking at the episome's conjugal origin, *oriT*, initiates recombination (29), which suggests that the initiating event for adaptive mutation to Lac⁺ in FC40 is likewise a nick at *oriT*. This nicking occurs even in the absence of a conjugal signal and persists in stationary-phase cells (45,66). Kuzminov (99) proposed that a double-strand end is created when a replication fork initiated at one of the episome's vegetative origins collapses at the nick at *oriT* (Figure 1A, B). The exonuclease and helicase activities of RecBCD create an invasive 3' end that initiates *recA*-catalyzed recombination (Figure 1C, D), accompanied by the restoration of a replication fork (Figure 1E). Adaptive mutations are then the result of replication errors produced by the reassembled fork. Indeed, this type of DNA synthesis may be particularly error prone (39,111,149). In addition, the new fork differs from a normal fork in that it is accompanied by a four-stranded recombination intermediate (a Holliday junction). Translocation of the Holliday junction towards the fork (e.g. by RuvAB) would create a tract of doubly unmethylated DNA in which polymerase errors would be randomly repaired by MMR (Figure 1F, left). This tract would thus contain a higher than normal number of mutations. Translocation of the junction away from the fork, or resolution of the Holliday junction before DNA synthesis begins (e.g. by RecG), would preserve the hemimethylated state of the DNA, allowing polymerase errors to be correctly repaired (Figure 1F, right). This model accounts not only for the opposite effects of the branch migration enzymes on adaptive mutation, but also for the fact that the mutational spectrum bears the mark of MMR deficiency (63).

Alternative Models

Conjugation can be mutagenic (31,98) and therefore it is possible that the adaptive Lac⁺ mutations are produced by conjugal replication initiated by nicking at *oriT* (61,67,126). But this hypothesis provides no clear role for recombination. In particular, the involvement of RecBCD implicates a double-strand end, the loading point for this enzyme (97), and it is not obvious when during conjugal replication an unprotected double-strand end would be created.

Alternatively, some cells may undergo RecA-dependent amplification of the Lac⁻ allele during lactose selection (2,53,159). A Lac⁺ mutation occurring during the DNA synthesis that produces the amplification would necessarily be retained, and the amplification would be resolved during growth of the Lac⁺ cells. A role for RecBCD can be provided by supposing that the amplified arrays are produced by rolling-circle replication initiated during aberrant double-strand end repair (135). Such events may be stimulated by a conjugal origin (131).

Arrays of tandem alleles are unstable. Using a different, leakier, Lac⁻ derivative of the episomal *lacI-lacZ* fusion (*lacIX13*), Tlsty et al (159) found that 60% of the Lac⁺ colonies that appeared after plating Lac⁻ cells on lactose minimal medium were composed mainly of unstable Lac⁺ cells. Instability was assayed by purifying the Lac⁺ cells on lactose, then streaking isolates on lactose-MacConkey plates (a nonselective medium on which Lac⁺ colonies turn red) and showing loss of the Lac⁺ phenotype. When a similar assay was used, only 2% of late-arising Lac⁺ colonies of FC40 were found to be unstably Lac⁺ (49). But when the *lac-pro* episome from FC40 was transferred to *Salmonella typhimurium*, Andersson et al (2) found that 0.5 to 8% of the Lac⁺ cells in every late-arising Lac⁺ colony

were unstable. They proposed that amplifying cells, which have up to 50-fold more *lac* DNA than normal, could proliferate enough to account for all adaptive Lac⁺ mutations by the normal growth-dependent mutation rate.

To be consistent with the constant rate of adaptive mutation in FC40, the replication rate of the *lac* region during lactose selection must also be constant. So, if adaptive mutations arise from amplified alleles, amplification and deamplification (or degradation of the amplified region) must always be in equilibrium (49). It is hard to reconcile this requirement with a growing population of cells. In addition, we could not detect an increase in the amount of *lac* DNA in Lac⁻ cells during incubation in lactose for 4 days, although a doubling occurred between days 4 and 5. However, this doubling could have been due to the inapparent growth of just a few amplified clones (58;WA Rosche & PL Foster, unpublished observations).

Experiments are under way to determine why such different results were obtained. The most likely explanation is that amplification is responsible for a majority of Lac⁺ mutants in the *S. typhimurium* strain, but a minority in the *E. coli* FC40 strain. One obvious difference between the strains is the amount of residual β -galactosidase made by Lac⁻ cells. As mentioned above, Lac⁻ cells of FC40 make 2 Miller units of β -galactosidase and cannot grow on lactose (49). The same episome in *S. typhimurium* results in two- to fourfold more β -galactosidase (PL Foster, unpublished observations), an amount approaching the 16 units made by the strain used by Tlsty et al (159). Unlike FC40, the *S. typhimurium* strain proliferates on lactose medium and Lac⁺ colonies appear at an ever-increasing rate (2,67; PL Foster, unpublished observations). It appears that the *lac* allele either is leakier or it exists in higher copy number in *S. typhimurium* than in *E. coli*. Either of these possibilities could result in a greater potential for amplification.

Nonselected Mutations Appear During Lactose Selection

Lac⁺ mutations do not arise when FC40 cells are starved in the absence of lactose, nor do nonselected mutations in the chromosomal *rpoB* gene giving a rifampicin-resistant (Rif^R) phenotype appear in the Lac⁻ population during lactose selection (26,49). Thus, mutation to Lac⁺ in FC40 appeared to be adaptive as originally defined. However, because the Lac⁻ allele is on an episome, it was possible that the mutational process in nondividing cells affected only the episome, giving the appearance that the Lac⁺ mutations were adaptive. This hypothesis was tested with tetracycline-sensitive (Tet^S) Tn10 elements on the episome. The two mutants studied were similar to the Lac⁻ allele, carrying +1 bp frameshifts in runs of G:C bps in *tetA*. Unlike the chromosomal *rpoB* gene, the mutant *tetA* alleles reverted to Tet^R when the cells were under lactose selection. Tet^R mutations accumulated at nearly the same rate and occurred by the same recombination-dependent mechanism as did the Lac⁺ mutations (50). That Tet^R mutations appear and persist in the Lac⁻ population eliminates the hypotheses that nonselected mutations are transitory, or that the cells (or episomes) bearing them are eliminated from the population. The previous failure to observe mutants in *rpoB* was likely due to the lower mutation rate on the chromosome and the relatively small number of mutational events that give a Rif^R phenotype.

Transient Mutation

To explain the specificity of adaptive mutation, Hall (74) proposed that during selection most cells in the population do not mutate, but a minority transiently experience a mutation rate so high that they die unless a useful mutation occurs. A specific prediction of this "hypermutable state model" is that nonselected mutations should occur at a higher frequency among cells that bear adaptive mutations than among cells that do not (74). This prediction has been confirmed in five cases (9,50,74,132,160). However, whether hypermutators are important contributors to a population of mutants depends on the proportion of cells that are

hypermutators and the degree to which their mutation rate is elevated (25,122). By using a broad mutational screen, loss of motility, we determined the frequency of nonselected mutations in starved Lac^- cells, in Lac^+ revertants, and in Lac^+ revertants carrying another nonselected mutation, and used these frequencies to solve Cairns' algebraic model of hypermutation (25,132). The calculations indicated that when FC40 is plated on lactose, about 0.1% of the cells are hypermutators and their mutation rate is increased about 200-fold, estimates remarkably similar to those predicted by Ninio on theoretical grounds (122). Although these hypermutators account for nearly all multiple mutations, they account for only about 10% of the adaptive Lac^+ mutations. Thus 90% of the adaptive Lac^+ mutations are not arising from hypermutators. Surprisingly, the proportion of Lac^+ clones bearing second mutations increased linearly during lactose selection, which suggests that most of the hypermutators do not die during the course of an experiment (132).

Loss of MMR had a far smaller effect on the hypermutators than on the population at large, which implies that hypermutators owe their high mutation rate to a complete or partial deficiency in MMR (132). The spectra of Lac^+ mutations did not appear to differ between the hypermutators and the normal population (WA Rosche & PL Foster, unpublished observations). The simplest hypothesis is that the mechanism that generates Lac^+ mutations on the episome is the same in all cells in the population, but that a deficiency in MMR allows more of these to be retained in the hypermutators. Other, nonselected mutations on the episome and elsewhere are also retained, although these may occur by different mechanisms. Hypermutators were also found when the $\Phi(\text{lacI33-lacZ})$ allele was on the chromosome; hence, the episome is not required to induce the hypermutable state (132).

OTHER LAC^- ALLELES

Episomal Lac^- Alleles

Many Lac^- strains used for mutagenesis studies originated with Miller and have similar genetic backgrounds (112,114). These strains carry a large deletion of the chromosomal *lac-pro* region, $\Delta(\text{lac-proB})_{\text{XIII}}$, also known as $\Delta(\text{gpt-lac})5$. Although its endpoints have not been precisely mapped, the deletion extends from about minute 5.5 through the *lac* operon at minute 7.9. For ease of genetic manipulation, the *lac-pro* region is carried on the F'128 episome. The episome is roughly 200 Kb (58), and carries the chromosomal region from about minute 5.25 through minute 8.05, interrupted by the approximately 100 Kb F factor (PL Foster & WA Rosche, unpublished observations).

A *LacZ* Amber Allele—SM195, the Lac^- *E. coli* strain originally used by Cairns et al to illustrate adaptive mutation (27), carries on the F'128 episome the complete *lac* operon, not the *lacI-Z* fusion described above. It is $\text{LacI}^+ \text{LacZ}^- \text{LacY}^-$ because of an amber mutation at codon 17 of *lacZ* that is polar on *lacY*. SM195 also carries a deletion of the chromosomal *uvrB-bio* region (minute 17) that was generated by selecting for chlorate resistance under anaerobic conditions (S Miller & J Cairns, personal communication). This is the same technique used to make the *S. typhimurium* Ames tester strains *uvrB*⁻ (1). The presence of the deletion enhances adaptive Lac^+ mutation on minimal lactose plates (27) but prevents papillation on lactose-MacConkey plates even though Lac^+ revertants are being produced (S Miller & J Cairns, personal communication; papillae are small colonies that grow on older colonies). Cairns has suggested that the strain is defective in its ability to enter and exit from stationary phase (J Cairns, personal communication). The gene or genes responsible have not been identified. Adaptive Lac^+ mutation in SM195 is not *recA*-dependent (PL Foster, unpublished observations).

Because the Lac^- allele carried by SM195 has an amber mutation, it can revert by both intragenic and extragenic (suppressor) mutations (27). After plating on lactose plates, early-

arising Lac^+ mutants are dominated by suppressors that arose during nonselective growth before plating. But then both classes of revertant appear continuously, so that by day 5 after plating, intragenic and suppressor mutants are arising at about the same rate of 1 per 10^8 cells per day (53). In reconstruction experiments, Prival & Cebula (124) demonstrated that many of the Lac^+ revertants of SM195 that arise after day 2 were slow-growing, and most of these were ochre suppressors (which can suppress amber mutations). These slow-growing revertants could have been due to mutations that occurred prior to plating. However, the proportion of slow-growing revertants declined so that by day 5, 70% of the Lac^+ revertants were due to fast-growing intragenic and amber suppressor mutants (124). These fast-growing revertants must have occurred after plating. Although Prival & Cebula argued that the fast-growers were due to cryptic growth of Lac^- cells on the lactose plates, the Lac^- population would have had to increase sevenfold during the experiment to give rise to so many mutants. Not only would such growth have been clearly visible, it also would have produced more ochre mutants (55). Therefore, lactose reversion in strain SM195 is not an artifact and still qualifies as an example of adaptive mutation.

Reddy & Gowrishankar (129) developed a method to eliminate Lac^+ mutants that appear during growth. In one method, colonies were grown at high temperature on nonselective plates supplemented with lactose and Xgal (5-bromo-4-chloro-3-indolyl- β -D-galactopyranoside, which turns Lac^+ colonies blue). Any Lac^+ mutants that appeared were killed because the strain carried a temperature-sensitive *galE* allele. Then the plates were shifted to permissive temperature, and blue Lac^+ papillae appeared. Because a colony grows from the center out, papillae appearing in the middle of a colony were presumed to be due to mutations occurring in non-dividing cells. Reddy & Gowrishankar used this technique to confirm many of the aspects of adaptive mutation in FC40. They also looked at other *lac* alleles, including the amber mutation described above. Their strain was not $\Delta(\text{uvrB-bio})$, and so centrally located papillae did appear, which confirms that reversion occurred in nondividing cells (129).

The Cupples-Miller Detector Strains—Cupples, Miller, and coworkers (34,36) created a set of strains with mutations in *lacZ* that alter important amino acids in the active site of β -galactosidase. Each allele reverts only by one specific event, and together the set can identify all six base changes and five different frameshifts. Two studies have determined the reversion rates of these strains to Lac^+ during lactose selection (76,107). Although the absolute rates differed by as much as 20-fold between the studies, both groups agreed that among the six base-substitution mutations, GC to TA and GC to AT gave the highest rates of adaptive mutation (0.4 to 5 per 10^9 cells per day). Hall (76) also tested the frameshift alleles and found that during lactose selection $-G$, $+G$, and $-A$ mutations occurred at rates about tenfold higher than the base substitutions, but the $+A$ did not occur. None of these events was *recA*-dependent (PL Foster, unpublished observations).

It has been argued that leakiness, i.e. partial activity of the allele under selection, is an essential element of adaptive mutation (68,95). Based on their ability to hydrolyze the lactose analogue *o*-nitrophenyl- β -D-galactoside (ONPG),³ the alleles that revert by GC to TA and GC to AT are, indeed, the leakiest among the alleles with base-substitutions (35). However, the allele that reverts by GC to CG produces virtually no adaptive mutations but is twofold leakier than FC40 by the same assay. Thus leakiness is not the only determinant of adaptive mutations (also see below).

³These mutations affect the active site of β -galactosidase and change the enzyme's substrate specificity. So, the degree to which the enzymes encoded by these alleles hydrolyze ONPG does not correlate with their abilities to hydrolyze X-gal. Their relative abilities to hydrolyze lactose are unknown (35).

Chromosomal Lac⁻ Alleles

Φ(LacI33-LacZ)—When the Φ(*lacI33-lacZ*) allele from FC40 is at its normal place on the chromosome, the adaptive mutation rate drops 100-fold to about 1 per 10⁹ cells per day (126). These adaptive reversions are not affected by the recombination functions RecA, RecD, or RecG, and the rate is not increased by supplying conjugal functions in *trans* (PL Foster & WA Rosche, unpublished observations; 61). The mutations that revert the chromosomal allele during lactose selection are not simple –1 bp frameshifts, and are thus completely different from the mutations that revert the episomal allele. However, in the absence of MMR, the adaptive mutation rate is increased 100-fold and the episomal mutational spectrum is obtained (WA Rosche & PL Foster, unpublished observations).

Other LacZ Alleles—Galitski & Roth (68) constructed 30 different strains of *S. typhimurium* carrying mutant *lacZ* alleles on a defective *Mu* element inserted in the chromosomal *hisC* gene. The ends of the *Mu* element flank the *E. coli lac* operon, but no *Mu* functions are active. About ten of the strains produced significant numbers of Lac⁺ revertants during lactose selection; these included some, but not all, of the strains with nonsense mutations and single-base frameshifts, but none of the strains carrying insertion mutations.⁴ All the strains that reverted during lactose selection carried “leaky” alleles and were able to grow on lactose. But the converse was not true—some strains with leaky alleles that could grow on lactose did not produce revertants during lactose selection. The degree of leakiness and the amount of growth were only poorly correlated with the number of adaptive mutations that appeared (the rank correlation was about 0.6 in both cases). Thus, some ability to slowly metabolize, but not necessarily to divide, appears to be required for cells to produce adaptive mutations, but the rate at which mutations appear while the cells are in this state is determined by other factors.

Mu-MEDIATED Ara-Lac⁺ FUSIONS

What appears to be the first documentation of adaptive mutation was described by Shapiro in 1984 (141). His strain was *E. coli* carrying a *Mu* prophage with a temperature-sensitive repressor (*cts62*). The prophage is inserted into the *araB* gene (encoding L-ribulokinase) and the *lacZ* and *lacY* genes are downstream of the *Mu* element with both translational and transcriptional blocks preventing their expression. When the *Mu* element excises, *lacZY* can be fused to *araB*, yielding a cell that encodes a hybrid AraB-LacZ protein; such a cell is Lac⁺ when arabinose is present as an inducer (designated Ara-Lac⁺). Shapiro reported that Ara-Lac⁺ fusion occurred at an undetectable rate during normal growth at noninducing temperatures or when cells were held unagitated in buffer. However, Ara-Lac⁺ mutants started appearing four days after 10⁸ cells were plated on lactose minimal medium supplemented with arabinose, and continued to appear for about 3 weeks until the plates were saturated with a few hundred colonies. These results were confirmed by Cairns et al (27), who further showed that starving cells in a moderately enriched medium with arabinose but not lactose did not induce fusion formation. Thus, Ara-Lac⁺ fusion appeared to occur only in the presence of lactose.

Subsequently, several studies demonstrated that Ara-Lac⁺ fusion formation could be induced by aerobic starvation in the absence of lactose (54,109,117,145). Interestingly, it was only the techniques of classical bacterial genetics—fluctuation analysis (54), sib-selection (109), and replica-plating (145)—that demonstrated that actual fusion, not just the propensity for fusion formation, was induced by starvation. Fusion formation, like *Mu* transposition, is

⁴In contrast, Reddy & Gowrishankar (129) found that a *lacZ::dTn10Kan* allele did revert to Lac⁺ in nondividing cells. Defective transposons do not have transposase activity, so their excision depends on host functions, some of which are apparently expressed in stationary cells, at least in *E. coli*.

under complex physiological controls (70,101,142,143). Fusion formation requires *Mu* derepression, and the *Mucts62* repressor undergoes Lon- and ClpXP- dependent inactivation in stationary-phase cells. In addition, some Crp-dependent functions appear to be required to antagonize the activity of MuB, which would otherwise promote replication of *Mu*, killing the cells (101). Hence, the conflicting results previously obtained are explained by the subtly different conditions used by various laboratories, some of which induced fusion formation and some of which did not. Interestingly, and still unexplained, the structures of the Ara-Lac⁺ fusions occurring during aerobic starvation and on lactose-arabinose medium are different, which indicates that the selective condition is influencing the mechanism by which the fusions occur (108,109).

THE EVOLVED β -GALACTOSIDASE (*ebg*) OPERON

The *ebg* operon of *E. coli* encodes a repressor, EbgR, and a β -galactosidase, EbgAC. The true substrate of EbgAC is unknown, but the repressor is 25% homologous to LacI and the enzyme is 34% homologous to the product of *lacZ* (86). If a strain is deleted for *lacZ* but makes lactose permease, mutations in the *ebg* operon can allow growth on lactose (Lac) or related sugars, such as lactulose (Lu). Various mutations in *ebgA* change the substrate specificity of the EbgAC enzyme. For example, class I mutations (Lac⁺ Lu⁻) allow good growth on lactose but only poor growth on lactulose, whereas class II mutations (Lac⁺ Lu⁺) allow growth on both lactose and lactulose. However, for these substrates to support growth, the operon's repressor must also be mutated so that it is either inactive or inducible [reviewed in (84)].

When a strain that is wild type for *ebgR* but *ebgA*^{II} was incubated on lactulose, EbgR⁻ mutants allowing growth on lactulose appeared continuously at a rate of about 2 per 10⁷ cells per day over the course of a week (85). Comparison of the early and late mutational spectra revealed that 6% of the early mutations but 39% of the late mutations were due to insertion of IS30 in *ebgR*. Over half of the late-appearing IS30 insertions were in one region of *ebgR*, apparently a hotspot for IS30 insertion. If the IS30 insertions are eliminated, the early and late mutational spectra do not differ ($\chi^2 = 3.3$, $P = 0.35$).

E. coli strains typically carry zero to three copies of IS30 (40). Although normally quiet, IS30 can undergo bursts of transposition stimulated by the formation of a tandem dimer of the element (123). In a study of the evolution of an *E. coli* K12 strain stored in agar slabs for up to 30 years, such bursts were found to have increased the variation among the cells sporadically but dramatically (121). In contrast, an *E. coli* B strain kept growing in batch culture by serial dilutions for 10,000 generations showed no IS30 movement, although other IS elements were active (123a). Thus, it appears that IS30 transposition is responsive to some conditions pertaining to prolonged starvation or slow metabolism.

A screen for genes that decrease adaptive mutation of *ebgR* yielded mutations in *phoP* and *phoQ*, a two-component regulatory system that in *Salmonella* species regulates several virulence genes (83). Although PhoP/Q appears to respond to a variety of environmental signals, the true effector may be Mg²⁺ concentration (72). The effect of *phoP/Q* mutations appears to be specific to the *ebgR* selection, as they did not affect adaptive mutation of the *bgl* operon (many of which are also due to IS insertions) or of *trpA* (83). Thus, it appears likely that PhoP/Q is a regulator, directly or indirectly, of IS30 transposition.

Hall also investigated the specificity of *ebgA* mutations to the selective conditions (80). When *ebgR*⁻ *ebgA*⁺ (wild-type *ebgA*) cells were incubated on lactulose, Lu⁺ colonies due to Class II mutations appeared at a rate of about 2×10^{-10} per cell per day for a week. During this time, new Lac⁺ Lu⁻ mutants (due to Class I mutations) did not appear among the population, even though they would have done so had the cells been incubated on lactose.

THE β -GLUCOSIDE (*bgl*) OPERON

The *bgl* operon encodes genes for the utilization of β -glucosides such as salicin and arbutin (104). The operon is maintained in a silent state in *E. coli* K12 strains by interactions of DNA-binding proteins [e.g. H-NS (93)] with sequences upstream of the promoter (the *bglR* site). Starting with a strain that was *bglR*^o (silent) and that also had an insertion of *IS103* in *bglF* (which encodes a phosphotransferase required for salicin utilization), Hall (73) showed that after 10 days of incubation on salicin MacConkey plates, Sal⁺ papillae started to appear on Sal⁻ colonies and continued to appear until 60% of the colonies had papillae. To become Sal⁺, two rare events had to occur: precise excision of the IS element from *bglF* and mutation from *bglR*^o to *bglR*⁺. The excision event occurred first within the aging colonies, followed by the *bglR* mutation. Subsequent study showed that the excision event alone allows cells within the colony to grow slowly on salicin, reaching a population large enough to give rise to *BglR*⁺ mutants (78,118).

Although no anticipatory mutagenesis (150) occurred in these experiments, as Hall originally thought, there is still a discrepancy in the literature. Mittler & Lenski claim that the excision event occurs in starving cells in the absence of salicin (118), whereas Hall claims that it does not, or at least is undetectable in the absence of salicin (78). Given the complex physiological influences on the *bgl* operon, it might be that in this case, as in the case of the Ara-Lac⁺ fusion, different results are obtained owing to subtle differences in the treatment protocols.

Mutations in several genes have been shown to activate the wild-type (silent) *bgl* operon, but the most common spontaneous mutation is insertion of *IS1* and *IS5* elements into the *bglR* region (130). Recently, Hall (81) showed that *Bgl*⁺ mutants appear at a rate of about 3 per 10⁷ cells per day during 5 days of incubation of wild-type *E. coli* on minimal arbutin plates. *Bgl*⁺ mutants did not appear when cells were incubated on minimal medium with limiting glucose; however, the *bgl* operon is catabolite-repressed, so this result could be due to a failure of *Bgl*⁺ mutants to be expressed when switched from glucose to arbutin. Hall compared the mutational spectra of *Bgl*⁺ mutants arising early (day 2) and late (days 3–5). The proportion of *Bgl*⁺ mutants due to IS insertions decreased from 98% to 79%, while the number due to mutation of *hns* increased from 0% to 21%, differences that are statistically significant (81).

REVERSION OF AMINO ACID AUXOTROPHIES

Hall's Experiments with *trp* Mutants

When *E. coli* K12 strains with missense mutations in either *trpA* (*trpA46*) or *trpB* (*trpB9578*) (encoding the α and β subunits of tryptophan synthase) are plated on medium with a limiting amount of tryptophan, small colonies form within 3 days. Hall reported that over the next 10 days these colonies gave rise to Trp⁺ papillae. Papillae formed at a linear rate per colony so that after 2 weeks about 8% of the colonies of the *trpA* strain and about 20% of the colonies of the *trpB* strain had papillae (74). The specificity of reversion to Trp⁺ was shown in two ways. First, starvation for other amino acids did not induce *trpA46* reversion; second, starvation for tryptophan did not induce *lacI*⁻ or Val^R mutations (74,79). However, 2 of 110 Trp⁺ revertants of *trpA46* carried auxotrophic mutations (versus 0/4530 aged *trpA46* cells), in support of the “hypermutable state” model (see above).

The adaptive reversion rate of the *trpA46* allele was unaffected by a defect in *recA*, but was increased about 100-fold by defects in MMR or in nucleotide excision repair (79). The last result is unusual because defects in nucleotide excision repair do not to affect other cases of adaptive mutation (further discussed below).

Hall reported that Trp⁺ papillae also arose from a population of doubly mutant *trpA46 trpB9578* cells at 10⁸-fold the expected rate (75). However, this turned out to be an artifact, as the two mutations most likely occurred sequentially. *trpA*[−] *trpB*⁺ mutants are able to grow and reach a population size of 10⁵ inside *trpA*[−] *trpB*[−] colonies (77). This population size is sufficient to account for the observed appearance of double revertants by the time-dependent mutation rate of *trpA46* to *trpA*⁺. The *trpA trpB*⁺ mutants were probably growing on indole, an intermediate in tryptophan biosynthesis, which could be produced by spontaneous breakdown of indoleglycerol phosphate, by the tryptophan synthase α subunit produced by the leaky *trpA46* allele, and by *trpA*⁺ *trpB*[−] mutants.

Reversion of Nonsense (Ochre) Mutations

Bridges used a more standard protocol to investigate reversion of amino acid auxotrophies; cells are plated on glucose minimal medium missing the required amino acid and revertants appear as colonies. Most of his experiments used *E. coli* B/r strains carrying ochre mutations in *trpE* (encoding a subunit of anthranilate synthase) (*trpE65*) or in *tyrA* (encoding T-protein that is required for tyrosine biosynthesis) (*tyrA14*); however, some experiments used an *E. coli* K12 strain carrying a missense mutation in *trpA* (*trpA23*). The *tyrA14* strain is also *leu*[−], and starvation for leucine induces reversion of *tyrA14* (14). Because in this case the reversion is not specific to the selection, Bridges prefers to call the process stationary-phase mutation instead of adaptive mutation.

The population dynamics of these strains on the selective plates is complex. When *tyrA14* strains are plated at high density the number of viable cells declines for 3 days or so, but then recovers (17). The development of this population of slow-growing cells is required for the appearance of late prototrophs. The initial decline in viability can be prevented by catalase, which suggests that it is due to oxidative damage (17). The revertants of both *trpE65* and *tyrA14* appear from days 5 to 15 at a rate of about 1 per 10⁷ cells per day. These mutants are not true revertants or any of the known vigorous tRNA suppressors, but unknown weak suppressors (13,14). In the *tyrA14* strain, a gene called *tas*, which in multiple copy partially complements *tyrA14*, is required for the development of the slow-growing population and late reversion to Tyr⁺. However, mutations in *tas* are not responsible for the Tyr⁺ phenotype, although mutations increasing *tas* expression may be (157). In the case of *trpA23*, about 9% of the slow-growing prototrophs had small in-frame deletions, which were able to restore some activity to the TrpA protein (23).⁵ The remainder of the late-appearing *trpA23* revertants consist mainly of fast-growing mutants presumed on the basis of other experiments to carry reversions at the *trpA23* site (BA Bridges, personal communication).

Stationary-phase reversion of these auxotrophies is unaffected by defects in nucleotide excision repair, DNA polymerase I, reverse transcriptase, SOS functions, or RecA⁶ (13,16). Although preliminary results indicated that transcription-coupled repair was required [a personal communication reported in (48)], another as yet unidentified gene in the strain background was responsible (17). However, mutations in *mutY* and *mutT* increase stationary-phase mutation 10- and 100-fold (15,18,22,23). MutY is a DNA glycosylase that removes adenines paired with guanine or with 8-oxo-guanine residues, and MutT hydrolyzes 8-oxo-dGTP, preventing its incorporation into DNA. The third member of this repair pathway is MutM, a DNA glycosylase that removes 8-oxoG and ring-open forms of oxidized purines from the DNA (115). Although a *mutM* defect had no effect on its own, it

⁵The *trpA23* allele is a change from Gly to Arg at residue 210 (codon 211) of TrpA and the *trpA46* allele is a change from Gly to Glu at the same residue (165). Thus it is likely that in-frame deletions may also have occurred in Hall's experiments (described above). Indeed, Berger et al (8) reported that both alleles could be reverted by high levels of the frameshift mutagen ICR191.

⁶The induction of some Trp⁺ revertants by oxidative damage appears to require the SOS-mutagenesis UmuDC or MucAB proteins (BA Bridges, personal communication; RG Fowler, personal communication).

was synergistic with a *mutY* defect in stimulating late reversion of *tyrA14*, and overproduction of MutY and MutM each reduced late reversion of *tyrA14* about twofold (22). So, oxidative damage appears to be involved in producing the class of mutations responsible for late-appearing revertants in these strains (further discussed below). Defects in RpoS did not affect stationary-phase reversion of *trpE65* or *trpA23*, which is curious because RpoS regulates genes encoding proteins that protect against oxidative damage in stationary phase. However, production of carotenoids, which are oxygen scavengers, reduced late reversion of *trpE65* and *trpA23* about twofold (24), but carotenoids are foreign to *E. coli*, and so there may be other explanations for this effect.

Mutations in the Histidine Operon of *Salmonella typhimurium*

Two of the *hisG* alleles (encoding ATP phosphoribosyl transferase) in common use in the Ames assay have also been used for adaptive mutation studies. *hisG46* is a missense mutation, changing CTC to CCC, and *his428* is an ochre mutation. Both can revert by intrasite mutations and extragenic suppressors, but ochre suppressors are far more common than missense suppressors. When 10^9 cells are plated on minimal glucose plates without histidine, the auxotrophic strains produce 10 to 20 His⁺ colonies over 10 days (125). Most of these appear within 2 days (TA Cebula, personal communication), but a rescreening experiment indicated that even the colonies that appear early are due to mutations that occurred during the first 24 h after the cells were plated without histidine (69). Starvation for histidine for 6 h did not induce mutations giving arabinose or rifampicin resistances, nor were His⁺ mutations induced by 6 h starvation for threonine. These time periods were short, but correspond to the period of residual DNA synthesis detected after cells were starved for histidine (69) (further discussed below). Although reversion to His⁺ was *recA*-independent, it was enhanced by SOS mutagenic functions (69).

PSEUDOMONAS PUTIDA AS A MODEL SYSTEM

Pseudomonas species are known for their genomic plasticity and their ability to detoxify and metabolize a wide range of unusual and toxic chemicals. The genes encoding the relevant metabolic pathways are often associated with mobile genetic elements, which makes *Pseudomonas* species attractive subjects for studying environmentally induced mutational processes. To date, three studies of *P. putida* have documented the occurrence of stress- and substrate-associated genetic changes. The genetic events involve cryptification and decryptification of catabolic pathways, and appear to be influenced by factors associated with both starvation and the specific substrates of the pathways.

P. putida strain PP3 is capable of metabolizing halogenated alkanolic acids (HAAs), but because of this is inhibited by certain toxic HAAs. When strain PP3 was incubated in the presence of the inhibitory dichloroacetic acid (DCA), DCA-resistant mutants appeared with time, approaching frequencies of $>10^{-3}$ per viable cell after 8 days (144). The strain carries two dehalogenase genes, *dehI* and *dehII*; DCA^R mutants result from the silencing of one or both of these by DNA rearrangements involving the transposon that carries *dehI*. In some cases, the silenced genes could be decryptified in the presence of a metabolizable nontoxic substrate. Although mutation to DCA^R and some of the reversion events occurred when the cells were merely starving, in most cases the mutation rates were greatly increased by the presence of the substrates of the genes involved (156).

A similar result was obtained with *P. putida* strain 54G. In batch cultures grown on toluene, Tol[−] mutants of 54G accumulated with time, reaching a frequency of 10% by 15 days. The Tol[−] phenotype was associated with DNA rearrangements resulting in loss of the *meta* catabolite pathway but, in contrast to the DCA^R mutants discussed above, Tol[−] mutants could not revert to Tol⁺. Other mutations did not accumulate during toluene exposure, or

when toluene catabolism was suppressed, but Tol⁻ mutants accumulated in the presence of benzyl alcohol (the first oxidation product of toluene) even when cell proliferation was limited by nitrogen deprivation. Thus to produce Tol⁻ mutants toluene must be metabolized but the cells need not be able to use it to grow. In this rather peculiar case, it is not clear what the selective pressure producing the Tol⁻ phenotype might be (102).

The third report of unusual *Pseudomonas* adaptation involved utilization of phenol. *P. putida* strain PaW85 carrying a plasmid with a promoterless *pheBA* operon is Phe⁻, but Phe⁺ mutants appeared after 3 days of incubation on phenol and continued to appear for approximately 1 week. Phe⁺ mutants did not accumulate when cells were starved in the absence of phenol. The Phe⁺ mutants had insertions, base substitutions, and deletions that created active promoters. The distributions of these various events among early-arising and late-arising Phe⁺ mutants were significantly different—insertion of Tn4652 was exclusively a late event, and the proportion of mutations due to a specific C to A mutation also increased in late-arising mutants (96).

QUESTIONS OF INTEREST

The controversy surrounding adaptive mutation originally centered on whether and how selected mutations could arise when neutral or deleterious mutations did not. The two criteria for this selectivity were: (a) nonselected mutations did not occur during selection; and (b) the selected mutations did not arise under nonspecific stress, such as starvation. As discussed above, several prominent examples of adaptive mutation have failed one or the other of these tests. Nonetheless, there are still interesting questions about how mutations arise in cells that are not proliferating, whether the mutational process is a regulated response to stress or simply the result of pathology, and what the evolutionary significance of adaptive mutation might be.

Are the Movements of Insertion Elements Adaptive?

The movements of live insertion elements, such as *Mu*, and the IS elements that activate the *bgl* and *ebg* operons are mediated by functions encoded by the element and by its host (33). In contrast, usually only host functions are required for precise excision of IS elements (7). Such functions clearly can be, and in some cases are, responsive to environmental conditions. But is this activation part of a programmed response? And, if so, has the response evolved because it benefits the host, or only the element? In the case of the Ara-Lac⁺ fusion discussed above, *Mu* functions are modulated by the host's physiological state, but this is, at least in part, because the *Mu* repressor is mutant—the pathway identified does not affect the wild-type repressor (101).

How Much DNA Synthesis Occurs in Static Cells?

Transposition requires only very limited DNA synthesis (33), but other types of genetic change involve more extensive DNA synthesis. It was previously estimated that nondividing cells of *E. coli* synthesized each day an amount of DNA equivalent to 0.5 to 5% of a genome (48). However, Bridges (19) uncovered a study that had not been previously considered. Tang et al (154) measured the amount of radioactive thymidine incorporated into, and lost from, the DNA of *E. coli* B/r cells held in buffer for 24 h. Tang et al estimated that during this time an amount of DNA equivalent to 20% of a genome was turned over. This number corresponds well to the 25% of a genome per day turnover during amino acid starvation estimated from the mutation rate of a *mutT* mutant (assuming that the mutations were due to incorporation of 8-oxo- dGTP during de novo DNA synthesis) (21). Although fivefold higher than previously estimated (48), this amount of DNA synthesis would account for less than 10% of the mutations in most cases of adaptive mutation if the mutations occur at the

normal growth-dependent mutation rate [see Table 2 in Reference (48)]. There are several explanations for this discrepancy, all of which may be true: (a) the conditions under which DNA turnover has been measured do not mimic the conditions under which mutations are occurring; (b) DNA synthesis is restricted by the selective conditions to only certain parts of the genome; or (c) DNA synthesis during the selective conditions is more error prone than normal DNA synthesis.

What Initiates DNA Synthesis in Nondividing Cells?

In the section describing FC40 above, the initiation of DNA synthesis by recombination was discussed (see Figure 1). However, mutations can arise in nondividing cells in the absence of RecA, so there must be other ways in which DNA synthesis is initiated. Most DNA repair pathways result in de novo DNA synthesis, the amount of which varies from one to thousands of bases. Although the DNA of static cells is well protected (92), it may be subjected to insults from exogenous and endogenous sources. DNA polymerase I (Pol I) is the major repair enzyme in *E. coli*, and the DNA turnover observed by Tang et al, mentioned above, was reduced 90% in a strain defective for the polymerase activity of Pol I (154). However, Pol I is not known to be required for any case of adaptive mutation in *E. coli*. Indeed, a defect in this enzyme increases adaptive mutation in FC40 (PL Foster, unpublished observations), probably because loss of Pol I induces the SOS response (4). The role of Pol I in adaptive mutation should be further investigated.

The fact that adaptive mutation rates are often increased when a DNA repair pathway is inactivated also argues against repair synthesis as a source of adaptive mutation. These pathways include nucleotide excision repair (79), oxidative damage repair (6,20), and methyl-directed mismatch repair (see below). In contrast, both growth-dependent (41) and adaptive mutations are increased by overproduction of the Vsr endonuclease, which initiates very short patch repair. But this is because Vsr titrates proteins required for mismatch repair (106); loss of Vsr has no effect on adaptive mutation in FC40 (57). Not every repair pathway affects every type of mutation, but, taken together, it would appear that adaptive mutations are not due to DNA synthesis initiated by DNA repair. Thus, with the exception of recombination, the origin of DNA synthesis in nondividing cells remains a mystery.

Is Transcription Mutagenic?

True directed mutation could be achieved if transcription were mutagenic because mutation rates would then be enhanced in the very genes that were relevant to the selective conditions (38,46). Although gratuitous transcription of *lac* does not produce Lac⁺ mutants in the absence of lactose (26,53), transcription may nonetheless be mutagenic. Transcription produces single-stranded DNA, and single-stranded DNA may be more vulnerable to damage than is double-stranded DNA. For example, the rate at which cytosine deaminates to uracil is 100-fold higher in single-stranded DNA than in double-stranded DNA (although the half-life is still 200 years) (64). In addition, transcribed genes are subjected to more repair than are nontranscribed genes, which increases the chance of polymerase errors (87).

A direct link between transcription and spontaneous mutation was first reported in *Saccharomyces cerevisiae* (37), and has been extended to *E. coli* (5,164) and *Bacillus subtilis* (136). The most persuasive results were obtained when transcription was gratuitously induced in the absence of selective pressure on the mutating gene (5,37,164). In other cases, transcription was modulated by starvation for amino acids and manipulation of the global regulator ppGpp (136,163). These conditions induce the stringent response that has pleiotropic effects, only one of which is to enhance the transcription of certain classes of genes (30). In the case of one gene, *leuB*, the spontaneous mutation rate was roughly proportional to the level of its transcript (164). Starvation for amino acids does not appear to

be globally mutagenic (74,136,163), but starvation for one amino acid can increase the reversion rate of the genes required for the biosynthesis of other amino acids (163). So this mutational process is “directed” to classes of genes, not the particular gene under selection.

Are Adaptive Mutations Due to DNA Lesions?

Because elimination of DNA repair pathways often increases adaptive mutation rates, certain endogenous DNA lesions must accumulate in nondividing cells and, if not repaired, cause mutations. The greatest danger appears to be alkylation and oxidative damage.

In the absence of the enzymes that repair *O*⁶-alkylguanine and *O*⁴-alkylthymine, base substitution mutations (particularly G:C to A:T transitions) increase as cells enter into stationary phase (128); in static cells during lactose selection these mutations are increased nearly 100-fold (53,107). The endogenous mutagens that are most likely responsible are nitrosated peptides and polyamines (140,155).

As mentioned above, failure to prevent or repair oxidative damage increased adaptive reversion of amino acid auxotrophies (6,20). Adding an exogenous SOD mimic (6) or endogenous carotenoids (24), but not exogenous catalase (17), reduced mutations. In several cases, adaptive mutations were reduced under anaerobic conditions (2,6,147; RG Fowler, personal communication), but the complications of anaerobiosis make these results difficult to interpret. By a process of elimination, Bridges has argued that the actual mutagen is singlet oxygen, which can produce 8-oxo-guanine (24).

These results indicate only that alkylation and oxidation of DNA are potentially mutagenic in stationary cells. They do not mean that adaptive mutations are actually due to this damage. In wild-type cells DNA repair pathways may be entirely adequate to prevent the accumulation of DNA damage. Indeed, MutM is supposed to decline in stationary phase, yet its overproduction, or overproduction of MutY, reduced adaptive mutations only modestly (22). In addition, this effect, as well as the effects of overproduction of other proteins and oxygen scavengers, could be indirect or pathological.

SOS mutagenesis functions in *E. coli* are required when the DNA contains lesions that block replication. But they are not required in most cases of adaptive mutation. Therefore, if adaptive mutations are due to DNA lesions, these must be easily bypassed miscoding lesions, such as 8-oxo- and *O*⁶-alkyl guanine. If such lesions are in the transcribed strand of the DNA, they could give rise to a mutant transcript, which could then give rise to a mutant protein without the need for DNA replication. If the protein relieved the selective pressure, the cell could start to replicate and immortalize the mutation. This mechanism for adaptive mutation was first hypothesized by Stahl (148) (see below), but has been periodically reinvented (9,15,128,152). Recently, the experiment was actually done—a uracil substituted for an adenine in the transcribed strand of DNA gave rise to a mutant transcript and a mutant protein in nondividing *E. coli* cells (161).

What Is the Role of Mismatch Repair in Adaptive Mutation?

The mismatch repair system (MMR) recognizes mismatches in newly replicated DNA and corrects them in favor of the template strand, which increases the fidelity of replication 50- to 500-fold. MMR also participates in some other DNA repair pathways and inhibits recombination between diverged sequences. Thus MMR plays a major role in insuring genomic stability (119).

The hypothesis that adaptive mutation is due to a decline in mismatch repair was first proposed by Stahl (148). And, indeed, the levels of some MMR proteins do decline in stationary-phase and starving *E. coli* (43). However, in several, perhaps all, cases of

adaptive mutation, defects in MMR greatly increase the mutation rate (9,18,26,79,94,129).⁷ In FC40, MMR defects have as large an effect on adaptive mutation as they do on growth-dependent mutation (52). Therefore, if MMR declines during selection, the repair capacity that remains must nonetheless be sufficient for the amount of replication that takes place. Note also that the conditions under which a decline of MMR has been documented do not mimic adaptive mutation conditions. The strain that was subjected to starvation on lactose medium was deleted for *lacZ* (43,89). In FC40, the residual β -galactosidase activity produced by the $\Phi(lacI33-lacZ)$ allele may insure that adequate levels of MMR proteins are maintained during lactose selection.

Nonetheless, the mutational spectrum of adaptive mutations in FC40, -1 bp frameshifts in runs of repeated bases, is exactly what would be produced by a deficiency in MMR (60,133). For this reason, I favor the hypothesis that MMR is evaded, not inoperative, during adaptive mutation in FC40 (63). Alternatively, the type of DNA synthesis that occurs during adaptive mutation may simply make more -1 bp frameshifts in runs. For example, the same spectrum would be obtained if the proofreader subunit of DNA polymerase III were defective. Indeed, the type of replication fork hypothesized (see Figure 1E) is functional in vitro in the absence of the proofreading subunit (111).

In addition to the physical evidence mentioned above, it has been argued that MMR is limiting for adaptive mutation because (a) when MMR is defective the adaptive mutation spectrum can be obtained in growing cells (105); and (b) overproduction of MMR proteins reduces adaptive mutations but not growth-dependent mutations (89). However, we found that overproduction of MMR proteins reduces both growth-dependent and adaptive mutations (52,56). All of these results can be explained if MMR capacity remains the same relative to the amount of DNA synthesized, but the fraction of mutations that are refractory to MMR is greater during growth. Then, during growth, excess MMR would have only a modest effect and different laboratories might easily get different results. But elimination of MMR would reveal those -1 bp mutations that are normally repaired. During lactose selection a smaller fraction of the mutations are refractory, and thus excess MMR would have a larger and reproducible effect.

How Important Are Mutators, Transient or Otherwise?

Because most mutations should be deleterious, the disadvantage of being a mutator would seem always to outweigh the advantage. Yet, continuous selection for phenotypes that increase competitive fitness (32,146), or repeated selection for a succession of novel phenotypes (110,116), selects for stable mutators. About 1 to 3% of natural isolates of *E. coli* and *S. typhimurium* appear to be heritable mutators (115). So, even in the real world, mutators sometimes may be advantaged. Several mathematical models have been developed to explain these results (3,103,153). In general, these models predict that small population sizes and rapidly changing conditions favor mutators.

If, when confronted with a barrier to growth, cells could increase their mutation rate transiently, they might achieve a solution to their current problem without continuing to be burdened with a potentially deleterious mutation rate. Leaving aside how such a mechanism might evolve, the obvious mechanisms by which it could be achieved is by downregulating DNA repair pathways or upregulating mutagenic pathways (74,151). Both Ninio (122) and Boe (10) suggested that hypermutators could be created as the result of occasional errors in

⁷It was reported that defects in *mutS* increased the mutation rate of starving cells but also accelerated their death rate (9,26). However, at least in the case of FC40, the decrease in survival was due to an attenuated *rpoS* allele that had been unintentionally introduced with the *mutS* allele (PL Foster, unpublished observations).

translation or transcription leading to defects in the proteins used for DNA replication or repair.

Whether by design or accident, about 1% of the cells under lactose selection are hypermutators and their mutation rate is increased about 200-fold. However, they contribute only about 10% to the adaptive Lac⁺ mutations (132). This is exactly as predicted by Ninio (122), and, indeed, a little thought indicates it must be true. If all mutation were confined to a hypermutating minority, every Lac⁺ mutation would have arisen in a hypermutator. Then the frequency of a given nonselected mutation would be higher in Lac⁺ single mutants than in the population as a whole, but it would not be still higher in Lac⁺ double mutants. But we found that the frequency of an additional mutation was 20-fold higher in Lac⁺ single mutants than in the Lac⁻ population, and tenfold higher yet in Lac⁺ doubles (132). Therefore, most Lac⁺ revertants must arise in the general population rather than in the hyper-mutating minority. However, 95% of double mutants and 100% of triple mutants arise from hypermutators (Figure 2). Therefore, when the solution to a particular challenge requires several mutations, transient mutators may have a significant evolutionary advantage.

CONCLUSIONS

The research on adaptive mutation has revealed a plethora of mutagenic mechanisms that may be important in evolution. The recombinational mechanism utilized by FC40 could be an important source of spontaneous mutation in cells that are not undergoing genomic replication. Recombination is often, perhaps always, associated with DNA synthesis (100), and this synthesis may have a high error rate (39,149). Thus recombination increases variation both by recombining existing alleles and by creating new ones.

The case of the Ara-Lac⁺ fusion shows that the movement of insertion elements can, in principle, be responsive to environmental conditions. Insertion elements not only activate and inactivate genes, they provide sequence homology that allows large-scale genomic rearrangements. Organisms like *P. putida* have invested large parts of their metabolic capacity in movable elements, making whole metabolic pathways contingent on plasmids and transposons.

Conjugative plasmids are common in natural isolates of enteric bacteria (12). On an evolutionary time scale, F and related plasmids frequently recombine and are passed among the major groups of *E. coli* and *Salmonella enterica* (11,12). Because F can recombine with its host's chromosome, it may acquire chromosomal genes that would then be exposed to its high mutation rate and be free to diverge from the chromosomal copies. These diverged alleles could then spread through the population and even to other species.

Finally, a subpopulation of nutritionally deprived cells entering into a state of transient hypermutation could be a source of multiple variant alleles. Whether an evolutionary strategy or simply a manifestation of pathology, transient hyper-mutation could provide a mechanism for rapid adaptive evolution under adverse conditions.

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Figure 1.

A model for recombination-dependent mutation. Panels A to B, collapse of the replication fork at *oriT*; panels C to D, RecABCD catalyzed recombination; panel E, restoration of the replication fork; panel F, translocation of the Holliday junction in opposite directions by RuvAB and RecG; panel G, resolution of the Holliday junction by RuvC (not shown). See text for details. Dashed lines are newly synthesized DNA; * at *oriT* indicates TraI.

Figure 2.
Venn diagram illustrating the role of transient mutators in adaptive mutation.