Adaptive Reversion of a Frameshift Mutation in *Escherichia coli*

John Cairns*1 and Patricia L. Foster†

*The Department of Cancer Biology, Harvard School of Public Health, Boston, Massachusetts 02115, and
†Department of Environmental Health, Boston University School of Public Health, Boston University School of Medicine, Boston, Massachusetts 02118

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ABSTRACT

Mutation rates are generally thought not to be influenced by selective forces. This doctrine rests on the results of certain classical studies of the mutations that make bacteria resistant to phages and antibiotics. We have studied a strain of *Escherichia coli* which constitutively expresses a lacI-lacZ fusion containing a frameshift mutation that renders it Lac−. Reversion to Lac+ is a rare event during exponential growth but occurs in stationary cultures when lactose is the sole source of energy. No revertants accumulate in the absence of lactose, or in the presence of lactose if there is another, unfulfilled requirement for growth. The mechanism for such mutation in stationary phase is not known, but it requires some function of RecA which is apparently not required for mutation during exponential growth.

RECENT experiments have shown that certain spontaneous mutations in *Escherichia coli* seem to occur at a higher frequency when they are beneficial (SHAPIRO 1984; CAIRNS, OVERBAUGH and MILLER 1988; HALL 1988, 1990). Although there have been rather few studies of the influence of circumstances upon the production of spontaneous mutants, it is a tenet of neo-Darwinism that the frequency of mutational events should be independent of their immediate utility. For that reason, the new experiments are controversial and are unlikely to gain wide acceptance until the molecular biology of adaptive mutation is understood. The investigation of mechanism might be easier if there were an example of a spontaneous mutation where the stimulatory effect of usefulness had been quantitated. It should then be possible to find out if the process that produces adaptive mutations under conditions of selection requires a special set of gene products that are not necessary for the random, nonadaptive mutations that occur during normal growth.

Frameshift mutations in *E. coli* lacZ have been found to undergo reversion at high frequency when lactose is the sole source of energy (BRUCE LEVIN, personal communication; JEFFREY MILLER, personal communication). We have studied one such case and show here that the majority of the Lac+ revertants are arising after the cells have been exposed to lactose. These adaptive revertants do not accumulate in the absence of lactose nor do they accumulate in the presence of lactose if there is some other, unfulfilled requirement for growth. Certain mutations in recA and lexA greatly reduce the rate of adaptive reversion under conditions of selection but seem to have no effect on the rate of nonadaptive reversion during growth. This suggests that the two classes of revertant arise by different mechanisms.

MATERIALS AND METHODS

**Bacterial strains:** All the strains used in this study were derivatives of P9OC [F′ ara Δ(lacI-lacP) add thi] (COULONDRE and MILLER 1977) or a spontaneous rifamycin-resistant (RifR) isolate of P90C. Into this background we transferred F′ episomes carrying the lac-pro region with mutant alleles of lacZ but with lacF′ lacI′ lacP′. FC29 is RifR and carries an F′ Δ(lacI lacZ) (obtained from J. BECKWITH); this strain cannot revert to Lac+. FC40 is RifR and bears an F′ with a lacI lacZ fusion that eliminates the coding sequence for the last four residues of lacI, all of lacP and lacO, and the first 23 residues of lacZ (MÜLLER-HILL and KANIA 1974); transcription is initiated constitutively from the *lacI* promoter but the construct is Lac− because of a polar +1 frameshift changing CCC to CCCC at the 320th codon of lacI (CALOS and MILLER 1981). FC82 is RifR and carries the same episome as FC40 but has been made trpE9777 by P1vir transduction; the trpE9777 allele has a +1 frameshift creating a run of six A:T base pairs (BRONSON and YANOFSKY 1974). Alleles of recA were transduced into these backgrounds via linkage to srl:Tn10 or srl*: lexA3 was transduced via linkage to mal*: Tn10. The transposons were removed by subsequent transduction to srl* or mal*.

**Genetic techniques:** were as described by MILLER (1972).

**Media:** The bacteria were routinely cultivated in roller tubes at 37° in M9 minimal medium (MILLER 1972) containing 20 μg/ml thiamine, 0.001% gelatin and 0.1–0.4% glycerol or glucose and supplemented where necessary with tryptophan. The frequency of Lac+ revertants was usually determined on M9 plates containing 0.1–0.4% lactose, either by spreading or by overlaying in 2.5 ml M9 agar containing lactose. The frequency of Trp+ revertants was determined on plates containing 0.2–0.4% glucose.

1 Present address: Clinical Trial Service Unit, The Harkness Building, Radcliffe Infirmary, Oxford OX2 6HE, England.
change, over time, in viable counts on spread plates was determined by removing small circular plugs, vortexing these in M9 and assaying the suspended bacteria on 1% peptone or LB plates containing 0.1 mg/ml rifamycin when necessary.

RESULTS

The timing of appearance of Lac\(^+\) revertants: The experiments presented here were conducted with a strain that constitutively transcribes a fusion of lacI and lacZ containing a frameshift mutation (MÜLLER-HILL and KANIA 1974; CALOS and MILLER 1981). This strain (FC40) was chosen because under conditions of selection it has a high rate of reversion to Lac\(^+\). When a loopful of a saturated liquid culture is spread on a lactose-M9 plate, Lac\(^+\) revertants continue to be produced for a period of a week or two, until the plate is covered with colonies. Our first task was to exclude various trivial explanations for this effect.

Like many frameshift mutations in lacZ (ATKINS, ELSEVIERs and GORINI 1972) the +1 frameshift in FC40, although slightly leaky, does not allow cultures to synthesize enough \(\beta\)-galactosidase to undergo any measurable increase in optical density in M9-lactose (data not shown). There are, however, contaminating sources of energy in agar, and M9-lactose plates will support a certain amount of growth of Lac\(^-\) cells. For this reason, the final number of Lac\(^+\) colonies present a week after plating \(10^6\sim10^9\) FC40 cells is almost independent of the number of cells plated. To limit the amount of growth that can occur on plates, we routinely added to each aliquot of FC40 about \(10^9\) scavenger cells (FC29) bearing a deletion of lacZ, which compete for the contaminating source of energy (SMITH and SADLER 1971; ALBERTINI et al. 1982). Figure 1 shows that, when this is done, the number of Lac\(^+\) colonies arising each day is proportional to the number of FC40 cells that were plated. Because the proportionality is maintained even when only one or two Lac\(^+\) colonies are formed per plate, the continued appearance of colonies with time cannot be attributed to local growth of Lac\(^-\) cells in the neighborhood of the first colonies to be formed (cross-feeding).

Because FC40 is Rif\(^R\), we could measure the amount of growth of FC40 in the presence of the Rif\(^R\) scavenger cells by punching out small plugs from between the Lac\(^+\) colonies and assaying them for viable count on plates containing rifamycin. Figure 2 shows that in a typical experiment, the population of FC40 cells undergoes less than one doubling in the first five days after plating on M9-lactose. During this period, however, the number of Lac\(^+\) colonies is rising approximately linearly from near zero to more than \(40\) per plate. This suggests that the revertants arise after plating as the result of some process that is time-dependent but not growth-dependent.

If these revertants do indeed arise after plating, the numbers found among different cultures should form a Poisson distribution (LURIA and DELBRÜCK 1943). We therefore measured the distribution of revertants among 120 saturated cultures of FC40, plated with scavengers and counted each day. The cultures produced an average of less than one Lac\(^+\) revertant colony by day two, but the number rose to about 20 by day 7. Two cultures were plainly exceptional; one
produced 25 colonies by day 2, and the other about 60 between days 5 and 7. This tells us two things. First, the presence of these “jackpots” means that some of the revertants were the result of mutations that occurred during the prior growth of the cultures; if 120 cultures included two jackpots containing at least 25 more mutants than the average culture, the mean number of mutational events during prior growth of the cultures was roughly \((2 \times 25)/120 = 0.4\) per culture (Luria 1951). Second, the two jackpots differed in the speed with which they produced colonies, meaning that individual Lac⁰ revertants of FC40 can differ in their rate of growth on lactose. To get a better measure of this, we checked 104 independently arising revertants that produced colonies on day 5 or 6. Seventy of these produced visible colonies on lactose plates within 48 hr but the remaining 34, although growing normally on glycerol, were slow to produce colonies on lactose.

It is convenient to describe distributions of numbers of mutants in a fluctuation test as the relation between the logarithm of the proportion of cultures with \(x\) or more colonies (points) compared to the expected number (lines) for the various values of \(x\) (mutations per culture during growth) and \(\mu\) (mutations per culture after plating) that are given in Table 1.

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It is convenient to describe distributions of numbers of mutants in a fluctuation test as the relation between the logarithm of the proportion of cultures with \(x\) or more mutants and \(\log x\) (Luria 1951; Cairns, Overbaugh, and Miller 1988). Figure 3 shows that the distributions of revertants in this experiment after day two were much narrower than would be expected if all the mutations had occurred before the cultures were plated. To find some combination of a Luria-Delbrück distribution (with a mean of \(m\) mutations per culture before plating) (Lea and Coulson 1949) and a Poisson distribution (with a mean of \(\mu\) mutations per culture after plating) that would fit each of the

![Figure 3](image_url)

**Figure 3.** The distribution of Lac⁰ revertants among different cultures of FC40. A culture of FC40 in M9-glycerol was diluted 10⁵-fold to give 120 0.5-ml cultures in M9-0.015% glycerol. These were grown to saturation (1.8 \(\times\) 10⁹ viable cells per culture). Each then received 9 \(\times\) 10⁸ washed scavenger cells and was plated with top agar onto M9-lactose. The number of Lac⁰ colonies on each plate was counted each day, starting at 42 hr (when the first colonies are just visible). The figure shows the observed number of plates with \(x\) or more colonies (points) compared to the expected number (lines) for the various values of \(x\) (mutations per culture during growth) and \(\mu\) (mutations per culture after plating) that are given in Table 1.

The values for \(m\) and \(\mu\) were chosen by trial and error to minimize the value of \(\chi^2\), with the sole restriction that the initial value of \(\mu\) on day 2 should be zero. To calculate \(\chi^2\), the counts were divided into groups so that the expected number in each group (number of cultures containing between \(x\) and \(x+r\) mutants) was never less than about 5. Because an arbitrary value of \(m\) was chosen for the first set of counts, and arbitrary values of \(m\) and \(\mu\) for all subsequent sets, the number of degrees of freedom (d.f.) was respectively 2 and 3 less than the number of groups in each set. The final value of \(m\) was 0.5 for populations of 1.8 \(\times\) 10⁹ cells, which corresponds to a mutation rate of 1.4 \(\times\) 10⁻⁹ per cell per generation. The final value of \(\mu\) was 4 per day, which corresponds to a mutation rate of 0.9 \(\times\) 10⁻⁹ per cell per hr.

**TABLE 1**

<table>
<thead>
<tr>
<th>Day</th>
<th>(m)</th>
<th>(\chi^2)</th>
<th>d.f.</th>
<th>(P)</th>
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<tr>
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<td>0.30</td>
<td>4.48</td>
<td>3</td>
<td>0.2</td>
</tr>
<tr>
<td>3</td>
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<td>5.84</td>
<td>5</td>
<td>0.4</td>
</tr>
<tr>
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<td>13.16</td>
<td>8</td>
<td>0.1</td>
</tr>
<tr>
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<td>0.45</td>
<td>7.94</td>
<td>9</td>
<td>0.6</td>
</tr>
<tr>
<td>6</td>
<td>13.50</td>
<td>8.76</td>
<td>11</td>
<td>0.2</td>
</tr>
<tr>
<td>7</td>
<td>17.90</td>
<td>18.53</td>
<td>12</td>
<td>0.1</td>
</tr>
</tbody>
</table>

Comparison of the observed and expected values shown in Figure 3. The values for \(m\) and \(\mu\) were chosen by trial and error to minimize the value of \(\chi^2\), with the sole restriction that the initial value of \(\mu\) on day 2 should be zero. To calculate \(\chi^2\), the counts were divided into groups so that the expected number in each group (number of cultures containing between \(x\) and \(x+r\) mutants) was never less than about 5. Because an arbitrary value of \(m\) was chosen for the first set of counts, and arbitrary values of \(m\) and \(\mu\) for all subsequent sets, the number of degrees of freedom (d.f.) was respectively 2 and 3 less than the number of groups in each set. The final value of \(m\) was 0.5 for populations of 1.8 \(\times\) 10⁹ cells, which corresponds to a mutation rate of 1.4 \(\times\) 10⁻⁹ per cell per generation. The final value of \(\mu\) was 4 per day, which corresponds to a mutation rate of 0.9 \(\times\) 10⁻⁹ per cell per hr.

The results of this curve-fitting can be summarized as follows. Before plating, the cultures appear to have experienced an average of 0.5 mutational events (representing a mutation rate of 1.4 \(\times\) 10⁻⁹ per cell division); because \(m\) increased slowly from 0.3 to 0.5, it seems that about half of these pre-plating mutants were able to form colonies within 42 hr and the rest grew rather more slowly. After plating, the cultures averaged about four mutational events per day (representing a mutation rate of 0.9 \(\times\) 10⁻⁹ per cell per hr); again, because \(\mu\) increased slowly from two per day to four per day, it seems that about half of these post-plating mutants could form colonies in 2 days, and the rest grew more slowly. In other words, the way the distribution of colony numbers changed with time suggests that more than 95% of the colonies appearing in the first week after plating were the result of mutations that occurred after the cells had been put out onto lactose-minimal plates.

**Conditions required for the formation of Lac⁰ revertants:** The first requirement is the presence of lactose. When FC40 is plated on plain M9 agar without an energy source, no Lac⁰ revertants arise until the plates are overlaid with agar containing lactose (Figure 4).

FC40 is constitutive for transcription of the lacI-
The results, up to this point, may be summarized as follows. During normal growth, FC40 and FC82 undergo a low rate of spontaneous reversion to Lac<sup>+</sup>, presumably due to errors in replication. Revertants also accumulate when the bacteria are deprived of an energy source, but only under conditions where the mutation allows the cell to resume multiplication. It is as if cells in stationary phase are continually producing changes in sequence that are preserved if the cell starts multiplying but otherwise are discarded (perhaps by destruction or repair of the mutated sequence, or by death of the cell).

The idea that mutation in stationary phase is a distinct process would be strengthened if we could show that it required some gene products that are not required for mutation during normal growth. We have not yet succeeded in identifying such genes simply by a direct screen of a mutagenized stock of FC40, but we have found that some known gene functions are required.

**Reversion rates in strains with mutations in recA and lexA:** Various alleles of recA and lexA were moved into FC40 by transduction, and we measured their effect on the rate of appearance of Lac<sup>+</sup> colonies on lactose-M9 plates. (We avoided using a recA(Def) mutant in these experiments because of the poor viability of such strains.) Given the complex interactions between RecA, LexA and the many other genes of the SOS pathway (Walker 1984), several combinations of alleles had to be tested in order to determine which...

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**Figure 4:** The effect of delaying the addition of lactose upon the production of Lac<sup>+</sup> revertants by FC40. Six cultures of FC40 were grown to saturation in M9-0.2% glycerol. Aliquots of 0.1 ml, containing 2 x 10<sup>8</sup> cells, were mixed with 3 x 10<sup>9</sup> scavenger cells and plated with 2.5 ml top agar on M9 plates lacking any energy source. Each plate was then overlaid with a further 2.5 ml top agar. Finally, the plates were overlaid with 2.5 ml top agar containing 1% lactose, half of the plates immediately and half after 4 days of incubation. Each point is the average for 18 plates. The arrows mark the times of addition of lactose.

**Figure 5:** The effect of delaying the addition of tryptophan upon the production of Lac<sup>+</sup> revertants by a Lac<sup>-</sup> Trp<sup>-</sup> strain (FC82) after plating on lactose. Six 20 ml cultures of FC82 were grown to saturation in M9 containing 0.4% glycerol and 3 μg/ml tryptophan, and then centrifuged and suspended in 0.25 ml M9. Aliquots of 0.05 ml were then spread onto one lactose plate and plated with 2.5 ml top agar onto three lactose plates. The spread plates were assayed for viable counts in the same way as for Figure 3 (closed circles). The top-agar plates were overlaid with a further 2.5 ml and finally overlaid with top agar containing 0.1 mg/ml tryptophan, either immediately or on day 4; the counts of Lac<sup>+</sup> colonies (open circles) are the averages for six plates. The arrows mark the times of addition of tryptophan.
was the important function. The main results are shown in Figures 6 and 7.

A mutant allele of lexA (lexA3), which produces a LexA protein that is resistant to RecA-mediated cleavage (LITTLE et al. 1980), conferred about a 3-fold reduction in reversion rate; this suggested that there is a requirement for at least one of the many genes controlled directly or indirectly by LexA, such as recA, umuD and umuC. But it is not umuD or umuC and the pathway for SOS-mutagenesis that is involved, because the reversion rate was unaffected by a mutant umuC- allele (data not shown) or by mutant alleles of recA, such as recA433 (Figure 6), that are deficient in processing Umud to its active form but are not deficient in processing LexA (ENNIS, OSSANNA and MOUNT 1989). To show that the reversion rate depends on the expression of recA itself, rather than any of the other genes repressed by LexA, we showed that the lexA3 allele has no effect when recA carries an operator-constitutive mutation (recAo281) (VOLKERT, MARGOSSIAN and CLARK 1981).

One allele of recA, recA430, that is deficient in processing both LexA and Umud (ENNIS et al. 1985; NOHMI et al. 1988; SHINAGAWA et al. 1988) showed a 5-10-fold reduction in Lac+ reversion rate (Figure 6). Although the reversion rate of the recA430 strain was not further lowered by the lexA3 allele, the effect of the recA430 mutation is probably not due simply to the poor ability of RecA430 to process LexA; since the lexA3 mutation, on its own, did not confer as great a decline in reversion rate as the recA430 mutation, it seems likely that RecA430 is deficient in some other RecA-function that is required for the production of Lac+ revertants of FC40.

These results indicate that most of the Lac+ revertants of FC40, that arise after plating on lactose, arise by a process that depends on certain functions of RecA. In contrast, the rate of reversion during prior growth, before the application of selection, is not markedly affected by the presence of the recA430 allele rather than wild type recA. A fluctuation test showed that in each case two cultures out of 40 contained a jackpot of more than 100 mutants, and the right-hand end of the distribution of mutant numbers, counted at 47 hours, was roughly the same for the two strains (Figure 7).

DISCUSSION

In their natural habitat, organisms like E. coli live under extreme nutritional constraint (OZAWA and FRETER 1964) and can divide only once or twice a day (MATIN et al. 1989). But E. coli is able to accumulate mutations even when it is not dividing (RYAN, OKADA and NAGATA 1963) or replicating its DNA (RYAN, NAKADA and SCHNEIDER 1961), and this is presumably its main source of genetic variation in nature. The process of mutation in stationary phase has the unusual property that it tends to produce whatever adaptive changes are needed to allow the resumption of growth, rather than random mutations that confer no benefit (SHAPIRO 1984; CAIRNS, OVERBAUGH and MILLER 1988; HALL 1988, 1990). To determine how this is accomplished, it would be helpful to have an example where the adaptive mutations arising in a culture in stationary phase can be distinguished from the random changes that arose earlier in the culture's history when the cells were still multiplying.
In this paper, we describe the spontaneous reversion of a frameshift in a constitutively expressed lacI-lacZ fusion. During exponential growth the rate of reversion is about $10^{-9}$ per cell generation (Table 1); most of the revertants are fully restored to the wild type growth rate on lactose and produce visible colonies on lactose plates in about 40 hr; their distribution among independent cultures is roughly the Luria-Delbruck distribution that would be expected if the mutations had occurred randomly throughout the history of each culture (Figures 3 and 7). After plating on lactose, revertant colonies continue to appear at a rate of about $10^{-9}$ per cell per hour (Table 1); the cells in these late-arising colonies grow on lactose at the same rate as the other revertants; the distribution of late arising colonies among independent cultures is close to the Poisson distribution that would be expected if these mutations are occurring on the plates (Figure 3). The late mutations that arise after plating cannot be attributed to growth on the plates (Figure 2) or to cross-feeding from the first colonies to be formed (Figure 1). Interestingly, they do not occur in the absence of lactose (Figure 4) or in its presence when there is some additional growth requirement (Figure 5), suggesting that mutations in stationary phase are preserved only if they quickly lead to the resumption of growth. The late mutations differ from those that occur during exponential growth because they have an almost absolute requirement for at least one of the many functions of recA. A mutation in recA, recA430, that affects both the expression and function of RecA shows normal Lac+ reversion rates during growth (Figure 7), but its subsequent rate of reversion on lactose plates is greatly reduced (Figure 6) even though the cells are surviving perfectly well. A decrease in spontaneous mutation rates in the presence of certain alleles of recA has been reported for other systems (Kondo et al. 1970; Sargentini and Smith 1981; Albertini et al. 1982; Whoriskey, Schofield and Miller 1991) although there was no thought at that time that some of the spontaneous mutations might be arising after the onset of selection.

Adaptive mutations could be acquired in stationary phase in many different ways (Cairns, Overbaugh and Miller 1988) but perhaps the most conservative explanation is the following (Stahl 1988; Boe 1990). The enzymes for DNA repair seem capable of resolving almost any form of damage and correcting any copying error provided that they have time to take the process to completion. Therefore, as long as a bacterium refrains from duplicating its DNA, it may be protected against spontaneous mutation. So the only spontaneous sequence changes that become irreversibly fixed in a stationary-phase population may be those that happen to be uncorrected in cells when they resume growth, and these changes will necessarily include any mutation that allowed the cell to start growing. Of course, the moment a cell is successful and resumes growth it may have no way of singling out for preservation the particular change in sequence that brought success, which could explain why mutants arising under selective conditions show a somewhat increased frequency of additional mutations in genes unrelated to the selective pressure (Hall 1990). So far we have not found a way to test this general model. Defects in certain repair pathways, such as mutHLS-dependent mismatch repair (Boe 1990) and ada-ogt-dependent alkylation repair (Rebeck and Samson 1991), are known to increase the rate of mutation in stationary phase, but this does not mean that these are the systems that are normally eradicating the unsuccessful sequence changes. To show that repair functions are preventing nonadaptive mutations, we would have to show that Lac+ revertants accumulate in the absence of lactose when these pathways are defective. So far our experiments along those lines have been indecisive. There is, however, less room for doubt when a defect in some DNA-processing system is found to decrease the rate of mutation. Our results with lexA3, which lowers the expression of recA, and with recA430, which is known to be deficient in several functions of recA, suggest that RecA plays (directly or indirectly) some role in the production or fixation of adaptive sequence changes, or perhaps in the creation or termination of a special state of high mutability.

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