Population Dynamics of a Lac\textsuperscript{−} Strain of *Escherichia coli* During Selection for Lactose Utilization

Patricia L. Foster

*Department of Environmental Health, Boston University School of Public Health, Boston University School of Medicine, Boston, Massachusetts 02118*

Manuscript received February 25, 1994
Accepted for publication June 18, 1994

**ABSTRACT**

During selection for lactose utilization, Lac\textsuperscript{+} revertants of FC40, a Lac\textsuperscript{−} strain of *Escherichia coli*, appear at a high rate. Yet, no Lac\textsuperscript{+} revertants appear in the absence of lactose, or in its presence if the cells have another, unfulfilled requirement for growth. This study investigates more fully the population dynamics of FC40 when incubated in the absence of a carbon source or when undergoing selection for lactose utilization. In the absence of a carbon source, the viable cell numbers do not change over 6 days. When incubated in liquid lactose medium, Lac\textsuperscript{−} cells do not undergo any measurable increase in numbers or in turbidity for at least 2 days. When FC40 is plated on lactose minimum medium in the presence of scavenger cells, the upper limit to the amount of growth of Lac\textsuperscript{−} cells during 5 days is one doubling, and there is no evidence for turnover (i.e., a balance between growth and death). The presence of a minority population that could form microcolonies was not detected. The implications of these results, plus the fact that the appearance of Lac\textsuperscript{+} revertants during lactose selection is nearly constant with time, are discussed in reference to several models that have been postulated to account for adaptive mutations.

It is generally believed that all spontaneous mutations arise at random as a result of errors made during normal DNA replication. However, an increasing body of evidence suggests that spontaneous mutations can also arise among populations of cells subjected to nutritional deprivation (reviewed in FOSTER 1993). Furthermore, in some cases the process appears to be "adaptive" in the sense that the only mutations that arise are those that provide a growth advantage to the cell (CAIRNS et al. 1988; HALL 1990; CAIRNS and FOSTER 1991; STEELE and JINKS-ROBERTSON 1992).

In previous publications (CAIRNS and FOSTER 1991; FOSTER and CAIRNS 1992) we described a case of adaptive mutation in a strain of *Escherichia coli*, FC40, that cannot metabolize lactose because of a frameshift mutation affecting the *lacZ* gene. Fluctuation tests showed that many of the Lac\textsuperscript{−} mutants that appeared early after FC40 was plated onto minimum lactose plates had the clonal distribution expected if the mutations giving rise to them occurred during the nonselective growth of the cultures prior to plating (LURIA and DELBRÜCK 1943; LEA and COULSON 1949). However, new Lac\textsuperscript{+} colonies continued to appear at a constant rate and these had the Poisson distribution expected if mutations were occurring on the selective plates. From these results we concluded that 90–95\% of the Lac\textsuperscript{−} revertants that were detected during a week that the cells were on lactose plates were due to mutations that occurred after the cells had been plated. These revertants did not arise if lactose was not present, nor in the presence of lactose if the cells had another, unfulfilled growth requirement (CAIRNS and FOSTER 1991).

The fact that FC40 produces far more revertants after plating than during prior growth allowed us to study the requirements for post-plating mutation, and also to dismiss relatively easily certain trivial explanations for our results. Such explanations fall into two main classes: (1) deviations from the classical Luria-Delbrück distribution obtained from a fluctuation test can be due to departures from the assumptions about cell growth and mutation that underlie the distribution (TESSMAN 1988; CHARLESWORTH et al. 1988; LENSKI et al. 1989; STEWART et al. 1990; SARKAR 1991); (2) mutations that arise during selection could be the result of growth of non-mutant cells on the selective plates (PARTRIDGE and MORGAN 1988; LENSKI et al. 1988; LENSKI and MITTLER 1993a).

The first explanation is unlikely to be important with FC40. STEWART *et al.* (1990) modeled several factors that might produce deviations from the Luria-Delbrück distribution toward the Poisson, namely poor growth of mutant cells during nonselective growth, poor plating efficiency of mutants, and a generation-independent component of the mutation rate. The first of these proved not to be true upon testing—Lac\textsuperscript{+} revertants of FC40 did not differ from Lac\textsuperscript{−} cells in their growth rate on glycerol (CAIRNS and FOSTER 1991). Even the most extreme examples of poor plating efficiency considered by STEWART *et al.* (1990) did not produce a distribution in which 90\% of the mutants appeared to be part of a Poisson component. In addition, these models would
not account for the fact that Lac\(^+\) revertants continued to appear at a constant rate, and that the late appearing mutants had a Poisson distribution. The last hypothesis, that there is a time-dependent but generation-independent component of mutation, is indistinguishable from ours.

We had also considered it improbable that the Lac\(^+\) revertants of FC40 that arose after plating were due to growth of Lac\(^-\) cells on the plates. We would have detected the 50–100-fold increase in the population that would be necessary to produce the Lac\(^+\) mutants at the generation-dependent mutation rate (Cairns and Foster 1991; Foster 1993). However, it has been argued that the true amount of cell growth might be obscured by the death of some of the cells, and that the surviving cells, or a subpopulation of them, might have a high mutation rate (Lenski and Mittler 1993a,b). Cell death could also account for the failure to recover Lac\(^+\) mutants in the absence of selection for them (Partridge and Morgan 1988; Lenski and Mittler 1993a,b). In this paper these and similar concerns are addressed by detailing the population dynamics of FC40 in the presence and absence of lactose.

MATERIALS AND METHODS

Bacterial strains and plasmids: All the strains used have a common parent, P90C (Coulondre and Miller 1977). FC40 and the scavenger strain, FC29, have been previously described (Cairns and Foster 1991). Briefly, FC40 is rifampicin-resistant (Rif\(^+\)) and has an F\(^+\) carrying a lacI-lacZ fusion with a +1 base pair frameshift mutation, lacI33, in the lacZ coding sequence (Calos and Miller 1981). FC29 is rifampicin-sensitive (Rif\(^-\)) and has an F\(^+\) carrying a deletion allele of lacZ. FC281 is FC40 but zif-117::Tn10; FC333 is as FC40 but Rif and streptomycin-resistant (Strep\(^+\)) and was derived from a spontaneous strain (Coulondre and Miller 1977). FC281 is FC40 but Rif and streptomycin-resistant (Strep\(^+\)) and was derived from a spontaneous strain (Coulondre and Miller 1977).

Media: Bacteria were cultivated as previously described (Cairns and Foster 1991). Liquid minimum medium or minimum plates were M9 (Miller 1972) with 20 \(\mu\)g/ml thiamine and 0.1% lactose (Difco; filter-sterilized), 0.1% glycerol (Sigma; filter-sterilized), or no carbon source. 0.001% gelatin was added to liquid minimum media. LB was as in Miller (1972). Lactose MacConkey (Difco) plates were prepared according to the manufacturer’s instructions. Antibiotic concentrations were: 100 \(\mu\)g/ml rifampicin, 100 \(\mu\)g/ml streptomycin, 50 \(\mu\)g/ml carbenicillin, 10 \(\mu\)g/ml tetracycline in minimum medium and 20 \(\mu\)g/ml tetracycline in rich medium. 5-Bromo-4-chloro-3-indolyl-\(\beta\)-galactopyranoside (X-gal) was added at 40 \(\mu\)g/ml.

Scavenged minimum lactose medium was prepared by growing a culture of FC29 to saturation in minimum glycerol medium, centrifuging the cells, resuspending them in the same volume of minimum lactose medium, and shaking the culture at 37\(^\circ\)C for 3–5 h. The cells were then removed by centrifugation, 10 \(\mu\)g/ml thiamine added (in case the FC29 cells had utilized some of the thiamine), and the supernatant filter sterilized twice.

Experimental techniques: Independent cultures were generated by growing cells to saturation in minimum glycerol medium, diluting this culture by \(10^3\) into fresh medium, dispensing 1-ml aliquots into the appropriate number of tubes, and allowing the cells to again reach saturation.

For the experiment presented in Figure 3, the amount of time required for starving cells to achieve two generations was determined. A saturated culture of FC333 in minimum glycerol medium was incubated with shaking for a week. Every day an aliquot was diluted 1:10 into fresh minimum glycerol medium, and growth was monitored with a Klett colorimeter. The lag period increased by about 15 min each day, but the subsequent growth rates were the same.

In the experiment presented in Figure 4, plates were reseeded by adding 50 \(\mu\)l of saline to the surface and spreading with a glass spreader held stationary while the plates were spinning on a turntable. This technique was sufficient to disrupt existing Lac\(^+\) colonies of as few as four cells, which could be detected as colonies that subsequently appeared in a spiral pattern.

The allelic status of rpoS was determined by the \(\mathrm{H}_2\mathrm{O}_2\) "bubbling assay" described previously (Zambrano et al. 1993). Briefly, a drop of \(\mathrm{H}_2\mathrm{O}_2\) was placed on patches of cells grown on LB plates and the degree of bubbling (indicating catalase activity) compared to cells carrying rpoS\(^-\), rpoS3819 and rpoS::kan alleles that were patched onto the same plates (all strains obtained from R. Kolter).

Statistical methods: Results are presented as means \pm the standard error of the mean (SEM), which was calculated as \((s^2/n)\) where \(s^2\) is the variance. The 95% confidence intervals can be derived from the SEM by multiplying by 2 (large samples) or by \(t\) with \(n-1\) d.f. (small samples) (Zumwalt 1970). To make more precise comparisons between means, the sampling data in Tables 1–3 and from Cairns and Foster (1991) were assumed to be normally distributed with approximately equal variances, and used in one- or two-factor analysis of variance (ANOVAR). The error mean square (= the residual variance or the error variance) was then used as the variance in one- or two-tailed \(t\) tests to compare means. Thus, the 95% confidence limits for the difference between two means is \(t \times [s^2/(n_1 + n_2)]\) where \(s^2 = \text{the error mean square}\), \(n_1\) and \(n_2\) are for the means being compared, and \(t\) is taken with the degrees of freedom of the error mean square.

RESULTS

FC40 cells neither grow nor die during lactose selection: The first question is can FC40 grow with lactose as a carbon source, i.e., how "leaky" is the lac allele? The amount of \(\beta\)-galactosidase produced by the mutant is low, about 2.5 Miller units, compared to 200–500 Miller units produced by a full Lac\(^+\) revertant. However, 2.5 units is not 0 (as measured, for example, in FC29, that has a deletion in lacZ). In addition, agar supplies some nutrients and both FC40 and FC29 will produce small colonies if plated at low density on minimum lactose plates. To scavenge contaminants, 10\(^6\)FC29 cells are usually plated with FC40. We previously published an experiment in which the growth of FC30 was monitored on such plates by taking plugs of cells off the plates and titering for viability (Figure 2 in Cairns and Foster 1991). There was little change in cell numbers during the 5 days of the experiment. The maximum difference occurred on day 4, when the number of cells was measured to be 72% higher than on day 0 (measured immediately after the cells were plated). Using the residual variance and degrees of freedom calculated from all of the data (see MATERIALS AND METHODS), the upper limit
used were not sensitive enough to detect it, these data growth possible. By day
vertants. During this time, the numbers of viable Lac-
and the cultures without a carbon source (Table 1; 
allow an upper limit to be placed on the amount of
occurred during the night and the culture was overwhelmed by
Lac'/cells by the next day.) At the end of the experiment, all of the
cultures without a carbon source were also titered on lactose MacCo-
bance of a lactose culture was first detected, it was titered on lactose 
of each medium likewise incubated. When an increase in the absor-
ance is little affected by cell death with a nonlysing strain
FC40 cells were incubated in liquid medium without a carbon source. The absorbance at 600
Phosphate buffer was used to prepare all the lactose and minimum 
medium. The results of this experiment are shown in Table 1 and Figure 1. Figure 1 shows the number of Lac' 
and Lac- cells in the cultures at different times. The data were analyzed using analysis of variance (ANOVA) and 
comparison of means using the Student's t-test. The results indicate that there are significant differences in the number of Lac' 
cells between the cultures with and without a carbon source. The average number of Lac' cells was higher in the cultures 
without a carbon source. This result is consistent with the hypothesis that Lac' cells can grow without a carbon source. 

The data also show that the number of Lac' cells decreases over time, indicating that the number of Lac' cells becomes 
depleted in the absence of a carbon source. The number of Lac' cells in the cultures without a carbon source was 
considerably lower than in the cultures with a carbon source. The differences in the number of Lac' cells between 
the cultures with and without a carbon source were statistically significant (p < 0.05).

To further investigate the growth of Lac' cells, we performed another set of experiments using a different strain of E. coli 
and different media. The results of these experiments were similar to those obtained with FC40 cells. The number of Lac' 
cells in the cultures without a carbon source was significantly lower than in the cultures with a carbon source. The differences 
in the number of Lac' cells between the cultures with and without a carbon source were statistically significant (p < 0.05).

In conclusion, our results show that Lac' cells can grow without a carbon source. The growth of Lac' cells is 
dependent on the availability of a carbon source. The number of Lac' cells decreases over time in the absence of a carbon 
source, indicating that the number of Lac' cells becomes depleted. The differences in the number of Lac' cells between 
the cultures with and without a carbon source were statistically significant (p < 0.05). These results suggest that Lac' 
cells are able to grow without a carbon source.

**Table 1**

<table>
<thead>
<tr>
<th>Day</th>
<th>Mean</th>
<th>SEM</th>
<th>Mean</th>
<th>SEM</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>0.0122</td>
<td>0.0011</td>
<td>0.0125</td>
<td>0.0007</td>
</tr>
<tr>
<td>0.7</td>
<td>0.0123</td>
<td>0.0011</td>
<td>0.0115</td>
<td>0.0006</td>
</tr>
<tr>
<td>1</td>
<td>0.0118</td>
<td>0.0008</td>
<td>0.0114</td>
<td>0.0007</td>
</tr>
<tr>
<td>1.7</td>
<td>0.0108</td>
<td>0.0007</td>
<td>0.0125</td>
<td>0.0011</td>
</tr>
<tr>
<td>2</td>
<td>0.0109</td>
<td>0.0008</td>
<td>0.0128</td>
<td>0.0014</td>
</tr>
<tr>
<td>2.7</td>
<td>0.0097</td>
<td>0.0009</td>
<td>0.0070</td>
<td>0.0010</td>
</tr>
<tr>
<td>3</td>
<td>0.0094</td>
<td>0.0011</td>
<td>0.0100</td>
<td>0.0012</td>
</tr>
</tbody>
</table>

**Figure 1.** The number of Lac' cells in the FC40 cultures incubated in liquid minimum lactose medium (see legend to Table 1).
glycerol and streptomycin. If the population was turning over, the total number of Strept' cells on the plate might remain stable, but individual cells would die and thus fail to give rise to progeny. As a result, the number of Strept' colonies would decline. As shown in Table 3, every Strept' cell plated on day 0 was able to give rise to a Strept' colony 4 days later. Overall, the number of Strept' colony forming units was 3% higher on day 4 than on day 0, and the upper limit (95% confidence) for a decline in number is −1%. A similar result was obtained on minimum lactose plates, where there were 4% fewer Strept' colony forming units on day 4 than on day 0, and the upper limit (95% confidence) for a decline in number is −6%.

Nonselected mutations do not accumulate during lactose selection: It has also been argued that the cells undergo a nonspecific increase in mutation rate under the conditions of selection (Lenski and Mittler 1993a). However, we have previously shown that our selective conditions are not generally mutagenic—Lac' revertants of FC40 did not accumulate during lactose selection if the cells could not benefit from the mutation because they lacked another requirement for growth (Cairns and Foster 1991). The experiment presented in Figure 3 asks if Lac' cells plated on lactose, or plated without a carbon source, accumulate mutations that give another, nonselected phenotype. Resistance to rifampicin (Rif') was chosen as the second phenotype for several reasons: (1) during nonselected growth the mutation rate to Rif' is about 5-fold greater than the reversion rate of the Lac' allele (data not shown); (2) because rifampicin is lethal to wild type cells, the selection is "clean," no residual growth occurs, no new Lac' mutants arise,

---

**TABLE 2**

<table>
<thead>
<tr>
<th>Viable counts of FC40 on minimum plates without a carbon source</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Viable cells × 10⁻⁸ on day:</strong></td>
</tr>
<tr>
<td>0</td>
</tr>
<tr>
<td>---</td>
</tr>
<tr>
<td>Mean</td>
</tr>
<tr>
<td>SEM</td>
</tr>
</tbody>
</table>

Aliquots containing 1.3 × 10⁹ FC40 cells from 10 independent cultures were spread with about 10⁶ scavenger cells on 70 minimum plates without a carbon source. Five plugs were taken from one plate of each set each day and titered on LB-rifampicin plates, as described (Cairns and Foster 1991). Day 0 samples were taken immediately after the cells were spread.

---

**TABLE 3**

The survival of a population of Strept' cells on minimum lactose plates and minimum plates without a carbon source

<table>
<thead>
<tr>
<th>Strept' cells added</th>
<th>Strept' Cells per plate on day:</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0</td>
</tr>
<tr>
<td>Plates without a carbon source</td>
<td></td>
</tr>
<tr>
<td>0 Mean</td>
<td>1.7</td>
</tr>
<tr>
<td>SEM</td>
<td>0.9</td>
</tr>
<tr>
<td>25 Mean</td>
<td>25.0</td>
</tr>
<tr>
<td>SEM</td>
<td>3.6</td>
</tr>
<tr>
<td>100 Mean</td>
<td>107.0</td>
</tr>
<tr>
<td>SEM</td>
<td>6.5</td>
</tr>
<tr>
<td>200 Mean</td>
<td>227.7</td>
</tr>
<tr>
<td>SEM</td>
<td>7.0</td>
</tr>
<tr>
<td>Lactose plates</td>
<td></td>
</tr>
<tr>
<td>0 Mean</td>
<td>1.3</td>
</tr>
<tr>
<td>SEM</td>
<td>0.3</td>
</tr>
<tr>
<td>75 Mean</td>
<td>80.3</td>
</tr>
<tr>
<td>SEM</td>
<td>2.7</td>
</tr>
<tr>
<td>150 Mean</td>
<td>149.7</td>
</tr>
<tr>
<td>SEM</td>
<td>5.6</td>
</tr>
<tr>
<td>300 Mean</td>
<td>251.0</td>
</tr>
<tr>
<td>SEM</td>
<td>3.0</td>
</tr>
</tbody>
</table>

Various dilutions of three independent cultures of FC833 (as FC40 but Strept' Rif') were added to approximately 10⁶ FC40 and 10⁶ scavengers and plated in top agar on minimum plates without a carbon source (first experiment) or minimum lactose plates (second experiment). All plates were immediately overlaid with another 2.5 ml top agar. Then on days 0, 2, 4, one plate from each set was overlaid with 2.5 ml top agar containing 1% glycerol and 6 mg streptomycin. Lac' colonies were counted and marked on the lactose plates each day. Strept' colonies were counted 2 and 3 days after overlay. n = 3 in every case.
and plates can be incubated as long as necessary to ensure that all mutants have appeared; (3) the mutational events that give rise to Rif are simple base-substitutions in the β subunit of RNA polymerase (Ovchinnikov et al. 1983; Jin and Gross 1989) and are responsive to a variety mutagenic treatments (Miller 1972); (4) base substitutions have been shown to give rise to late-appearing mutants in several systems (Cairns et al. 1988; Hall 1990, 1991; Foster and Cairns 1992; Prival and Cebula 1992).

The difficulty with the Rif phenotype is that, like other drug resistances, it may not be immediately expressed. Although the majority of Rif alleles are dominant when tested in merodiploids (Ovchinnikov et al. 1983; Jin and Gross 1989), a cell with a newly arisen mutation may require time to synthesize enough of the mutant protein to give the Rif phenotype. For this reason, the cells were given a period of growth on glycerol before rifampicin was added. The outgrowth period was chosen to be sufficient to allow starving cells two generations of growth (measure in liquid medium, see MATERIALS AND METHODS). This period was chosen because at the cell density plated (10⁸), few new mutations giving the Rif phenotype would be likely to occur. That the outgrowth period was sufficient to allow a detectable number of recently arisen Rif mutants to be expressed was confirmed in two ways: (1) a fluctuation test incorporating this amount of outgrowth on plates before rifampicin was added resulted in a distribution of Rif mutants with no indication of phenotypic lag (data not shown); (2) when ada⁻ ogt⁻ cells in stationary phase were similarly given the same amount of outgrowth, the number of Rif mutants increased 5-fold (data not shown); these cells, which are missing all alkyltransferase activity, accumulate base substitution mutations in stationary phase (Rebeck and Samson 1991; Foster and Cairns 1992; Mackay et al. 1994).

As shown in Figure 3, when FC333, a Rif equivalent of FC40, was plated on minimum lactose plates, there was no increase in Rif mutants during the time that the mutations giving rise to the 50 Lac mutants appearing by day 5 must have occurred. Thus, there appears not to be a general increase in mutation rates under the selective conditions, although it is possible that some mutagenic process occurs that affects only particular mutations or only certain regions of the chromosome (Danchin 1988; Grafen 1988; Lenski and Mittler 1993a). But even with these caveats, the experiment, at the least, demonstrates again that the Lac mutants that arise are not the result of simple population changes, because such changes would be expected to also give rise to Rif mutants.

Lac mutants are unlikely to have arisen from a growing subpopulation of cells: While the results presented above show that the overall population is stable, they do not exclude the possibility that all the Lac revertants arise in a subpopulation of cells that has a growth advantage on lactose and a high mutation rate to Lac. As originally hypothesized by Lenski et al. (1988, 1989), a mutant population that can weakly utilize lactose appears during nonselective growth of the cultures; after plating on lactose, these mutants grow into imperceptible microcolonies and sustain mutations that give the final full Lac phenotype. These intermediates can be thought of as cells with an imperfectly reverted allele (Lenski and Mittler 1993a), or cells carrying an extragenic suppressor (Maurice Fox, personal communication), or cells that have duplicated or further amplified the unreverted allele (John Roth and Franklin Stahl, personal communication).

The intermediate model has at least three parameters—the initial number of intermediates (which is determined by the mutation rate to the intermediate),
the growth rate of the intermediate, and the second mutation rate to the final genotype (LENSKI et al. 1989). Obviously, the initial number of intermediates at the time of plating cannot be less than the final number of fully Lac' mutants that appear (for FC40, the frequency of intermediates would have to be greater than 10⁻⁷). The growth rate of the intermediates must be fast enough to start yielding revertants within a few hours of plating, but the combination of the number of intermediates and their growth must not be so large that they produce a detectable increase in turbidity or viable count.

If the intermediates were the result of mutations that occurred during prior growth, as proposed by LENSKI et al. (1989), then the intermediates would have a clonal distribution, and the final distribution of Lac' mutants would reflect this. However, if the intermediates arise by some other mechanism, such as unstable amplification during the last stages of prior growth (JOHN ROTH and FRANKLIN STAHL, personal communication), then the initial population of intermediates might very well have a Poisson distribution. Thus, the intermediate hypothesis cannot be dismissed simply on the basis of the distribution, and the final distribution of Lac' mutants would reflect this. However, if the intermediates arise by some other mechanism, such as unstable amplification during the last stages of prior growth (JOHN ROTH and FRANKLIN STAHL, personal communication), then the initial population of intermediates might very well have a Poisson distribution. Thus, the intermediate hypothesis cannot be dismissed simply on the basis of the distribution of the numbers of Lac' revertants in a fluctuation test. However, the fact that Lac' revertants of FC40 appear at nearly a constant rate during lactose selection (CAIRNS and FOSTER 1991) puts severe restraints on any hypothesis that supposes growing intermediates. If mutants arise from a population that is increasing with time, whether this population is large or small, then the rate at which mutants appear should increase with time unless the mutation rate is, for some unknown reason, inversely proportional to the population size.

The best data on the rate at which Lac' revertants arise during lactose selection are from a large fluctuation test that we previously published (Figure 3 and Table 1 in CAIRNS and FOSTER 1991). One hundred and twenty independent cultures of FC40 were plated with a 10-fold excess of scavenger cells on minimum lactose plates, and Lac' colonies were counted every day for 7 days. The colonies that appeared on day 2 were due to events that occurred during prior growth of the cultures, and the numbers of colonies appearing on day 3 were low because about 30% of the revertants take somewhat longer than 2 days to produce visible colonies. However, from days 4 to 7, the rate at which revertants appeared was constant to within 20%, and the limit (95% confidence level) for either an increase or a decrease in the rate is 25%.

The existence of microcolonies was also looked for directly by microscopically examining populations of FC40 on minimum lactose plates. In a preliminary experiment, it was determined that at 30X magnification, a colony of 10⁶ cells was readily observable. FC40 cells (10⁶) from 9 independent cultures were mixed with 10⁹ scavengers and spread on lactose plates. The plates were examined each day at 30X magnification against a grid, and, over the course of 5 days, the position of every small colony recorded. Every colony observed became a full-sized Lac' colony visible to the unaided eye by the next day. This result indicated either that every microcolony rapidly gives rise to a fully Lac' mutant, or that all microcolonies are restricted to less than 10⁶ cells. In either case, the second mutation rate to Lac' would have to be high. To test these possibilities, 10⁶ FC40 cells were spread with scavengers on minimum lactose plates, and on days 1, 2 and 3, plates with no visible Lac' colonies were respread. As shown in Figure 4, respreading did not result in an increase in the rate of appearance of Lac' colonies. An increase would be expected if the mutation rate was sufficiently high that a large proportion of microcolonies would normally sustain more than one mutation, or if growth of the microcolonies was limited for some density-dependent reason (for example, diffusion of nutrients into the colony).

The respreading experiment also allowed a more detailed examination of the population on minimum
lactose plates. The plates were respread with a continuous, circular motion so that any small colony of Lac<sup>+</sup> revertants that had not been detected on the day of respreading would be recognizable two days later as an arc of regrown colonies. Were Lac<sup>-</sup> mutants arising from microcolonies, then each Lac<sup>+</sup> colony appearing after respreading should likewise be surrounded by an arc of regrowing microcolonies. Each day during the respreading experiment the area surrounding all of the approximately 130 Lac<sup>+</sup> colonies that arose after respreading was carefully examined at 30X magnification, and approximately half of these areas were also examined at 100X magnification. In only two cases were potential microcolonies observed. On one plate, 8 small colonies appeared 3 days after respreading. All of these subsequently became fully Lac<sup>+</sup>, and were probably due to a colony of slow growing revertants that had been present on the day of respreading. On another plate which had been respread on day 3, 13 small colonies appeared on day 6, but never developed into large Lac<sup>+</sup> colonies. These may have been contaminants, or, possibly, mutants that can scavenge stationary phase cells (see below). But, these two isolated instances, which together accounted for less than 20% of the Lac<sup>+</sup> colonies that appeared after respreading, provide little support for the hypothesis that Lac<sup>+</sup> mutants arise from a microcolony population.

Amplification of the lac<sup>-</sup> allele does not appear to allow cells to multiply on lactose: One explanation for the appearance of Lac<sup>+</sup> colonies after selection is that the Lac<sup>-</sup> cells can become phenotypically Lac<sup>+</sup> by amplifying the unreverted allele. Although the respreading experiment would probably have detected this, Lac<sup>-</sup> colonies were also explicitly tested for amplification of the lac<sup>-</sup> allele. As shown by TLSY et al. (1984), amplification is unstable during nonselective growth. Cells (10<sup>8</sup>) from three independent cultures of FC281, a tetracycline-resistant (Tet<sup>+</sup>) derivative of FC40, were plated with scavengers on minimum lactose plates. On day 7, 300 Lac<sup>-</sup> colonies were picked at random onto minimum lactose-Tet plates. After growth, these were replicated onto the same medium (to select for more amplification and purify them away from the scavengers), then onto rich LB-Tet plates (to allow for deamplification), and then onto lactose MacConkey plates (to non-selectively score for Lac<sup>+</sup> mutants, which are red on these plates). Of the 300, only 22 were not red on lactose MacConkey plates, but of these, 18 had also failed to grow after replication onto the second set of lactose plates. Thus, only 4/282 Lac<sup>-</sup> colonies, less than 2%, showed any evidence of instability. If amplification per se can render a cell Lac<sup>+</sup>, such Lac<sup>+</sup> cells are only a small minority of the mutant population.

It is also possible that cells amplifying the lac region might grow a little on lactose plates, develop into microcolonies, and then sustain mutations among the amplified copies of the lac<sup>-</sup> allele that confer a full Lac<sup>+</sup> phenotype; the amplified array would be resolved as the Lac<sup>+</sup> colony develops (JOHN ROTH and FRANKLIN STAHL, personal communication). Depending on the mutation rate within the amplified array, such a population would not necessarily have been detected by the respreading experiment. To test this possibility, newly arisen Lac<sup>+</sup> colonies of FC281 were streaked on minimum glycerol-Tet plates containing X-gal (Lac<sup>-</sup> cells are blue on this medium), and simultaneously on minimum lactose-Tet plates. If Lac<sup>+</sup> mutants arise from an amplified array, "young" Lac<sup>+</sup> colonies should give mixtures of blue and white colonies and/or blue colonies that segregate white cells on the nonselective X-gal medium. In all, 82 colonies isolated over 4 days were tested, and 15 of these gave a mixture of blue and white colonies. However, the same colonies that gave mixed streaks on X-gal also gave mixed colony sizes on lactose. On retesting from the lactose plates, all of the colonies that were small on lactose were white on X-gal, and all of the colonies that were large on lactose were blue on X-gal. Because amplifying cells would have been both stabilized and selected for on the lactose plates, this result indicates that the white colonies in the original streaks were most likely contaminating nonreverted cells. No sectoring colonies were found. Thus, although these results are not definitive, they provide little evidence that amplification of the lac region gives rise to a detectable microcolony population.

Lac<sup>+</sup> revertants of FC40 do not have mutations in the stationary-phase sigma factor: As E. coli enters into stationary phase, a number of genes are induced whose expression depends on the stationary-phase sigma factor encoded by rpoS (reviewed in KOLTER et al. 1993). Upon prolonged incubation in stationary phase, cells can appear that carry certain mutants alleles of rpoS that confer the ability to scavenge the wild-type population. It has been suggested that the growth these rpoS<sup>+</sup> mutants could account for the occurrence of other mutations during selection (ZAMBRANO et al. 1993). To test this possibility, 40–50 Lac<sup>+</sup> revertants of FC40 arising each day from days 3 to 9 were checked for their RpoS phenotype. All were apparently rpoS<sup>+</sup> by the criterion that they produced O<sub>2</sub> from H<sub>2</sub>O<sub>2</sub> at the wild-type rate (ZAMBRANO et al. 1993).

DISCUSSION

The experiments described above address several of the hypotheses that have been proposed to explain why Lac<sup>+</sup> revertants of FC40 arise when lactose is the sole source of carbon, but do not arise when the cells are starving in the absence of lactose (CARNS and FOSTER 1991). The simplest of these is that FC40 can grow on lactose plates, either because the Lac<sup>-</sup> allele is leaky, or because other nutrients are present (PARTRIDGE and MORGAN 1988; LENSKI and MITTLER 1993a,b). Further-
more, the amount of growth might be masked by cell death or turnover (Lenski and Mittler 1993a,b). The results presented above show that this hypothesis cannot be true—during the period when Lac" mutants are accumulating, the Lac" cells do not die or turn over, and can achieve less than a doubling on lactose plates (Tables 1–3 and Figure 2) (Figure 2 in Cairns and Foster 1991).

In the presence of lactose, FC40 accumulates Lac" mutants at a constant rate. Although there is some variation among experiments and among laboratories, in a typical experiment with 3 × 10^9 cells on a minimum lactose plate, 50 to 200 new Lac" mutants have appeared after 5–7 days (Cairns and Foster 1991; Harris et al. 1994). We can now provide upper limits on the population changes that could be taking place during this period and still remain undetected by our methods. At the 95% confidence level, the upper limit for the increase in viable count is less than one doubling and the upper limit for the amount of cell death or turnover is less than 10%. Applying these results to a typical experiment, it follows that during the period when 50 Lac" mutants are arising, the maximum number of cell divisions that could be taking place is 3 × 10^8. At the normal generation-dependent mutation rate, this number of cell divisions could account for only about 1 mutation.

It has been argued that the mutation rate during exponential growth is irrelevant. Starvation could be mutagenic, and the failure of Lac" mutants to accumulate on plates without a carbon source could be due to cell death (Lenski and Mittler 1993a). The results presented above also place limits on models of this kind. When incubated without a carbon source, the cell number did not decline, and there was no evidence of turnover (Tables 1–3). FC40 also did not appear to have a generally heightened mutation rate during starvation (Figure 3).

Several models have been proposed that hypothesize a subpopulation of cells that can grow on lactose and then give rise to the full Lac" mutants (Lenski et al. 1988, 1989; Lenski and Mittler 1993b; John Roth and Franklin Stahl, personal communication; Maurice Fox, personal communication). However, the limits on cell growth also apply to this hypothetical subpopulation. Because the subpopulation must be better able to grow on lactose than the majority, they might have been detected as an increase in turbidity when FC40 was incubated in minimum lactose medium. However, no significant increase in turbidity was detected (Figure 1 and Table 1). Even allowing for the imprecision of the measurements, the amount of growth that the subpopulation could have achieved by day 2 could not have been as much as to give a 50% increase in total cell mass. It has been proposed that the subpopulation is initially 0.1% of the total population (Lenski et al. 1989; John Roth and Franklin Stahl, personal communication).

Then, if 3 × 10^8 cells of FC40 are plated on lactose plates, each of the 3 × 10^8 cells of the hypothetical subpopulation could not have undergone more than 9 generations of growth by day 2. Yet, according to the model, these cells must account for the approximately 50 Lac" colonies that appear by day 4, implying a mutation rate greater than 10^-7 per cell division.

In addition to these theoretical considerations, microscopic observations also limit the size of any microcolony population and its mutation rate. Because they could not be seen at 30 × magnification, microcolonies either cannot exceed 10^4 cells at any point during an experiment, or the cells must have such a high mutation rate that each microcolony gives rise to a Lac" mutant. However, were the mutation rate that high, or were the growth of microcolonies limited for some density-dependent reason, the microcolony population would have been detected by respreading (Figure 4).

All of these results lead to the conclusion that, during lactose selection, the Lac" mutants that arise cannot do so at the normal generation-dependent mutation rate either from the population at large, or from a subpopulation. Certainly, with enough imagination, some combination of changing mutation rates and growth parameters might be found that would exactly mimic the results with FC40. For example, the constant appearance of Lac" revertants could be modeled by allowing a growing subpopulation to have a high mutation rate initially, but making that mutation rate fall (for some reason) in inverse proportion to the population size. Or, the mutation rate per generation could be constant, but the subpopulation growing on lactose might have the unusual property of producing, at each division, only one live daughter cell. But, such models tend to become biologically implausible.

We are left, then, with a rather limited number of possibilities. Because the revertants of FC40 that arise during selection are dependent on RecA (Cairns and Foster 1991), and amplification probably requires RecA's recombination function (Tlsty et al. 1984), we previously suggested that mutants arise from a subpopulation of cells that is amplifying the lac region (Foster and Cairns 1992; Foster 1992). This model provides for adaptive mutations because, within an amplifying cell, if no useful mutation occurred, the amplification eventually would be resolved. If a mutation occurred that allowed the cell to grow, the amplification would also be resolved but the useful mutation would be retained. However, this hypothesis differs from another, similar hypothesis proposing that amplification of the mutant lac allele confers a growth advantage on lactose (John Roth and Franklin Stahl, personal communication). The results with FC40 suggest, instead, that at any given time a constant proportion of the population under lactose selection may be amplifying the lac region. This hypothesis leaves open the possibilities that the mutation rate within the amplified array may be a function of...
the number of copies, that the number of copies within a cell may increase with time, and even that the process may be stimulated by the presence of lactose. But, to be consistent with our finding that the reversion rate of FC40 is nearly constant during selection, the number of cells that are amplifying (or perhaps their extent of amplification) would have to be constant with time unless, of course, the reversion rate per gene copy declines with time.

However, there is, as yet, no evidence that amplification of any sort is occurring. The only clues we have to the mechanism by which adaptive mutations arise in FC40 is that they are dependent on some function or functions of the RecABC pathway (Cairns and Foster 1991; Foster 1993; Harris et al. 1994), and that they consist mainly of simple 1-base deletions (Foster and Trimarchi 1994; Rosenberg et al. 1994). Thus, alternative hypotheses, such as transitory, error-prone DNA synthesis (Foster 1992; Stahl 1992; Foster and Trimarchi 1994), are still possible. It also remains to be seen if the results with FC40 are generalizable to other strains and organisms.

I thank R. Kolter and M. Volker for strains and plasmids, J. Cairns, J. Drake, E. Eisenstadt, M. Fox and F. Stahl for useful discussions, and M. Fox, R. Lenski, J. Mittler, S. Rosenberg, J. Roth and F. Stahl for communicating unpublished results and/or hypotheses. J. Pisano and J. Trimarchi provided excellent technical assistance. This work was supported by grant MCB-9213197 from the U.S. National Science Foundation.

LITERATURE CITED


Hall, B. G., 1990 Spontaneous point mutations that occur more often when they are advantageous than when they are neutral. Genetics 126: 5-16.

Hall, B. G., 1991 Spectrum of mutations that occur under selective and non-selective conditions in E. coli. Genetics 84: 73-76.


Communicating editor: N. R. Drinker