Nonadaptive Mutations Occur on the F’ Episome during Adaptive Mutation Conditions in Escherichia coli

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One of the most studied examples of adaptive mutation is a strain of Escherichia coli, FC40, that cannot utilize lactose (Lac–) but that readily reverts to lactose utilization (Lac+) when lactose is its sole carbon source. Adaptive reversion to Lac+ occurs at a high rate when the Lac– allele is on an F’ episome and conjugal functions are expressed. It was previously shown that nonselected mutations on the chromosome did not appear in the Lac– population while episomal Lac+ mutations accumulated, but it remained possible that nonselected mutations might occur on the episome. To investigate this possibility, a second mutational target was created on the Lac– episome by mutation of a Tn10 element, which encodes tetracycline resistance (Tet+), to tetracycline sensitivity (Tet–). Reversion rates to Tet+ during normal growth and during lactose selection were measured. The results show that nonselected Tet+ mutations do accumulate in Lac– cells when those cells are under selection to become Lac+. Thus, reversion to Lac+ in FC40 does not appear to be adaptive in the narrow sense of the word. In addition, the results suggest that during lactose selection, both Lac+ and Tet+ mutations are created or preserved by the same recombination-dependent mechanism.

Mutations occurring in nondividing cells have been called adaptive when they allow cells to escape from nonlethal selective pressure (5, 7, 20). A narrower definition of adaptive mutation is that only useful, not deleterious or neutral, mutations occur during selection (7, 12, 29). Most current theories for adaptive mutation propose that cells under selection produce genetic variants at random, but these variants are transient, or the cells producing them die, unless a useful mutation occurs and the cells start to grow (3, 7, 22, 43). All of these models predict that nonselected mutations may appear in the successful cells (because all variants that exist when a cell begins to grow will be captured), but nonselected mutations should not appear in the unsuccessful cells (reviewed in reference 12).

One of the most studied examples of adaptive mutation is a strain of Escherichia coli, FC40, that cannot utilize lactose (Lac–) but that readily reverts to lactose utilization (Lac+) when lactose is its sole carbon source (5). This high level of adaptive reversion occurs when the Lac– allele is on an F’ episome and conjugal functions appear (17, 19, 39). Although mutations in a chromosomal gene, recA, do not appear in the Lac– population while episomal Lac– mutations accumulate (13), the question remained whether nonselected mutations might occur on the episome. Here, I report that, indeed, nonselected mutations at another site on F’ accumulate in Lac– cells when those cells are under selection to become Lac+. Thus, any given gene on the episome may be subjected to a highly mutagenic process in cells under nonlethal selective pressure. Although this could provide an effective strategy for adaptive evolution, mutation to Lac+ in FC40 does not appear to meet the narrow definition of adaptive mutation.

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MATERIALS AND METHODS

Bacterial strains. FC40 has the lac operon deleted from its chromosome but carries a mutant lac allele, ΦlacI33-lacZ2, on the lac-pro episome, F129 (5, 8, 37). A defective Tn10 element (ΦTn1831-Tn10Tet [obtained from J. Roth]) that confers tetracycline resistance (Tet+) was placed on this episome with P1 bacteriophage transduction by selection for Tet+ and screening for Lac–. ΔTn10 was mapped by cotransduction with ΔΔlacY::Cam100 (obtained from C. Manoil). Pulsed-field gel electrophoresis (performed essentially as described in references 28 and 38) located ΔTn10 on the same SfiI fragment as the lac operon. The Tet+ cells were mutated with ICR191, which creates frameshift mutations (36), and tetracycline-sensitive (Tet–) cells were selected (34). Two Tet+ alleles were obtained, and the episomes carrying them were mated back into FC36, the F’ parent of FC40 (5), giving strains FC722 and FC723. The two strains have similar spontaneous reversion rates to Tet+ and Tet– alleles are recessive to Tet+, indicating that they contain mutations in the structural gene for tetracycline resistance, tetA. The two tetA alleles were amplified (the 5′ primer had the sequence of bp 1574 to 1596, and the 3′ primer was complementary to bp 2820 to 2838 of the TRN10TETR locus [GenBank accession number 2838] and sequenced with an ABI 373A sequencer.

The following alleles were transduced into FC36 by selection for the indicated drug resistances and screening for increased sensitivity to UV light: recA938: Tn1000 (45), ΔstrA::AC65, edaC7::Cam (18, 35), and recG22S::Tn10::Kan (30). The Lac– Tet+ episome was then mated into these strains by selection for proline prototrophy (Pro+), inducible Lac–::Kan was placed on the Lac– Tet+ episome by recombination, and the conjugation-defective phenotype was confirmed (17). The nonrevertible Lac– scavenger strain, FC29, has been described previously (5). Standard genetic and molecular biological techniques were used (1, 36).

Media. M9 glycerol medium (5, 36) was supplemented with 22.5 mg of kanamycin per liter, 17 mg of chloramphenicol per liter, 10 mg of tetracycline per liter, 40 mg of nalidixic acid per liter, or 10 mg of ampicillin as a selection required. M9 lactose plates were as described previously (5). Scavenged M9 lactose medium was prepared by resuspension of a saturated culture of FC29 in an equal volume of M9 medium containing 0.1% lactose, incubation of the mixture for 3 h at 37°C, and then removal of the FC29 cells (13). S-Bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-Gal, a chromogenic substrate of β-galactosidase which stains Lac– cells blue) was added at 40 mg/liter. Luria-Bertani (LB) plates and lactose-teetrizolium plates (on which Lac– colonies are white and Lac+ colonies are red) were as described previously (36).

Experimental techniques. To produce independent cultures for mutation experiments, each strain was grown to saturation in M9 glycerol medium, diluted 10-fold into fresh medium, divided into several cultures, and allowed to again reach saturation. The cultures were then centrifuged and resuspended in saline and titers were determined on LB plates. For the overlay experiments (Fig. 1 and 2), about 106 cells (or 106 cells of the recG22S strain) from each independent culture were mixed with 106 cells of the scavenger strain and plated in minimal top agar on several M9 lactose plates (5). These plates were then overlaid with another 2.5 ml of minimal top agar and incubated at 37°C. New Lac– colonies were counted and marked every day. To determine the number of Tet+ mutants...
present on a given day, one set of lactose plates was overlaid with 5 ml of minimal top agar containing 1.2% glycerol and 0.4 mg of tetracycline, and new (Tet') colonies were counted 3 days later. At the end of the overlay experiments, the tetracycline plates were overlaid with an additional 2.5 ml of top agar containing 1 mg of X-Gal to identify Tet' colonies that were also Lac'. These colonies were then streaked onto M9 lactose-tetracycline plates to confirm that they were composed mostly of Tet' Lac' cells.

There were two exceptions to this overlay protocol. In the experiment with the trnD mutant strain (Fig. 2), the lacO plates were overlaid with top agar containing glycerol, allowed 1.5 h for expression, and then overlaid with top agar plus tetracycline. This procedure was subsequently found to be unnecessary, because the Tet' phenotype proved to be immediately expressed. In the experiment with the ruvAC mutant strain (Fig. 2), the scavenger strain was also mutated in ruvAC to eliminate the possibility that episomes transferred into ruvAC scavengers could give rise to mutants (26).

To show that the Tet' phenotype was due to reversion of the episomal Tet' allele, 150 Tet' colonies in the wild-type background were purified on minimal glycerol medium plus tetracycline and grown on the same medium. The epimorphs from these isolates were replica plated into strain XA100 [lacI33 trnD (arg mso Nal'R Rfi')] (10) by selection for nalidixic acid resistance (Nal') and Pro'. The exconjugants were then replica plated to minimal medium plus arginine, methionine, nalidixic acid, and tetracycline to score for transfer of the Tet' phenotype with the episome.

For the first experiment in liquid lactose medium (see Table 1, experiment A), the titers of five independent cultures of FC722 were determined for Tet' cells on M9 glycerol-tetracycline plates and for total cell number on LB plates. Each was diluted 50-fold into scavenge M9 medium-0.1% lactose (13) plus X-Gal. One hundred-microliter aliquots were dispensed into five 96-well microtiter dishes, for a total of 476 wells, and incubated for 3 days. At that point, 310 wells were neither visibly turbid nor blue because of growth of Lac' cells. Twenty clear wells from each dish were plated on M9 glycerol-tetracycline-X-Gal plates; Tet' Lac' and Tet' Lac' colonies were counted 3 days later. To determine the titer of the cells, an additional five clear wells from each dish were pooled, and the dilutions were plated on lactose-tetratolizium plates. For the second experiment (see Table 1, experiment B), the protocol was initially the same. A total of 480 wells were inoculated; after 3 days, 225 wells were clear. However, instead of being plated directly on tetracycline plates, the contents of 100 microtiter wells (20 from each original culture) were inoculated into 10 ml of M9 glycerol medium and allowed to grow overnight. A total of 0.1 ml of each of these 100 cultures was then plated on M9 glycerol-tetracycline-X-Gal plates; Tet' Lac' and Tet' Lac' colonies were counted 3 days later. The titers of five cultures were determined for total and Lac' cells numbers on lactose-tetratolizium plates. A summary of the results from experiments A and B is given in Table 2.

The growth-dependent mutation rate to Tet' of FC722 and FC723 was determined in fluctuation tests. Aliquots (0.1 ml) containing 10^6 cells in M9 glycerol medium were inoculated into microtiter wells; after 8 h of incubation at 37°C, the entire contents of 90 wells for each strain were plated on M9 glycerol-tetracycline-X-Gal; Tet' colonies were counted 3 days later. Dilutions of six wells were plated onto LB plates to determine the total cell number.

Throughout this paper, the reported Tet' counts represent the colonies appearing within 3 days of plating or overlay. A few additional Tet' colonies continued to appear for several more days, but these were not included because of the possibility that the potency of the tetracycline was declining.

RESULTS

A second mutational target was created on the Φ(lac33-lacZ) episome by first placing on it a defective Tn10 element, which confers Tet'. Based on cotransduction frequencies, dTn10 is about 40 kb from the lac operon, and pulsé field gel electrophoresis indicated a probable gene order of tra lac dTn10. The Tet' cells were mutated with a frameshift mutagen, and Tet' isolates were selected. Strains FC722 and FC723 carry the Lac' episome with two of these Tet' alleles. Each allele has a 1 frameshift in the tetA gene that increases a run of 4 G:C bp to 5 bp; thus, they are similar to lacI33, which has a +1 frameshift that increases a run of 3 G:C bp to 4 bp (8). The mutations are at bp 331 (FC722) and bp 51 (FC723) of the coding sequence of the tetA gene (which is 1,203 bp long), and the alleles are predicted to encode truncated proteins with sizes of 88 and 31 amino acids, respectively. The mutants reported here were obtained with FC722, but the occurrence of nonselected Tet' mutations during lactose selection was confirmed with FC723.

Based on a 90-culture fluctuation test, during nonselective exponential growth, FC722 has a reversion rate to Tet' of 3 × 10^-3/cell/generation, similar to the growth-dependent reversion rate to Lac' of FC40 (4 × 10^-3/cell/generation) (18). On minimal lactose plates, the reversion rate to Lac' of FC722 was about 3 × 10^-7/cell/day (Fig. 1). Every day during the experiment shown in Fig. 1, subsets of plates were overlaid with top agar containing tetracycline plus a utilisable carbon source. The numbers of new colonies subsequently appearing on the overlaid plates gave the number of Tet' cells present at the time of overlay. This technique showed that, after a 1-day delay, Tet' cells accumulated on lactose plates at about 80% of the rate that Lac' cells accumulated (Fig. 1). A total of 150 Tet' colonies were purified and proved to transfer the Tet' phenotype with their episomes; thus, the episomal Tet' allele had reverted. These 150 mutants were also tested for their Lac phenotype on lactose plates, and at the end of the experiment, the tetracycline plates were overlaid with top agar containing X-Gal to identify any remaining Tet' colonies that were also Lac'. By these two assays, 4 of the 850 Tet' colonies that arose in this experiment were Lac'.

The simple explanation of the results in Fig. 1 is that during selection for Lac', Tet' mutants accumulate in the Lac' population, even though tetracycline resistance should confer no growth or survival advantage. However, it is possible that during starvation, cells develop the ability to respond adaptively to selection, and the experiment is therefore measuring the increase in that ability instead of the number of actual Tet' mutants. The classical proof that a population contains mutants before selection is imposed is to show that clones of mutants arise from that population during nonselective growth (9, 14, 31, 33). However, the test is complicated in this case because mutant clones will be produced not only by preexisting Tet' mutations, but also by new Tet' mutations that arise during the nonselective growth. Therefore, experiments were designed to test if, after incubation in lactose, the Lac' population would produce more Tet' clones than would be expected from growth-dependent mutation rate.

About 2 × 10^6 FC722 cells in minimal lactose medium were inoculated into microtiter wells and incubated for 3 days. At that point, 100 wells that did not show visible growth of Lac' cells were plated on minimal glycerol plates containing tetracycline and X-Gal. The number of Lac' cells per well was the
same on day 3 as on day 0, and the number of Lac" Tet" mutants is shown in Table 1, experiment A. Only 44% of the wells did not contain Lac" Tet" cells, suggesting about 0.7 new Tet" mutant per well had appeared during the 3 days of incubation in lactose (Table 2, experiment A). Assuming that the appearance of Tet" mutants lagged for a day as it did on lactose plates (Fig. 1), the mutation rate to Tet" was 2 × 10⁻⁷/cell/day, in agreement with the results in Fig. 1. Thus, incubation in liquid lactose medium reproduced the results obtained on lactose plates.

A surprising outcome of this experiment was that, although the Lac" population was static, about 10% of the wells contained more Lac" Tet" cells than would be predicted from a Poisson distribution (Table 1). This could be because mutant epipodes are occasionally replicated and passed to other cells, as has also been observed when the cells are on solid medium (16, 39). A different possibility is that some Lac" cells contained both Lac+ Tet" and Lac+ Tet" epipodes, and these segregated when the cells began to grow on the lactose.

Next, the experiment was repeated, but after incubation in lactose, the cells were inoculated into nonselective medium and allowed to grow for 12 generations before being assayed for Lac" and Tet" (Table 1, experiment B). One minor problem in the interpretation of this experiment is that after several days of starvation, the ability of individual cells to initiate growth varies; thus, some clones are overrepresented and some are underrepresented in the final population (14). Nonetheless, the results were clear. At least half of the cells produced more Lac+ Tet" mutants than could have arisen during the nonselective growth (Table 2, experiment B). Therefore, Tet" mutants existed in the static Lac" population before selection for tetracycline resistance was imposed, meaning nonadaptive Tet" mutants accumulate during lactose selection.

Unlike growth-dependent reversion, efficient adaptive reversion to Lac" in FC40 requires both conjugal and recombination functions (5, 17–19, 25, 26). The same functions were required for the appearance of Lac" Tet" mutants during lactose selection. Figure 2 shows the effects on reversion to Lac" and Tet" of mutant alleles affecting conjugation (traD), the RecABC pathway for recombination (recA), and the RuvABC pathway for resolution of recombination intermediates (rvaAC). In addition, a mutant allele of recG that disables an alternative recombination intermediate-resolving function (46) and enhances adaptive mutation to Lac" in FC40 (18, 26) also enhanced mutation to Tet" (Fig. 2). Therefore, the Tet" mutations that arise during lactose selection appear to be created or preserved by the same mechanism as the Lac" mutations.

**DISCUSSION**

The experiments described above show that during lactose selection, both selected mutations, conferring the Lac" phenotype, and nonselected mutations, conferring the Tet" phenotype, rise on the F' episome. That Tet" mutations appear and persist in the Lac" population during lactose selection eliminates, for FC40, the hypotheses that nonselected mutations are necessarily transitory (3, 7, 43) or that the cells (or epipodes) bearing them are necessarily eliminated from the population (22, 40). Based on the results presented here, mutation to Lac" in FC40 does not meet the narrow definition of adaptive mutation. This conclusion cannot be extended to other cases of apparent adaptive mutation, each of which must be evaluated individually. In addition, it is possible that some fraction of Tet" Lac" epipodes originate in Lac" cells, which would be consistent with the adaptive mutation hypothesis. Nonetheless, there is a clear nonadaptive component to postplating mutation in FC40.

The most conservative explanation for the occurrence of both selected and nonselected mutations on the episome during lactose selection is that the Φ(lacI33-lacZ) allele is sufficiently "leaky" to provide the energy for DNA synthesis and recombination on the episome, but not leaky enough to allow cell division (17, 19). This hypothesis predicts that partial activity is an essential property of alleles that mutate under

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**TABLE 1. Distributions of Tet" mutants among the Lac" population after incubation in lactose medium**

<table>
<thead>
<tr>
<th>No. of Lac&quot; Tet&quot; mutants (X)</th>
<th>No. of cultures with X Lac&quot; Tet&quot; mutants</th>
</tr>
</thead>
<tbody>
<tr>
<td>Expt A</td>
<td></td>
</tr>
<tr>
<td>0</td>
<td>44</td>
</tr>
<tr>
<td>1</td>
<td>24</td>
</tr>
<tr>
<td>2</td>
<td>16</td>
</tr>
<tr>
<td>&gt;2</td>
<td>16</td>
</tr>
<tr>
<td>Expt B</td>
<td></td>
</tr>
<tr>
<td>&lt;200</td>
<td>14</td>
</tr>
<tr>
<td>200–299</td>
<td>10</td>
</tr>
<tr>
<td>300–449</td>
<td>5</td>
</tr>
<tr>
<td>450–4,000</td>
<td>46</td>
</tr>
<tr>
<td>&gt;4,000</td>
<td>25</td>
</tr>
</tbody>
</table>

* For experiment A (direct assay on tetracycline), these results represent the expected number of wells with x mutants for a Poisson distribution with a mean of 0.82 mutant per well (see Table 2). For experiment B (assay on tetracycline after nonselective growth), these results represent the expected number of cultures with x mutants for a Luria-Delbrück distribution with a mean of 54 mutations per culture (7). The number 54 is the expected number of mutations that would have occurred during growth given a growth-dependent mutation rate to Tet" of 3 × 10⁻⁶ per cell per generation. (Only 1/1000th of each culture was plated, so the six cultures in the 400 to 499 range have been evenly distributed into the 300 to 449 and 450 to 4,000 ranges).

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**TABLE 2. Summary of results from Table 1**

<table>
<thead>
<tr>
<th>Expt</th>
<th>Lac&quot; cells/culture</th>
<th>Lac&quot; Tet&quot; mutants/culture</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Initial</td>
<td>Final</td>
</tr>
<tr>
<td>A</td>
<td>1.6 × 10⁶</td>
<td>1.7 × 10⁶</td>
</tr>
<tr>
<td>B</td>
<td>2.3 × 10⁶</td>
<td>9 × 10⁶</td>
</tr>
</tbody>
</table>

* The number of mutants per culture = −ln(Po). The mutation rate, (−ln(Po)/N), was 4.5 × 10⁻⁷/cell over the 3 days.

* Roughly half of the cultures had more Lac" Tet" mutants than would be expected from the growth-dependent mutation rate, and thus they contained Lac" Tet" mutants at the start of nonselective growth. This gives a minimum number of Lac" Tet" mutants per well after 3 days of incubation in lactose of 0.7.
selection. Among a collection of chromosomal Lac alleles in Salmonella typhimurium, only leaky alleles reverted to Lac during lactose selection, but their reversion rates were only poorly correlated with the amounts of β-galactosidase that the unreverted alleles produced (20). Likewise, among several different Lac alleles on the same F episome as studied here, there is no particular correlation between residual β-galactosidase levels and reversion rates to Lac during lactose selection (6, 11, 23, 32). Thus, in order for a gene that is under selection to mutate, it may be necessary that it have some partial activity to allow limited DNA synthesis; however, partial activity is not sufficient for mutation, and other unknown factors are important. Whether nonselected mutations also occur during selection may depend on the extent of the DNA synthesis allowed by the leaky allele.

That mutations to Lac and to Tet during lactose selection share genetic requirements and occur at nearly the same rate suggests that the mutations do not arise independently. If so, the frequency of Lac Tet double mutants should be higher than predicted from the individual mutation rates to Lac and Tet. In the overlay experiments, Lac Tet colonies appeared at a frequency of about 1/109 Lac Tet cells. Although this is 103-fold greater than expected on the assumption of independence, not all of the Lac Tet colonies necessarily arose from a double mutant. At any given time, approximately 106 growing but undetected Lac cells are present in the lawn of Lac cells on a lactose plate, and these will give rise to Tet mutants at the growth-dependent mutation rate. Allowing for these Lac cells, the frequency at which Lac Tet mutants appeared during lactose selection was about 50-fold higher than expected. Significantly, however, only 1 of 238 Tet mutants that appeared in the recG mutant strain (Fig. 2) was also Lac+, a frequency of about 1/107 Lac Tet cells. Mutation rates to Lac and Tet during lacose selection, but not during growth, are each increased about 100-fold in recG mutant cells (18, 26) (Fig. 2). If the recG defect were independently increasing the frequency of each mutation, the frequency of double mutants should have been 106-fold higher in recG mutant cells than in wild-type cells. That it was not implies that the recG defect affects the frequency of a mutagenic process, but not the probability of any given mutation occurring during this process. For example, both Lac and Tet mutations may be produced during DNA synthesis initiated or preserved by recombination (15, 41); the recombination functions may alter the frequency of such events, but the probability of one or both mutations being produced would depend only on the accuracy of the DNA synthesis. A less specific hypothesis is that both Lac+ and Tet+ mutations occur within a subpopulation of highly mutating cells, and the recombination functions determine the number of these cells but not their mutation rate.

The highly mutagenic process that takes place on F may play a role in bacterial evolution. F and related conjugal plasmids can recombine with all regions of the E. coli chromosome (27). Thus, within a population, any gene may be carried both on the episome and on the chromosome. Because the chromosomal copy would be preserved, a gene on F would be free to mutate and diverge and could be immortalized if a useful activity evolved. This mechanism would be more efficient if a cell contained several copies of all or part of the episome and these copies tended to segregate when the cell began to grow. Then, the mutation that was responsible for growth would be preserved, but nonselected mutations would be relegated to a nongrowing minority and lost from the population (4). Duplicated genes have been proposed to play a similar role in evolution (42). Recently it was found that 17% of natural isolates of E. coli contain F or related conjugal plasmids, a frequency higher than previously thought (2). These conjugal plasmids show evidence of a high rate of recombination and frequent transfer between the major groups of E. coli. Although conjugal functions are not mutationally derepressed on the naturally occurring plasmids, as they are on the laboratory F (2), conjugal functions may be regulated by environmental conditions. If nutritional deprivation or other kinds of stress prove to be such conditions, F could be an important element in adaptive evolution.

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FIG. 2. Genetic requirements for mutation to Lac+ (C) and to Tet+ (II) during incubation of Lac− Tet− cells on lactose plates. The increases (per 106 cells plated) in Lac− and Tet− mutants after 3 days on lactose are shown. The results are from three separate experiments (see Materials and Methods). WT, wild type. Three independent cultures of each of the strains were used. Results are means, and error bars represent standard errors.
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