Title
Pathways of genetic adaptation: multistep origin of mutants under selection without induced mutagenesis in Salmonella enterica.

Permalink
https://escholarship.org/uc/item/9m29p44b

Journal
Genetics, 192(3)

ISSN
0016-6731

Authors
Quiñones-Soto, Semarhy
Reams, Andrew B
Roth, John R

Publication Date
2012-11-01

DOI
10.1534/genetics.112.142158

Peer reviewed
Pathways of Genetic Adaptation: Multistep Origin of Mutants Under Selection Without Induced Mutagenesis in Salmonella enterica

Semarhy Quiñones-Soto, Andrew B. Reams, and John R. Roth

Department of Microbiology, University of California, Davis, California 95616

ABSTRACT In several bacterial systems, mutant cell populations plated on growth-restricting medium give rise to revertant colonies that accumulate over several days. One model suggests that nongrowing parent cells mutagenize their own genome and thereby create beneficial mutations (stress-induced mutagenesis). By this model, the first-order induction of new mutations in a nongrowing parent cell population leads to the delayed accumulation of visible colonies. In an alternative model (selection only), selective conditions allow preexisting small-effect mutants to initiate clones that grow and give rise to faster-growing mutants. By the selection-only model, the delay in appearance of revertant colonies reflects (1) the time required for initial clones to reach a size sufficient to allow the second mutation plus (2) the time required for growth of the improved subclone. We previously characterized a system in which revertant colonies accumulate slowly and contain cells with two mutations, one formed before plating and one after. This left open the question of whether mutation rates increase under selection. Here we measure the unselected formation rate and the growth contribution of each mutant type. When these parameters are used in a graphic model of revertant colony development, they demonstrate that no increase in mutation rate is required to explain the number and delayed appearance of two of the revertant types.

IN many biological systems, genetic adaptation is extremely rapid. This is seen when mutants arise during growth under selection and increase their frequency in the population. In contrast, laboratory genetic practice uses stringent selection to detect preexisting mutants without affecting their frequency. In laboratory experiments, mutants formed during nonselective growth in liquid medium and are detected as colonies on solid selective medium. Stringent selection conditions prevent growth of both parent cells and common small-effect mutants, but allow preexisting large-effect mutants to form colonies. The validity of using strong selection as a pure detection scheme was verified by classic experiments of Luria and Delbruck (1943), Newcombe (1949), and Lederberg and Lederberg (1952). These laboratory selection methods are central to the practice of bacterial genetics.

Several bacterial genetic systems seem to violate the expectations of laboratory selections. In these systems, mutant colonies accumulate over several days on selective medium above a lawn of nongrowing parent cells (Hall 1982; Shapiro and Brinkley 1984; Cairns et al. 1988; Steele and Jinks-Robertson 1992; Taddei et al. 1997). Some models attribute behavior of these exceptional systems to stress-induced mutagenesis. Cells may have mechanisms to upregulate their mutation rate when growth is blocked and thereby generate new variability in times of need (Cairns et al. 1988; Taddei et al. 1995). This general mutagenesis (Torkelson et al. 1997; Galhardo et al. 2007) is thought to affect only a subset of the stressed population (Hall 1990). If stress is generally mutagenic, then selected mutants would be expected to show secondary nonadaptive changes in their genomes, but only one system shows evidence of this (Torkelson et al. 1997). Other models propose that stress directs mutations preferentially to sites that improve fitness (Foster and Cairns 1992; Foster 2007). All of these models propose that stress induces new large-effect mutations in nongrowing cells. An alternative possibility is that colonies are initiated by preexisting small-effect mutant cells that grow and improve under selection.
The exceptional selection systems that originally suggested stress-induced mutagenesis may also be explained entirely by natural selection, acting on mutants that arise at standard mutation rates. The original interpretation of these systems assumed that a stringent laboratory selection was in place, when in fact the laboratory paradigm may have broken down. In the Cairns system, for example, conditions block growth of the bulk parental cell population, but may not prevent growth of common small-effect mutants. Selective conditions in many exceptional systems may be relaxed enough to allow growth of small-effect mutants. These cells initiate slow-growing colonies whose populations adapt to include cells with fully fit genotypes. Thus, the mutant colonies appearing under selection may reflect preexisting small-effect mutants that adapted and improved during colony growth. The number of cells in the colony may allow a secondary mutation with no increase in mutation rate. The initiating mutants are common under all growth conditions, with or without selection. The high frequency of small-effect mutants may be unexpected, because they are not detected in standard laboratory selections, where selection is very stringent.

The selection-only model proposes that relaxed selection stringency allows small-effect mutants to initiate slow-growing clones on selective plates. Within each clone, cell number increases enough to allow a secondary improving mutation. The secondary mutations form at a standard mutation rate, but are made more likely on selective medium by the increasing number of cells in the colony. The exponential growth of the improved cell type expands the clone and speeds appearance of a visible colony. The overall process looks like time-dependent mutagenesis of resting cells because mutant colonies appear late above a nongrowing lawn. The selection-only model attributes the delay in colony appearance to the time required for growth of the initial mutant, formation of the secondary improvements, and overgrowth by the improved cell types.

To test feasibility of the selection-only model, one must identify the preexisting mutant types and measure their formation rate during nonselective growth and their growth rate under selection. This has not been done in most exceptional systems. In the Ara-lac system, Lac+ clones appear under selection and carry deletions that fuse the ara promoter to a silent lac structural gene. It is not known whether other mutation types precede deletion formation. A Mu prophage is located between ara and lac genes and its transposition functions contribute in an unknown way to deletion formation (Shapiro 1984; Shapiro and Brinkley 1984). In the system of Cairns and Foster, a mutant lacZ gene is located on an F′ plasmid (Cairns and Foster 1991), where recombination is intense (Seifert and Porter 1984) and the lac genes amplify at high rates (Reams et al. 2010). These amplifications improve growth under selection (Roth et al. 2006). Furthermore, the gene for an error-prone repair polymerase (DinB) is located close enough to lacZ to coamplify and thereby increase mutation rates during growth under selection (Slechta et al. 2003). Testing the “selection-only” model is difficult in the Cairns system, because the frequency of lac amplifications in unselected cultures is maintained at a steady state, making it impossible to use fluctuation tests to determine whether lac duplications that initiate revertant colonies arise before or after plating (Reams et al. 2010). Features of the several exceptional systems have made it difficult to demonstrate clearly whether revertant colonies are initiated by cells that preexist selection (Slechta et al. 2003; Foster 2007; Galhardo et al. 2007; Andersson et al. 2011).

To test the selection-only model, we have characterized a simpler system (Pur-Lac), which shows the same general behavior as the pioneer systems and was used initially to support stress-induced mutagenesis (Yang et al. 2001, 2006). In this system, a lawn of phenotypically Lac− mutant cells is plated on selective lactose medium and gives rise to ∼100 revertant colonies that accumulate above a lawn of nongrowing cells over a period of 6 days. Adaptation in the Pur-Lac system involves standard chromosomal mutations with no involvement of either phage or plasmid functions. If one can explain the revertant colonies arising under selection without any stress-induced increase in mutation rate, then a principle has been demonstrated that may encourage experimental tests of other systems.

The Pur-Lac system employs a tester strain of Salmonella enterica (serovar Typhimurium) with a complete lac operon inserted into the purD gene of the chromosomal purHD operon, which encodes purine biosynthetic genes (diagrammed in Figure 1). Expression of the inserted lac genes is reduced by (1) the native PurR repressor, (2) a polar purD nonsense (UGA) mutation, and (3) a stem-loop structure that contributes to premature termination of lac transcripts (Quiñones-Soto and Roth 2011). The inserted lac genes are part of a transposon (Mudlac) derived from a phage Mu that lacks transposition functions (Castilho et al. 1984). The mutations that confer a Lac+ phenotype during growth under selection (Slechta et al. 2003). Testing the “selection-only” model is difficult in the Cairns system, because the frequency of lac amplifications in unselected cultures is maintained at a steady state, making it impossible to use fluctuation tests to determine whether lac duplications that initiate revertant colonies arise before or after plating (Reams et al. 2010). Features of the several exceptional systems have made it difficult to demonstrate clearly whether revertant colonies are initiated by cells that preexist selection (Slechta et al. 2003; Foster 2007; Galhardo et al. 2007; Andersson et al. 2011).

To test the selection-only model, we have characterized a simpler system (Pur-Lac), which shows the same general behavior as the pioneer systems and was used initially to support stress-induced mutagenesis (Yang et al. 2001, 2006). In this system, a lawn of phenotypically Lac− mutant cells is plated on selective lactose medium and gives rise to ∼100 revertant colonies that accumulate above a lawn of nongrowing cells over a period of 6 days. Adaptation in the Pur-Lac system involves standard chromosomal mutations with no involvement of either phage or plasmid functions. If one can explain the revertant colonies arising under selection without any stress-induced increase in mutation rate, then a principle has been demonstrated that may encourage experimental tests of other systems.

The Pur-Lac system employs a tester strain of Salmonella enterica (serovar Typhimurium) with a complete lac operon inserted into the purD gene of the chromosomal purHD operon, which encodes purine biosynthetic genes (diagrammed in Figure 1). Expression of the inserted lac genes is reduced by (1) the native PurR repressor, (2) a polar purD nonsense (UGA) mutation, and (3) a stem-loop structure that contributes to premature termination of lac transcripts (Quiñones-Soto and Roth 2011). The inserted lac genes are part of a transposon (Mud-lac) derived from a phage Mu that lacks transposition functions (Castilho et al. 1984). The mutations that confer a Lac+ phenotype during growth under selection (Slechta et al. 2003). Testing the “selection-only” model is difficult in the Cairns system, because the frequency of lac amplifications in unselected cultures is maintained at a steady state, making it impossible to use fluctuation tests to determine whether lac duplications that initiate revertant colonies arise before or after plating (Reams et al. 2010). Features of the several exceptional systems have made it difficult to demonstrate clearly whether revertant colonies are initiated by cells that preexist selection (Slechta et al. 2003; Foster 2007; Galhardo et al. 2007; Andersson et al. 2011).

To test the selection-only model, we have characterized a simpler system (Pur-Lac), which shows the same general behavior as the pioneer systems and was used initially to support stress-induced mutagenesis (Yang et al. 2001, 2006). In this system, a lawn of phenotypically Lac− mutant cells is plated on selective lactose medium and gives rise to ∼100 revertant colonies that accumulate above a lawn of nongrowing cells over a period of 6 days. Adaptation in the Pur-Lac system involves standard chromosomal mutations with no involvement of either phage or plasmid functions. If one can explain the revertant colonies arising under selection without any stress-induced increase in mutation rate, then a principle has been demonstrated that may encourage experimental tests of other systems.

The Pur-Lac system employs a tester strain of Salmonella enterica (serovar Typhimurium) with a complete lac operon inserted into the purD gene of the chromosomal purHD operon, which encodes purine biosynthetic genes (diagrammed in Figure 1). Expression of the inserted lac genes is reduced by (1) the native PurR repressor, (2) a polar purD nonsense (UGA) mutation, and (3) a stem-loop structure that contributes to premature termination of lac transcripts (Quiñones-Soto and Roth 2011). The inserted lac genes are part of a transposon (Mud-lac) derived from a phage Mu that lacks transposition functions (Castilho et al. 1984). The mutations that confer a Lac+ phenotype during growth under selection (Slechta et al. 2003). Testing the “selection-only” model is difficult in the Cairns system, because the frequency of lac amplifications in unselected cultures is maintained at a steady state, making it impossible to use fluctuation tests to determine whether lac duplications that initiate revertant colonies arise before or after plating (Reams et al. 2010). Features of the several exceptional systems have made it difficult to demonstrate clearly whether revertant colonies are initiated by cells that preexist selection (Slechta et al. 2003; Foster 2007; Galhardo et al. 2007; Andersson et al. 2011).

To test the selection-only model, we have characterized a simpler system (Pur-Lac), which shows the same general behavior as the pioneer systems and was used initially to support stress-induced mutagenesis (Yang et al. 2001, 2006). In this system, a lawn of phenotypically Lac− mutant cells is plated on selective lactose medium and gives rise to ∼100 revertant colonies that accumulate above a lawn of nongrowing cells over a period of 6 days. Adaptation in the Pur-Lac system involves standard chromosomal mutations with no involvement of either phage or plasmid functions. If one can explain the revertant colonies arising under selection without any stress-induced increase in mutation rate, then a principle has been demonstrated that may encourage experimental tests of other systems.

The Pur-Lac system employs a tester strain of Salmonella enterica (serovar Typhimurium) with a complete lac operon inserted into the purD gene of the chromosomal purHD operon, which encodes purine biosynthetic genes (diagrammed in Figure 1). Expression of the inserted lac genes is reduced by (1) the native PurR repressor, (2) a polar purD nonsense (UGA) mutation, and (3) a stem-loop structure that contributes to premature termination of lac transcripts (Quiñones-Soto and Roth 2011). The inserted lac genes are part of a transposon (Mud-lac) derived from a phage Mu that lacks transposition functions (Castilho et al. 1984). The mutations that confer a Lac+ phenotype during growth under selection (Slechta et al. 2003). Testing the “selection-only” model is difficult in the Cairns system, because the frequency of lac amplifications in unselected cultures is maintained at a steady state, making it impossible to use fluctuation tests to determine whether lac duplications that initiate revertant colonies arise before or after plating (Reams et al. 2010). Features of the several exceptional systems have made it difficult to demonstrate clearly whether revertant colonies are initiated by cells that preexist selection (Slechta et al. 2003; Foster 2007; Galhardo et al. 2007; Andersson et al. 2011).

To test the selection-only model, we have characterized a simpler system (Pur-Lac), which shows the same general behavior as the pioneer systems and was used initially to support stress-induced mutagenesis (Yang et al. 2001, 2006). In this system, a lawn of phenotypically Lac− mutant cells is plated on selective lactose medium and gives rise to ∼100 revertant colonies that accumulate above a lawn of nongrowing cells over a period of 6 days. Adaptation in the Pur-Lac system involves standard chromosomal mutations with no involvement of either phage or plasmid functions. If one can explain the revertant colonies arising under selection without any stress-induced increase in mutation rate, then a principle has been demonstrated that may encourage experimental tests of other systems.
phenotype on this tester strain are standard chromosomal changes, including point mutations and deletions (see Figure 1). Point mutations (purO) damage the repressor-binding site. Loss-of-function purR mutations (unlinked to the purHD operon) reduce the level or quality of the repressor protein. The deletion mutation (mDEL1) removes a transcription-blocking stem-loop structure located promoter-proximal to the lac genes. Amplification of the purHD-lac region may also contribute to improved growth on lactose.

The course of a reversion experiment is diagrammed in Figure 2. In the standard experiment, 10^8 Lac^- tester cells are plated on selective medium where they divide about six times during the first day, using nutrients other than lactose that contaminate the agar, and thereafter show no further growth. Above this lawn, the earliest Lac^- revertant colonies appear on day 3.

The process of reversion to Lac^+ is initiated by preexisting singly mutant cells (purO, purR, or mDEL1) that arise during nonselective growth prior to plating. This was shown previously by reconstruction experiments and Luria–Delbrück fluctuation tests. Any of these single mutants can initiate a slow-growing colony on selective medium (Quiñones-Soto and Roth 2011). Within each clone, a second mutation arises that enhances growth and contributes to development of a visible colony. The earliest revertant colonies appear on day 3 and more colonies become visible over several subsequent days.

All colonies that become visible on day 6 include two mutant cell types—singly mutant cells of the type that initiated the colony and doubly mutant cells that carry an additional secondary mutation acquired during colony development under selection. This mixture of cell types reflects the sequence in which mutations appeared. The majority of revertant colonies are initiated by an mDEL1 mutant that later acquires either a purR or a purO mutation during growth under selection. A few colonies initiated by an mDEL1 deletion mutant include cells with a larger stem deletion that removes the polar UGA mutation. Other revertant colonies have simple mDEL1 mutant cells plus cells with an amplification of the mutant purHD-lac region. Revertants have not experienced general mutagenesis and the yield of revertant colonies is unaffected by either a dinB (Quiñones-Soto and Roth 2011) or a lexA^IND mutation (our unpublished results), which eliminates general mutagenesis and reduces revertant yield in the Cairns system (Slechta et al. 2003).

Only double mutants (mDEL1, purR or mDEL1, purO) are able to form a colony under selection by day 2. These colonies are not seen in the standard reversion experiment but are seen in reconstruction experiments or when 10-fold more tester strains are plated. By counting only colonies that appear on day 2, the stringency of the selection is increased and only rare preexisting double mutants are detected. Under these conditions, Luria–Delbrück fluctuation tests can be used to determine unselected mutation rates. This fact is used here to measure the formation rate of each mutant type that contributes to reversion in the Pur-Lac system.

Previous results, summarized above, demonstrate that revertants in the Pur-Lac system arise by a series of two events. The colony is initiated by a mutant that arose prior to plating and thus cannot be stress induced. A second mutation arises during subsequent growth of the clone under selection. No general genome-wide mutagenesis was evident prior to the appearance of the secondary mutation (Quiñones-Soto and Roth 2011). It was suggested that the probability of a secondary mutation is enhanced only by the increase in cell number within the developing colony and selection acts only to favor growth of the new double mutants within each colony. However, while general mutagenesis has been eliminated, it is still possible that stress induces and directs mutations to beneficial sites as originally suggested by Cairns et al. (1988). Here evidence is provided that behavior of the Pur-Lac system can be explained using standard mutation rates measured during nonselective growth. No stress-induced mutagenesis (general or directed) is needed to account for the behavior of this system.

Materials and Methods

Bacterial strains and plasmids

All strains are derived from S. enterica (serovar Typhimurium) strain LT2. The Pur-Lac tester strain was a generous gift from Z. Yang, Z. Liu, and A. Wang (Yang et al. 2001, 2006) and has been given the strain number TT25154. The origin of the F^purR^+ plasmid is described below. Genotypes of bacterial strains are in Table 1.

Media and chemicals

The rich media were Luria–Bertani (LB) medium (Difco Laboratories, Detroit) and nutrient broth (NB) (Difco Laboratories). Minimal medium was no-citrate-E (NCE) medium (Berkowitz et al. 1968; Davis et al. 1980). These media were solidified with 1.5% agar (Baltimore Biological). Prior to selection, cells were

![Figure 2](image_url)
grown overnight in liquid NCE minimum medium with 0.2% glycerol supplemented with 10 μg/ml adenine and 0.06 mM thiamine. This medium is referred to as glycerol (adenine) medium. Revertants were selected on NCE minimal plates supplemented with 10 μg/ml adenine, 0.06 mM thiamine, and 1% lactose (Yang et al. 2001). This medium is referred to as lactose (adenine) medium. The chromogenic β-galactosidase substrate X-gal (5-bromo-4-chloro-3-indolyl-D-galactopyranoside) was added to selection plates at a final concentration of 50 μg/ml. Antibiotic concentrations in rich media were 20 μg/ml tetracycline (Tc) and 20 μg/ml chloramphenicol (Cm). Antibiotic concentrations in minimal medium were 10 μg/ml Tc and 10 μg/ml Cm.

**Transductional methods**

Transductional crosses were mediated by a high-transducing mutant of phage P22 (HT105/1, int−) (Schmiegler 1972). Transductant colonies were purified by single-colony isolation on green indicator plates (Chan et al. 1972) and tested for phage sensitivity with P22 clear-plaque mutant H5.

**Construction and transfer of the F' purR plasmid**

The F' purR plasmid carries a Salmonella purR gene inserted in an *Escherichia coli* F' his plasmid (T. Guo-Ming, unpublished results). The purR gene was derived from a strain with Tn10dTc and Tn10dCm elements inserted on either side of the chromosomal purR+ gene. This unit (Tn10dTc-purR-Tn10dCm) was allowed to transpose to an F' plasmid that carried the his region of *E. coli*. Thus, the plasmid carries his+ (*E. coli*) and purR+ (*S. enterica*) plus both a tetracycline and a chloramphenicol resistance determinant. This plasmid was transferred by conjugation into the Pur-Lac tester strain to prevent detection of recessive purR mutations.

**Determining mutation rates**

The formation rates (μ) of the mutations were determined by Luria–Delbrück fluctuation tests (Luria and Delbruck 1943). Parallel cultures were pregrown in nonselective minimal glycerol (adenine) medium and were then tested for mutant frequency either selectively or by nonselective screening. The distribution of mutant frequencies among the parallel cultures was used to estimate the most likely number of mutational events per culture (m). The value of m is then used to calculate the mutation rate (μ) by dividing the observed number of mutants per culture by the total number of cells (N) as μ = m/N (Luria and Delbruck 1943). In several determinations, m was estimated by the p0 method (Luria and Delbruck 1943; Rosche and Foster 2000), where the proportion of cell cultures without mutants is used to calculate the number of mutations per culture:

\[
p_0 = \frac{\text{number of cultures without mutants}}{\text{total number of cultures}} \quad (1)
\]

\[
m = -\ln p_0. \quad (2)
\]

In experiments for which only a portion of the cell cultures was plated, the mutant frequency (f) in the whole culture was expressed as a function of the total cell number (N) (Drake 1991), and the mutation rate per replication (μ) was calculated as

\[
\mu = \frac{f}{\ln N} = \frac{0.4343f}{\log N} \quad (3)
\]

Rates were estimated only for experiments in which cultures showed a Luria–Delbrück distribution of mutant frequencies, thereby ensuring that measured rates were for mutations that arose under nonselective conditions. The frequency distribution was tested by plotting the logarithm of the number of mutants per tube (x) against the logarithm of the probability of tubes (p_x) with >x mutants. This plot gives a slope of −1 when mutant frequencies show a Luria–Delbrück

---

**Table 1 Strain list of Salmonella strains used in this study**

| Strain           | Genotype                                                                 |
|------------------|---------------------------------------------------------------------------|
| TR10000          | Wild-type *Salmonella enterica* serovar Typhimurium strain LT2            |
| TT25154          | Tester strain purO+ purD2380(UGA), purD2145::MudJ purR+                 |
| TT12360          | zda-1891::Tn10dCm (90% linked to purR+)                                   |
| TT26169          | purD2380 (UGA) purD2145::MudJ purR2379::Cm(sw)                           |
| TT26173          | purD2380 (UGA) purD2145::MudJ fl- purR+ (zda-1891::Tn10dCm purR+        |
|                  | (zda-3730::Tn10dCm)                                                     |
| TT26174          | purD2380 (UGA) purD2145::MudJ mDEL1(DL2070)                              |
| TT26175          | purD2380 (UGA) purD2145::MudJ mDEL1(DL2070)purO2381::G3A                 |
| TT26176          | purD2380 (UGA) purD2145::MudJ purO2382::C14T                              |
| TT26177          | purD2380 (UGA) purD2145::MudJ mDEL1(DL2070)purR2379::Cm(sw)              |
| TT26178          | purD2380(UGA) purD2145::MudJ gaikT::Tn10dTc Tn10dTc                      |
| TT26203          | purD2380 (UGA) purD2145::MudJ mDEL1(DL2070)purR+ fl- purR+ (zda-1891::Tn10dCm purR+ |
|                  | (zda-3730::Tn10dCm)                                                     |
| TT26204          | purD2380 (UGA) purD2145::MudJ purO2382::C14T zda-1891::Tn10dTc          |
| TT26205          | purD2380 (UGA) purD2145::MudJ mDEL1(DL2070)purO2381::G3A zda-1891::Tn10dTc |
| TT26206          | purD2380 (UGA) purD2145::MudJ mDEL1(DL2070) zda-1891::Tn10dTc            |
| TT26207          | purD2380 (UGA) purD2145::MudJ purO+                                     |
| TT26254          | purO+ purD2380(UGA), purD2145::MudJ (lacY4643::Tn10d-cam) purR+         |
distribution (Luria 1951). A steeper negative slope is seen for a Poisson distribution, which is expected if mutants arise on the selection plate and are subject to the possibility of stress-induced mutagenesis.

Measured mutation rates (μ) were verified using an Excel spreadsheet simulation in which a virtual single cell grows and produces new mutant cells of equal fitness at a given mutation rate (μ). The spreadsheet keeps track of the entire population of cells. The value of μ in this spreadsheet was varied until the simulation produced a frequency of mutant cells equal to the median frequency observed experimentally (after a number of virtual generations equal to those required to produce the number of cells that initiate the selection experiment). The optimal value of μ is then compared to the value estimated from the fluctuation test.

A second Excel spreadsheet simulation was used to calculate the number of mutants plated based on the measured formation rate of each type. The simulation started with a single parent cell lacking the mutation of interest and was applied to estimate the frequency of cells carrying a purR null, mDEL1, or purO single mutation in the nonselectively grown parent population just prior to plating. After each doubling, the mutant cell number increased at the constant growth rate, using Equation 3 above. The optimal value of μ was then compared to the value estimated from the fluctuation test.

Determination of stem-loop deletions by PCR amplification

Primer pairs were designed to amplify the deletion junctions of the stem-loop deletion (mDEL1). Strain TT26174 (an mDEL1 deletion mutant) was grown to saturation in 2.0 ml LB liquid medium at 37°C. Twenty cell cultures were started with 10^8 cells and grown overnight at 37°C. Cells in each culture were pelleted and resuspended in 1.0 ml NCE medium. Aliquots (0.1 ml or ~10^8 cells) were plated on selective medium (NCE, 1% lactose, X-gal, adenine, and thiamine) and plates were incubated at 30°C. The number of Lac^+ revertant colonies was scored for use in the fluctuation test.
Determining growth rates of single and double mutants on selective plates

The isogenic strains tested were TT26177 (purR mDEL1), TT26205 (purO mDEL1), TT26169 (purR), TT26204 (purO), TT26206 (mDEL1), TT26254 (Pur-Lac tester strain, CmR in lacA), and TT25154 (simple Pur-Lac tester strain). Each strain was grown to saturation in 2.0 ml NCE medium with 0.2% glycerol and the appropriate supplements. Approximately 100 cells (in 0.1 ml) were spot-plated on a lawn of $10^8$ Pur-Lac tester cells on minimal lactose (adenine) medium. Each mutant strain was tested in duplicate.

Multiple spots were made for each strain and the whole population of one spot was determined every 24 hr for 144 hr (the aggregate population of ~100 colonies growing above a tester lawn) by excising an entire spot and suspending cells in 1.0 ml of NCE medium. Each cell suspension was plated for single colonies on rich medium with X-gal. For strains TT26205, TT26204, and TT26206, cells were plated on terracycline plates, while strains TT26169 and TT26177 were plated on chloramphenicol. This eliminated lawn cells and assessed the aggregate number of mutant cells (in all colonies together) present at each time point. The assay medium also contained X-gal to allow visual screening of mixed clones. Generation times were calculated from the number of viable cells present at each time.

An Excel spreadsheet simulation was used to model graphically the many pathways that lead to the various types of revertants (described in Supporting Information, File S1). This method was used to predict the development of a single revertant colony, using the unselected mutation rates and growth rates determined above for each mutant type. The simulation started with a singly mutant cell lacking the second mutation needed to become fully Lac+. After each doubling, the singly mutant cell number increased at the constant growth rate measured above. Double-mutant cells appeared after the singly mutant cells reached the number needed to acquire the second mutation. A second simulation started with the appearance of the secondary mutation. New double-mutant cell numbers increased at the constant growth rate previously determined.

Results

Measuring unselected mutation rates

The goal here is to determine the formation rate of the three mutation types (purR, mDEL1, and purO) under nonselective conditions. Because selection is used to detect these mutants, it is particularly important to show that the detected mutants arose before exposure to selection. Rates were calculated using fluctuation tests in which it was ensured that variation in revertant number reflected a Luria–Delbrück distribution and all detected mutations arose before selection. To test this, the logarithm of the number of mutants in a tube ($x$) was plotted vs. the logarithm of the probability of tubes with $x$ or more mutants. For a Luria–Delbrück distribution, this plot shows a line of slope $-1$ (Luria and Delbrück 1943). All of the rates reported here were based on data sets that showed a Luria–Delbrück fluctuation (see Figure 3). The reported rate measurements rely heavily on methods described by Rosche and Foster (2006).

Determining the formation rate of purR mutations

It is expected that purR mutations will have a range of phenotypes, only some of which are sufficiently severe to enhance growth in the Pur-Lac system. Thus only a subset of mutations is relevant to the reversion under selection. This is clear from the set of purR characterized from Pur-Lac revertants (Quiñones-Soto and Roth 2011). Of 25 selected purR mutations, only 7 were missense mutations; the other 18 were rarer types expected to cause more extreme loss of gene function (deletions, frameshifts, nonsense, and promoter changes).

Initially purR mutants were detected by their colony color on rich medium containing X-gal. The strain used was a purR+ tester strain (purHD-lac) with an added mDEL1
deletion. This strain makes light blue colonies on NB X-gal that are distinguishable from mDEL1, purR double mutants whose colonies are darker blue. For each of 20 parallel cultures, ~5000 cells were plated on LB X-gal medium. Five of the cultures showed one mutant each and one culture had two mutants (for a total of six mutants among 100,000 cells screened). We estimated that purR mutants are present in the unselected cultures at a mean frequency of 0.35 mutants per culture or ~2.7 × 10⁻⁵/10⁸ cells. Using the median mutant frequency by the method of Drake (1991), this median frequency indicated that purR mutations form at a rate of 1.3 × 10⁻⁶ mutations per cell per division (Table 2). This rate seemed extremely high in the sense that if the mutation rate per base pair is ~10⁻¹⁰, then we are detecting ~10% of all base changes at this locus, which disagrees with the spectrum of mutation types recovered after selection. The visual screen seems to detect a higher fraction of total purR mutants than the selective regimen. (Note that this method also detects purO mutants, but their contribution was ignored because they are ~1000-fold rarer than purR mutants.)

The fluctuation tests were repeated using selection to detect purR mutants. To do this, 10⁶ cells of an mDEL1 mutant were plated on lactose and Lac⁺ colonies were counted on day 2. All of the colonies appearing on day 2 were homogeneous and were composed entirely of double mutants (purR, mDEL1) carrying a purR mutation that formed prior to selection. The fluctuation in revertant number was shown to be a Luria–Delbrück distribution (slope = −1) in Figure 3 and the estimated purR mutation rate was 3.5 × 10⁻⁸ per cell per division. This suggests that selection detects ~1% of spontaneous purR mutants, in agreement with the spectrum of purR mutations arising under selection (strongly biased toward null types).

The plausibility of this rate of purR mutation formation was tested in a spreadsheet simulation in which parallel spreadsheet runs are initiated with mDEL1 testers and allowed to grow nonselectively with the above mutation rate. Fractional mutants arise in the simulation so the problem of Luria–Delbrück fluctuation is avoided. The frequency of purR mutants seen after 10 generations of simulation was very close to the median number seen in the fluctuation test. The rate based on selective identification of revertants is used below to model behavior of the Pur-Lac system.

### The formation rate of mDEL1 deletion mutations

Both colony color and selective plating were used to detect mDEL1 mutants in fluctuation tests. In this case, the visual and selective detection of mutants should reveal the same number of mutants since all mDEL1 mutations are identical. The starting strain used for visual detection carried two copies of the purR⁺ gene (one on the chromosome and one on an F’ plasmid). In this strain, new mDEL1 mutations are detected as blue colonies among a field of white colonies formed by parental cells. New purR mutations are not detected and purO mutations can be ignored since they form at a 10⁵-fold lower frequency (see below). Thus, the distribution of mDEL1 mutant numbers among cultures was used to calculate mutation rate by the Drake method (Drake 1991). This showed that mDEL1 mutants are present at a median frequency of 4.2 × 10⁻³ and formed at a rate of 5.3 × 10⁻⁴ mutations per cell per division.

To determine the unselected formation rate of mDEL1 mutations using mutants detected selectively, the starting tester strain carried a purR mutation and mutants were detected as colonies appearing on day 2. These colonies contained mDEL1 mutations that had formed prior to plating. This method gave a rate of 1.9 × 10⁻⁴ per cell per generation, very close to that estimated by visual scoring. The high formation rate of the mDEL1 deletion suggests that the palindromic structure being removed contributes to the rate of deletion formation as described previously (Sinden et al. 1991; Leach 1994). In the analysis of the Pur-Lac reversion system, we use 2 × 10⁻⁴ as the formation rate of the mDEL1 mutation under nonselective conditions.

### The formation rate of purO mutations

Because of their small mutational target size, purO mutations are too rare to be detected by nonselective visual

---

**Table 2 Unselected formation rates for each mutation type**

| Mutation type      | Strain used                                     | Method for detecting mutants | Median mutant frequency | Formation rate per cell per division |
|--------------------|-------------------------------------------------|------------------------------|-------------------------|--------------------------------------|
| mDEL1              | Tester with purR⁺·purR⁺                          | Visual: blue colony on X-gal | 4.2 × 10⁻³               | (5.3 × 10⁻⁴)                        |
|                    | Tester with purR mutation                       | Selected day 2 colonies from 10⁸ plated cells | 2.3 × 10⁻³               | (4.2 × 10⁻⁴)                        |
| purR               | Tester with mDEL1 deletion                      | Visual: dark blue colony on X-gal | 2.7 × 10⁻⁵               | (1.3 × 10⁻⁵)                        |
|                    | Tester with mDEL1 deletion and purR⁺·purR⁺      | Selected day 2 colonies from 10⁸ plated cells | 8.0 × 10⁻⁷               | (3.5 × 10⁻⁷)                        |
| purO               | Tester with mDEL1 deletion                       | Selected day 2 colonies from 10⁸ plated cells | 9.0 × 10⁻¹⁰              | (1.5 × 10⁻¹⁰)                       |
| purR, mDEL1 double | Standard tester (10⁹ cells plated)              | Selected day 2 colonies from 10⁸ plated cells | 6.70 × 10⁻³              | (1.1 × 10⁻⁸)                        |

a Calculated from median mutant frequency by the method of Drake (1991).
b Calculated by a spreadsheet simulation as described previously (Reams et al. 2010).
c Calculated by the y-intercept method as described by Rosche and Foster (2000).
d Calculated by the zero-tube method as described by Rosche and Foster (2000).
and an F plasmid with an extra copy of the purR+ gene to prevent detection of purR mutants. Parallel cultures were grown overnight in rich medium and plated on lactose plates, where fully Lac+ purO, mDEL1 double mutants were counted on day 2. To show that this selection method operated as intended, 20 independent Lac+ clones appearing on day 2 were tested by sequencing of PCR fragments. All proved to carry a purO mutation (see Appendix, Figure A1 for sequence changes). The frequency of purO mutants among independent nonselective cultures showed a Luria-Delbrück distribution, ensuring that mutations arose prior to plating (see Figure 3). The tests revealed a formation rate of 6.4 × 10⁻¹⁰ mutations per cell per division (Table 2), consistent with a target of only a few base pairs.

### Table 3 Growth rates of the Pur-Lac tester strain and its derivative mutant strains in liquid and solid media

| Strain         | Growth on glycerol (hr/gen) | Growth on lactose (hr/gen) |
|----------------|----------------------------|---------------------------|
|                | Liquid medium              | Solid medium              |
|                | (hr/gen)                   | (first 24 hr)             |
|                | Liquid medium              | Solid medium              |
|                | (hr/gen)                   | (after 24 hr)             |
| LT2 strain     | 1.2 (0.02)                 | NA                        |
| Pur-Lac tester | 1.3 (0.05)                 | 5.5 (0.6)                 |
| purR           | 1.5 (0.25)                 | 2.5 (0.2)                 |
| purO           | 1.7 (0.4)                  | 2.2 (0.03)                |
| mDEL1          | 1.3 (0.01)                 | 3.6 (0.6)                 |
| purR mDEL1     | 1.5 (0.07)                 | 1.3 (0.02)                |
| purO mDEL1     | 1.3 (0.04)                 | 1.2 (0.07)                |

NA, not available. Numbers in parentheses are standard deviations.

The selection-only model suggests that preexisting cells with one mutation initiate a slow-aa full Lac+ phenotype. This model predicts that singly mutant cells should grow less well on lactose than those carrying two mutations. The model also predicts that populations of singly mutant cells in nascent colonies become sufficiently large (and standard mutation rates are sufficiently high) to allow secondary mutations to arise without any stress-induced increase in mutation rate. To test these predictions, the growth rates of single and double mutants were tested under nonselective and selective conditions.

Growth rates were first measured in liquid glycerol (adenine) medium and lactose (adenine) medium and are presented in Table 3 (leftmost two columns). The rates on lactose medium seemed too high to account for the long delay in colony appearance seen on the selection plate. To make more realistic estimates, growth rates were determined directly on selection plates, where single cells are forced to grow above a lawn of competing parent cells.

To determine growth rate of the several mutant types on the selective plate, 100 cells of each mutant were spotted (on a 2-cm spot) above a lawn of parent (10⁸ cells) on selective lactose medium. The added mutant cells carried a drug-resistance marker that allowed them to be selectively distinguished from cells in the parent lawn in determining growth rate. Each of the 100 cells within the spot initiated a colony. A series of identical spots were used to estimate growth rate. A plug of agar with an entire spot (100 nascent colonies) was removed and cells were suspended. Many time points were taken before individual colonies were visible in the spot. Each suspension contains all of the marked cells in 100 nascent colonies plus cells from the unmarked parent lawn. The suspensions were plated on rich medium with an antibiotic that allowed only cells from the 100 mutant clones to grow. Different spots (100 colonies each) were sampled daily over several days and the aggregate number of mutant cells revealed the initial growth rate of cells on the selection plates. The growth rates were determined before any colony achieved a size large enough to permit occurrence of mutations providing secondary growth improvements. Growth rates of the Pur-Lac tester strain and the different mutant types are presented in Table 3 (rightmost two columns). Rates are presented for day 1 (when cells had access to nutrients contaminating the agar) and for subsequent days (during which growth was on lactose alone).

It should be noted that within each spot on selective plates, individual colonies of the mutant ultimately became visible above the parent lawn. In spots inoculated with double-mutant types (purR, mDEL1 or purO, mDEL1) colonies became visible by 48 hr of incubation. That is, each double mutant cell formed a visible colony (~10⁷ cells) within 2 days on selective medium. In spots initiated by single mutants, the purO colonies appeared after 72 hr of incubation and the purR colonies after 96 hr. However, the mDEL1 single mutant formed no visible colonies even after 120 hr of incubation. This suggested the mDEL1 deletion mutants grew substantially more poorly than the other singly mutant types under conditions of the selective plate.

### Pathways of genetic adaptation—a graphic model to explain delayed appearance of revertant colonies above a nongrowing parent lawn

The selection-only model proposes that colonies appearing under selection are initiated by mutant cells that arose during nonselective growth before plating and that two mutations are required to give full growth ability leading to
a colony that becomes visible within 6 days. Each preexisting singly mutant cell type plated on selective medium grows slowly with a characteristic generation (doubling) time. The increase in mutant cell number within a colony enhances the likelihood that some cell in that colony acquires a secondary mutation. The time at which the second mutation occurs depends on the doubling time of the initial single mutant and the formation rate of the secondary genetic change. A revertant colony becomes visible when its population exceeds 10^6 cells. Thus, the time at which a new colony first becomes visible depends on the growth rate of the single mutant, the time at which the second mutation arises, and the growth rate of doubly mutant cells within the colony.

In the Pur-Lac system, 10^8 plated tester cells give rise to 100 Lac^+ colonies by the end of day 6. Before plating, these cells grow overnight under nonselective conditions where Lac^− parental cells can acquire a mutation that improves growth on lactose: mDEL1, purR or purO. The mutation type formed at the highest rate (mDEL1) is most frequent in the plated population (10^8 mutants per 10^8 parental cells), but grows so slowly under selection that it is unlikely to acquire a rare improving mutation (purO or purR) on the timescale of the experiment. Clones initiated by the rarer mutant types are expected to grow more rapidly under selection and contribute heavily to early-appearing revertant clones. To assess behavior of the system, the unselected mutation rates and growth rates estimated above were used in a graphic model to evaluate the predicted behavior of the several pathways that lead to various types of revertants. These graphs test the selection-only model to the extent that they show whether revertants can be explained with no increase in mutation rate over that found in nonselectively grown cells. Various versions of the stress-induced mutagenesis model predict that revertant appearance requires either directed or general mutagenesis. We showed previously that no general mutagenesis accompanies reversion in the Pur-Lac system (Quiñones-Soto and Roth 2011). The graphic method used here can eliminate the need for directed mutagenesis.

Figure 4A presents the predicted development of a revertant colony initiated by a preexisting purR single mutant. When a purR mutant is first plated on lactose (adenine) medium, it grows nonselectively at a rate of 2.3 hr/generation for the first 24 hr of incubation, using contaminants present in the agar (see Table 3). Once these contaminants are exhausted, cells grow at a slower rate [4.5 hr/generation (gen)]. As the colony population approaches 10^4 cells, one cell in each purR colony population may acquire an mDEL1 mutation (an event that occurs at 10^-6 per cell per division). The newly formed purR mDEL1 double mutant within the colony grows at the faster rate of 1.3 hr/gen and eventually takes over the colony. The whole colony (composed of a mixture of single and double mutants) reaches 10^7 cells and becomes visible by the third day of incubation on the lactose reversion plate. Variable times of colony appearance are expected since different purR mutations may have different growth rates under selection and because the formation time of the second mutation (mDEL1) is expected to be subject to stochastic variation. The graph in Figure 4A is based on a spreadsheet simulation that uses the measured parameters to show that colonies following this pathway of selection can appear well within the time of the experiment, using standard unselected mutation rates (see File S1).

We observed a similar projection for a plated purO single mutant (Figure 4B). The frequency of purO mutants in the unselected population is very low, so while this trajectory is reasonable, this type is unlikely to contribute heavily to the number of revertant colonies initiated by plated singly mutant cells.

The origin of mutants initiated by a preexisting mDEL1 mutant poses a problem. As seen in Figure 4C growth of a plated mDEL1 mutant starts at a doubling time of 3.5 hr/gen at expense of contaminants present in the agar plates. After growth becomes dependent on lactose, the growth rate slows to 17 hr/gen. The projection in Figure 4C shows that the clone initiated by a single plated mDEL1 mutant cell cannot reach the population size required for visibility (10^7) within the experimental period. It also cannot reach a population size sufficient to ensure occurrence of the most frequent second mutation type (purR, 10^8 cells). Thus, the simplest form of the selection-only model does not explain how a common mDEL1 single mutant could ever initiate a revertant colony. This is a serious problem, because half of the observed revertant colonies were initiated by an mDEL1 mutant (Quiñones-Soto and Roth 2011). Clearly something extra must be involved. Stress-induced general mutagenesis was eliminated previously (Quiñones-Soto and Roth 2011), but directed mutagenesis is still a possibility (Foster and Cairns 1992). However, several aspects of mDEL1 mutant behavior make it likely that a more mundane explanation will prove correct. First, mDEL1 is the most frequent mutation type, with ~10^5 such mutants included in the plated population. Second, the mDEL1 mutant allele produces a substantial amount of β-galactosidase (Quiñones-Soto and Roth 2011). Finally, the purD::lac region is subject to frequent duplication. Below we apply these properties in explaining the origin of mDEL1-initiated revertants.

The large number of plated mDEL1 mutants may compensate for their slow growth

While each mDEL1 clone has a very small probability of generating a visible colony within 6 days, many such clones are initiated because this mutation arises at a high rate. The plated population includes 10^5 mDEL1 mutants (calculated using the spreadsheet method), each of which initiates a growing clone with a low probability of acquiring a second mutation (purR or purO). Half of the 100 revertants appearing on day 6 were initiated by an mDEL1 mutation. That is, ~50 of the 10^5 initiated clones succeed in generating a visible colony. Perhaps the aggregate number of cells in all the colonies together is sufficient to ensure that a few of those colonies get a secondary mutation. This is tested by the graph in Figure 4D.
The graph in Figure 4D presents the expected total number of mDEL1 cells present on one plate in the $10^5$ growing clones. While the total number of mDEL1 cells on the plate reaches $10^7$ within 1 day, no visible colony is expected because each clone has only $\frac{1}{100}$ cells. By day 5, the aggregate cell number is expected to reach $10^8$, a number sufficient to allow a purR mutation somewhere on the plate (formed at $10^{-2}$ per cell per division). Such a colony grows rapidly and achieves visibility by day 6. Thus, the high formation rate of the mDEL1 mutant may compensate for its slow growth under selection and allow a few clones to develop to contribute heavily to the number of final revertant colonies. This pooling effect should explain some but not all mDEL1-initiated revertants.

**Amplification of the lac region may enhance growth of mDEL1 mutants under selection**

The purHD-lac region lies between two ribosomal RNA genes (rrnB and rrnE). These loci have nearly identical 5-kb sequence separated by $\sim$40 kb. Duplications of the regions between rrn genes form at a very high rate of $10^{-2}$ per cell per generation (Anderson and Roth 1981) and rapidly come to a steady-state frequency of $\sim$1% in an unselected population (Reams et al. 2010). Thus 1000 of the $10^8$ mDEL1 mutations are expected to be in cells with a purD-lac duplication at the time they are plated. Additional amplification steps occur at $\sim 10^{-1}$ per cell per division. Thus many mDEL1 mutants are likely to grow faster.
under selection than that estimated for cells with a single copy of the mutant purHD-lac locus. Amplification of the rrnB-rrnE region adds targets for purO mutations, which arise within the amplified purHD operon. Thus amplification enhances both growth and the likelihood of a purO mutation. This process of adaptation by amplification under selection underlies the accelerated appearance of Lac+ revertants in the Cairns system (Cairns and Foster 1991; Hendrickson et al. 2002; Slechta et al. 2003) and has recently been demonstrated to explain rapid adaptation of poxviruses (Elde et al. 2012).

In the course of work on the Pur-Lac system, revertant colonies were found that included only one mutation type (mDEL1). Many cells from these colonies have multiple copies of the mDEL1 mutant allele and give rise to segregants that lose copies and some of their Lac+ phenotype. Appearance of revertants in the purHD-lac system is reduced substantially in the absence of RecA, which is required for gene amplification. The contribution of amplification to reversion in the Pur-Lac system will be described elsewhere (S. Quinones-Soto, unpublished results).

Discussion

In the Pur-Lac selection system, Lac+ revertant colonies appear over time above a lawn of nongrowing parent lawns. While this behavior was initially attributed to stress-induced mutagenesis (Yang and Polisky 1993; Yang et al. 2001), it can be explained by selection alone acting on mutants that arise during pregrowth and initiate clones that grow and adapt under selective conditions. We have characterized four pathways of adaptation, each initiated by a single mutant present in the preselection culture prior to plating on selective medium. The unselected formation rate of each mutant type in the pregrowth dictates the frequency of that mutant in the plated population. The probability of a plated partial revertant developing into a visible Lac+ colony within 6 days is determined by the growth rate of the initial clone and the rate at which secondary improving mutations arise during colony development. For two mutant types (purR and purO), the appearance of colonies can be explained using standard mutation rates determined under non-selective conditions. Growth limitation acts purely as an agent of selection and contributes to neither general nor directed mutagenesis.

Clones initiated by the mDEL1 mutation are harder to explain. The slow growth rate of the single mutant under selection and the low formation rate of the subsequent mutation types suggest that individual mDEL cells should not generate visible colonies within the 6-day period. However, revertants of this type are in fact common, representing roughly half of the observed colonies. A simple explanation of this conflict is that the high rate of mDEL1 mutation formation ensures that many (10^3) mDEL1 mutants are plated. Their aggregate growth is sufficient to ensure that a few of these clones acquire the secondary mutation needed for the observed revertant colonies. To explain the observed colonies, only ~50 of the 10^5 clones need to succeed.

Two other explanations can be considered. One could imagine that the stress of slow growth directs formation of the needed secondary mutations, allowing them to arise despite the small size of the initial clones as originally suggested by Cairns et al. (1988). We have shown previously that no general mutagenesis occurs during colony growth in the Pur-Lac system, but it is harder to eliminate directed mutagenesis. In the light of the behavior of the other revertant types, we are reluctant to suggest directed mutagenesis, but suggest that gene amplification may be a better explanation.

The behavior of these clones may be explained by amplification of the purHD-lac region during growth under selection. This region duplicates at the astounding rate of 10^{-3} per cell per division (Reams et al. 2010), due to its location between directly repeated copies of the rrnE and rrnB genes. This suggests that 100 of the 10^5 mDEL1 mutants in the plated population should already have a duplicated mDEL1 mutant allele of the purHD-lac region. In these cells, further amplifications (occurring at 10^{-1} per cell per generation) would enhance growth of the nascent colony under selection and increase the likelihood of a purO mutation by providing more copies of the target sequence. Evidence for this will be presented elsewhere.

The general conclusion from the behavior of this system is that populations can respond rapidly to selection, when mutants with small improvements are common. There may be many equivalently adaptive end states and many routes to each state. The particular pathway taken is dictated stochastically by the frequency of the several mutation types and the growth improvement caused by each new mutation. The pathway used by most developing clones is likely to be initiated by the mutation type that arises at the highest rate. Many different pathways may be followed simultaneously by various lineages within a single population (in different colonies), but for each developed cell type in that population (each colony), a single particular pathway should be definable.

Acknowledgments

We thank Zhiwei Yang, Zhong Lu, and Aoquan Wang for generously providing the tester strain they developed. We thank laboratory members Eric Kofoid, Sophie Maisnier-Patin, Emiko Sano, Doug Huseby, and Natalie Duleba for advice and helpful comments on the preparation of the manuscript. This work was supported in part by National Institutes of Health grant GM27068.

Literature Cited

Anderson, R. P., and J. Roth, 1981 Spontaneous tandem genetic duplications in Salmonella typhimurium arise by unequal recombination between rRNA (rrn) cistrons. Proc. Natl. Acad. Sci. USA 78: 3113–3117.
Andersson, D. I., D. Hughes, and J. R. Roth (Editors), 2011 *The Origin of Mutants under Selection: Interactions of Mutation, Growth, and Selection*. ASM Press, Washington, DC.

Berkowitz, D., J. M. Hushon, H. J. Whitfield, J. R. Roth, and B. N. Ames, 1968 Procedures for identifying nonsense mutations. J. Bacteriol. 96: 215–220.

Cairns, J., and P. L. Foster, 1991 Adaptive reversion of a frameshift mutation in Escherichia coli. Genetics 128: 695–701.

Cairns, J., J. Overbaugh, and S. Miller, 1988 The origin of mutants. Nature 335: 142–145.

Castilho, B. A., P. Olfson, and M. J. Casadaban, 1984 Plasmid insertion mutagenesis and lac gene fusion with mini-mu bacteriophage transposons. J. Bacteriol. 158: 488–495.

Chan, R. K., D. Botstein, T. Watanabe, and Y. Ogata, 1989 DNA-based microbes. Proc. Natl. Acad. Sci. USA 86: 7160–7164.

Cairns, J., and P. L. Foster, 2000 Determining mutation rates in bacterial populations. Methods 20: 4–17.

Cairns, J., and P. L. Foster, 2006 Methods for determining spontaneous mutation rates. Methods Enzymol. 409: 195–213.

Chan, R. K., D. Botstein, T. Watanabe, and Y. Ogata, 1989 DNA-based microbes. Proc. Natl. Acad. Sci. USA 86: 7160–7164.

Cairns, J., J. Overbaugh, and S. Miller, 1988 The origin of mutants. Nature 335: 142–145.

Castilho, B. A., P. Olfson, and M. J. Casadaban, 1984 Plasmid insertion mutagenesis and lac gene fusion with mini-mu bacteriophage transposons. J. Bacteriol. 158: 488–495.

Chan, R. K., D. Botstein, T. Watanabe, and Y. Ogata, 1972 Specialized transduction of tetracycline resistance by phage P22 in Salmonella typhimurium. II. Properties of a high frequency transducing lysate. Virology 50: 883–898.

Davis, R. W., D. Botstein, and J. R. Roth, 1980 *Advanced Bacterial Genetics*. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.

Drake, J. W., 1991 A constant rate of spontaneous mutation in DNA-based microbes. Proc. Natl. Acad. Sci. USA 88: 7160–7164.

Elde, N. C., S. J. Child, M. T. Eickbush, J. O. Kitzman, K. S. Rogers et al., 2012 Poxviruses deploy genomic accordions to adapt rapidly against host antiviral defenses. Cell 150: 831–841.

Foster, P. L., 2007 Stress-induced mutagenesis in bacteria. Crit. Rev. Biochem. Mol. Biol. 42: 373–397.

Foster, P. L., and J. Cairns, 1992 Mechanisms of directed muta- tion. Genetics 131: 783–789.

Galhardo, R. S., P. J. Hastings, and S. M. Rosenberg, 2007 Mutation as a stress response and the regulation of evolvability. Crit. Rev. Biochem. Mol. Biol. 42: 399–435.

Hall, B. G., 1982 Evolution of a regulated operon in the laboratory. Genetics 101: 335–344.

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

Hendrickson, H., E. S. Slechta, U. Berghthorsson, D. I. Andersson, and J. R. Roth, 2002 Amplification-mutagenesis: evidence that “directed” adaptive mutation and general hypermutability result from growth with a selected gene amplification. Proc. Natl. Acad. Sci. USA 99: 2164–2169.

Leach, D. R., 1994 Long DNA palindromes, cruciform structures, genetic instability and secondary structure repair. Bioessays 16: 893–900.

Lederberg, J., and E. M. Lederberg, 1952 Replica plating and in- direct selection of bacterial mutants. J. Bacteriol. 63: 399–406.

Luria, S. E., 1951 The frequency distribution of spontaneous bacteriophage mutants as evidence for the exponential rate of phage reproduction. Cold Spring Harb. Symp. Quant. Biol. 16: 463–470.

Luria, S. E., and M. Delbruck, 1943 Mutations of bacteria from virus sensitivity to virus resistance. Genetics 28: 491–511.

Newcombe, H. B., 1949 Origin of bacterial variants. Nature 164: 150.

Quiñones-Soto, S., and J. R. Roth, 2011 Effect of growth under selection on appearance of chromosomal mutations in Salmo- nella enterica. Genetics 189: 37–53.

Reams, A. B., E. C. Kofoid, M. Savageau, and J. R. Roth, 2010 Duplication frequency in a population of Salmonella en- terica rapidly approaches steady state with or without recombina- tion. Genetics 184: 1077–1094.

Rosche, W. A., and P. L. Foster, 2000 Determining mutation rates in bacterial populations. Methods 20: 4–17.

Rosche, W. A., and P. A. Foster, 2006 Methods for determining spontaneous mutation rates. Methods Enzymol. 409: 195–213.

Roth, J. R., E. Kugelberg, A. B. Reams, E. Kofoid, and D. I. Andersson, 2006 Origin of mutations under selection: the adaptive muta- tion controversy. Annu. Rev. Microbiol. 60: 477–501.

Schmiegler, H., 1972 Phage P22-mutants with increased or de- creased transduction abilities. Mol. Gen. Genet. 119: 75–88.

Seifert, H. S., and R. D. Porter, 1984 Enhanced recombination between lambda plac5 and F42lac: identification of cis- and trans-acting factors. Proc. Natl. Acad. Sci. USA 81: 7500–7504.

Shapiro, J. A., 1984 Observations on the formation of clones con- taining araB-lacZ cistron fusions. Mol. Gen. Genet. 194: 79–90.

Shapiro, J. A., and P. M. Brinkley, 1984 Programming of DNA rearrangements involving mu prophages. Cold Spring Harb. Symp. Quant. Biol. 49: 313–320.

Sinden, R. R., G. X. Zheng, R. G. Brankamp, and K. N. Allen, 1991 On the deletion of inverted repeated DNA in *Escherichia coli*: effects of length, thermal stability, and cruciform formation in vivo. Genetics 129: 991–1005.

Slechta, E. S., K. L. Bunny, E. Kugelberg, E. D. I. Andersson et al., 2003 Adaptive mutation: general mutagenesis is not a pro- grammed response to stress but results from rare coamplification of dinB with lac. Proc. Natl. Acad. Sci. USA 100: 12847–12852.

Steele, D. F., and S. Jinks-Robertson, 1992 An examination of adaptive reversion in *Saccharomyces cerevisiae*. Genetics 132: 9–21.

Taddei, F., I. Matic, and M. Radman, 1995 cAMP-dependent SOS induction and mutagenesis in resting bacterial populations. Proc. Natl. Acad. Sci. USA 92: 11736–11740.

Taddei, F., J. A. Halliday, I. Matic, and M. Radman, 1997 Genetic analysis of mutagenesis in aging Escherichia coli colonies. Mol. Gen. Genet. 256: 277–281.

Torkelson, J., R. S. Harris, M.-J. Lombardo, J. Nagendran, C. Thulin et al., 1997 Genome-wide hypermutation in a subpopulation of stationary-phase cells underlies recombination-dependent adaptive mutation. EMBO J. 16: 3303–3311.

Yang, Y. L., and B. Polisky, 1993 Suppression of ColE1 high-copy- number mutants by mutations in the polA gene of Escherichia coli. J. Bacteriol. 175: 428–437.

Yang, Z., Z. Lu, and A. Wang, 2001 Study of adaptive mutations in Salmonella typhimurium by using a super-repressing mutant of a trans regulatory gene purR. Mutat. Res. 484: 95–102.

Yang, Z., Z. Lu, and A. Wang, 2006 Adaptive mutations in Salmo- nella typhimurium phenotypic of purR super-repression. Mutat. Res. 595: 107–116.

Communicating editor: J. Lawrence
Figure A1  Spontaneous operator (purO) mutations. Twenty independent Lac\(^+\) mutants appearing in an mDEL1 parent on day 2 were assumed to carry purO mutation based on linkage of their Lac\(^+\) phenotype to the purHD operon. These mutations were shown by fluctuation tests to have arisen during nonselective pregrowth and were used in estimating the formation rate of purO mutations able to provide the Lac\(^+\) phenotype. The purO region was sequenced by amplifying the region with primers TP1833 (5’-AACAGGCGGGCGGTGCCCCC-3’) and TP1834 (5’-GTGAAGTGATTCACATCCGC-3’). The changes are noted above. Mutations at the hotspots (base pairs 3 and 14) were also found in the initial set of selected mutants (Quiñones-Soto and Roth 2011).
Pathways of Genetic Adaptation: Multistep Origin of Mutants Under Selection Without Induced Mutagenesis in *Salmonella enterica*

Semarhy Quiñones-Soto, Andrew B. Reams, and John R. Roth
Available online is an Excel file with the spreadsheet simulations for growth trajectories described in Figure 4. The unselected formation rates of the three single mutant types and the growth rates of all single and double mutants are described in the Results and are used in these simulations.

Spreadsheet 1 describes growth of a pre-existing purR single mutant (with a growth rate of 2.5 h/gen shifting to 5.3 h/gen after day 1). A secondary mDEL1 mutation is expected when the population within the colony reaches about $10^4$ cells. At this point (after 1.7 days of incubation), a new purR mDEL1 double mutant appears in the population and grows at a different, faster rate (see Spreadsheet 4). The sum of these two trajectories represents the number of cells in the developing Lac$^+$ colony.

Spreadsheet 2 describes growth of a pre-existing purO single mutant (with a growth rate of 2.2 h/gen shifting to 2.6 h/gen after day 1). An additional mDEL1 mutation is expected when the population within the colony reaches about $10^4$ cells. At this point (after 1.4 days of incubation), a new purO mDEL1 double mutant appears in the population and grows at a different, faster rate. Spreadsheet 5 describes growth of the double purO mDEL1 mutant. The total of these two trajectories (growths) represent the number of cells in the Lac$^+$ colony.

Spreadsheet 3 describes growth of a pre-existing mDEL1 single mutant (with growth rate of 3.6 h/gen shifting to 17.0 h/gen after day 1). At the sixth day after incubation (end of the reversion experiment), a colony started by a pre-existing mDEL1 single mutant accumulated about $10^4$ cells. At this point, there are not enough cells in the colony to allow the formation of a new purR mutation (formation rate of $3.3 \times 10^{-8}$) or a new purO mutation (formation rate of $1.2 \times 10^{-10}$).

Spreadsheet 6 describes the contribution of all single mDEL1 mutants ($10^5$ cells plated) starting independent, slow-growing colonies. A secondary purR mutation is expected when the population within the colony reaches about $10^8$ cells (after 5.3 days of incubation). Spreadsheet 7 describes growth of the double purR mDEL1 mutant originated from one of the many single mDEL1 mutants plated.
File S1

Supporting Information

Available for download at http://www.genetics.org/lookup/suppl/doi:10.1534/genetics.112.142158/-/DC1.

Available online is an Excel file with the spreadsheet simulations for growth trajectories described in Figure 4. The unselected formation rates of the three single mutant types and the growth rates of all single and double mutants are described in the Results and are used in these simulations.

Spreadsheet 1 describes growth of a pre-existing purR single mutant (with a growth rate of 2.5 h/gen shifting to 5.3 h/gen after day 1). A secondary mDEL1 mutation is expected when the population within the colony reaches about $10^4$ cells. At this point (after 1.7 days of incubation), a new purR mDEL1 double mutant appears in the population and grows at a different, faster rate (see Spreadsheet 4). The sum of these two trajectories represents the number of cells in the developing Lac^- colony.

Spreadsheet 2 describes growth of a pre-existing purO single mutant (with a growth rate of 2.2 h/gen shifting to 2.6 h/gen after day 1). An additional mDEL1 mutation is expected when the population within the colony reaches about $10^4$ cells. At this point (after 1.4 days of incubation), a new purO mDEL1 double mutant appears in the population and grows at a different, faster rate. Spreadsheet 5 describes growth of the double purO mDEL1 mutant. The total of these two trajectories (growths) represent the number of cells in the Lac^- colony.

Spreadsheet 3 describes growth of a pre-existing mDEL1 single mutant (with growth rate of 3.6 h/gen shifting to 17.0 h/gen after day 1). At the sixth day after incubation (end of the reversion experiment), a colony started by a pre-existing mDEL1 single mutant accumulated about $10^4$ cells. At this point, there are not enough cells in the colony to allow the formation of a new purR mutation (formation rate of $3.3 \times 10^{-8}$) or a new purO mutation (formation rate of $1.2 \times 10^{-10}$).

Spreadsheet 6 describes the contribution of all single mDEL1 mutants ($10^5$ cells plated) starting independent, slow-growing colonies. A secondary purR mutation is expected when the population within the colony reaches about $10^8$ cells (after 5.3 days of incubation). Spreadsheet 7 describes growth of the double purR mDEL1 mutant originated from one of the many single mDEL1 mutants plated.