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

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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
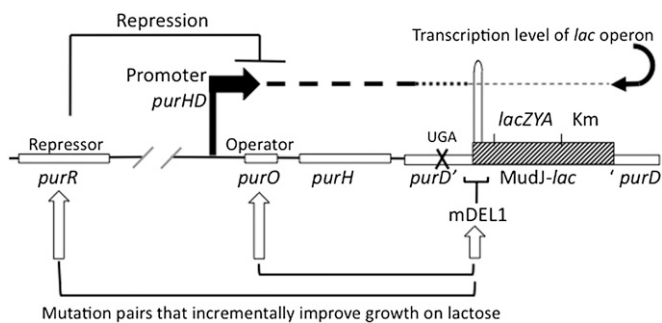

Figure 1 Mutations that provide a Lac$^+$ phenotype in the Pur-Lac system. An intact *lac* operon is inserted within the purine biosynthetic operon (*purHD*) and is transcribed from the *purHD* promoter. Transcription of *lac* is repressed when PurR protein binds to the *purO* operator site and is further reduced by a polar *purD* nonsense mutation (UGA), whose effect on transcription is increased by a terminator stem-loop structure within the MudJ element. The Lac$^-$ phenotype of the parent tester strain is partially corrected by any of the three single mutations: a *purR* loss-of-function mutation, a *purO* (operator) mutation, or a deletion of the terminator stem (mDEL1). Expression of *lac* is further enhanced in double mutants: *purR*, mDEL1 or *purO*, mDEL1.

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 (MudJ-*lac*) derived from a phage Mu that lacks transposition functions (Castilho *et al.* 1984). The mutations that confer a Lac$^+$
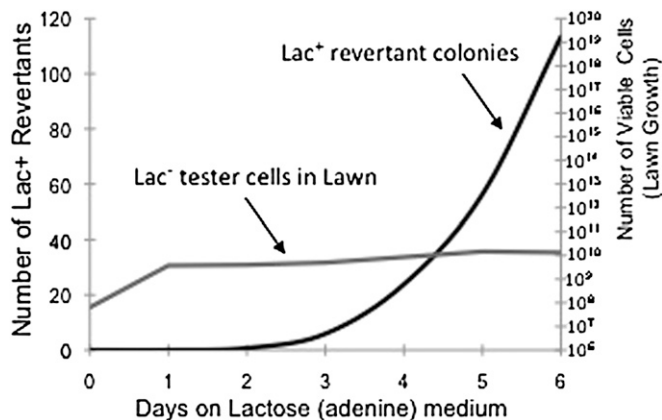
Figure 2 Course of a reversion experiment in the Pur-Lac system. Phenotypically Lac⁻ tester cells (10^8) are plated on lactose (adenine) selective medium and Lac⁺ revertant colonies accumulate over a period of 6 days of incubation. The lawn of tester cells grows (about six generations) during the first day of the incubation period and then remains constant.

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

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*::Tn*10*dTc (90% linked to *purR⁺*)
TT26169	*purD2380* (UGA) *purD2145*::MudJ *purR2379*::Cm(sw)
TT26173	*purD2380* (UGA) *purD2145*::MudJ/F′ *his⁺* (*zda-1891*::Tn*10*dTc *purR⁺* *zda-3730*::Tn*10*dCm)
TT26174	*purD2380* (UGA) *purD2145*::MudJ mDEL1(DEL2070)
TT26175	*purD2380* (UGA) *purD2145*::MudJ mDEL1(DEL2070)*purO2381*::G3A
TT26176	*purD2380* (UGA) *purD2145*::MudJ *purO2382*::C14T
TT26177	*purD2380* (UGA) *purD2145*::MudJ mDEL1(DEL2070)*purR2379*::Cm(sw)
TT26178	*purD2380*(UGA) *purD2145*::MudJ *galKT*::Tn*10*d-Tet
TT26203	*purD2380* (UGA) *purD2145*::MudJ *mDEL1*(DEL2070)/F′ *his⁺* (*zda-1891*::Tn*10*dTc *purR⁺* *zda-3730*::Tn*10*dCm)
TT26204	*purD2380* (UGA) *purD2145*::MudJ *purO2382*::C14T *zda-1891*::Tn*10*dTc
TT26205	*purD2380* (UGA) *purD2145*::MudJ mDEL1(DEL2070) *purO2381*::G3A *zda-1891*::Tn*10*dTc
TT26206	*purD2380* (UGA) *purD2145*::MudJ mDEL1(DEL2070) *zda-1891*::Tn*10*dTc
TT26207	*purD2380* (UGA) *purD2145*::MudJ *purD⁺*
TT26254	*purO⁺ purD2380*(UGA), *purD2145*::MudJ (*lacY4643*::Tn*10*d-cam) *purR⁺*

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-β-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⁻*) (Schmieger 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 Tn*10*dTc and Tn*10*dCm elements inserted on either side of the chromosomal *purR⁺* gene. This unit (Tn*10*dTc-*purR*-Tn*10*dCm) 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 Delbrück 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 $\mu = m/N$ (Luria and Delbruck 1943). In several determinations, m was estimated by the p_0 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:

$$p_0 = \frac{\text{number of cultures without mutants}}{\text{total number of cultures}} \qquad (1)$$

$$m = -\ln p_0. \qquad (2)$$

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

$$\mu = \frac{f}{\ln N} = \frac{0.4343 f}{\log N}. \qquad (3)$$

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

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 mutation rate measured previously. Mutant cells started accumulating as fractional cells at the same rate as parent cells. Mutant cells continued to accrue until the total population size reached 10^8 cells (27 generations), the number of parent cells plated onto selective medium. The frequency of mutants accumulated after 27 generations in the simulation agreed closely with the measured mutant frequency.

Determining mutation rates: (1) estimating purR formation rate

The formation rate of *purR* mutations was measured in two ways. Both of these methods also detect *purO* mutants, whose contribution is ignored because their formation rate and frequency in the population are ~1000-fold lower than those of *purR* mutations.

In the first method, the frequency of *purR* mutants was scored unselectively, using a tester strain that carried a stem-loop deletion (mDEL1). Strain TT26174 (an mDEL1 deletion mutant) was grown to saturation in 2.0 ml LB liquid medium at 37°. Twenty cultures were inoculated in LB liquid medium by ~10^3 mDEL1 tester cells and grown to saturation. Each saturated cell culture was diluted 10^{-5}-fold and 0.1-ml aliquots were plated on rich medium containing X-gal. Five replicas were plated for each of the 20 cultures. The plates of single colonies were screened visually for dark blue colonies among a field of light blue parental colonies after 1 day of incubation. The mutant frequencies were used to calculate the number of mutation events and the mutation rate, using Equation 3 above.

The second method detects *purR* mutants selectively by using the same mDEL1 mutant (TT26174) grown to saturation at 30° in 2.0 ml NCE with 0.2% glycerol and the appropriate supplements. Cells 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°. The number of day 2 Lac$^+$ revertant colonies was scored for use in the fluctuation test.

Determining mutation rates: (2) estimating mDEL1 formation rate

Strain TT26173, which has two copies of the *purR$^+$* gene (R^+/R^+), was grown to saturation in 2.0 ml NCE medium, 0.2% glycerol (adenine) at 37°. Twenty cultures were inoculated in rich liquid medium with 10^3 R^+/R^+ cells and grown to 10^9 cells/ml. Each saturated cell culture was diluted 10^5-fold and 0.1-ml aliquots were plated on rich medium containing X-gal. Plates were visually screened for blue colonies after 1 day of incubation. In determining the mDEL1 formation rate, only a portion of the cultures was plated so some of the mutant cells were lost. To compensate for this, the formation rate was calculated using Equation 3.

The formation rate of the stem deletion mutation was verified by a second method, in which mDEL1 mutants were detected selectively by using a *purR* mutant tester strain (TT26169). Strain TT26169 was grown to saturation in 2.0 ml NCE medium with 0.2% glycerol and the appropriate supplements at 30°. Cells 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, Xgal, adenine, and thiamine) and plates were incubated at 30°. The number of Lac$^+$ colonies was scored on day 2.

Determining mutation rates: (3) estimating purO formation rate

Strain TT26203 (mDEL1, R^+/R^+) was grown to saturation in 2.0 ml NCE medium, 0.2% glycerol (adenine) at 37°. Twenty cell cultures were started with 10^3 cells and grown overnight at 37°. Cells in each culture were pelleted and washed in 200 μl of 1× NCE salt solution. Aliquots from 5 of the cultures were used to determine the total cell number. Aliquots of 0.1 ml were plated on 1% lactose minimal medium, supplemented with 10 μg/ml adenine and 0.06 mM thiamine, and X-gal. The number of Lac$^+$ colonies was scored 2 days after plating. The *purO* formation rate was calculated using Equations 1 and 2. The low formation rate of *purO* mutations precluded measuring their formation by nonselective visual screens.

Identification of stem-loop deletions by PCR amplification

Primers TP2569 (5′-TTCCAGCTGGCGCAGAGCAG-3′) and TP2570 (5′-TGGTGATCGCCGCGGGCGGT-3′) were used to determine deletions of the stem loop from the MudJ insert (Quiñones-Soto and Roth 2011). In parental cells lacking this deletion, the amplified fragment was 300 bp. Cells carrying the mDEL1 deletion gave a 200-bp PCR fragment.

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, Cm^R 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 tetracycline 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–
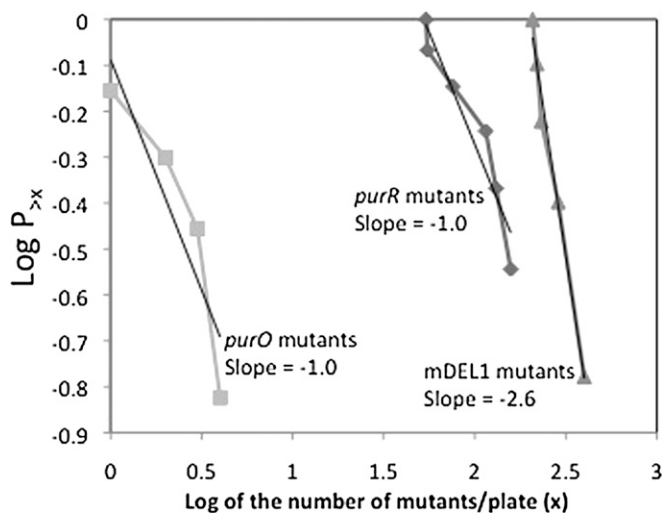
Figure 3 Fluctuation tests for the three mutation types in the Pur-Lac system. The formation rates of the three mutation types (mDEL1, *purR*, and *purO*) were determined by Luria–Delbrück fluctuation tests in which independent cultures were grown nonselectively and then assayed for the frequency of a preexisting single mutation type. To measure the formation rate of *purR* mutations, 10^8 mDEL1 tester cells from 10 independent cultures were plated on lactose (adenine) medium and the frequency of Lac^+ revertants was scored on day 2. The slope (−1.0) suggests that the detected double mutants (*purR*, mDEL1) arose before plating. To measure formation of the *purO* mutation type, 10^9 mDEL1, R^+/R^+ cells from 50 independent cultures were plated on selection plates and Lac^+ colonies were counted on day 2. The slope (−1.0) suggests that double mutants (*purO*, mDEL1) arose before plating, but are rare (since we had to plate 10-fold more cells than used in a standard reversion experiment). To measure formation rate of the mDEL1 deletion type, 10^5 *purR* mutant cells were plated on selection plates and day 2 colonies were counted. The distribution of new mDEL1 mutants (slope −2.6) suggests a deviation from the classical Luria–Delbrück distribution (−1), probably reflecting occurrence of a larger fraction of these common mutations during day 1 on the selection plate (where some residual growth occurs). Mutants arising on the plate are expected to show Poisson distribution.

Delbrück distribution, this plot shows a line of slope −1 (Luria and Delbruck 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

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}	$(5.3 \times 10^{-4})^a$
				$(4.2 \times 10^{-4})^b$
	Tester with $purR$ mutation	Selected day 2 colonies from 10^8 plated cells	2.3×10^{-3}	$(1.9 \times 10^{-4})^a$
				$(1.6 \times 10^{-4})^b$
$purR$	Tester with mDEL1 deletion	Visual: dark blue colony on X-gal	2.7×10^{-5}	$(1.3 \times 10^{-6})^a$
	Tester with mDEL1 deletion	Selected day 2 colonies from 10^8 plated cells	8.0×10^{-7}	$(3.5 \times 10^{-8})^a$
				$(5.9 \times 10^{-8})^b$
$purO$	Tester with mDEL1 deletion and $purR^+$/$purR^+$	Selected day 2 colonies from 10^9 plated cells	9.0×10^{-10}	$(1.5 \times 10^{-10})^c$
				$(6.5 \times 10^{-10})^d$
$purR$, mDEL double	Standard tester (10^9 cells plated)	Selected day 2 colonies from 10^9 plated cells	6.70×10^{-9}	$(1.1 \times 10^{-9})^c$

[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).

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}/10^8 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, 10^8 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 10^5-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 3 Growth rates of the Pur-Lac tester strain and its derivative mutant strains in liquid and solid media

Strain	Growth on glycerol		Growth on lactose (hr/gen)	
	liquid medium (hr/gen)	Liquid medium	Solid medium (first 24 hr)	Solid medium (after 24 hr)
LT2 strain	1.2 (0.02)	NA	NA	NA
Pur-Lac tester	1.3 (0.05)	14.7 (12)	5.5 (0.6)	19.3 (1.1)
purR	1.5 (0.25)	4.8 (0.2)	2.5 (0.2)	5.3 (1.1)
purO	1.7 (0.4)	3.1 (0.8)	2.2 (0.03)	2.6 (0.01)
mDEL1	1.3 (0.01)	3.6 (0.2)	3.6 (0.6)	17.0 (2.8)
purR mDEL1	1.5 (0.07)	1.2 (0.1)	1.3 (0.02)	1.4 (0.01)
purO mDEL1	1.3 (0.04)	1.2 (0.07)	1.3 (0.01)	1.4 (0.0)

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

screening. Therefore, *purO* mutations were detected selectively in a tester strain carrying an mDEL1 deletion mutation 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.

The growth rates of the several mutant types on selective (lactose) medium

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 (10^8) 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 ($\sim 10^7$ 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 (10^5 mutants cells 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^{-4} 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.
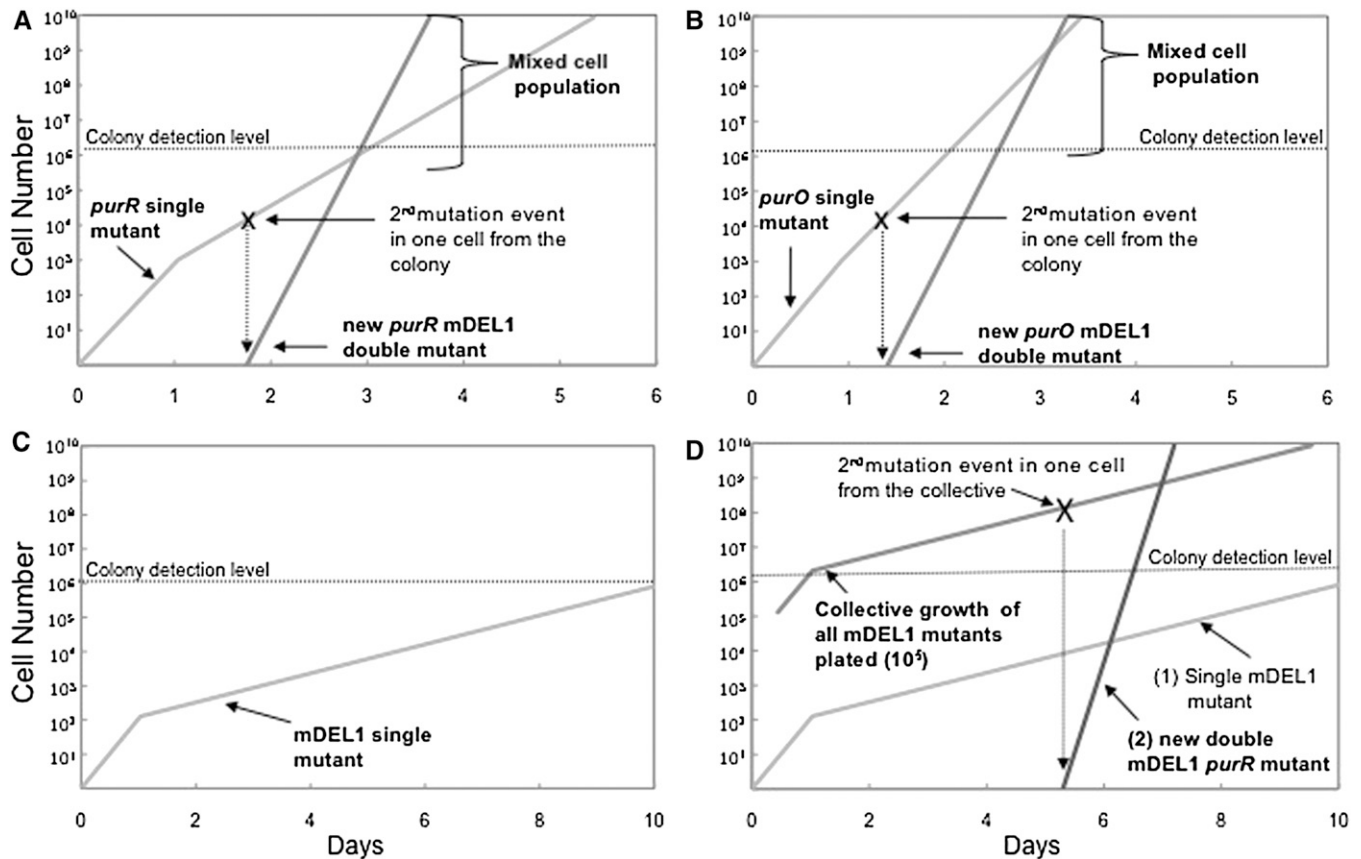
Figure 4 Development of single revertant colonies by various adaptation pathways: graphic tests of the selection model. Shown is the predicted development of a single Lac+ colony by the three main pathways of adaptation, using rates of mutation and growth described above. A colony becomes visible when the population reaches between 10^6 and 10^7 cells, shown by a dashed line (colony detection level). In the spreadsheet simulations (see File S1), independently determined mutation rates and growth rates are propagated with time to generate the predicted increase in cell number within one colony. (A) A *purR* single mutant initiates a colony that grows at a rate of 2.5 hr/gen for the first 24 hr and then shifts to a slower rate of 4.5 hr/gen. As the colony population approaches 10^4, the formation of an mDEL1 mutation occurs (2.5×10^{-4} mutations per cell per gen). The newly formed *purR* mDEL1 double mutant then grows under selection at 1.3 hr/gen and soon dominates the colony population. (B) A *purO* single-mutant cell starts a colony that grows at a rate of 2.2 hr/gen for the first 24 hr and then shifts to a slower rate of 2.6 hr/generation when contaminating nutrients are exhausted. While very few of these colonies are expected, they can quickly acquire a frequent secondary mDEL1 mutation. The *purO*, mDEL1 double mutant grows at a doubling time of 1.3 hr/gen and overtakes the colony. (C) A single mDEL1 mutant starts slowly at a rate of 3.5 hr/gen during the first day of the reversion experiment. After 24 hr, the doubling time slows to a rate of 17.0 hr/gen. An mDEL1 mutant cell needs to acquire a second mutation (either a *purR* null or a *purO*) to become fully Lac+, but on the timescale of this experiment, it never reaches a population size sufficient to allow independent acquisition of the second mutation event (the *purR* mutant forms at a rate of 3.3×10^{-8} mutations per cell per gen and the *purO* mutant forms at 1.2×10^{-10} mutations per cell per gen). (D) When all of the 10^5 plated mDEL1 mutant cells are considered together, it is possible for a secondary *purR* to appear in one of the many clones.

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 ~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^{-8} 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 ~40 kb. Duplications of the regions between *rrn* genes form at a very high rate of ~10^{-3} per cell per generation (Anderson and Roth 1981) and rapidly come to a steady-state frequency of ~1% in an unselected population (Reams *et al.* 2010). Thus 1000 of the 10^5 mDEL1 mutations are expected to be in cells with a *purD-lac* duplication at the time they are plated. Additional amplification steps occur at ~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 nonselective 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 (10^5) 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

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, 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 mutation. 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. Bergthorsson, 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 indirect 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 Salmonella 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 enterica rapidly approaches steady state with or without recombination. 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 mutation controversy. Annu. Rev. Microbiol. 60: 477–501.

Schmieger, H., 1972 Phage *P22*-mutants with increased or decreased 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 containing 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. Kofoid, D. I. Andersson *et al.*, 2003 Adaptive mutation: general mutagenesis is not a programmed 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 Salmonella typhimurium phenotypic of purR super-repression. Mutat. Res. 595: 107–116.

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Appendix
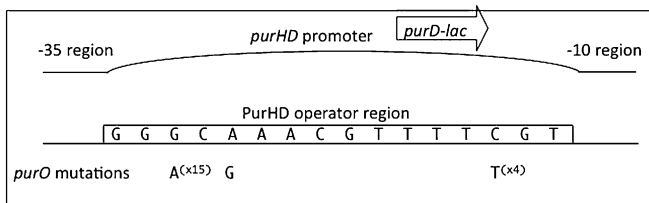
Figure A1 Spontaneous operator (*purO*) mutations. Twenty independent Lac+ mutants appearing in an mDEL1 parent on day 2 were assumed to carry *purO* mutation based on linkage of their Lac+ phenotype to the *purHD* operon. These mutations were shown by fluctuation tests to have arisen during nonselective 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).

GENETICS

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

File S1

Supporting Information

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 3.3×10^{-8}) or a new *purO* mutation (formation rate of 1.2×10^{-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.

 S. Quiñones-Soto, A. B. Reams, and J. R. Roth

Supporting Information

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