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Features of *Escherichia coli* mutant colonies appearing on nonlethal conditions of selection

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Abstract

Spontaneous mutagenesis in bacteria is understood as the product of genome-wide mutations occurring in the absence of selection during active cell proliferation in which mutant cells survive lethal selection while non-mutants cells die. However, when non-lethal selection is used to identify mutants, cells that have not yet mutated are still alive but unable to actively proliferate. This report addresses whether the first mutant colonies appearing on non-lethal selection plates are the product of spontaneous mutations occurring during exponential growth or whether they are produced by mutagenesis during selection. We infer, based on evidence obtained by using two unique methods, that most of these colonies are the product of mutation events happening soon after saturated bacterial cultures are deposited on plates. This mutability takes place irrespective of the selective allele location, whether chromosomal or extrachromosomal. This result demonstrates the fallibility of fluctuation analysis under non-lethal conditions of selection because this method assumes that the first mutants on a selection plate have occurred during exponential growth. A process of mutagenesis, such as the one observed here, occurring during starvation and in the absence of cell division, provides insights into how bacteria may quickly adapt to an ever-changing environment, and rapidly evolve new traits, such as antibiotic resistance. Furthermore, this is a demonstration of the strength of mutagenic processes, perhaps evolutionary conserved, that occur in the absence of active cell proliferation that might partly explain the acquisition of chemotherapy resistance in transformed cells.

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Introduction

It has been established that mutations occur without selection during the growth of bacterial cultures, and that the mutants grow as clones within such cultures. Selective conditions allow the identification of these pre-existing mutants (Luria and Delbruck, 1943). It is also known that if a single clonal mutation is responsible for all mutants, the approximate timing of a given mutational event can be estimated. This is based on the number of mutant colonies on a selection plate. For example, a large number of mutants is likely the product of sibling multiplication and suggests that the original mutation occurred in a single event that happened quite early in the growth of the culture (Hayes, 1968). These observations were made approximately 70 years ago (Cavalli-Sforza, 1950; Lederberg, 1952; Luria and Delbruck, 1943; Novick and Szilard, 1951), under conditions in which cells lacking the selected mutation were killed by the selective agent, i.e. lethal selection. Measurements of mutation rates under these conditions sometimes made use of the fluctuation test (Foster, 1994, 1999, 2006; Hall et al., 2009; Stewart, 1994; Stewart et al., 1990). In this assay, a small inoculum of bacteria is introduced into independent parallel liquid cultures that are grown without selection and then plated on selective medium for the mutant phenotype. The mutant colonies are counted, resulting in a distribution of colony numbers on the plates where the probability of having no mutants is large, a few plates yield a variable number of mutant colonies while occasional plates show a large number of mutants (so-called "jackpots"; Fig S1). Because mutations occur without selection, it is likely that the timing during growth of the original mutational event is random since mutagenesis is independent of environmental signals (Luria and Delbruck, 1943). Thus, the variation in the number of mutant colonies on selective plates derived from independent parallel cultures strengthens the idea of mutation randomness. Basic statistical analyses of such samples generate an extremely large variance, a term that measures sample spread (Sokal and Rohlf, 1995). Such large variances are reflected in "jackpots" (Fig S1), which greatly deviate in magnitude from the mean. Since most of the selective plates have none or few mutant colonies, the mean tends to be of small magnitude. Such mutant colony distribution does not fit a typical Poisson distribution in which the mean and the variance are similar in magnitude (Sokal and Rohlf, 1995). Thus, a fluctuation analysis of mutants from a large number of independent cultures (usually >50) (Foster, 2006) that follows the findings of Luria and Delbruck (Fig S1) (Luria and Delbruck, 1943) typically displays two parameters: a large variance (due to "jackpots") and a majority of cultures with none or few mutants. Because there is still no mathematical description for this type of distribution, a large variance and cultures with none or few mutants define the distribution. The work of Lea and Coulson (Lea and Coulson, 1949) provided an experimental handle on the fluctuation test that is widely used to estimate mutation rates (mutations/cell/generation) in bacterial cultures and other organisms (Boesen et al., 1994; Gos et al., 2000; Watanabe et al., 2001).

Two types of selection are used experimentally to identify mutants: lethal and non-lethal selection (Fig S2). In lethal selection (Fig S2 A) non-mutant cells die and only mutant cells survive. Classic examples of this kind of selection are the isolation of Escherichia coli mutants that are phage resistant (Fig S1 B) (Luria and Delbruck, 1943) or rifampicin resistant (Rif^R) (Miller, 1974).

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Conversely, under non-lethal conditions of selection, all cells survive (Fig S2 B), but only mutant cells (those that have mutations in gene products that permit growth under the selective conditions) proliferate and form colonies. Thus, under non-lethal conditions of selection, bacteria that can not grow (i.e. have not mutated yet or have mutated somewhere else on the genome) are still alive (Cairns and Foster, 1991; Foster and Cairns, 1992, 1998; Godoy et al., 2000; Slechta et al., 2003).

An example of non-lethal selection is when selection is imposed by a carbon source (e.g. lactose) that bacteria are unable to use for growth, and the gene product(s) in question are present but inactivated on the genome. Thus, bacteria that are unable to use lactose (Lac-) are plated on minimal medium with lactose as the sole carbon source. Such cells are Lac- because β-galactosidase, the enzyme responsible for the cleavage of lactose into glucose and galactose, is inactive due to mutations in the enzyme active site (Cupples and Miller, 1989), elsewhere in the enzyme, or in regulatory gene products (Andersson et al.; Quinones-Soto and Roth, 2011). Gain of function mutations in the lacZ gene (encoding β-galactosidase), in the regulatory regions or in the regulatory gene products (Quinones-Soto and Roth, 2011) will enable cells to become Lac$^+$, i.e. to metabolize lactose as sole carbon source, and form colonies on minimal lactose medium.

In one of these assays, cells lacking the *lac* genes on the chromosome and bearing an episomal (low copy number extrachromosomal element) lac- allele (lacI33) consisting of an out of frame *lacZ* gene fusion (Cairns and Foster, 1991) are grown in glucose/glycerol minimal medium and then deposited on plates with lactose as sole carbon source. Lac$^+$ colonies (or revertants) start to appear on day 2 and accumulate with time (Cairns and Foster, 1991). These Lac+ colonies usually have gain of function -1 frameshift mutations restoring the reading frame of the *lacZ* gene (Foster and Trimarchi, 1994; Rosenberg et al., 1994). It has been shown that the Lac- population remains static, i.e. they neither divide nor die during the duration of the experiment (Cairns and Foster, 1991). These conditions of selection permit, in addition to mutations to Lac$^+$, the accumulation of non-selected mutants including antibiotic resistant (Godoy et al., 2000; Perez-Capilla et al., 2005; Petrosino et al., 2009; Torkelson et al., 1997). Thus, it is clear that mutations occur and accumulate with time during non-lethal selection even in the absence of cell division (Quinones-Soto and Roth, 2011). This is contrary to what would be seen if mutagenesis could occur only during cell proliferation. The early-appearing mutants are thought to be the product of DNA replication errors occurring during growth in exponential phase (Rosenberg et al., 1994). In contrast, late-appearing mutants are attributed to gene amplification, the activity of error prone DNA polymerases, or both (Andersson et al.; Bull et al., 2001; Foster, 1994; Foster and Trimarchi, 1994; Galhardo et al., 2009; Galitski and Roth, 1995, 1996; Harris et al., 1997; Harris et al., 1999; Layton and Foster, 2003; Radicella et al., 1995; Rosenberg et al., 1998; Slechta et al., 2002a; Tompkins et al., 2003).

Other lac- alleles under similar conditions of selection behave differently, in that new Lac$^+$ colonies either do not accumulate with time or do so quite poorly (Cupples and Miller, 1989; Godoy et al., 2000) for as yet unknown reasons. Moreover, the Lac$^+$ colonies appearing at day 2 (i.e. at least 35 h of incubation at 37ºC) are presumed to be the result of mutational events happening during the growth of the culture (before selection) as classically seen in fluctuation tests.

Indeed, many investigators assume that, irrespective of the conditions of selection, the first mutant colonies to appear on plates after the imposition of selection, are the result of mutations that have arisen prior to selection during the growth of the culture [(Foster, 2006) and references therein]. The strength of this assumption is under investigation in the work described here.

The mutational target that we investigate consists of a *lacZ* gene (CC104) with a single base pair substitution in a codon that is essential for ß-galactosidase activity (Cupples and Miller, 1989), i.e. it encodes a catalytically disabled ß-galactosidase. CC104 cells can become Lac$^+$ only by a gain of function mutation in which a C to A transversion converts a G**C**G to a G**A**G codon. Amplification of this gene can not directly result in Lac$^+$ because the encoded ß-galactosidase is catalytically inactive. In the absence of a mutagen, the mutation frequency of the episomal CC104 lac- allele from Lac- to Lac$^+$ is ~1x10-^{8}, which is high enough to permit detection of Lac$^+$ (Cupples and Miller, 1989). We were intrigued by CC104 because it has a high frequency of reversion to Lac$^+$ considering that only a single base pair substitution (from a G**C**G to a G**A**G) would render Lac$^+$. To put this in perspective, selection for Rifampicin resistant mutants (Rif^R) in the absence of a mutagen, have a similar frequency (~1x10-^{8}), but many different base pair substitutions give rise to the Rif^R phenotype (Jin and Gross, 1988). Furthermore, cells bearing the CC104 *lac*- allele accumulate Lac$^+$ poorly with time (Godoy et al., 2000). These data suggested to us that CC104 is likely to be different to other *lac*- alleles previously studied (Andersson et al., 1998) (Cupples and Miller, 1989).

We devised two approaches to determine whether the observed number of CC104 Lac$^+$ obtained after at least 35 h of incubation at 37ºC on lactose selective medium were the product of mutational events happening during the growth of the culture. These unique approaches rely on a culture's observed frequency of Lac$^+$ and predict the number of Lac$^+$ that should be obtained upon re-growth in either non-selective (end-point dilution, Fig S3) or selective medium (respreading, Fig S4). The experiments show that only a small fraction of the Lac+ mutants had originated during the growth of the culture. Thus, we infer that the CC104 Lac$^+$ mutants appearing between 35-48 h incubation at 37ºC on lactose selective medium are, for the most part, not the product of mutational events that occur during exponential growth of the culture, but rather arise while the cells are experiencing the imposed non-lethal selection. Notably, we have evidence that this mutability happens irrespective of the lac allele location. Any mutational event happening after the threshold of time required for us to detect a Lac$^+$ colony (i.e. after 48 h) is not part of this investigation. The presented findings are important

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for practical purposes when studying mutagenesis under non-lethal conditions of selection by fluctuation analysis. Furthermore, the presented evidence suggests a process of mutagenesis occurring in stationary phase, without prolonged starvation, and thus different to adaptive mutagenesis (Andersson et al., 1998; Bull et al., 2001; Foster, 1994; Foster and Trimarchi, 1994; Galhardo et al., 2009; Galitski and Roth, 1995, 1996; Harris et al., 1997; Harris et al., 1999; Layton and Foster, 2003; Radicella et al., 1995; Rosenberg et al., 1998; Slechta et al., 2002a; Tompkins et al., 2003). This mechanism provides important insights into the ability of bacteria to survive many environmental challenges, and quickly evolve new traits, one of which might be antibiotic resistance.

Materials and Methods

Bacterial strains and reagents. The strain CC104 has been previously described (Cupples and Miller, 1989). CC104 carries a derivative of the F'128 episome carrying the CC104 lac- allele. The same allele was transferred by P1 transduction to the chromosome of the strain M182 (M.S. Fox collection), which is isogenic with CC104 (*ara* Δ(*gpt-lac*)*thi-1*), except that the deletion in the M182 strain encompasses only the lac operon.

M9 was used as the defined medium (Miller, 1974) with either glucose (0.1%) or lactose (0.1%) as carbon source. Rifampicin (Rif, Sigma) was used at the final concentration of 100 µg/mL.

Determination of Lac$^+$ mutation frequencies. Cultures of strain CC104 (Cupples and Miller, 1989), with the extrachromosomal episomal CC104lac- allele, and those of the strain M182 with the same lac- allele on the chromosome were grown to saturation in 0.1% glucose M9 minimal medium (Miller, 1974) at 37ºC. These cultures routinely give rise to approximately 1x10^8 to 1x10^9 cells/mL (Radicella et al., 1995) after incubation at 37ºC with shaking for at least 20 h. The saturated cultures were diluted a million-fold and used to inoculate independent liquid cultures (3-20 mL) with a total of ~ 1x10^4 cells. This inoculum size was chosen based on the estimated spontaneous mutation frequency of the CC104 lac- allele in the presence and absence of mutagens (~1-2.4 x10-^{8}, (Cupples and Miller, 1989)) and ensures a low probability of cultures with preexisting Lac$^+$ mutants (Luria and Delbruck, 1943). Once the parallel independent liquid cultures reached saturation (usually at 24 h), a sample was taken to determine colony-forming units (CFUs) and 1 mL was used to measure the frequency of Lac- to Lac$^+$ mutations as described previously (Godoy et al., 2000).

End-point dilution experiments. See diagram in Fig S3. Lac$^+$ mutation frequencies were determined for a number of (>50) parallel independent liquid cultures. These independent cultures were diluted (1:30 or 1:100) into 30-100 one-mL volume cultures in minimal medium with 0.1% glucose. These were incubated until saturation. The average CFUs of the diluted and regrown bacterial cultures was determined by sampling at least 10 one-mL cultures by taking a small aliquot (20-50µL) to serially dilute and estimate CFUs. The remaining volume of each culture was mixed with 3 mL of minimal medium lacking carbon source (0.5% agar), and poured onto the surface of minimal medium plates with 0.2% lactose, as previously described (Godoy et al., 2000). Lac$^+$ colonies were counted between 35-48 h (but not more than 48h) incubation at 37ºC. The same procedure was carried out for the end-point dilution experiment where the antibiotic rifampicin (100 µg/mL), instead of defined lactose medium, was used as selective agent in rich LB medium (Miller, 1974), except that plates were incubated for at least 20 h at 37ºC.

Respreading experiments. The independent parallel cultures were inoculated as described above and grown in 20-30 mL of defined M9 0.1% glucose medium. After 24 h incubation at 37ºC, cultures were concentrated ten times in 1x M9 salts and 100 µL of the concentrate (~1x10^9 bacteria) used to spread on replicate plates (~10-15) containing M9 defined medium with 0.2% lactose as sole carbon source. At various times (see figure legends) sets of the replicate plates were taken out of the incubator and the plate surface was re-spread each with a new glass spreader, followed by further incubation of the plates for at least 35 h at 37ºC (but no more than 48 h; see diagram in Fig S4). In the reconstitution experiment 10-20 Lac$^+$ cells were added per 1x10^9 cells before the suspension was concentrated. In the experiments where only Lac$^+$ cells were used, the cultures were appropriately diluted and spread on lactose-containing minimal medium and re-spread at the times indicated in the corresponding figure legend.

Determination of cell proliferation on non-lethal selective plates. This was carried out as described by Cairns and Foster (Cairns and Foster, 1991) and others (Galitski and Roth, 1996; Godoy et al., 2000; Petrosino et al., 2009).

Statistical analyses. Statistical analyses were performed using the SPSS software (spss.com, IBM). Non-parametric tests were chosen for sample analyses since these tests do not require samples to be normally distributed. The different tests and their significance are explained in the text. The variance was estimated using the function that shows that the square root of the variance is equal to the standard deviation of the mean (Luria and Delbruck, 1943). A Poisson distribution was assumed whenever the variance was similar in magnitude to the mean.

Results

Measurement of the frequency of Lac$^+$ mutants of the lac- allele located either on the episome or the chromosome. Frequencies of mutation are used throughout this report because we can measure these directly for each independent culture and are thus operationally easier to handle. The mutation that must occur for CC104 *lac*- cells to revert to Lac$^+$ is a change from G:C to T:A in a codon encoding an active site glutamic acid residue essential for ß-galactosidase activity (Cupples and Miller, 1989). Second site suppressors are plausible, but unlikely in this assay because usually multiple mutations (Hall, 1991, 1998; Parker and Hall, 1988) are required to supply a ß-glucosidase activity to break down lactose into glucose and galactose, which will in turn provide a carbon source for bacteria to proliferate. Lac$^+$ mutants (revertants) appearing between 35-48 h of incubation on selective lactose minimal medium at

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37ºC are the source of our data. The mutation frequency from Lac- to Lac$^+$ of 85 independent cultures of the strain bearing the CC104 lac- allele on an episome was first determined. This is approximately 1x10-^{8}. The distribution of Lac$^+$ mutant colonies resulting from plating 85 parallel independent CC104 cultures is shown in Fig 1 A. One of the first intriguing observations when counting CC104 Lac$^+$ revertants appearing on minimal lactose plates is that they fail to follow a classic Luria and Delbruck distribution (Luria and Delbruck, 1943). They are distributed evenly around a given interval, closely resembling a Poisson distribution (Fig 1 A). However, unlike a typical Poisson distribution, where the mean is similar in magnitude to the variance, here there is one culture that contained 110 Lac$^+$ revertants (a jackpot). Therefore, it appears that these Lac$^+$ mutants have a distinct distribution that conforms to neither a typical Poisson nor a Luria and Delbruck distribution.

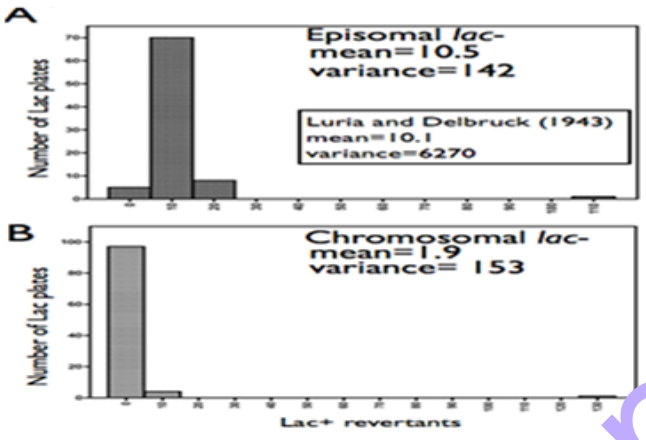

Figure 1. Lac$^+$ mutants derived from a non-leaky *lac* allele located either the episome or the chromosome are mostly distributed around the mean. A. At odds with a Luria and Delbruck typical random distribution (1943), Lac$^+$ mutants originated from reversion of the episomal CC104 lac- allele are mostly distributed around the mean, except for 1 culture with a "jackpot". The box inside the graph is shown as a comparison with data from experiment 22, Luria and Delbruck (1943). 85 parallel independent cultures were grown to saturation and 1x10^9 cells were deposited on lactose minimal medium selective plates. Lac$^+$ colonies were counted between 35-48 h of incubation at 37ºC and the distribution plotted. B. The distribution of Lac$^+$ colonies arising from the chromosomal non-leaky *lac*- allele is also mostly distributed around the mean following a similar pattern to that of the same allele on an episome. 102 parallel independent cultures of the strain harboring the chromosomal *lac*- allele were grown, plated and Lac$^+$ colonies counted and plotted as described in A.

In the case of the CC104 lac- allele present on the chromosome, we find fewer Lac$^+$ colonies compared to the same allele present in the episome (Fig 1 B). Here, we also identified a jackpot of 130 Lac$^+$ colonies. The number of CC104 Lac$^+$ revertants, except for the jackpot, is approximately 1/10 of that found in strains bearing the same allele on an episome. This is consistent with a previous report, in which the number of Lac+ revertants, the product of a frameshift mutation, was directly compared for the same allele located either on an episome or the chromosome (Godoy et al., 2000). The chromosomal CC104 lac- allele becomes Lac$^+$ at a frequency of 1.5x10-^{9} (102 independent cultures analyzed). Notably, revertants of the chromosomal CC104 lac- allele have a similar distribution pattern compared to episomal revertants (Fig 1B). Again, we observed neither a typical Poisson nor a Luria and Delbruck distribution for these mutants.

A Poisson distribution would be expected if a constant number of mutants arose over time (the magnitude of the mean and the variance being similar), and a Luria and Delbruck distribution would be expected had the mutants arisen exclusively during the growth of the culture. As a means of comparison, we provide the mean and the variance of one of the experiments described by Luria and Delbruck (Luria and Delbruck, 1943); clearly much larger than the ones we describe here (Fig 1). Our findings suggest two possibilities. First, that some of the chromosomal Lac$^+$ mutants might be the product of mutational events that occurred after, not during growth; or second, that these mutants did arise during growth but grow slower than Lac- cells. We decided to test these possibilities.

End-point dilution experiment. In this set of experiments we assumed that the CC104 Lac$^+$ revertants appearing on the non-lethal lactose selection plates had originated during the growth of the independent cultures, and thus we devised a strategy consisting of an end-point dilution experiment based on mutation frequencies (diagram in Fig S3). In these experiments, to determine the frequency of mutation to Lac$^+$ for a given culture, independent cultures of CC104 are deposited on at least 10 lactose minimal medium replicate plates. The Lac$^+$ colonies appearing on these plates are counted and divided by the number of viable counts for each culture, which is also determined by plating appropriate dilutions on rich LB medium. Independent cultures are then diluted into a number of one-mL cultures and regrown for 20 h in liquid glucose minimal medium. Each of these one-mL cultures are, upon saturation, deposited on lactose selective plates and incubated as indicated in Materials and Methods. We expect that a fraction of the one-mL cultures would contain a predicted number of Lac$^+$ founders (or pre-existing mutants) based on the frequency of mutation to Lac$^+$ of a given culture. These founders come from the original parallel independent culture. For example, if the Lac$^+$ mutation frequency for a given culture were 10 Lac$^+$ mutants/10^9 cells (i.e. 1x10-^{8}), dilution of this culture 1:100 into 100 one-mL cultures should result in 10 one-mL cultures (10 Lac$^+$/100=0.1 or 10:100) containing 1 founder (i.e. a Lac$^+$ mutant), which upon growth for approximately 6-7 doublings, would render approximately 100 Lac$^+$ colonies upon plating under lactose selective conditions. The remaining one-mL cultures should

Table I End point dilution method detects Rif^R mutants occurring during growth

Original Cultures	Ct 1	Ct 2	
Dilution	1/50	1/50	
Frequency of Rif^R original culture	14 ± 3	20 ± 6	
Number of one-mL cultures	50	68	
*Expected **founders	15	27	
Observed founders	12	21	
&Observed:Expected	12:15	21:27	#33:42

*Expected= Frequency x Number of one-mL cultures x Dilution factor
** A"founder" plate with >70 Rif^R colonies; a pre-existing mutant.
& Number of observed one-mL cultures with Rif^R founders : Number of one-mL tubes expected to have Rif^R founders
# Total number of one-mL tubes expected to have Rif^R founders : Number of observed one-mL cultures with Rif^R founders

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contain no founders and lead to an average of 10 Lac+/plate (i.e. the number of Lac+ mutants determined experimentally for a given independent culture). To assess whether the end-point dilution method is sufficiently robust to detect growth-dependent mutants, an experiment was carried out with two parallel independent cultures of CC104 selecting for Rif resistance (100 µg/mL) in rich LB medium (Table 1). We easily detected 79% of the predicted founders (Table 1, bottom row), suggesting that preexisting Rif^R mutants are readily recovered with the end-point dilution method.

Table 2 End point dilution method suggest that most Lac+ mutants occur during selection

Original Cultures	Ct1	Ct1	Ct2	Ct2	Ct3	Ct4	Ct5	Ct6	Ct7	Ct8	Ct9	
Dilution	1/100	1/30	1/100	1/30	1/50	1/50	1/50	1/50	1/50	1/50	1/50	
Frequency of Lac+ original culture	11±2	11	22±3	22	7±3	6±3	6±3	7±3	10±3	9±2	11±4	
Number of one-mL cultures	92	30	102	27	72	72	72	60	60	60	60	
*Expected **founders	10	11	22	20	10	9	9	8	12	11	13	
$Adjusted Expected	7-8	8-9	17-18	15-16	7-8	7-8	7-8	6-7	9-10	8-9	10-11	
Observed founders	1	1	2	None	None	2	None	2, 4	3, 4	1	12	
&Observed:Expected	1:10	1:11	2:22	0:20	0:10	2:9	0:9	2:8	3:12	1:11	1:13	*12:135
								4:8	4:12		2:13	**16:135

* Expected= Frequency x Number of one-mL cultures x Dilution factor
** A "founder" plate with >70 Lac+ colonies.
$ Adjusted Expected is calculated based on the yield of growth-derived mutants in the experiment shown in Table 1 (79%).
& Number of observed one-mL cultures with Lac+ founders : Number of one-mL tubes expected to have Lac+ founders
* Total number of one-mL tubes expected to have Lac+ founders : Number of observed one-mL cultures with Lac+ founders
** This ratio is as in *, but "founder" plates are defined as those with >50 Lac+ colonies.

Then the experiment was attempted for the CC104 lac-allele located on the chromosome (Table S1). However given that the reversion to Lac+ is approximately 10-fold lower in this configuration of the mutant allele (Fig 1), the dilution method proved impractical. Even an experiment in which we used 10 independent cultures diluted into 100 one-mL cultures each (data not shown) showed no statistically meaningful difference between one-mL cultures with and without founders. The experiment was then repeated for nine independent cultures of the strain with the episomal CC104 *lac* allele varying the dilutions for some of the cultures. In Table 2, we compared the number of observed founders with the number of expected founders. In every case, the number of observed founders is dramatically less than expected. These two numbers differ because they depend upon the number of diluted cultures chosen to represent a yield of Lac+ colonies that were the product of founder multiplication. Either determination shows that approximately 90% of the Lac+ colonies appearing upon plating on non-lethal selective lactose medium are not the product of mutational events occurring during growth. This strongly suggests that only approximately 10% of the Lac+ mutants were present in the CC104 culture before plating. Because the control experiment for Rif^R mutants detected 79% of the growth-derived mutants (assuming an error of approximately 20%), an adjusted expect of founders was calculated in the experiments shown in Table 2. This is 79% of the theoretical expected value. The result of that calculation shows that in general between 80-90% of the Lac+ colonies observed originally are most likely the result of mutagenesis on the non-lethal selective conditions.

To assess the robustness of the end-point dilution method in an independent fashion we inoculated sixty parallel independent cultures as indicated in Materials and Methods and selected for Lac+ mutants. We found two "jackpot" cultures with 41 Lac+ colonies in one case and 43 in the other. "Jackpots" refer to cultures in which a mutational event happens early during the growth of the culture resulting in the clonal expansion of this early mutational event giving rise to a higher number of mutants compared to most cultures (Hayes, 1968). Therefore, since the Lac+ colonies in these two cultures are the product of growth-derived mutational events one would predict that using the endpoint dilution approach, a 1/50 dilution of these jackpot cultures into 50 one-mL cultures (42 Lac+/50 = 0.84) would result in approximately one Lac+ per diluted one-mL culture. Thus, 1/50 dilution and re-growth in a **single** one-mL culture (rather than in 50) should suffice for easy detection of such growth-derived Lac+ mutants. Plating of the contents of such one-mL culture should yield between 100-200 Lac+ colonies after incubation. This is indeed the result that we obtained with these two cultures (150 and 220 Lac+ respectively). Therefore, we have shown that for non-lethal selection, if the mutations occur during growth, the end-dilution method will be able to detect it.

We then experimentally determined whether there exist alternative explanations for the results obtained with the end-point dilution method. Lac+ revertants originating from the episomal lac- allele are neither slow growing nor the product of last generation of growth. One model that would account for the results without requiring mutagenesis of static bacteria on non-lethal selective plates is slower growth by the Lac+ bacteria. If proliferation of Lac+ bacteria were somehow slower in the context of a larger Lac- population the end-point dilution experiment would yield the observed results. To test this model, Lac- and Lac+ bacteria were mixed at different rations in minimal glucose medium and grown to saturation. If Lac+ were growing slower, we expected that the ratio of Lac+ to Lac- would not be constant. CFUs were measured at the beginning of the experiment, every hour for 6 h, and at 24 h for all ratios of Lac-/Lac+ used. We show in Fig 2 representative experiments in

Table 3 Lac+ mutants are not the product of mutation events happening during the last generation of growth

Total number of Lac+ colonies on selective plates

Independent Culture*	T=0	T=1	T=2	T=3
A	42	51	39	36
B	66	35	46	28
C	64	32	38	25
D	51	45	64	37
E	44	29	53	27
F	40	37	30	30
G	61	79	31	28
H	11	36	29	30
I	53	62	25	32
J	16	35	24	24
K	26	50	26	24
L	47	36	18	34
Totals	521	527	423	355
CFUs/mL	7×10^8	8.5×10^8	8×10^8	8.2×10^8

* Total cells from each culture were plated in 5 replicate plates. Totals numbers of Lac+ from the 5 plates are shown.

which the ratios of Lac+ to Lac- remain constant indicating that there is no growth rate difference for Lac+ compared to Lac-in these experiments. This leads us to favor the hypothesis that many of the Lac+ revertants are arising on the non-lethal lactose plates.

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We have determined that Lac+ mutants grow as well as the parental Lac- in minimal glucose medium, therefore it is expected that adding a small number of Lac+ cells (10-20) to parallel independent Lac- cultures, and carrying out the end-point dilution experiment, as described above, would result in the expected number of diluted cultures with a founder (a yield of >70 Lac+ colonies after incubation). We performed this experiment and obtained the expected result (data not shown). Since Lac+ have no growth disadvantage *versus* Lac- bacteria, the simplest explanation of the end-point dilution experiment is that the mutation to Lac+ did not occur during the growth of the culture. An alternative hypothesis is that mutagenesis occurs not in static cells on the plate, but in growing cells during the last generation of growth. This requires hypothesizing a highly mutagenic "last generation". If this were the case, then giving cells the opportunity to experience this "last generation" again and again would amplify mutagenesis. We therefore, recreated the "last generation" conditions by diluting saturated cultures in minimal glucose liquid medium 1:4, expecting that further regrowth might amplify the number of revertants per total CFUs. Repetitions of the cycle (i.e. repeated 1:4 dilutions and regrowth) should amplify the number of revertants each time, while the number of viable bacteria in the culture would remain constant. Thus, after three cycles the number of Lac+ revertants should be larger than the appraisal done before starting the cycling. The results in Table 3 show instead that the number of Lac+ colonies per CFUs is constant throughout the repetitions of dilution and growth. Furthermore, since it has already been shown that Lac+ mutants have no growth disadvantage under these conditions the results suggest that Lac+ revertants could not be the result of mutations taking place during the last generation of growth in liquid medium. Lac+ revertants appearing on lactose selective plates two days after plating, we propose, are the product of mutational events occurring while bacteria are on the non-lethal selective plate. If, however, the Lac- indicator cells were able to divide enough times on the selective plates during the first 12 h after plating, the Lac+ revertants will not strictly be the result of post-plating events. To address this possibility, we used the approach used by Foster (Foster, 1994) and measured CFUs from agar plugs of a known diameter taken from the selective plates. We found no significant increase or decrease in the number of viable bacteria. Summing up our findings, the evidence supports the concept that most (roughly 80-90%) of the Lac+ mutants that are derived from the episomal lac- allele have not arisen during growth. Our findings are all consistent with the model that mutagenesis occurred during non-lethal lactose selection. Chromosomal mutational events of Lac- to Lac+. As shown above, the reversion of the CC104 lac- allele located in the chromosome occurs at a lower frequency than transversion of the same allele on an episome, but displays a similar distribution pattern (Fig 1B). We were unable to use the end-point dilution method (Fig S3; Table S1) to characterize the mutagenesis of the chromosomal CC104 *lac*- allele because the numbers of colonies obtained are of small magnitude and thus are intrinsically less robust.

Therefore, to analyze the reversion of the chromosomal CC104 *lac*- allele, we took advantage of the fact that a bacterial colony is the product of a single bacterium, and use a re-plating assay to amplify the signal (i.e. Lac+ cells). In this experiment replicate minimal lactose plates are spread with approximately $1x10^9$ cells from a single saturated culture (i.e. with the same mutational history). After at least 35 h of incubation (but no more than 48 h) full-sized colonies are observed with the naked eye in these plates. Further spreading (or respreading) of these plates' surface earlier, after a time interval that would permit formation of a microcolony invisible to the naked eye, would result in the physical separation of the cells making up microcolonies at the time of respreading. Incubation of the re-spread plates as indicated in Materials and Methods results in Lac+ colony counts that corresponds to the number of cells making up the original Lac+ microcolony. If mutations to Lac+ were happening during the growth of the culture, rather than during non-lethal selection, and the chromosomal Lac+ reversion frequency is approximately 1.5 $x10^{-9}$ (i.e. between 1 and 2 Lac+ in ~ $1x10^9$ Lac- cells), spreading of ~$1x10^9$ total cells on a non-lethal lactose selective plate would result in depositing between 1-2 Lac+ bacteria in ~$1x10^9$ Lac- cells. These would start dividing after time while on the surface of the non-lethal lactose minimal medium plate, forming a microcolony of predictable CFUs that is invisible to the naked eye. Further spreading of the cells already present on such a plate after an appropriate interval of time (enough to permit cell division) would result in physical separation of the individual cells in the Lac+ microcolonies, which after further incubation would result in a larger count of Lac+ colonies. In contrast, if the mutagenesis is occurring during non-lethal selection instead of during growth, we predict that Lac+ microcolonies will not yet have formed; in this case, whether or not respreading is done, the same small number of Lac+ colonies will ultimately appear on the plates.

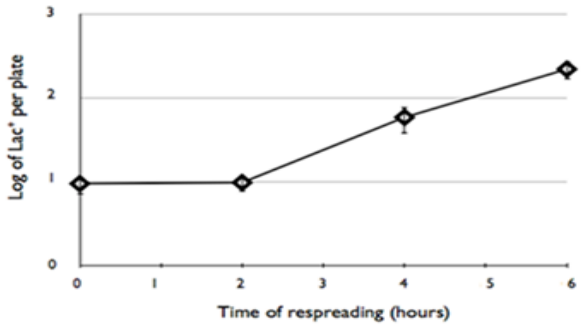

Figure 3. Respreading microcolonies of Lac+ bacteria on the surface of a lactose plates permits measurement of growth. Approximately 10 Lac- cells were spread on the surface of 20 lactose minimal medium plates, 5 of which were either not respread or respread at the times shown. The error bars shown represent the standard deviation of the mean. 20 Lac- cells were also plated in a similar set, where twice as many Lac- colonies were obtained at each time of respreading.

To test the principles of this experiment, we performed two experiments. In the first, we addressed both whether respreading is likely to physically separate individuals within a microcolony, and whether respreading could be used quantitatively to estimate growth of a microcolony on the surface of a plate. Approximately 10 to 20 Lac+ were spread on the surface of lactose minimal plates, and then respread at the times shown (Fig 3). Visible Lac+ colonies were scored without respreading and after respreading and further incubation (at

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least 35 h, but no more than 48 h). The data demonstrate that Lac+ cells, at this density, lag for approximately 2 hours before starting to divide, and then divide with a doubling time of ~40 minutes (Fig 3). Thus, respreading is a useful technique to separate microcolonies into individual cells and estimate growth on the surface of plates. We predict that the maximum resolution of the method is the separation of a microcolony composed of approximately 1500 cells, since respreading of a larger microcolony would result in an uncountable lawn of growth upon further incubation. Therefore, respreading should be performed during the time interval when the microcolonies are composed of approximately 250 to 1000 cells. Thus, assuming some lag time before Lac+ cells start to divide on the plate surface

Table 4

Time of respreading in hours	No respreading	10	24
Lac+ colonies	9	604	TMTC
	15	621	TMTC
	12	641	TMTC
	17	307	TMTC
	12	792	TMTC
Average number of Lac+ obtained per respreading time point	13±3.0	593±177	TMTC
Average number Lac+ expected per respreading time point	10	640[a]	TMTC

Table 4 Reconstruction experiment displays the expected number of Lac+ cells at different times of respreading. An estimated 10 Lac+ bacteria per 1x10⁹ Lac- cells were added to the culture, which was then spun down and concentrated 10 fold. 0.1 mL of the mixture, which is expected to have on average10 Lac+ per 1x10⁹ Lac- cells (i.e. per plate), were spread on 15 replicate lactose minimal plates and incubated at 37°C for at least 35 h. At the times shown 5 plate replicates were taken out of the incubator and respread to separate Lac+ microcolonies invisible to the naked eye. The plates were then further incubated as before to allow for Lac+ cells to develop a macrocolony visible to the naked eye. a, expected number of Lac+ with a lag time between 2-3 hours; TMTC, too many to count. The error shown is the standard deviation of the mean.

and ~ 1h per generation after the initial lag, respreading of the plates seeded with 1-2 Lac+ after, for example, 10-12 h of incubation, would result in physical separation of ~ 250-1000 cells (CFUs) and thus in a detectable number of Lac+ colonies after respreading and further incubation (to allow for visible colony counts, usually between 35- 48 h; diagram Fig S4).

In the second experiment, although we have previously shown that there is no growth disadvantage between the Lac+ and Lac- cells (see above Fig 2), we still needed to assess whether the assay is viable when the individual Lac+ cells are mixed with a much larger population of *lac*- cells. To test this, we added ~ 10 Lac+ per 1x10⁹ Lac- cells, and the mixture was spread on replicate lactose minimal medium plates. Sets of replicates were respread at 10 and 24 hours. Control plates that were not respread showed as expected between 9-17 Lac+ colonies, as seen in Table 4. Respreading 10 hours after plating resulted in a large number of Lac+ colonies, consistent with our expectation that respreading separates existing Lac+ microcolonies. The data are consistent with cells having a 2 h lag time and a doubling every hour.

We performed a series of non-parametric statistical analyses on the data that we obtained from respreading (Table 4). One of these assays measures whether there is relationship between samples (Sokal and Rohlf, 1995). Statistically, it is possible to determine whether a sample is dependent on another as would be expected for the Lac+ colonies appearing with no respreading compared to those with respreading (Table 4) because these plates are replicates of a single culture with a similar source of Lac+. The test calculates a coefficient of concordance; the closer this is to 1, the better the agreement between samples. The coefficient of concordance (Kendall's, (Sokal and Rohlf, 1995) that we calculated on the data obtained from Table 4 is 1, which indicates that the compared samples are dependent upon each other.

Thus, the respreading assay is likely to enable us to detect Lac+ mutants that are the product of reversion of the chromosomal lac- allele because their number is amplified. Furthermore, statistical analysis suggests that it is possible to assess whether the number of Lac+ revertants obtained with respreading is dependent, as expected, on the Lac+ cells with no respreading. The respreading experiment, shown in Table 5, was performed with a single culture with the chromosomal CC104 *lac*- allele. We observe on average 1 Lac+ mutant (with no respreading), consistent with the predicted frequency of Lac+ mutation of 1±0.5 x10-⁹. However, if the Lac+ had been pre-existing in the population that was originally spread on the plate (i.e. if reversion occurred during growth), respreading should have resulted in large numbers of colonies. In particular, as a result of the respreading at times greater than 16 hours, we expected to have too many colonies to count, i.e. a lawn of Lac+. Notably, that is not the case. In fact, the numbers of colonies resulting from respreading were dramatically less than would be predicted for Lac+ mutations arising during growth (see Table 4).

Moreover, we find that there is no correlation between the time of respreading and the number of Lac+ colonies produced by the respreading, which represents the number of Lac+ composing the microcolonies at the time of respreading. The observed data is inconsistent with a single culture experiencing the same mutational history, and in short, these data are what we might expect if plate replicates were coming from independent cultures. We have already shown the lack of variability observed as a result of seeding Lac+ in the culture (Table 4), though we could not have determined whether any new mutants had arisen during selection because of the large number of Lac+ colonies produced as result of respreading.

These data therefore support our hypothesis that reversion of the chromosomal CC104 *lac*- allele to Lac+, like reversion of the same *lac*- allele on the episome, is occurring during non-lethal selection, and not during growth.

Table 5

Time of respreading in hours	No respreading	16	18	20	23
Lac+ colonies counted after respreading and at least 35 h of further incubation	1	13	327	0	0
	0	0	0	27	88
	1	0	0	284	1
	1	0	0	220	1
	0	0	3	96	30
Average number of Lac+ obtained per respreading time point	1±0.5	3±0.5	66±14	125±151	24±28
Average number Lac+ expected per respreading time point	1	2x10[3a]	TMTC ($4x10^3$)	TMTC ($8x10^3$)	TMTC ($\sim 3x10^4$)

Table 5. The mutational events leading to Lac+ mutants on the chromosome appear to occur while bacteria experience non-lethal selection. An independent culture of the strain harboring the chromosomal CC104 *lac*- allele was spun down and concentrated 10 fold as described in Table 4 . 0.1 mL of cells presumably containing a mixture of 1 Lac+ in 10⁹ Lac- cells (based on the estimated frequency, Fig 1) were spread each on the surface of 25 lactose plate replicates. Plates were incubated at 37°C and five plates were taken out of the incubator and respread at the times shown to physically separate Lac+ microcolonies invisible to the naked eye. Lac+ colonies obtained as the result of the respreading were counted after at least an additional 35 h of incubation. Shown per respreading time point are the independent Lac+ counts per plate, the obtained average of Lac+ counted and the average expected number of Lac+. TMTC; too many to count. Numbers in parenthesis are the numerical estimate of Lac+ revertants. The error shown is the standard deviation of the mean. [a] estimated based on between 2-3 h lagging plus 2 hours doubling time.

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The respreading experiment was then repeated using 25 independent cultures with 10 replicates each per time point. One set of plates was not respread and the other set was respread at 10 h after plating. Table 6 shows the results obtained from representative 9 parallel independent cultures since the data are the same for all, except one, of these cultures. This time point was chosen based on the previous experiment (Table 5), which shows that respreading at 10 h results in a measurable number of Lac$^+$ colonies. We expected the number of Lac$^+$ colonies without respreading to be numerically different to plates respread 10 h after plating due to growth of the Lac$^+$ bacteria after 10 h as shown in Table 4, but dependent on each other because all of these replicates are from a culture that has gone through a similar mutational history.

If the average frequency of Lac$^+$ for a given culture were numerically close to 1, as expected if the Lac$^+$ mutation occurred during the growth of the culture, we predicted that 10 h later the Lac$^+$ microcolony would contain approximately between 250-1000 cells (Table 4), which would result in a similar number of Lac$^+$ colonies after respreading in most of the plate replicates. One of the cultures (out of 26) displayed the predicted number of Lac$^+$ colonies had the Lac$^+$ mutation arisen during the growth of the culture (i.e. before plating, Fig 4). In this case, as expected, after 10 h of incubation counts of Lac$^+$ colonies have similar distributions but larger counts of Lac$^+$ colonies representing the number of Lac$^+$ cells within a microcolony; without respreading (mean of 0.25) and with respreading (mean of 220). Thus, the evidence suggests that a fraction of the Lac$^+$ mutants are the product of mutational events occurring during the growth of the culture and prior to plating. However, as observed in Table 1 (respreading column) most of the tested cultures showed a different result. The average number of Lac$^+$ colonies after respreading 10 h after plating is lower than expected even taking into account the largest possible number of Lac$^+$ colonies as predicted by the standard deviation. This finding is contrary to expectations and to what we had observed earlier (Table 4). Moreover, and contrary to the coefficient obtained when Lac$^+$ were seeded to a culture (Table 4), the analysis shows that the Lac$^+$ counts without and with respreading 10 h later seem to be independent of each other (Kendall's coefficient is 0.02 where dependence is shown by values equal or closer to 1).

In order to address the possibility of plating error (defined as significant differences in the number of cells from the same culture deposited on each replicate plate) in these respreading experiments, we employed a classic analysis used by Luria and Delbruck (Luria and Delbruck, 1943), and based on the standard that whenever the variance is closest to the mean, the plating error is considered small. They applied this test to replicates of independent cultures used in a phage resistance assay (Luria and Delbruck, 1943). Thus, we used the data already gathered and determined the variance of the number of Lac$^+$ obtained from representative 9 independent cultures each replicated 10 times. Notably, the variance in the number of Lac$^+$ counted among replicate lactose selective plates with no respreading from each of the cultures is similar to the mean (Table 6), indicating that indeed the plating error is small.

Table 6 Respreading experiments suggest that chromosomal Lac$^+$ mutants occur during selection

	No respreading		Respreading after 10 h	
Culture	Mean±SD	Variance	Mean±SD	Variance
1	0.7±0.8	0.6	9±12	159
2	0.3±0.4	0.2	4±4	15
3	0.8±0.6	0.4	6±15	223
4	0.3±0.4	0.2	18±38	1429
5	1±1	0.9	3±6.5	42
6	0.6±0.4	0.2	12±20	413
7	0.9±0.9	0.9	35±40	1628
8	0.1±0.3	0.1	11±12	137
9	0.4±0.6	0.4	18±25	635

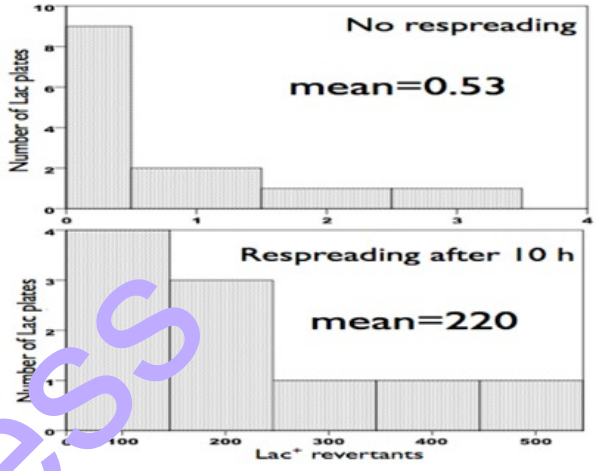

Fig 4. Lac$^+$ colonies obtained from the culture shown are distributed as expected if the mutation to Lac$^+$ occurred during active growth. A Distribution of Lac$^+$ revertants with no respreading. The number of Lac+ mutants obtained from this culture is consistent with a frequency of ~1x10^{-9}. Procedure as indicated in Fig 3 and in Materials and Methods. B. The number of Lac$^+$ colonies obtained through mechanical separation (respreading) of *lac*$^+$ microcolonies 10 h after plating is consistent with cell division of 1 Lac$^+$ cell in 10^9 bacteria deposited on the surface of plates. The distribution of Lac+ colonies in all plates respread is indicative of a mutational event occurring during active growth. The respread plates shown in B are replicates of the plates shown in A.

However, examination of the number of Lac$^+$ colonies counted after respreading, which represents the number of cells in a Lac$^+$ microcolony after 10 h of incubation, shows disparity among plate replicates from the same culture. In stark contrast to the data without respreading, 10 h after the variance is high and variable (Table 6, 10 h respreading column). This is not likely to be due to plating error, since we have already established that not to be the case for the parallel independent cultures studied here (no respreading). The inconsistent distribution displayed by the Lac$^+$ colonies respread 10 h later could instead be caused by the randomness inherent in mutagenesis occurring on the plate, during non-lethal selection.

Taking all of these experiments together, we conclude that there are mutational events occurring in the chromosome of cells that are not actively proliferating in a short time interval after experiencing non-lethal conditions of selection. It appears that most of the chromosomal Lac$^+$ mutants are not the result of mutagenesis processes occurring during the growth of the culture.

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Discussion

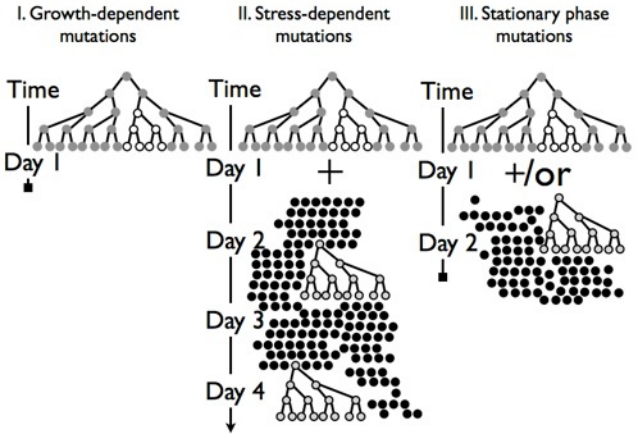
We have examined a particular mutation that must occur at a specific codon of the lacZ gene, encoding the ß-galactosidase enzyme, to enable bacteria to grow in minimal medium with lactose as the sole carbon source. We have used a Lac- strain of bacteria which only become Lac$^+$ due to a specific transversion mutation in *lacZ* (Cupples and Miller, 1989). Unlike some other *lacZ* alleles, this allele has no residual activity, which may be reason it does not accumulate mutants with time (Godoy et al., 2000) as in adaptive mutagenesis. Typically, adaptive *lac*- or *trp*- alleles (Cairns and Foster, 1991; Godoy et al., 2000; Petrosino et al., 2009) encode proteins with residual enzymatic activity, which allows selection for gene amplifications. Furthermore, localization of the gene on extrachromosomal elements may facilitate accumulation of mutants with time (Hastings et al., 2004; Slechta et al., 2002b) (Slechta et al., 2002a).

We studied the non-leaky lac- allele in two distinct locations: either on an episome (CC104) (Cupples and Miller, 1989) or on the chromosome. The Lac$^+$ mutant colonies analyzed appear between 35-48 h of incubation at 37ºC, which is the same time frame that is required for Lac$^+$ cells to form visible colonies on lactose minimal medium. The Lac- population does not appear to divide once deposited on non-lethal selective conditions, i.e. lactose minimal medium, in agreement with previous findings (Cairns and Foster, 1991; Petrosino et al., 2009). A small increase in cell viability has been observed previously (Quinones-Soto and Roth, 2011), but even in that case, it is not large enough to explain the elevated mutagenesis. In this report we show that, remarkably, this static Lac- population is able to produce Lac+ revertants during non-lethal selection. We are able to verify that only a small minority of the Lac$^+$ bacteria arose in the growing culture before selection.

This is the first experimental demonstration that most of the first mutants detected in non-lethal selection conditions are not the product of mutagenesis occurring during exponential growth. These results highlight the difficulty of either experimentally measuring and of estimating mutation rates (defined as mutants per cell per generation). Currently, there is clear evidence of at least two different mechanisms to explain mutagenesis in a bacterial population, represented by two distinct classes of mutants. One class of mutations occurs during the growth of a culture, likely via rare DNA replication errors leading and subsequent clonal expansion (e.g. jackpots; Fig 5-I). The products of such mutational events are detected through lethal or non-lethal selection (Fig 1) (Hayes, 1968; Luria and Delbruck, 1943). A second class of mutations is represented by late-appearing mutants in leaky-Lac- to Lac$^+$ reversion assays. These Lac$^+$ mutants continuously appear during 5-7 days of incubation on non-lethal selective minimal lactose medium, while the general population of Lac- bacterial cells are not multiplying (Foster, 1994) (Fig 5-II). Similar results are obtained in revertible alleles encoding enzymes of amino acid biosynthetic pathways (Godoy et al., 2000). This second class of mutagenesis has been called "adaptive" or stress-induced mutagenesis (Bjedov et al., 2003; Galhardo et al., 2007; Hall, 1998; Rosenberg et al., 1995; Slechta et al., 2003). Sequence analysis of the late-appearing (and therefore adaptive) Lac$^+$ mutants shows that they are for the most part different from the first crop of mutants to grow into colonies (Rosenberg et al., 1994). The mechanisms that underlie these late appearing mutants are still controversial, but it is likely that allele amplification and Y family DNA polymerases play a role in the process (Andersson et al., 1998; Galhardo et al., 2009; Godoy et al., 2007; Hendrickson et al., 2002).

We propose here a third class of events leading to mutants under non-lethal selective conditions (Fig 5-III). The present report shows that bacterial cells can also mutate by a mechanism that occurs after cells carrying a non-leaky allele are deposited on a non-lethal selective medium. Our data shows that, of revertants arising in the CC104 lac- allele located on the episome, 80-90% are not present in the culture before selection, although they appear earlier than classic adaptive revertants. Instead, the mutational events that led to the majority of Lac$^+$ revertants in our assay occurred later, while bacteria are on the plate (Table 2). These Lac$^+$ mutants accumulate poorly with time as has been previously shown (Godoy et al., 2000). Our data therefore implies that a third category of mutagenesis is occurring in the 35 to 48 hour window of time after cells are deposited on non-lethal selective media. This is the same time window in which a Lac$^+$ cell becomes a visible colony on minimal medium. They are neither the result of population growth on the plate, nor the product of the last generation of growth (Table 3). Lac$^+$ mutants that are plated as a minority to a Lac- population divide and grow with no disadvantage in this mixture (Fig 2), thus establishing the feasibility to interpret the results that we obtained in the end-point dilution (Table 2) and respreading experiments (Fig 4 and Tables 5,6). These findings are consistent with those found for other Lac- bacteria bearing non-leaky alleles, which do not divide while on the plates and do not accumulate late-appearing mutants (Galitski and Roth, 1996; Godoy et al., 2000).

Figure 5



We cannot yet define the underlying mechanism(s) for the observed mutagenesis. Allele amplification (Andersson et al., 1998; Roth et al., 2006; Slechta et al., 2002a) is thought of as elevated gene copy number of a leaky allele able to provide a selective advantage to cells, because each copy of the defective *lacZ* produces some level of ß-galactosidase (Andersson et al., 1998). Amplification could play a part in the mutagenesis demonstrated here, because multiple copies of the Lac- allele would increase the chance for a mutation to

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occur (Pranting and Andersson, 2011; Quinones-Soto and Roth, 2011); however, in our system, there can be no selective advantage conferred by amplification itself.

Alternatively, it is also possible that the amplification of a factor or factors that drive the mutagenesis (e.g. *dinB* gene) plays a role in the mutagenesis we observe. Such amplifications have not been observed; however, it seems unlikely that such amplifications would be stable once the mutation that allows for growth occurs (Quinones-Soto and Roth, 2011). Therefore the possibility cannot be ruled out.

The data for the lac- locus on the chromosome suggest that the mutagenesis is governed by mechanism(s) similar to those occurring on the episome. The evidence indicates that most of Lac$^+$ mutants appear while the cells are on the non-lethal selective plate (Tables S1, 5 and 6). In the respreading experiments only one culture was identified that had a signature distribution resembling what was expected based on our predictions (Fig 4). Notably, these experiments have permitted us to discriminate between mutations that likely occurred during growth and those happening during selection, suggesting that approximately 4% of the events happened during growth. It is likely that the other Lac$^+$ revertants that we observe after non-lethal selection are the result of mutational events happening during selection (Fig 5-panel III).

The existence of this class of mutations points out the inaccuracy of using fluctuation analysis to calculate mutation rates when the selection is not lethal. It also confirms the concept that bacterial cells that are not dividing at a detectable rate are nonetheless able to generate variants (Foster and Cairns, 1992; Galitski and Roth, 1996; Petrosino et al., 2009). Interestingly, a recent report shows evidence that supports this early timing of chromosomal mutational events (Zhang et al., 2011). *E. coli* was found to mutate to Ciprofloxacin resistance during the first 10 h of treatment with a gradient of the drug, a condition that can be considered a non-lethal selection since cell growth is inhibited in the conditions of the assay (Zhang et al., 2011). Indeed, Frisch and Rosenberg (Frisch and Rosenberg, 2011), point out that these conditions may favor stress-induced mutagenesis. It may be that in all cases where non-lethal selection is applied, the early-appearing mutant colonies arise from mutational events happening both during growth and during selection, but that our ability to find mutants at this early time after bacteria are exposed to non-lethal selection has been limited by both detection and frequency of occurrence. It has been previously suggested that early-appearing Lac$^+$ mutants (day 2) of the frameshift lac- *lacI33* allele may be part of early adaptive mutagenesis (possibly the product of the mutability discussed here) because the mutational signature of the early-appearing Lac$^+$ is different from those appearing later (Rosenberg et al., 1994). Interestingly, different treatments or other bacteria have also shown mutagenesis while cells are not actively proliferating (Godoy et al., 2006; Kunz, 1985; Wood et al., 2009), and others have suggested that there may be novel mechanisms at work in non-mutant cells which survive exposed to deleterious conditions (Galitski and Roth, 1996; Zhang et al., 2011).

The findings described in this report are important for practical purposes when studying mutagenesis under non-lethal conditions of selection by fluctuation analysis. It is likely these analyses will be flawed, because many of the mutants counted will have arisen while cells were under non-lethal selective conditions on plates. Our findings also have implications regarding our understanding of the ability of bacteria to survive and evolve under changing environmental conditions. An example of particular interest is the development of antibiotic resistance, especially since low concentrations of antibiotics, often a condition of non-lethal selection, abound in the environment (Gullberg et al., 2011).

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