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# A source of artifact in the *lacZ* reversion assay in *Escherichia coli*

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ABSTRACT

The *lacZ* reversion assay in *Escherichia coli* measures point mutations that occur by specific base substitutions and frameshift mutations. The tester strains cannot use lactose as a carbon source (Lac$^-$), and revertants are easily detected by growth on lactose medium (Lac$^+$). Six strains identify the six possible base substitutions, and five strains measure +G, −G, −CG, +A and −A frameshifts. Strong mutagens give dose-dependent increases in numbers of revertants per plate and revertant frequencies. Testing compounds that are arguably nonmutagens or weakly mutagenic, we often noted statistically significant dose-dependent increases in revertant frequency that were not accompanied by an absolute increase in numbers of revertants. The increase in frequency was wholly ascribable to a declining number of viable cells owing to toxicity. Analysis of the conditions revealed that the frequency of spontaneous revertants is higher when there are fewer viable cells per plate. The phenomenon resembles "adaptive" or "stress" mutagenesis, whereby lactose revertants accumulate in Lac$^-$ bacteria under starvation conditions in the absence of catabolite repression. Adaptive mutation is observed after long incubation and might be expected to be irrelevant in a standard assay using 48-h incubation. However, we found that elevated revertant frequencies occur under typical assay conditions when the bacterial lawn is thin, and this can cause increases in revertant frequency that mimic chemical mutagenesis when treatments are toxic but not mutagenic. Responses that resemble chemical mutagenesis were observed in the absence of mutagenic treatment in strains that revert by different frameshift mutations. The magnitude of the artifact is affected by cell density, dilution, culture age, incubation time, catabolite repression and the age and composition of media. Although the specific reversion assay is effective for quickly distinguishing classes of mutations induced by potent mutagens, its utility for discerning effects of weak mutagens may be compromised by the artifact.



## 1. Introduction

The lacZ specific reversion assay in *Escherichia coli* offers a simple means of detecting revertants that arise by known molecular mechanisms [1,2]. The tester strains cannot grow on medium containing lactose as the sole carbon source. Reversion from the Lac$^-$ phenotype to Lac$^+$ is detected by selection for growth on minimal lactose medium [3]. The target alleles in the tester strains are *lacZ* mutations carried on an F′ episome (F′ *proA*$^+$*B*$^+$ *lacI*$^-$ *lacZ*$^-$) in *E. coli* strain p90c. Growth of the bacteria on lactose depends on reversion of the episomal gene, because p90c contains a deletion (*Δgpt-lac*) of the entire *lac* operon as well as flanking genes. Thus, the tester strains all have the following genotype: *ara Δ*(*lac pro*)/F′ *lacI*$^-$ *lacZ*$^-$ *proA*$^+$*B*$^+$. There are 11 main strains in the assay, and each reverts by a single mutational mechanism. Together they detect all six base-pair substitutions [1] and frameshift mutations in different sequence contexts [2]. The assay is summarized in Fig. 1. An excellent review by Josephy [3] summarizes the use of the *lacZ* assay for detecting mutagens and describes derivative strains that enhance the versatility of the assay.

Two procedures for conducting the *lacZ* assay are a plate test and a quantitative reversion assay [3]. The former is analogous to the Ames test [4,5], in which bacteria are plated only on selective medium, and mutagenesis is detected on the basis of an increase in the number of Lac$^+$ colonies per plate. In a quantitative reversion assay, bacteria are plated on selective medium (i.e., minimal lactose) to detect revertants, and a diluted sample is plated on nonselective medium (i.e., minimal glucose) to quantify numbers of viable, colony-forming cells. A revertant frequency, which can be expressed as revertants per $10^8$ surviving cells, is calculated by dividing numbers of revertants by numbers of viable cells.

We recently attempted to use the *lacZ* assay to explore whether intercalation between DNA base pairs is sufficient for frameshift

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**The *lacZ* Reversion Assay**

Genotype of tester strains: *ara Δ(lac pro) / F' lacI⁻ lacZ⁻ proA⁺B⁺*
Chromosome: *ara Δ(gpt-lac)5*
F': *lacI⁻ lacZ⁻ proA⁺B⁺*

| Strain | Sequence of *lacZ⁻* tester strain | Sequence of *lacZ⁺* revertants | Reversion mechanism |
|---|---|---|---|
| CC101 | AAT TAG AGT | AAT GAG AGT | A:T → C:G transversion |
| CC102 | AAT GGG AGT | AAT GAG AGT | G:C → A:T transition |
| CC103 | AAT CAG AGT | AAT GAG AGT | G:C → C:G transversion |
| CC104 | AAT GCG AGT | AAT GAG AGT | G:C → T:A transversion |
| CC105 | AAT GTG AGT | AAT GAG AGT | A:T → T:A transversion |
| CC106 | AAT AAG AGT | AAT GAG AGT | A:T → G:C transition |
| CC107 | GGCGGGGGGCC | GGCGGGGGGGCC | +1 (+G) frameshift ($G_6 \rightarrow G_7$) |
| CC108 | GGGGGGCGGAGC | GGGGGCGGAGCC | −1 (−G) frameshift ($G_6 \rightarrow G_5$) |
| CC109 | TACGCGCGCGCG | TACGCGCGCGTG | −2 (-CG) frameshift ($CG_5 \rightarrow CG_4$) |
| CC110 | ATAAAAAATGG | ATAAAAAAATGG | +1 (+A) frameshift ($A_6 \rightarrow A_7$) |
| CC111 | ATCAAAAAAATG | ATCAAAAAATGG | −1 (−A) frameshift ($A_7 \rightarrow A_6$) |

**Fig. 1.** The *lacZ* specific reversion assay in *Escherichia coli*. The genotypes and reversion mechanisms are based on Cupples et al. [1,2]. Methods for the assay have been reviewed by Josephy [3].

mutagenesis or a precursor that may (or may not) be processed into frameshifts. We used compounds whose structures do not suggest intercalation but that are thought to intercalate on the basis of computer modeling [6,7], displacement of ethidium from DNA [8], and interaction with bleomycin in genotoxicity assays in Chinese hamster cells [9] and yeast [10]. We observed strong mutagenicity for reactive acridine derivatives, as expected, and weakly positive responses for the other compounds, some of which had earlier yielded negative results for mutagenicity in our laboratory and elsewhere. The positive responses in the *lacZ* assay were dose-dependent, statistically significant, and reproducible. However, analysis of the dose–response relationships led us to suspect that the evidence of weak mutagenicity was unreliable.

We proposed hypotheses that might cause an increase in the frequency of revertants under the assay conditions in lieu of bona fide chemical mutagenesis. These ideas centered on the following possibilities: 1) selection during treatment favoring pre-existing $Lac^+$ revertants; 2) lateral transfer of revertant plasmids by conjugation; 3) systematic technical error associated with treatment, dilution, and plating; and 4) elevated frequencies of spontaneous revertants under the assay conditions. Any of these factors, if correct, could explain a reproducible, dose-dependent, statistically significant increase in revertant frequency that could mimic chemical mutagenesis. Experimental analysis eliminated the first three of the four proposed sources of artifact. However, the fourth hypothesis – elevated frequencies of spontaneous revertants under the assay conditions – turned out to be correct. The evidence is presented in this paper.

## 2. Materials and methods

### 2.1. Strains

*E. coli* strains CC107, CC108, CC109, and CC111 (*ara Δ*(*lac pro*)/F' *lacI⁻ lacZ⁻ proA⁺B⁺*) were obtained from Dr. Claire G. Cupples, University of Victoria, Victoria, BC, Canada. *Saccharomyces cerevisiae* strain D7 (*a ade2-40 trp5-12 ilv1-92/α ade2-119 trp5-27 ilv1-92*) was obtained from F.K. Zimmermann (Technische Hochschule, Darmstadt, Germany).

### 2.2. Chemicals

Nitracrine dihydrochloride (ledakrin; 1-nitro-9-(3′-dimethylaminopropylamino)-acridine diHCl; Chemical Abstracts Registry number (CAS) 6514-85-8) was synthesized in the Auckland Cancer Society Research Centre, University of Auckland, New Zealand, and given to us by Dr. W.A. Denny. Diphenhydramine hydrochloride (DPH; CAS 147-24-0) was purchased from Sigma–Aldrich Chemical Company, St. Louis, MO. Stock solutions were frozen at −20 °C with no diminution of biological activity over several months. The methylated glucose derivative α-methylglucoside (αMG), also called methyl-α-D-glucopyranoside, was also from Sigma–Aldrich.

### 2.3. Media

Vogel–Bonner citrate (VBC) medium [11], also called Medium E, was used to make minimal lactose medium (VBCL), which contains α-lactose as the sole carbon source and can therefore be used to select for $Lac^+$ revertants. Its composition was 0.2% α-lactose, 15 μM thiamine, and 1.8% Difco Bacto Agar. Numbers of viable cells were determined on minimal glucose medium, which is VBC medium containing 0.5% D-(+)-glucose (VBCG). Chemical treatments were done in liquid LB medium [12]. When minimal lactose was supplemented with αMG, the concentration of αMG was 0.2%. Yeast strain D7 was grown in YEPD rich medium [10]. Gene conversion at the *trp5* locus was selected on yeast minimal medium containing adenine and isoleucine (YMAI), and numbers of viable cells were determined on the same medium supplemented with tryptophan (YMAIT) [10].

### 2.4. Mutation assay

Bacteria were streaked on VBCG plates without proline to select for the presence of the episome. Single colonies were picked from the streaks and grown to stationary phase in liquid LB medium. The time of incubation was 16–18 h, except where otherwise noted. Bacteria were spread on VBCL plates to determine the spontaneous revertant frequency while the original cultures were held at 4 °C. Revertant frequencies did not vary widely, and this step ensures that experiments are conducted with cultures whose spontaneous frequencies are consistent with our historical controls and published values [2].

For mutagenesis experiments, bacteria from these characterized cultures were subcultured in liquid LB for 16–18 h in a shaker, after which 10 μl aliquots were inoculated into 1 ml LB containing the compound to be assayed for mutagenicity. The treatments were terminated after 16–18 h incubation in a shaker at 37 °C by adding 10 ml cold saline (0.9% NaCl), centrifuging and resuspending the cells in 1 ml saline for plating. Approximately $1–2 \times 10^8$ cells were spread on VBCL plates to select for revertants, and dilutions giving 100–200 colonies per plate were spread on VBCG to quantify viable cells. Experiments to test for dilution artifacts were conducted similarly, except that cell concentrations were adjusted as explained for the design of the particular experiment. In both kinds of experiment, bacteria were plated in triplicate, except where it is noted that 6 replicate plates were used. Colonies were counted after 48 h at 37 °C. The significance of differences in mean numbers of revertants per plate and in revertant frequencies was determined by analysis of variance, and slopes of dose–response curves were determined by linear regression analysis.

## 3. Results

Results of a mutagenicity test of nitracrine and diphenhydramine are shown in Table 1. Nitracrine, a reactive acridine compound [13], induces −2 frameshift mutations, indicated by significant increases in numbers of revertants per plate and in revertant frequencies in strain CC109. The dose–response curves have significantly positive slopes for revertants per plate ($r^2 = 0.90$; $P < 0.0001^{****}$) and for revertant frequency ($r^2 = 0.99$; $P = 0.0019^{**}$). Diphenhydramine, which gave negative results in other microbial

**Table 1**
Apparent induction of −CG frameshift mutations by nitracrine and diphenhydramine (DPH) in strain CC109 of the *E. coli lacZ* reversion assay.

| Compound[a] | Dose (μM)[a] | Relative cell density | Lac$^+$ revertants per plate[a] | Revertant frequency (Lac$^+$/10$^8$)[b] |
|---|---|---|---|---|
| Nitracrine | 0 | 1.00 | 137.3 ± 7.31 | 73.32 ± 3.9 |
| | 0.03125 | 0.84 | 374.0 ± 7.55$^{***}$ | 238.22 ± 4.8$^{NS}$ |
| | 0.0625 | 0.66 | 532.7 ± 38.92$^{***}$ | 430.61 ± 31.5$^{**}$ |
| | 0.125 | 0.43 | 699.0 ± 43.51$^{***}$ | 866.17 ± 53.9$^{****}$ |
| DPH | 0 | 1.00 | 45.8 ± 3.73 | 37.36 ± 3.04 |
| | 256 | 0.60 | 58.7 ± 6.33$^{NS}$ | 79.63 ± 8.60$^{**}$ |
| | 512 | 0.42 | 45.3 ± 3.76$^{NS}$ | 87.18 ± 7.22$^{***}$ |
| | 1024 | 0.39 | 53.7 ± 5.70$^{NS}$ | 111.81 ± 11.87$^{***}$ |

[a] Treatment of growing cells at 35 °C for 18 h in LB in a shaker. Plating was in triplicate except that 6 replicate plates were used for the untreated control for DPH. Plating cell densities were ~10$^8$ cells per plate of minimal lactose medium to select for revertants and ~100 cells on minimal glucose medium to determine the relative cell density as a measure of toxicity.
[b] Frequencies are means ± SEM. The significance of differences from the untreated control was determined by ANOVA with a Bonferroni multiple comparisons test ($^{NS}$nonsignificant; $^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$; $^{****}P < 0.0001$).

mutagenicity assays [10,14], also caused a significant increase in revertant frequency ($P < 0.001^{***}$), albeit at much higher doses. One might conclude on the basis of the significant increases in revertant frequency that DPH is weakly mutagenic in CC109. However, the elevated revertant frequencies are not accompanied by an absolute increase in numbers of revertants per plate, and the slope for numbers of revertants per plate did not differ significantly from zero ($P = 0.76$ NS). Unlike the result with nitracrine, which is clearly mutagenic, there was a roughly constant number of revertants in a declining population of surviving cells after DPH treatment. The increase in revertant frequency therefore resided exclusively in the denominator. Results with other putative nonmutagens or weak mutagens gave similar increases in revertant frequencies. While this could reflect real mutagenesis, we were suspicious and explored it further.

Table 2 shows an experiment in which there was no chemical treatment but thinning of the bacterial population by dilution was used to simulate toxicity. Colony numbers on glucose medium are consistent with dilution, as expected. However, colony numbers for dilutions on minimal lactose, which preceded the glucose platings in the same dilution series, are not compatible with dilution. There is an increase in the frequency of Lac$^+$ colonies with increasing dilution. Thus, when there are fewer viable cells per plate, revertant frequencies are too high relative to expectations. The increase in revertant frequency with dilution is statistically significant and reproducible, and it resembles dose-dependent mutagenesis.

Because of the similarities between this dilution-associated increase in revertant frequency and the phenomenon known as adaptive mutation, we explored the parallels between the two phenomena. We evaluated the effect of catabolite repression on dilution experiments like that shown in Table 2 by incorporating αMG into the selection medium, in light of findings that this compound is strongly catabolite-repressing but non-utilizable as a carbon source by *E. coli* [15,16]. We used αMG to determine whether the dilution-associated increase in revertant frequency in the *lacZ* reversion assay is related to catabolite repression. Table 3 presents data from an experiment of the same design as that in Table 2 except that the bacteria were plated both on standard minimal lactose medium and on the same medium supplemented with 0.2% αMG. Catabolite repression by αMG, which suppresses adaptive stress mutagenesis [15], also suppresses the dilution-associated artifact in strain CC109. This result links the two phenomena and suggests that the artifact may arise through adaptive mutagenesis occurring under the assay conditions.

Because adaptive mutagenesis is reported to occur in stationary-phase cells in the absence of growth [17,18], we evaluated whether the same is true of the dilution artifact. Fig. 2 shows spontaneous

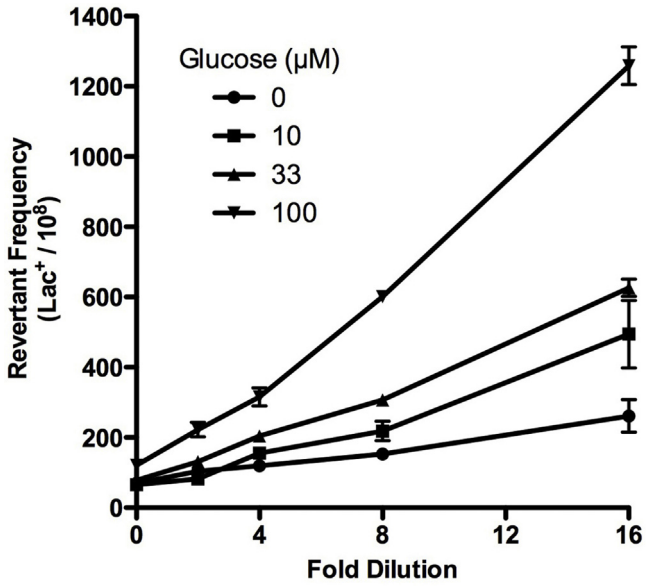


**Fig. 2.** Spontaneous revertant frequency in strain CC109: effects of dilution and a trace of glucose in lactose medium.

**Table 2**
A dilution-associated artifact in the measurement of revertant frequencies in strain CC109 of the *E. coli lacZ* reversion assay.

| Fold dilution[a] | Colonies per plate on glucose | Relative plating efficiency[b] | Revertants per plate on lactose | Revertant frequency (Lac$^+$/10$^8$)[b] |
|---|---|---|---|---|
| 1× | 274.7 | 1.00 ± 0.022 | 161.7 | 117.75 ± 4.50 |
| 2× | 149.0 | 1.08 ± 0.074$^{NS}$ | 90.7 | 131.98 ± 11.47$^{NS}$ |
| 4× | 76.7 | 1.12 ± 0.056$^{NS}$ | 55.3 | 161.32 ± 13.61$^{NS}$ |
| 8× | 34.7 | 1.01 ± 0.085$^{NS}$ | 45.3 | 263.57 ± 10.79$^{**}$ |
| 16× | 14.0 | 0.82 ± 0.089$^{NS}$ | 23.7 | 275.19 ± 44.70$^{**}$ |

[a] Strain CC109 was grown at 35 °C for 18 h in LB in a shaker. The cell density was 2.76 × 10$^9$ cells per ml. Plating was in triplicate. Bacteria were diluted in saline for spreading at cell densities of ~1.4 × 10$^8$ (1×), 7.0 × 10$^7$ (2×), 3.5 × 10$^7$ (4×), 1.75 × 10$^7$ (8×) and 8.75 × 10$^6$ (16×) cells per plate of minimal lactose medium (VBCL) to select for revertants and ~280, 140, 70, 35, and 17.5 cells per plate of minimal glucose medium (VBCG) to determine numbers of viable cells.
[b] Relative plating efficiencies and revertant frequencies are means ± SEM. The significance of differences from the reference point (1× = ~1.4 × 10$^8$ cells on VBCL; 280 on VBCG) was determined by ANOVA with a Bonferroni multiple comparisons test ($^{NS}$nonsignificant; $^{*}P < 0.05$; $^{**}P < 0.01$).

**Table 3**
Effect of catabolite repression by α-methylglucoside (αMG) on the dilution-associated artifact in *E. coli* strain CC109.

| Fold dilution[a] | Colonies per plate on glucose | Relative plating efficiency[b] | Revertants per VBCL plate | | Revertant frequency[b] ($Lac^+/10^8$) | |
|---|---|---|---|---|---|---|
| | | | no αMG | +αMG | no αMG | VBCL + αMG |
| 1× | 288.7 | $1.00 \pm 0.17$ | 69.0 | 13.7 | $47.82 \pm 4.23$ | $9.47 \pm 2.57$ |
| 2× | 136.0 | $0.94 \pm 0.06^{NS}$ | 43.7 | 5.7 | $64.22 \pm 6.14^{NS}$ | $8.33 \pm 4.01^{NS}$ |
| 4× | 69.7 | $0.96 \pm 0.01^{NS}$ | 40.0 | 2.7 | $114.94 \pm 9.24^{NS}$ | $7.66 \pm 2.53^{NS}$ |
| 8× | 28.0 | $0.78 \pm 0.06^{NS}$ | 31.3 | 1.0 | $223.81 \pm 29.0^{***}$ | $7.14 \pm 0.00^{NS}$ |
| 16× | 20.7 | $1.14 \pm 0.10^{NS}$ | 23.0 | 0.7 | $223.30 \pm 39.2^{**}$ | $6.47 \pm 6.47^{NS}$ |

[a] Strain CC109 was grown at 35 °C for 16 h in LB medium in a shaker. The cell density was $2.88 \times 10^9$ cells per ml. Bacteria were diluted in saline for spreading at cell densities of $\sim 10^8$, $5 \times 10^7$, $2.5 \times 10^7$, $1.25 \times 10^7$ and $6.25 \times 10^6$ cells per plate of minimal lactose medium (VBCL) and of VBCL + αMG. To determine numbers of viable cells ~280, 140, 70, 35, and 17.5 cells were spread per plate of minimal glucose (VBCG).

[b] Relative plating efficiencies and revertant frequencies are means ± SEM. The significance of differences from the reference point ($\sim 10^8$ cells on VBCL; 280 on VBCG) was determined by ANOVA with a Bonferroni multiple comparisons test ($^{NS}$nonsignificant; $^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$).

revertant frequencies in strain CC109 when a trace of glucose was added to minimal lactose medium. The glucose leads to a significantly increased spontaneous revertant frequency ($P = 0.002$) and positive slope in linear regression ($r^2 = 0.96$; $P < 0.02$). These increases are expected on the basis of there being more cell divisions, each of which carries a small probability of giving rise to a mutation. The slopes of the lines are significantly greater than zero ($r^2 > 0.97$ and $P < 0.0015$ in all cases), confirming the dilution-related increase in revertant frequency. The slopes with glucose supplementation are steeper than that for conventional minimal lactose ($P = 0.0007$ for 10 μM glucose and $P < 0.0001$ for the higher glucose concentrations). The slopes for the different glucose concentrations all differ significantly from one another except for the comparison of 10 and 33 μM glucose, which does not quite rise to the level of statistical significance ($P = 0.053$).

These results suggest that the dilution artifact is dependent on cell division, rather than being a division-independent increase in stationary phase as has been proposed for adaptive stress mutagenesis. Autoclaving lactose rather than filter sterilizing it or using aged lactose medium also increased the dilution artifact. This effect presumably reflects a trace of glucose being added to the medium through a low level of lactose cleavage [19]. The findings suggest that the artifact is ascribable to an accumulation of spontaneous genetic alterations when the bacteria have a minimal supply of glucose and less competition from other $Lac^-$ bacteria.

Fig. 3 shows changes in the scale of the dilution-associated artifact if the incubation time is extended beyond the standard 48-h incubation. Numbers of $Lac^+$ revertants increase with incubation time on minimal lactose medium. Thus, the accumulation of revertants depends both on time and dilution, in that it is greatest with long exposure to starvation conditions in the absence of catabolite repression at low cell densities. This result resembles the finding (Fig. 2) that a small amount of glucose in the lactose medium enhances the dilution artifact. A trace of glucose may permit cell division favoring spontaneous mutation, yet be insufficient for catabolite repression. A low level of lactose degradation and cross-feeding may contribute a small amount of utilizable carbon source.

The data in Table 4 show the dilution artifact in strains CC107, CC108, CC109 and CC111, which detect +G, −G, −CG and −A frameshift mutations, respectively. Colony counts on glucose are consistent with dilution, but those on minimal lactose show higher revertant frequencies when there are fewer cells per plate. The effect is most easily detected in CC109 because of its high spontaneous revertant frequency, but it is not restricted to a single strain or reversion mechanism. In strains CC107, CC108 and CC109, it is unequivocally detected within a standard 48-h incubation. In strain CC111 the artifact is a modest effect at 48 h, but it becomes appreciable when the duration of nutrient deprivation is extended.

To confirm our interpretation that the increase in revertant frequency is ascribable to there being fewer viable cells in the selection plates, we conducted an experiment of the same design as that shown in Table 2 but added nongrowing but viable cells to the bacterial lawn. Cells of strain WP2, which were unable to grow owing to their tryptophan auxotrophy (*trpE56*), were mixed with dilutions of CC109 in quantities sufficient to keep the total number of viable cells constant. When the mixtures were plated on minimal lactose medium, absolute numbers of revertants declined with dilution of CC109, and there were no significant differences in CC109 revertant frequencies. The data are shown in Table 5. Thus, the presence of the filler or scavenger cells suppressed the dilution artifact. As a control for the experimental design, the same procedure was followed using a sham mixture of CC109 with cells that had been rendered nonviable by incubation in saline without a carbon source. In this case, the increase in CC109 revertant frequency with dilution was comparable to that seen in Table 2 and other dilution experiments.

A critical assumption is made in diluting cultures to measure viable cells in fully supplemented medium and then using these values to calculate frequencies of genetic events detected on selective media at much higher cell densities. The dilution experiments reported here argue that such a practice is precarious for the *lacZ* reversion assay. However, this procedure has been much used without difficulty in other assays. To confirm that the apparent artifact is not a consequence of a flaw in our dilution

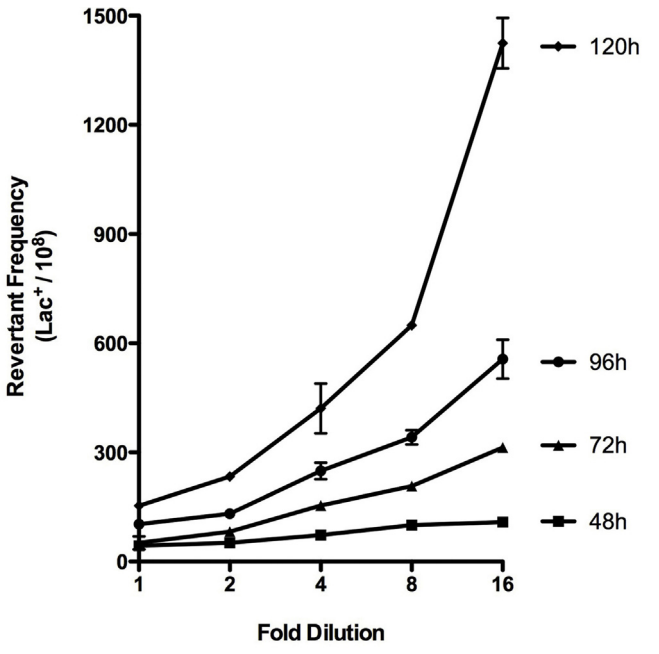


**Fig. 3.** Dilution-associated increase in revertant frequency in *Escherichia coli* strain CC109 after incubation for different times on minimal lactose medium.

**Table 4**
Dilution-associated artifact in the measurement of revertant frequencies in four frameshift strains of the *E. coli lacZ* reversion assay.

| Strain and incubation time[a] | Fold dilution[a] | Colonies per plate on glucose | Relative plating efficiency[b] | Revertants per plate on lactose[a] | Revertant frequency ($Lac^+/10^8$)[b] |
|---|---|---|---|---|---|
| CC107 | 1× | 518.7 | $1.00 \pm 0.048$ | 29.8 | $23.42 \pm 1.83$ |
| (48 h) | 2× | 252.3 | $0.97 \pm 0.023^{NS}$ | 20.2 | $31.66 \pm 1.96^{NS}$ |
| | 4× | 120.7 | $0.93 \pm 0.052^{NS}$ | 11.5 | $36.05 \pm 6.26^{NS}$ |
| | 8× | 70.8 | $1.09 \pm 0.100^{NS}$ | 8.5 | $53.46 \pm 11.79^{NS}$ |
| | 16× | 29.7 | $0.92 \pm 0.041^{NS}$ | 11.5 | $144.47 \pm 25.70^{***}$ |
| CC108 | 1× | 579.7 | $1.00 \pm 0.031$ | 10.2 | $6.44 \pm 0.60$ |
| (48 h) | 2× | 296.5 | $1.02 \pm 0.029^{NS}$ | 5.2 | $6.54 \pm 0.39^{NS}$ |
| | 4× | 168.2 | $1.16 \pm 0.044^{NS}$ | 3.5 | $8.86 \pm 1.70^{NS}$ |
| | 8× | 83.7 | $1.15 \pm 0.024^{NS}$ | 2.3 | $11.84 \pm 2.51^{NS}$ |
| | 16× | 40.2 | $1.11 \pm 0.115^{NS}$ | 3.0 | $30.43 \pm 7.86^{***}$ |
| CC109 | 1× | 308.2 | $1.00 \pm 0.24$ | 53.2 | $31.93 \pm 1.99$ |
| (48 h) | 2× | 157.5 | $1.02 \pm 0.019^{NS}$ | 33.7 | $40.42 \pm 1.01^{NS}$ |
| | 4× | 85.8 | $1.11 \pm 0.011^{NS}$ | 33.8 | $81.33 \pm 9.09^{***}$ |
| | 8× | 42.3 | $1.10 \pm 0.082^{NS}$ | 21.2 | $101.92 \pm 11.21^{***}$ |
| | 16× | 22.5 | $1.17 \pm 0.101^{NS}$ | 12.5 | $120.19 \pm 8.14^{***}$ |
| CC111 | 1× | 649.8 | $1.00 \pm 0.02$ | 14.7 | $12.68 \pm 1.49$ |
| (48 h) | 2× | 304.8 | $0.94 \pm 0.02^{NS}$ | 8.0 | $13.84 \pm 2.10^{NS}$ |
| | 4× | 171.7 | $1.06 \pm 0.04^{NS}$ | 6.0 | $20.76 \pm 4.38^{NS}$ |
| | 8× | 81.0 | $1.00 \pm 0.04^{NS}$ | 4.7 | $32.18 \pm 7.05^{*}$ |
| | 16× | 46.2 | $1.14 \pm 0.11^{NS}$ | 1.5 | $20.83 \pm 6.94^{NS}$ |
| CC111 | 1× | Cell density determined after 48-h | | 23.8 | $20.60 \pm 1.90$ |
| (72 h) | 2× | incubation (unchanged at 72 h) | | 14.5 | $25.09 \pm 2.44^{NS}$ |
| | 4× | | | 10.3 | $35.76 \pm 5.70^{NS}$ |
| | 8× | | | 8.8 | $60.92 \pm 6.99^{***}$ |
| | 16× | | | 4.0 | $55.56 \pm 11.34^{**}$ |

[a] Bacteria were grown at 35 °C for 16 h in LB in a shaker and diluted in saline for spreading on 6 replicate plates of minimal lactose medium at densities of $\sim 1.2 \times 10^8$, $6 \times 10^7$, $3 \times 10^7$, $1.5 \times 10^7$ and $0.75 \times 10^7$ cells per plate. A corresponding series of twofold dilutions was plated on minimal glucose to determine numbers of viable cells.
[b] Relative plating efficiencies and revertant frequencies are means ± SEM. The significance of differences from the reference point (1×) was determined by ANOVA with a Bonferroni multiple comparisons test ($^{NS}$nonsignificant; $^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$).

experiments, we conducted the same procedure with an assay for gene conversion in yeast strain D7. The data are shown in Table 6. Colony numbers in strain D7 are compatible with dilution both on fully supplemented minimal medium (YMAIT) and on medium without tryptophan (YMAI) that is used to select for *trp5* convertants. Thus, the D7 assay does not show a dilution-associated artifact like that observed in measurements of revertant frequencies in the *E. coli lacZ* assay. The artifact does not stem from some peculiarity of our procedure, and it is not generalizable to determinations of frequencies from colony counts at very different cell densities on selective and nonselective media in other assays.

## 4. Discussion

It was observed 25 years ago that extended incubation of $Lac^-$ *E. coli* on minimal lactose medium leads to an accumulation of $Lac^+$ revertants in the bacterial population [17]. The report was controversial because it suggested that such mutations represented "adaptive mutation," in which nongrowing bacteria produced $Lac^+$ revertants when the revertants were needed for survival. Even more controversial was the suggestion that this may also represent "directed mutation," in which the enhanced mutagenesis is restricted to a specific gene that confers the needed phenotype. Cairns and Foster [18] explored the phenomenon in detail

**Table 5**
Suppression of the dilution artifact by filler cells of *Escherichia coli* strain WP2 mixed with strain CC109.

| CC109 : filler ratio[a] | Colonies per plate on glucose[a] | Relative plating efficiency[b] | Revertants per plate on lactose[a] | Revertant frequency ($Lac^+/10^8$)[b] |
|---|---|---|---|---|
| A. Mixture of CC109 with viable but nongrowing cells of strain WP2 ($trp^-$): | | | | |
| 1:0 | 317.0 | $1.00 \pm 0.049$ | 62.0 | $39.12 \pm 5.50$ |
| 1:1 | 178.3 | $1.12 \pm 0.097^{NS}$ | 37.3 | $47.11 \pm 6.89^{NS}$ |
| 1:3 | 105.0 | $1.32 \pm 0.074^{*}$ | 19.3 | $48.79 \pm 9.92^{NS}$ |
| 1:7 | 50.0 | $1.26 \pm 0.058^{NS}$ | 12.0 | $60.57 \pm 5.05^{NS}$ |
| *p* value | | 0.047 | | 0.27 |
| B. Sham mixture CC109 with nonliving cells of CC109: | | | | |
| 1:0 | 222.0 | $1.00 \pm 0.072$ | 86.0 | $72.76 \pm 21.29$ |
| 1:1 | 102.0 | $0.92 \pm 0.036^{NS}$ | 46.3 | $78.40 \pm 3.43^{NS}$ |
| 1:3 | 60.3 | $1.09 \pm 0.064^{NS}$ | 32.7 | $110.40 \pm 19.63^{NS}$ |
| 1:7 | 29.0 | $1.04 \pm 0.062^{NS}$ | 24.3 | $164.40 \pm 12.54^{*}$ |
| *p* value | | 0.30 | | 0.01 |

[a] Strains CC109 and WP2 (*trpE56*) were grown at 35 °C for 18 h in LB in a shaker. Strain WP2, which was unable to grow in the minimal medium because of its tryptophan requirement, was added in numbers complementing the dilution of CC109, thereby providing for similar numbers of viable cells per plate in all cases. Plating was in triplicate at densities of $\sim 2 \times 10^8$, $1 \times 10^8$, $5 \times 10^7$ and $2.5 \times 10^7$ cells of CC109 per plate of minimal lactose medium. The same procedure was followed for part B, using a sham mixture of viable CC109 with dead cells of CC109, rendered nonviable by extended incubation in saline without a carbon source.
[b] Relative plating efficiencies and revertant frequencies are means ± SEM. The significance of differences from the reference point (CC109 without filler cells) was determined by ANOVA with a Bonferroni multiple comparisons test ($^{NS}$nonsignificant; $^{*}p < 0.05$).

**Table 6**
Absence of a dilution-associated artifact in an assay for gene conversion in *Saccharomyces cerevisiae* strain D7.

| Fold dilution[a] | Colonies per plate on supplemented minimal medium[a] | Relative plating efficiency[b] | Convertants per plate on selective medium[a] | Convertant frequency ($Trp^+/10^5$)[b] |
|---|---|---|---|---|
| 1× | 307.0 | 1.00 ± 0.019 | 184.0 | 0.56 ± 0.013 |
| 2× | 162.7 | 1.06 ± 0.037$^{NS}$ | 107.3 | 0.66 ± 0.030$^{NS}$ |
| 4× | 95.3 | 1.24 ± 0.067$^{NS}$ | 44.3 | 0.54 ± 0.023$^{NS}$ |
| 8× | 45.3 | 1.18 ± 0.102$^{NS}$ | 24.3 | 0.60 ± 0.036$^{NS}$ |
| 16× | 15.7 | 0.82 ± 0.035$^{NS}$ | 11.3 | 0.56 ± 0.066$^{NS}$ |

[a] Strain D7 was grown at 28 °C for 16 h in YEPD in a shaker. The cell density was $1.6 \times 10^8$ cells per ml. Plating was in triplicate. Yeast were diluted in saline for spreading at ~$3.2 \times 10^7$, $1.6 \times 10^7$, $8 \times 10^6$, $4 \times 10^6$ and $2 \times 10^6$ cells per plate of minimal medium without tryptophan (YMAI) to select for convertants and ~300, 150, 75, 37.5 and 18.8 cells per plate of fully supplemented minimal medium (YMAIT) to determine numbers of viable cells.

[b] Relative plating efficiencies and convertant frequencies are means ± SEM. The significance of differences from the reference point (~$3.2 \times 10^7$ cells on YMAI; ~300 on YMAIT) was determined by ANOVA with a Bonferroni multiple comparisons test ($^{NS}$nonsignificant).

and found the earlier findings to be reproducible. The "adaptive mutations" were frameshift mutations that arise in Lac⁻ stationary-phase cells under conditions of starvation when lactose is the only energy source [18]. The appearance of Lac⁺ revertants occurred after several days and depended on RecA⁺ gene function [18]. Such mutations in aging stationary-phase cells were unexpected, as the expression of new mutations normally occurs in dividing cells. Cairns et al. [17] suggested that this mutagenesis may be "directed," because the Lac⁺ mutations that relieved the starvation conditions appeared not to be accompanied by mutations that confer resistance to valine ($Val^R$).

The phenomenon of adaptive mutagenesis has been confirmed in many studies, but the suggestion that the mutations may be directed has not found experimental support. Ambrose and MacPhee [15] refuted the idea of directed mutation by showing that the difference between Lac⁺ and $Val^R$ mutations is explainable by conditions of catabolite repression. Catabolite repression is a regulatory mechanism by which *E. coli* preferentially uses glucose as a carbon source when other possible sources, such as lactose or glycerol, are also present. The presence of a glucose catabolite leads to lower levels of cyclic AMP, which is needed to generate cAMP-CAP, a regulatory molecule required for the utilization of lactose. The mechanism permits *E. coli* to use the better carbon source [20].

Unlike glucose and lactose, αMG cannot be used by *E. coli* as a carbon source. Ambrose and MacPhee [15] showed that αMG depresses cellular concentrations of cyclic AMP and thereby behaves similarly to glucose as a catabolite repressor. When they plated valine-sensitive ($Val^S$) or Lac⁻ strains of *E. coli* on medium selective for valine-resistance ($Val^R$) or lactose utilization (Lac⁺), they found that the numbers of $Val^R$ or Lac⁺ colonies were reduced on medium containing αMG along with a nonrepressing carbon source (i.e., glycerol or lactose). Thus, the initial failure to observe $Val^R$ mutagenesis [17] could be ascribed to the presence of a catabolite repressor in the valine medium (in that case, glucose), not mutagenesis directed to *lacZ* [15]. The adaptive/stress mutagenesis from Lac⁻ to Lac⁺ depended on the absence of catabolite repression. Our finding (Table 3) that αMG similarly suppresses the dilution-associated artifact links this phenomenon to adaptive mutagenesis. The dilution-associated artifact is clearly evident in the absence of catabolite repression. Prevention of the artifact by αMG is not absolute, in that we also observed it to occur weakly in the presence of αMG after long incubation.

Several hypotheses have been advanced to explain adaptive mutation from Lac⁻ to Lac⁺. Rather than being directed mutation, the evidence indicates that the process occurs by randomly occurring spontaneous mutagenesis. Explanations include cryptic growth models, in which conventional growth-dependent mutagenesis occurs in rare growing cells, and hypermutation models [21–22]. Enhanced mutagenesis could, in principle, occur by a small fraction of cells within a colony entering a hypermutable state [23,24] or by a more global process in which all cells are able to generate the mutations [25]. Foster [19] proposed that the stressed bacteria are primed for genetic change by a transient mutator state. Stationary-phase *E. coli* under strong selection pressure without catabolite repression is reported to accumulate spontaneous mutations through error-prone mechanisms that require recombination functions and are associated with polymerases of translesion synthesis, especially DNA polymerase IV encoded by *dinB*⁺ [16,21].

Roth et al. have argued that mechanisms other than a transient mutator state can explain the adaptive mutation phenomenon. They proposed that amplification of slightly leaky Lac⁻ alleles, minimal amounts of growth, and accumulating mutations in the elevated number of *lac* targets offer a likely explanation that requires only conventional processes of genetic alterations and selection [22,26–28]. Other studies have suggested that amplification, rather than being an intermediate that leads to point mutations through conventional means, may be an independent pathway of adaptive genetic change [29].

The Roth group has recently proposed an alternative to both the stress-induced mutation hypothesis and the selective amplification hypothesis. They presented data supporting the view that stable and unstable revertants both arise prior to selection in rare cells that have a higher than typical copy number of the F′ episome, not a tandem amplification in the *lac* region [30]. Like amplification within the F′, the higher copy number increases the number of targets and would lead to an elevated revertant frequency. Other recent studies have argued in favor of stress mutagenesis by showing that mutation rates can be elevated threefold to fourfold without selection for growth fitness by controlling the intracellular concentration of the transcription factor RpoS [31], which had earlier been reported to be required for adaptive stress mutagenesis [21]. Thus, the debate continues owing to the difficulty of cleanly differentiating whether the elevated revertant frequencies observed after long incubation on selective media are ascribable to stress-induced mutagenesis [19,32] that may involve error-prone DNA synthesis during recombination in nongrowing cells [33] or to the growth limitation serving exclusively as a selective agent for revertants that arise at conventional mutation rates [30,34–36].

Our data show that different frequencies of spontaneous revertants are recovered depending on the density of viable cells in the *E. coli lacZ* reversion assay. An accumulation of Lac⁺ revertants should be expected with long incubation under starvation conditions in light of earlier findings on adaptive/stress mutagenesis [19]. However, our observations were made under conventional conditions of a quantitative reversion assay with 48-h incubation of plates [3]. This phenomenon can cause dose-dependent increases in revertant frequency after chemical treatments that are toxic but not mutagenic. The elevated frequencies mimic chemical mutagenesis, and they also occur without chemical treatment when there are fewer viable cells per plate of selective medium. This phenomenon is a potentially serious source of artifact in the

measurement of chemical mutagenesis in the *lacZ* reversion assay, especially in cases of weak mutagenic responses.

The results that mimic mutagenicity are reproducible in strains that revert by frameshift mutations. Low revertant frequencies make it less practical to evaluate this phenomenon in the base-pair-substitution strains of the *lacZ* assay. However, reports of stress mutagenesis in other assays extend to base-pair substitutions [37]. A trace of glucose in minimal lactose medium increases the magnitude of the *lacZ* dilution artifact, suggesting that the phenomenon depends on growth. Thus, the artifact is not exclusively a division-independent increase in stationary phase, as has been proposed for adaptive mutation [38]. More than one of the mechanisms proposed to explain the accumulation of $Lac^+$ revertants under starvation conditions [18,19,21,22,26,30] may contribute to the phenomenon. Independently of the mechanism, the risk of an artifact in the measurement of induced mutation frequencies is clear.

It has been common practice to use survival plates at low cell density to calculate mutant frequencies in selection plates at high cell densities. While the assumption underlying this calculation may seem perilous, it has been done in many assays for decades. The dilution procedure that revealed a potential artifact in the *lacZ* reversion assay (Table 2) showed no comparable problem in an assay for gene conversion in yeast strain D7 (Table 6). Leaky alleles are prone to violation of the underlying assumption, and the validity of calculating revertant, mutant or recombinant frequencies from platings at very different cell densities should be evaluated independently for each assay.

The phenomenon that we described in the *lacZ* assay had been noticed by others many years ago. Miller et al. [39] stated in the methods section of a paper on *ndk* strains of *E. coli* that they plated derivatives of CC107 at high plating densities and that cells of a scavenger strain (J93) were added when it was necessary to plate $<10^9$ cells of a culture. Using scavenger cells, 10-fold dilutions of the culture being tested yielded the expected 10-fold fewer revertants [39]. Unfortunately, this finding received little attention in the literature. Our results using WP2 as filler cells (Table 5) support the same interpretation. The experiments of Miller et al. [39] suggest a means of avoiding the dilution artifact through the use of scavenger cells. In an earlier study, Waleh et al. [40] used filler cells to measure toxicity in the plate-test procedure of the Ames assay. The results revealed complex relationships between toxicity to the indicator strain and the density of the filler cell population. The use of filler cells offers the prospect for avoiding artifacts in the *lacZ* assay, but careful calibration of procedures would be required before accepting the assumption that survival data collected at low cell density in nonselective medium can be applied to revertant counts in selection plates. If appropriate accommodation is not made, the dilution artifact is a potentially serious source of error in using the *lacZ* reversion assay for quantitatively evaluating the genetic activity of weak mutagens.

## Conflict of interest

The authors declare that there are no conflicts of interest regarding this work.

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