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Stationary phase-induction of G → T mutations in *Escherichia coli*

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Abstract

A series of *Escherichia coli* mutants, constructed originally by Cupples and Miller [C.G. Cupples, J.H. Miller, A set of *lacZ* mutations in *Escherichia coli* that allow raid detection of each of the six base substitutions, Proc. Natl. Acad. Sci. U.S.A. 86 (1989) 5345–5349], provides a unique system for quantifying base-change mutations, and the repair processes that limit their establishment, in bacteria under selective and non-selective conditions. We focussed on one strain in which a T → G replacement inactivates the *lacZ* gene. Reversions of this strain can occur through oxidation of G, leading to G → T transversions. We show that spontaneous reversions occurred both in lactose (selective) and glucose (non-selective) medium. The number of revertants per viable cell was much greater in medium containing lactose or both sugars than glucose alone. In glucose medium, the rate of reversion was highest below 0.6% glucose and strongly inhibited at and above that level. Evidence that reversions occurred through G → T transversions in both lactose and glucose media came from two observations: by sequence analysis of a series of revertants and by comparing the reversion rates in strains possessing and lacking the *mutM* gene (encoding formamidopyrimidine DNA glycosylase, FPG). However, the rate of reversion was stimulated by reducing O_2 to 1% and inhibited or delayed by increasing O_2 to 90%. In *mutM*$^-$ cells grown on glucose medium, the proportion of revertants increased over a 5-day period. In contrast, in *mutM*$^+$ cells, revertants appeared primarily during the first 2–3 days after plating; few new revertants appeared in the following days. These data imply that base excision repair initiated by FPG was less effective in the first 2 days and more effective later in stationary phase.

Keywords: Adaptive mutations; *Escherichia coli*; FPG; G to T transversions; lacZ operon; MutM; Guanine oxidation

1. Introduction

Cairns et al. [1] noted that reversions of *Escherichia coli* auxotrophs to prototrophy occurred more rapidly under selective conditions than in the absence of selection. This effect has since been reported in other systems, including ones with base replacement and frame shift mutations. Cairns originally suggested that mutagenesis was being "directed" toward genes that could allow survival, but the prevailing theory is that the stress of the selective conditions increases mutation rate in non-advantageous and advantageous genes, including the gene(s) leading to prototrophy ("adaptive" mutagenesis). There are several hypotheses to explain the mechanism of adaptive mutagenesis, including base modification resulting in mispairing, polymerase errors, errors in recombination, and amplification of genes leading to increased expression of low-activity enzymes [2–5].

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Much of the previous work has involved strains with the FC40 plasmid, which has a frameshift in the *lacZ* gene and generally reverts by deletion mutation [6–8]. Mutations of other genes, including *lacZ* and *tet* genes on the F′ episome, also often involve frameshifts [9]. Studies by Bridges et al. with carotenoid scavengers, catalase, and *mutY* and *mutM* mutants indicated that reversions of tryptophan auxotrophs to prototrophy are caused by reactive oxygen species acting as mutagens [10,11]. They suggested that oxidation of guanine (forming 7,8-dihydro-8-oxoguanine) leads to many, although not all, of the mutations observed. Some of these mutations are caused by oxidized guanine mispairing with adenine, and some involve frameshifts [12,13]. There is also evidence implicating reactive oxygen in some FC40 *lacZ* frameshift mutations [14].

An alternative to using the FC40 frameshift mutant employs a set of revertible *lacZ* gene mutants that contain single-base replacements that inactivate the expressed β-galactosidase [15,16]. As restoration of β-galactosidase expression in these strains requires reversion of the mutant *lacZ* alleles for activity, this system permits the identification of specific base changes caused by suspected mutagens. For example, Palmer et al. [17] used these mutants to show that UV-A (but not UV-B) specifically increased the rate of reversions in mutants with a T → G mutation. Combining the *lacZ* replacements with *mutM* knock-out mutations, these authors also showed the influence of base excision repair on the rate of reversion.

Because the expression of the *lacZ* gene can be easily monitored by a color reaction, the Cupples and Miller strains provide a useful system to investigate the phenomenon of adaptive mutagenesis through base substitution, both under selective (lactose) and non-selective (glucose) conditions, and its relationship to starvation and stationary phase [16].

In this study, we compare the rates and time courses of reversion in lactose and glucose media and in *mutM*$^+$ and *mutM*$^-$ strains. We also examine the possible role of metabolic stress on mutagenesis by varying glucose and O_2 concentrations. Finally, we confirm the sequences of revertant genes from mutants arising in both lactose and glucose and in *mutM*$^+$ and *mutM*$^-$ strains.

2. Materials and methods

2.1. Strains and chemicals used

E. coli strains CSH104 and the HS1194 derivative were previously described [17]. Both strains are *ara* Δ(*gpt-lac*)5 and possess a F′104 *lacZ*(Ala-461) plasmid. In addition, in HS1194 the *mutM (fpg)* gene is transposon-inactivated (*fpg*::*kan*^{r}) [17]. HS1194 is thus isogenic to CSH104 with the exception of *fpg*::*kan*^{r}. Lactose was obtained from Sigma Chemical Co., which rated the preparation as 99% total lactose and 70% β-lactose.

2.2. Measurement of reversion

In a typical experiment, an overnight culture was grown in M9 salts (Gibco, BRL) plus 27 μg ml^{-1} thiamine HCl, 1 mM $MgSO_4$, and 2% glucose to a concentration of 3.9 (±0.8) × 10^9 cells ml^{-1} (mean ± S.D., $n = 6$). The high glucose concentration was used to limit reversion before the culture was plated (see Section 3.1 and Fig. 3). The overnight culture was centrifuged and resuspended in an equal volume of phosphate-buffered saline solution, and 100 μl were plated on 1.5% agar-solidified medium containing M9 salts supplemented with 27 μg ml^{-1} thiamine HCl, 1 mM $MgSO_4$, 0.2% glucose or lactose, 0.4 mM IPTG, and 0.4 mM 5-bromo-4-chloro-3-indole-β-D-galactoside (X-gal) in 10-cm diameter plates. Plates were incubated at 37 °C. Each day, blue colonies were counted, and the accumulated number of colonies was reported. To incubate plates in controlled atmospheres, the plates were placed in hermetically sealed, 25-l boxes and flushed with N_2 or O_2 at 500 ml min^{-1} for an appropriate length of time (N_2, 150 min; O_2, 104 min).

2.3. Cell viability

The cells were washed off plates with 1 ml of phosphate of either buffered saline or LB medium. The suspension was then collected and the washing was repeated; the two collected suspensions were combined. The suspensions were diluted immediately, and the number of viable cells was determined by dilution plating on LB medium. In one test for reproducibility, eight independent overnight cultures of CSH104 were plated on standard M9 medium containing 0.2% glucose and incubated overnight. The washing procedure gave a calculated mean number of cells per plate of 1.3 × 10^{10} with a S.D. of 2.0 × 10^9, representing a coefficient of variation of 16%. Because we placed a priority on measuring the accumulation of revertant colonies on each plate over time, calculations on a per-cell basis were made using colony counts from parallel experiments.

2.4. Sequences of revertant lacZ genes

Fragments of the *lacZ* genes from revertant colonies were amplified by polymerase chain reaction using forward and reverse primers 5′-CAAATAATATCGGTTGCGGAGGTG-3′ and 5′-AATATTGAAACCCACGGCATGGTG-3′, respectively. The fragments of 250 base pairs were isolated by agarose gel electrophoresis, and the sequences determined, using the forward primer, by the UC Davis Division of Biological Sciences Automated DNA Sequencing Facility.

3. Results

3.1. Reversion on lactose and glucose media

On M9 agar containing 0.2% lactose, IPTG, and X-gal, cells of CSH104 (*mutM*$^+$) and HS1194 (*mutM*$^-$) formed only discrete blue colonies. On M9, 0.2% glucose, IPTG, and X-gal, these cells formed a lawn with blue foci appearing later. In general, the foci seen in glucose plates were small and well separated, especially in plates with CSH104 (FPG proficient) colonies. In plates with HS1194 (FPG deficient) colonies, the numbers of foci were high, and it is likely that, because of superposition, the total number of revertants was even higher than the observed counts indicated.

The number of revertants per plate was normally lower in lactose- than in glucose-containing medium. For both HS1194 and CSH104, a combination of 0.05% glucose and 0.2% lactose gave a much greater number of revertants per plate than either sugar alone (Fig. 1). We hypothesized that this effect occurred because the glucose supported more growth than lactose alone, and

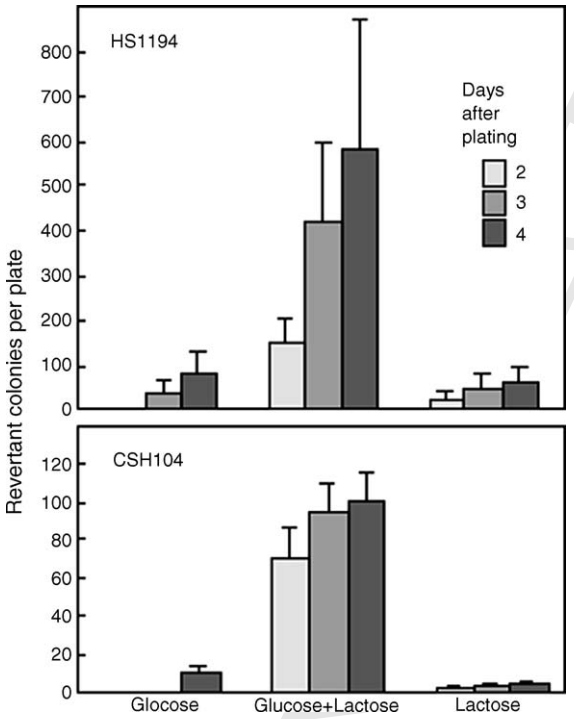

Fig. 1. Reversions of *lacZ*$^-$ cells to *lacZ*$^+$ in M9 medium containing 0.05% glucose, 0.2% lactose, or a combination of both 0.05% glucose and 0.2% lactose. Bars show the means from three independent experiments, ±S.E. Revertants only began to appear at day 2. Note the difference in the scales on the *y*-axis. Although variation among experiments was high, within each experiment the relationship among the three treatments was the same as shown by the means.

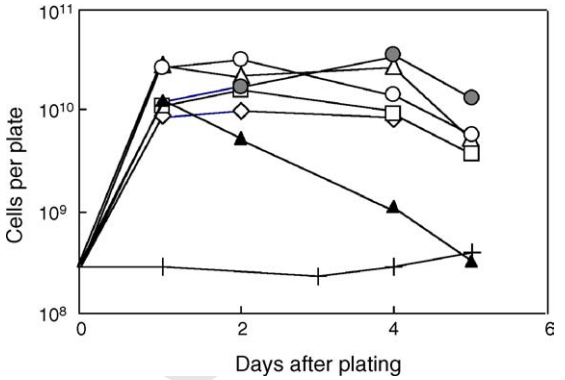

Fig. 2. Growth of CSH104 cells in lawns on plates of M9 medium containing glucose or lactose carbon source. Glucose (%): open diamond, 0.05; open square, 0.1; open triangle, 0.2; open circle, 0.4; filled circle, 0.6; filled triangle, 2.0. Lactose (%): +, 0.2. Cells were washed off the plates, diluted, and plated for counting as described in the Methods section. Results obtained with HS1194 were similar.

lactose allowed more revertants to survive, outcompete wild type, and thrive than did glucose alone. To test this hypothesis, we compared the number of revertants to the number of cells that could be recovered from the plates (Fig. 2). The number of cells in glucose medium was 30–100 times the number in lactose; the numbers of revertants per viable cell were approximately equal in media containing lactose alone and lactose plus glucose, but 10-fold lower in glucose medium (Table 1).

The number of revertants per 10^8 cells of CSH104 and HS1194 cells on M9 medium containing different amounts of glucose as carbon source peaked at 0.2–0.4% glucose (Fig. 3). There were few or no revertants at 0.6% and none were apparent at 2% glucose. Control experiments established that revertant cells produced blue colonies after an overnight incubation on 2% glucose, indicating that the cells had used up enough glucose to overcome catabolite repression. Thus the absence of revertants at 2% glucose was not an artifact due to inhibition of expression of the *lacZ* gene. The numbers of revertants per viable cell were approximately the same from 0.05% to 0.4% glucose, but lower at 0.6% glucose

Table 1
Frequencies of reversions of CSH104 and HS1194 cells growing on M9 medium containing 0.05% glucose, 0.2% lactose, or both sugars, for 4 days

Strain	Media		
	Glucose	Glucose + lactose	Lactose
CSH104	9.3×10^{-10}	1.1×10^{-8}	1.3×10^{-8}
HS1194	8.9×10^{-9}	6.4×10^{-8}	1.8×10^{-7}

Values represent revertants per viable cell, calculated from the data of Figs. 1 and 2.

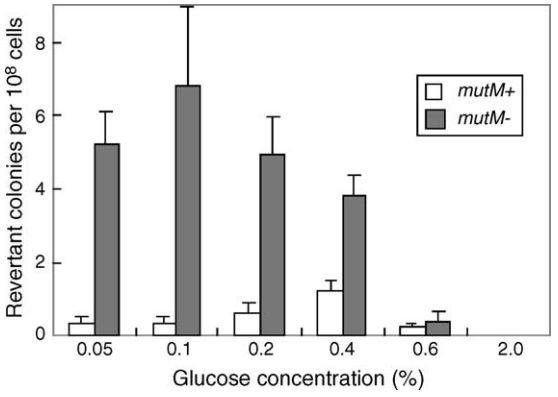

Fig. 3. Reversions of *lacZ*$^-$ cells to *lacZ*$^+$ in M9 medium containing different concentrations of glucose. Cells were plated on M9 medium containing IPTG and X-gal, and blue colonies were scored each day. Values shown represent the total *lacZ*$^+$ colonies (mean ± S.E.) at 4 days after plating. Data are from three independent experiments for CSH104 (*mutM*$^+$) and seven for HS1194 (*mutM*$^-$).

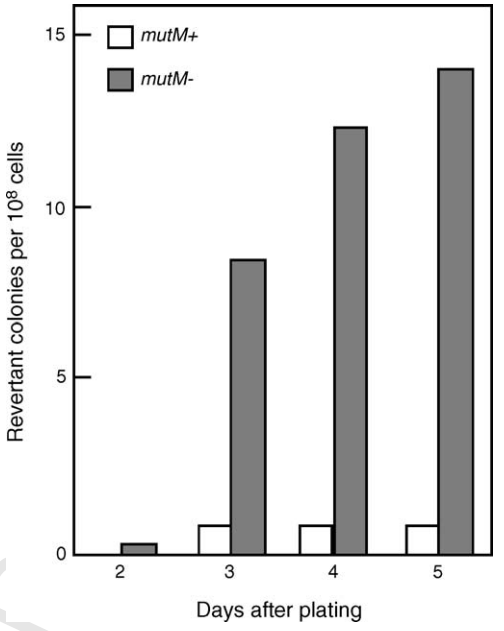

Fig. 4. Accumulation of *lacZ*$^+$ revertants in *mutM*$^+$ (CSH104) and *mutM*$^-$ (HS1194) plates over time. Cells were plated on M9 medium containing 0.2% glucose (plus IPTG and X-gal). A representative experiment is shown. Table 2 gives statistical information for eight independent experiments.

and very low at 2% glucose. In 2% glucose, the cells died quickly after reaching their maximum population density (Fig. 2), and this could have contributed to the lack of revertants, but this explanation does not hold for 0.6% glucose. The data suggest that the rate of reversion depends both on nutrient availability for cell division and is alleviated (or blocked, depending on point of view) by high glucose concentrations.

3.2. Rates and time courses of reversion

In glucose and lactose plates seeded with either CSH104 or HS1194, revertants did not start to appear until the second day of incubation (Fig. 1), well after the culture reached stationary phase (Fig. 2). Furthermore, for strain CSH104, plates of M9 medium with 0.2% glucose inoculated with 1×10^6 cells had the same number of revertants as those inoculated with 4×10^8 cells (mean 101%, minimum 45%, maximum 189%, nine comparisons). In HS1194, plates inoculated with 1×10^6 cells had approximately 60% the number of revertants as those inoculated with 4×10^8 cells (mean 62%, minimum 24%, maximum 101%, six comparisons). These observations are consistent with the interpretation that the reversions occurred after the cells reached stationary phase.

Table 2 shows the ratio in the number of HS1194 revertants to CSH104 revertants over 5 days, averaged from eight independent experiments. Although the number of revertants was two to three times higher in HS1194 than CSH104, even at 2 days, the ratio rose to over 10-fold higher by 5 days. For CSH104 (*mutM*$^+$), revertants appeared on M9 medium + 0.2% glucose in 2–3 days, but, after that period, few additional revertants appeared. In contrast, For HS1194 (*mutM*$^-$), revertants initially appeared at the same time, 2–3 days, but they continued to accumulate over a longer

Table 2
Ratio of *lacZ*$^+$ revertants on *mutM*$^-$ (HS1194) plates to the number on *mutM*$^+$ (CSH104) plates, averaged from eight independent experiments

	Days after plating			
	2	3	4	5
Revertants/cell:HS1194	$3.0\ (\pm 1.1) \times 10^{-9}$	$3.2\ (\pm 1.0) \times 10^{-8}$	$6.1\ (\pm 1.4) \times 10^{-8}$	$5.7\ (\pm 1.7) \times 10^{-8}$
Revertants/cell:CSH104	$1.0\ (\pm 0.4) \times 10^{-9}$	$4.6\ (\pm 0.8) \times 10^{-9}$	$5.7\ (\pm 0.9) \times 10^{-9}$	$5.4\ (\pm 1.4) \times 10^{-9}$
Ratio	3.3 ± 1.0	3.9 ± 1.0	7.2 ± 1.2	10.5 ± 3.1

In all cases, cells were plated on M9 medium containing 0.2% glucose (plus IPTG and X-gal) and incubated at 37 °C at ambient O_2 concentration. Values represent means ± S.E.; for "ratio," the table gives the mean of ratios (from different experiments), not the ratio of means.

Table 3
Reversion to *lacZ*$^+$ at different partial pressures of O_2

O_2 concentrations (%)	Strain	
	HS1194	CSH104
≤1	$8.4 (\pm 3.0) \times 10^{-7}$ a	$2.1 (\pm 0.5) \times 10^{-8}$ a
5	$2.4 (\pm 1.2) \times 10^{-7}$ b	$2.6 (\pm 0.7) \times 10^{-8}$ a
21	$2.0 (\pm 0.7) \times 10^{-7}$ b	$2.5 (\pm 0.6) \times 10^{-8}$ a
30	$3.6 (\pm 1.4) \times 10^{-7}$ ab	$2.5 (\pm 1.3) \times 10^{-8}$ a
90	$7.3 (\pm 7.3) \times 10^{-10}$ c	$2.8 (\pm 2.8) \times 10^{-9}$ b

Cells were plated on M9 medium containing 0.2% glucose (plus IPTG and X-gal) and incubated at 37 °C. Numbers indicate revertants per cell on day 4 (mean ± S.E.; $n = 3$, except 21% O_2 $n = 6$). In each column, means not followed by the same letter differ significantly at the 95% confidence level, as evaluated by *t* test applied to log transformed data.

period of time (at least 4–5 days, after which counting became impractical) (Fig. 4). Most revertants of CSH104 cells were robust (large and dark colonies), whereas many revertant colonies of HS1194 were very small.

3.3. Effect of O_2 concentration

Because G → T transversions can result from mispairing of adenine with 8 oxo-dG formed by the reaction of guanine with reactive oxygen species, we tested the effect of O_2 concentration on the rate of reversion. Between ≤1% and 30% O_2, we found high numbers of revertants on plates inoculated with HS1194 cells and lower, but still substantial, numbers of revertants with CSH104 cells. However, at 90% O_2 there were few or no revertants apparent in either CSH104 or HS1194 plates (Table 3). The fact that a few revertants appeared in 90% O_2 showed that the O_2 did not inhibit color formation. 90% O_2 plates that were moved to 21% O_2 after 5 days showed revertants within one additional day, and cells recovered from plates that had been in 90% O_2 for 4 days showed full viability (1.3×10^9 on 0.05% glucose to 1.4×10^{10} on 0.6% glucose), indicating that the cells had not been killed by high levels (90%) of O_2.

3.4. Revertant genes

Mutations could arise either through reversion or suppression by second site mutations. To confirm that the mutant *lacZ* allele in the indicator strains had truly reverted, a randomly selected set of *LacZ*$^+$ colonies was isolated from *mutM*$^+$ and *mutM*$^-$ strains plated on lactose and on glucose. In each case, a PCR fragment spanning the original mutant base was amplified, purified, and sequenced, and its sequence was compared to the sequence of the parental mutant *lacZ* allele. Every revertant possessed a G → T transversion at the predicted position (Fig. 5). There were no deletions or insertions identified in the amplified PCR fragments.

4. Discussion

4.1. Mechanism of reversion

Three possible mechanisms have been hypothesized to account for stimulation of mutagenesis in *E. coli* under stressful conditions. The lactose-competent cells of HS1194 and CSH104 can arise as a result of a G:C to T:A transversion in the *lacZ* gene [16]. The G → T transversions considered in this study may be caused by oxidation of guanine, followed by 8-oxo-G mispairing with A [2,11,18,19]. Other mechanisms of reversion include the induction of error-prone polymerase and the amplification of the *lacZ* gene leading to multiple adjacent copies of *lacZ* on the F′ plasmid [5,20].

The fact that the revertants all showed G → T transversions indicates that amplification by itself is not the mechanism of reversion. Although it does not rule out amplification in addition to transversion, we did not see mixtures of G and T in PCR fragments from pure revertant cultures. The fact that the revertants all showed G → T transversions does not rule out error-prone polymerase as the mechanism of reversion if the G → T

Fig. 5. Base sequences of a PCR fragment containing the relevant base of the *lacZ* gene of revertant and non-revertant strains of *mutM*$^+$ (CSH104) and *mutM*$^-$ (HS1194) cells. Revertant strains were obtained from lactose plates, using both growth and X-gal hydrolysis as criteria, and from glucose plates, using X-gal hydrolysis as the sole criterion. Selection from glucose plates required one or two colony purifications by dilution and re-plating.

transversion is the only change that can restore activity to the β-galactosidase [16].

The evidence that the *lacZ* reversions considered here occur through guanine oxidation and mispairing include: (a) the observation that all revertants showed G → T transversions. However, we recognize that selection, either naturally on lactose medium or artificially from glucose medium, may have enforced this situation; (b) the inhibition of reversion in *mutM*$^+$ cells. The *mutM* product, FPG, is known to excise 8-oxo-G from DNA. From our data showing that the difference in reversion rate between *mutM*$^+$ and *mutM*$^-$ cells was greater in the later phase of stationary phase (4–5 days after plating), we conclude that 8-oxo-G played a greater part in reversion at that time. Palmer et al. [17] also reported that G → T transversions in *lacZ* occurred more frequently in *mutM*$^-$ than *mutM*$^+$ cells, whereas the frequency of other transversions and transitions was similar. Bridges et al. [10] reported that a tyrosine auxotroph with a *mutM* deficiency failed to exhibit greater rates of mutation to prototrophy than its parent strain, although *mutY*$^-$ and *mutM*$^-$*mutY*$^-$ strains did show higher rates of mutation. Although the results with respect to *mutM* seem contradictory to ours, *mutY* (adenine-DNA glycosylase) is thought to work in the same transversion pathway, because A is the base that mispairs with 8-oxo-G.

The observation that reversions occurred in *mutM*$^+$ cells early in stationary phase (i.e., 2–3 days after plating) – at about the same time and rate as in *mutM*$^-$ cells – suggests that for this period base excision repair initiated by FPG was less efficient. Alternatively, the mechanism of mutation in these cells did not involve 8-oxo-G, and the pre-mutagenic lesions were not correctable by FPG. Saumaa et al. [21] also found a change in the types of mutations occurring during stationary phase. Working with mutations in the promoter of a phenol utilization operon of *Pseudomonas putida*, they found that 70% of the selected strains had G → T or C → A transversions at 2–3 days, but less than 50% at 6–7 days. At the later times deletions, insertions, and unidentified mutations became more important, and the effect of the absence of *mutY* function less important.

4.2. Conditions promoting reversion

The rate of appearance of *revertants per cell* was higher both on lactose alone and lactose together with glucose than on glucose alone. The similarity in lactose with and without glucose suggests that neither the glucose itself nor the glucose-dependent increase in population of cells, with consequent crowding and competition, influenced the reversion rate. Since glucose represses the SOS response [22], yet did not reduce the rate in the presence of lactose, the higher rate in the presence of lactose seems unrelated to error-prone polymerase. It is possible that cells on the lactose media acquire greater numbers of plasmids carrying the mutant β-galactosidase gene; an increase in the number of plasmids carrying mutant β-galactosidase increases the chance that one of the copies for β-galactosidase will mutate to produce a functional β-galactosidase gene. This hypothesis holds so long as cells grown on lactose media undergo greater rates of plasmid replication than do cells grown on glucose media [22].

4.3. Effect of O_2

The high rate of reversion in HS1194 cells in 1% O_2 and its inhibition by 90% O_2 atmosphere was unexpected. We were aware that re-aeration following transient hypoxia induces an increase in reactive oxygen species in animal cells, so we were careful not to open the N_2-flushed chambers until we were ready to count revertants. Thus the reversions occurred under hypoxic conditions. There is evidence for hypoxia-stimulated generation of reactive oxygen by NAD(P)H oxidase 1 in cultured mouse cells and hypoxia-stimulated mutations in mismatch-repair deficient human carcinoma cells [23,24]. We hypothesize that the formation of 8-oxo-G in low O_2 occurs through reactive oxygen generated by an inhibited electron transport system. This hypothesis can be tested: if reversion reflects the production of reactive oxygen species (by leakage of electrons from a blocked electron transport chain), then we would expect a higher number of C → T transitions, but not other, non-oxidative transitions or transversions, in 1% O_2. If reversion in low O_2 reflects the induction of error-prone polymerases, we would expect higher numbers of all base substitutions under the same conditions.

5. Conclusions

The complex process of bacterial adaptation in stationary phase is thought to include accumulation of selective beneficial mutations. In this study we have shown that G → T transversions accumulate during early stationary phase even in the presence of an active base excision repair system. As these mutations were stimulated by low oxygen tension and inhibited by high O_2, it is likely that reactive oxygen species are important in the formation of this type of mutation, which is known to be produced as a consequence of oxidative damage to cells.

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