Regulating General Mutation Rates: Examination of the Hypermutable State Model for Cairnsian Adaptive Mutation

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ABSTRACT

In the *lac* adaptive mutation system of Cairns, selected mutant colonies but not unselected mutant types appear to arise from a nongrowing population of *Escherichia coli*. The general mutagenesis suffered by the selected mutants has been interpreted as support for the idea that *E. coli* possesses an evolved (and therefore beneficial) mechanism that increases the mutation rate in response to stress (the hypermutable state model, HSM). This mechanism is proposed to allow faster genetic adaptation to stressful conditions and to explain why mutations appear directed to useful sites. Analysis of the HSM reveals that it requires implausibly intense mutagenesis (10^5 times the unselected rate) and even then cannot account for the behavior of the Cairns system. The assumptions of the HSM predict that selected revertants will carry an average of eight deleterious null mutations and thus seem unlikely to be successful in long-term evolution. The experimentally observed 35-fold increase in the level of general mutagenesis cannot account for even one Lac$^+$ revertant from a mutagenized subpopulation of 10^5 cells (the number proposed to enter the hypermutable state). We conclude that temporary general mutagenesis during stress is unlikely to provide a long-term selective advantage in this or any similar genetic system.

MUTATION rates vary widely among species (Drake 1999) in relation to a set of population parameters identified as significant by Muller (Muller 1932, 1964; Felsenstein 1974). Mutation rates tend to increase with population size and frequency of sexual recombination, and they tend to decline with increases in the information content of the genome. Thus species with high mutation rates generally have small genomes, large populations, and (in many cases, at least) high rates of sexual recombination (Lawrence and Roth 1999). Organisms with high mutation rates adapt genetically to rapidly changing conditions and minimize the costs of deleterious mutations by carrying very little genetic information and by having huge populations that include many individuals with impaired fitness along with some that remain largely free of deleterious mutations. Such species may be able to replicate faster by abandoning proofreading and repair. In contrast, species with low mutation rates have lifestyles that depend on maintaining a considerable body of genetic information that is placed at risk by mutation. They are more likely to adapt physiologically (rather than genetically) to stresses by regulating expression of their information. Consistent with these ideas, Drake (1991) provided evidence that for DNA-based microbes there is an inverse correlation between genome size and mutation rate—that is, organisms with a larger genome have lower mutation rates (per base pair).

In the context of these relationships, the apparently increased rate of reversion to Lac$^+$ that is seen in the experimental system of Cairns and Foster has suggested to several authors that organisms with low mutation rates might benefit from a mechanism that increased their mutation rates temporarily in response to stress, because such a mechanism would increase the production of beneficial mutations and thereby facilitate genetic adaptation (Rosenberg 1997, 2001; Radman *et al.* 1999, 2000; Giraud *et al.* 2001; McKenzie and Rosenberg 2001). However, such a mechanism would also increase the production of deleterious and lethal mutations. The quantitative implications of the resulting genetic load have not been evaluated for models based on the features and observed behavior of the Cairns system.

The costs of a temporary increase in general mutability can be divided into two categories. First, lethal mutations remove cells from the mutagenized population and reduce the potential yield of individuals with a beneficial mutation. Second, even genomes that acquire a beneficial mutation (without suffering a lethal mutation) are likely to acquire deleterious nonlethal mutations. Some of these associated mutations will cause

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growth defects under most conditions, but others will cause major growth defects under specific, relatively rare conditions for which the lost function is critical. In either case, deleterious mutations will compromise the long-term fitness of the genomes in which they occur. In the short run, it may seem better to survive the immediate stress, even at the cost of carrying such deleterious mutations ("If I'm facing death, why not roll the dice?"). But there is no way for a cell to know that the inducing stress will be fatal or if the immediate problem can even be solved by mutation; if it is not, then the costly mutagenesis is futile. If mutations can solve the problem, then a cost-benefit analysis is needed to determine whether a mutagenic mechanism can provide benefits sufficient to counterbalance its cost. To persist in evolution, any mechanism must be beneficial to its bearers *on average* under the full range of conditions experienced by the species. In organisms like *Escherichia coli* and Salmonella, which engage only rarely in sexual exchanges, it is difficult for a beneficial mutation and the mechanism that produced it to evade the cost of associated deleterious lesions. These problems of cost must be solved if any mechanism to increase general mutability is to persist. The difficulty of fixing beneficial mutations in asexual populations has been analyzed previously (Peck 1994; Orr 2000).

For *E. coli*, conditions that select in the short term for higher mutation rates have been identified both theoretically (Taddei *et al.* 1997) and experimentally (Sniegowski *et al.* 1997; Miller *et al.* 1999). When a small population is placed under conditions selecting for rare mutations that confer a large increase in fitness, genetic adaptation is limited by the supply of mutations; rare mutations provide a fitness benefit that can outweigh (in the short term and under the given conditions) the cost of associated deleterious mutations. But it is not clear how often such conditions appear in natural populations or how long they might persist. More importantly, it is not clear whether the mutable individuals (favored in the short term) are able to compete in the long term with the more prevalent (less mutable) types, especially in organisms with very little genetic exchange. The cost of mutagenesis is reduced only slightly by making mutagenesis temporary and limiting it to periods of selective stress; associated deleterious mutations remain in the genome and can impair growth long after the period of mutagenesis has ended.

The different mutation rates that characterize particular species have each evolved to fit a particular genetic lifestyle—with a characteristic genome size, population size, and recombination rate. Varying mutation rate under a temporary set of circumstances might be regarded as a strategy of moving between genetic lifestyles. Such shifts are likely to be difficult and costly because the parameters that constrain or are constrained by mutation rate are not likely to change rapidly (genome size, population size, and recombination rate). In the particular case of *E. coli*, with a large genome and a low mutation rate, even a temporary increase in mutation rate would be expected to provide favorable mutations only at a heavy cost of associated deleterious mutations. Could such a mechanism provide the long-term benefit required for it to evolve and to be maintained under selection?

In the system described by Cairns and Foster (1991; called the Cairns system below), a *lac* mutant is starved on medium containing lactose as the only available carbon source, and selective stress appears to induce and direct mutations to sites that restore a Lac^+ phenotype (Foster and Cairns 1992). The Lac^+ revertants arising under selection have been generally mutagenized during the reversion process since they have an increased probability of carrying associated mutations (Torkelson *et al.* 1997; Slechta *et al.* 2002b), but they are not stable mutators because they do not show an increased mutation rate during later nonselective growth. In the Cairns experiment, $\sim$100 Lac^+ revertants accumulate over 5–6 days. The bulk of the nongrowing parent population is neither killed nor mutagenized appreciably; that is, the number of viable starved parental cells does not change and the frequency of unselected mutations in that starved nonrevertant population increases very little (Bull *et al.* 2001). These observations suggest that stress induces a temporary mutagenic state in a subset of the initial plated population. These observations have been interpreted to explain why mutation appears to be directed to useful sites.

The hypermutable state model (HSM) devised by Hall (1990) seems to explain both the apparent directed mutation (Foster and Cairns 1992) and the general mutagenesis suffered by Lac^+ revertants (Torkelson *et al.* 1997). (See Figure 1.) A population of $\sim 10^8$ Lac^- cells is plated on minimal lactose medium. A subset of the starved nongrowing population, estimated at 10^5 cells (Torkelson *et al.* 1997), enters a hypermutable state in response to stress, while the bulk of the population remains viable but neither grows nor accumulates mutations. Cells in the hypermutable state will ultimately die due to lethal mutations unless they obtain a mutation (to Lac^+) that relieves the stress and allows cells to exit the hypermutable state before acquiring a lethal mutation. A few mutagenized cells can be detected that have not yet acquired either a Lac^+ reversion or a lethal, but do carry unselected nonlethal mutations (Bull *et al.* 2001). Mutations appear to be directed or focused toward useful sites because only a small subset of the population is mutagenized and all mutagenized cells that do not obtain the selected mutation are ultimately killed by lethal mutations. The HSM proposes that the behavior of the Cairns system reflects an evolved regulatory mechanism that responds to stress by inducing general (undirected) mutagenesis in a small subset of the stressed population (Hall 1992; Rosenberg 2001).

The behavior of the Cairns system appeared initially to support the hypermutable state model because revertants (Lac^+) do in fact show an increased probability of carrying associated unselected mutations while the bulk of the stressed population has suffered very little mutagenesis (Torkelson *et al.* 1997; Rosche and Foster 1999; Slechta *et al.* 2002b). The revertant cells show normal mutation rates during nonselective growth and therefore do not carry stable genetic mutators; thus they must have passed through a temporary hypermutable state. These observations have been interpreted as support for the idea that *E. coli* and many other organisms may increase their general mutability in response to selective stress (Foster 2000; Radman *et al.* 2000; Rosenberg 2001).

Here we examine quantitative predictions of the hypermutable state model. Previous mathematical descriptions of the model have not explicitly considered the cost of associated lethal or deleterious mutations (Lenski and Sniegowski 1995; Cairns 1998; Golding *et al.* 2001). We ask whether such a model can explain the number of Lac^+ revertants that arise under selection and the apparent direction of mutation to useful sites in the Cairns system. A broader question is whether stress-induced general mutagenesis (if it occurs) is likely to be the function of an evolved mechanism. These questions are distinct because some growth conditions may increase the mutation rate despite evolved mechanisms for preventing genetic change—not because of mechanisms that evolved to mutagenize the genome. The implausibility of the HSM is discussed in terms of an alternative model that explains the data without requiring regulated mutability.

RESULTS

Assumptions: In describing the HSM, we express the mutation rate m as the number of mutations/genome/time under selection. The mutations formed are divided into three fractions: a fraction a are Lac^+ reversion events; b are lethal mutations at other loci; and c are nonlethal null mutations at yet other loci. The sizes of these three fractions are related to each other by the relative target sizes of the three mutation classes. If induced mutagenesis is truly general, then all three mutant classes are expected to increase in parallel when the general mutation rate m is increased by the proposed regulatory mechanism. In comparing the mutation rate in starved nongrowing cells (mutations per cell per unit time) to the rate in unstressed, growing cells (mutations per cell per division), we treat the period of nongrowth under selection as one time unit. (It might be compared to a period of starvation occurring between two acts of cell division.) We assign relative target sizes to the three critical mutation types (Lac^+ reversions, lethal mutations, and nonlethal null mutations).

The parental *lac* mutation is a +1 frameshift that reverts (by a compensating −1 frameshift) at a rate of $\sim 10^{-8}$/cell/division during nonselective growth (Foster and Trimarchi 1994; Rosenberg *et al.* 1994). We consider only −1 frameshift mutations (needed for reversion to Lac^+) and ignore the fact that base substitutions and +1 frameshifts will also occur and contribute to deleterious associated mutations but not to reversion. These assumptions underestimate the cost of general mutagenesis and thus are generous to the HSM.

We estimate that reversion of the *lac* mutation is about one-tenth as likely as a null frameshift mutation in a typical gene. This is because the *lacZ* sequence in which reversion must occur is ~100 bp and the target for a null frameshift mutation in a typical gene is 1000 bp. We define lethal mutations as those that prevent growth on minimal medium containing a utilizable carbon source. We estimate that there are ~500 genes in the *E. coli* genome that give such mutations: 250 nonsupplementable essential genes and 250 biosynthetic genes (Schmid *et al.* 1989; Hughes and Andersson 1997). Thus the target for lethal mutation is ~5000 times larger than the target for reversion to Lac^+, implying $b = 5000a$. The genome contains ~4000 other genes that are nonessential for growth on minimal lactose medium in the laboratory. Most of these genes are expected to be important for long-term survival in a natural setting since they have been maintained by selection. Thus the fraction of mutations that are nonlethal but deleterious is ~40,000 times the frequency of Lac^+ revertants, implying $c = 40{,}000a$. Since we consider only these three classes of mutations, the accumulation of mutations per unit time or per division is $m(a + b + c) = m$. The units for m are mutations/cell/replication and during the starvation period, one replication corresponds to 5–6 days. The measured reversion rate to Lac^+ under nonselective conditions (10^{-8}/cell/division) is am and the total mutation rate is $(am + bm + cm) = 10^{-8} + (5000 \times 10^{-8}) + (40{,}000 \times 10^{-8}) = 45{,}001 \times 10^{-8}$ or 0.00045 mutations/cell/division (or per time under selection). If stress causes an increase in the general mutation rate, each component of this overall rate should increase by the same factor.

The basic problem: A set of random mutations large enough to include one Lac^+ reversion would on average include 5000 lethal mutations and 40,000 nonlethal null mutations. Thus, a set of mutations including 100 Lac^+ revertants would on average include 5×10^5 lethals and 40×10^5 nulls. According to the HSM, diagrammed in Figure 1, these mutations are imposed on a nongrowing subpopulation (N_m) that enters the hypermutable state. The mutagenized subpopulation must be small because the overall plated population (10^8 cells) shows very little increase in mutation rate (Bull *et al.* 2001) and is not noticeably killed by selection. The mutagenized population has been estimated at 10^5 cells (Torkelson *et al.* 1997; Rosenberg 2001). If a set of random mutations including 100 Lac^+ revertants is distributed among 10^5

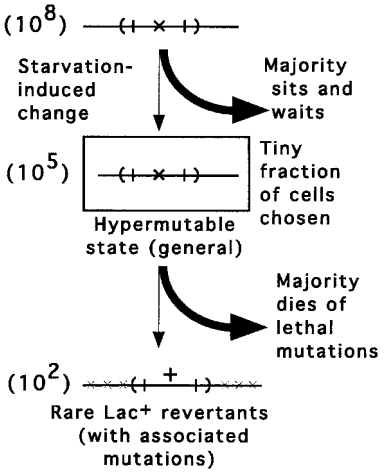

FIGURE 1.—Hypermutable state model (stress-induced mutations). When $\sim 10^8$ *lac* tester cells are plated on selective lactose medium, they are placed under stress. The model proposes that an evolved mechanism senses this stress and responds by placing a subset of cells (10^5) in a hypermutable state. The rest of the cells starve without dying, growing, or being mutagenized. Most cells in the hypermutable state suffer a lethal mutation and die, but $\sim$100 revert to Lac$^+$ and leave the mutable state before receiving a lethal mutation. These Lac$^+$ revertants carry associated, nonlethal mutations.

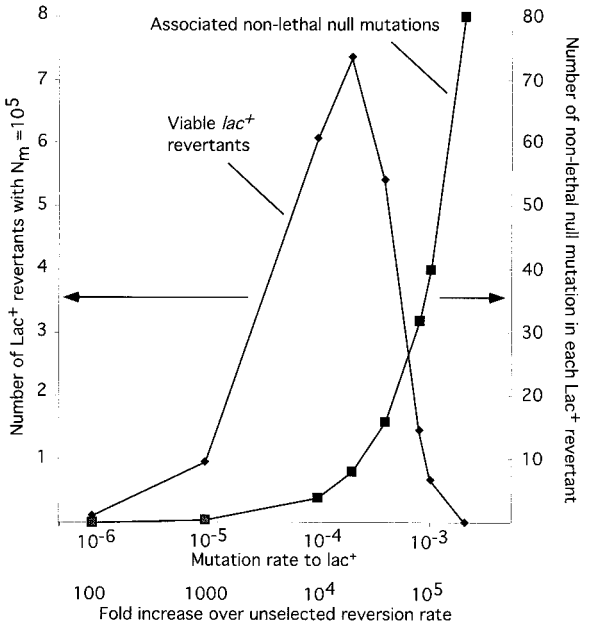

FIGURE 2.—Avoiding Poisson-distributed lethal mutations (HSM I). If cells in the hypermutable state receive a fixed dose of mutations and mutagenesis is not shut off upon reversion, there is very little chance of forming a revertant. The optimal dose of mutagenesis is very intense and produces only a few revertant cells.

cells, then each cell (and each Lac$^+$ revertant) would incur an average of 5 lethal mutations and 40 nonlethal null mutations. Any mutagenized cell that succeeds in initiating a revertant colony must avoid all of these expected lethal mutations. Long-term survival under natural conditions of such an induced beneficial mutation must occur despite the heavy load of associated nonlethal deleterious mutations.

Avoiding lethality by chance with constant mutagenesis: To estimate the magnitude of the problem of avoiding lethal mutations, we first consider what would happen if a fixed dose of mutations were distributed randomly and could be avoided only by chance (HSM I). According to HSM I, there is no way of shutting off the flux of mutagenesis following reversion. If each mutation type occurs independently, then its distribution among cells will follow a Poisson distribution. The probability of a reversion and no lethal mutation is

$$N_{\mathrm{Lac+}} = N_{\mathrm{m}} amte^{-bmt} = N_{\mathrm{m}} amte^{-5000amt}, \qquad (1)$$

where $N_{\mathrm{Lac+}}$ is the number of live revertants, N_{m} is the number of cells that enter the hypermutable state, m is the general mutation rate (mutations/cell/time under selection), a is the fraction of mutations that cause Lac$^+$ reversion, and b is the fraction of mutations that are lethal. The exponential term (the zero class of the Poisson distribution) gives the fraction of cells with no lethals when the expected dose of lethals per cell is bmt or 5000 times the frequency of Lac$^+$ revertants (amt). The yield of Lac$^+$ revertants varies as a function of the total mutagenic intensity mt. If we set $t = 6$ days (a time at which a typical Cairns experiment yields $\sim$100 revertants), then the relative mutagenic intensity is the ratio of m to the mutagenic intensity in unselected growing cells. As shown in Figure 2, the number of Lac$^+$ revertants reaches a maximum of about seven when the mutation intensity reaches a level 2×10^4-fold higher than that during nonselective growth. This is very intense mutagenesis. Further increases reduce the yield of revertants and ultimately result in death of the entire mutagenized population.

If reversion occurs as outlined above, then the load of associated nonlethal null mutations is large ($40{,}000a$). When 10^5 cells receive an optimum dose of mutations during selection (a 2×10^4-fold increase over the unselected rate), each of the seven viable Lac$^+$ revertants would carry an average of 8 associated nonlethal null mutations ($40{,}000 \times 10^{-8} \times 2 \times 10^4$). Increasing the mutagenesis intensity above this level is counterproductive. Increasing the size of the mutagenized population 10-fold provides 70 revertants but does not alter the expected load of associated mutations, which remains 8 nonlethal nulls per viable Lac$^+$ revertant. Thus the simplest form of the HSM neither solves the problem of producing 100 revertants nor seems likely to provide any revertants without a heavy load of associated mutations. Any revertants produced are unlikely to have credible long-term growth prospects in a natural population.

Avoiding lethality by shutting off mutagenesis after reversion: The model suggested by Hall (HSM II) is

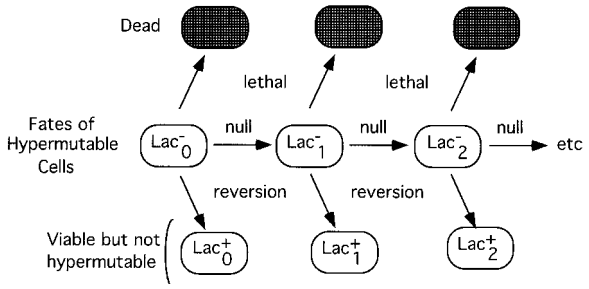

FIGURE 3.—The diagrammed process of reversion by the hypermutable state model with shutoff of mutagenesis following reversion (HSM II). This flow chart presents the process shown in Figure 1. Cells that enter the hypermutable state emerge only by reversion or death. They accumulate nonlethal null mutations during the time that elapses prior to reversion. Following reversion, mutagenesis stops and cells are no longer subject to killing.

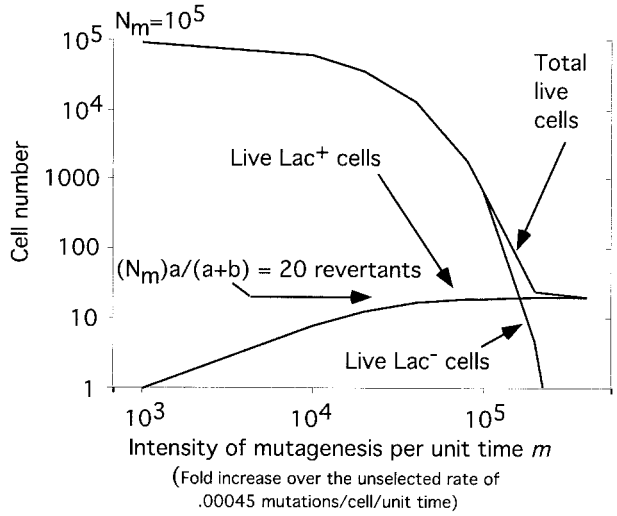

FIGURE 4.—Predictions of the HSM II model for killing and Lac^+ revertant formation. When 10^5 cells enter the hypermutable state, a maximum of 20 revertants arise and this requires very intense mutagenesis. Further revertant accumulation is prevented by mutational killing of the rest of the mutagenized population. This gives the appearance of directed mutagenesis because only revertant cells survive mutagenesis. If more cells are mutagenized, one can achieve more revertants with less intense mutagenesis, but then mutagenized Lac^- cells survive and contribute unselected mutants to the nonrevertant pool and the appearance of directed mutation is lost.

more sophisticated (HALL 1990, 1992). It proposes reducing the number of lethals by shutting off hypermutability as soon as a Lac^+ reversion occurs. Thus a Lac^+ revertant that arises prior to any lethal mutation avoids later mutagenesis and killing. This process is diagrammed in Figure 3. We represented HSM II as a set of differential equations. There are initially N_0 cells, N_m of which enter the hypermutable state. As described above, m is again the mutation rate (-1 frameshifts only), while a, b, and c are again the fractions of mutations that cause reversion, lethality, and nonlethal null phenotypes, respectively. Let X be the total number of live nonrevertant Lac^- cells in the hypermutable state, and let Y be the total number of live Lac^+ cells. Y increases (and X diminishes) as cells revert to Lac^+; X also diminishes as cells acquire lethal mutations. Loss of Lac^+ cells by lethal mutations is negligible since hypermutability ceases when stress is relieved by reversion.

Given the rates for reversion and lethal mutations (am and bm as described above) and ignoring, for the moment, the accumulating nonlethal null mutations, the time rates of change in number of live Lac^- cells in the hypermutable state and the number of produced Lac^+ cells are

$$dX/dt = -amX - bmX \quad (2)$$

$$dY/dt = amX. \quad (3)$$

The solution for initial conditions $X(t = 0) = N_m$ and $Y(t = 0) = 0$ is

$$X(t) = N_m e^{-(a+b)mt} \quad (4)$$

$$Y(t) = N_m[a/(a + b)][1 - e^{-(a+b)mt}]. \quad (5)$$

In Equation 5, the fraction is basically the probability that a cell obtains a Lac^+ reversion before it obtains a lethal. The right-hand term in brackets is the probability that all mutations (lethals + reversions) have not been avoided by chance. The number of live Lac^- cells drops as the number of Lac^+ revertants increases to a maximum of 20, at which point further accumulation is impossible because all other cells in the mutagenized population have been killed by lethal mutations. Thus by shutting off mutagenesis on reversion, HSM II allows only a few more revertants to be obtained from 10^5 mutagenized cells than were obtained by HSM I (20 rather than 7 revertants; compare Figures 2 and 4). With a small mutagenized population, the high ratio of lethal mutations to Lac^+ revertants (5000:1) strongly limits the yield of revertants.

Consequences of varying the size of the mutagenized population (N_m) and the intensity of mutagenesis are explored in Table 1, which shows the predicted numbers of Lac^+ revertants (column 4) and the increase in frequency of unselected mutations in the population of nonrevertant Lac^- cells (mutagenized plus unmutagenized; column 6). Lines 1–10 present conditions (described individually below) chosen to make predictions that best fit the experimental observations (line 12). Underlined values approximate those determined experimentally. Line 11 gives the results predicted by the null hypothesis that there is no mechanism for increasing mutagenesis during starvation. Below we consider the number of associated nonlethal mutations expected for each Lac^+ revertant.

Table 1, line 1, shows the case graphed in Figure 4 (mutagenesis intense enough to kill all of 10^5 mutagenized cells yields a maximum of 20 revertants). Since essentially no Lac^- cells survive mutagenesis (column

TABLE 1

Predictions of HSM II under various conditions

Line no.[a]	No. of cells entering the hypermutable state	Mutations/cell/ unit time = 6 days (fold increase over unselected rate)	Total number of Lac^+ revertants per 6 days	No. of Lac^- survivors of the hypermutable state	Fold increase in the frequency of unselected mutations in the total Lac^- population[b]
1	10^5	104 (2×10^5 times)	20	1	1×
2	10^5	18 (4×10^4 times)	17.3	1.3×10^4	6×
3	2×10^6	0.09 (200 times)	4	2×10^6	5×
4	5×10^5	22.5 (5×10^4 times)	92	4×10^4	22×
5	10^6	4.5 (10^4 times)	79	6×10^5	62×
6	10^6	6.26 (1.39×10^4)	100	5×10^5	71×
7	2×10^6	2.25 (5×10^3 times)	88	1.6×10^6	79×
8	10^7	0.45 (10^3 times)	98	9.5×10^6	96×
9	10^8	0.045 (100 times)	100	9.95×10^8	100×
10	10^8	0.016 (35 times)	35	9.9×10^7	35×
11[c]	10^8	0.00045 (1 time)	1	10^8	1×
12	Unknown	0.016 (35 times)[d]	100	Unknown	4×[e]

[a] Each line (1–10) presents the predictions of HSM II given one number of mutagenized cells and one intensity of mutagenesis (columns 1 and 2). Values were chosen so that the results agree with at least one of the three experimental observations shown in line 12. Values underlined show reasonable agreement with experimental observations.

[b] The values are the calculated increase in unselected mutations in the total Lac^- population, including both survivors of mutagenesis and unmutagenized parent cells.

[c] Line 11 presents the expectations if there were no increase in mutability during starvation.

[d] The value of 35 times is the average of two experimental estimates. Rosche and Foster (1999) estimated a 20-fold increase in the frequency of Mot^- mutants among Lac^+ revertants. An estimate in Salmonella suggested an ~50-fold increase in the frequency of unselected auxotrophic mutations in a 100-gene target (Slechta *et al.* 2002b).

[e] Initial experiments suggested no increase in associated mutagenesis (Torkelson *et al.* 1997), but more sensitive later tests suggested an ~4-fold increase (Bull *et al.* 2001).

5), there is no increase in the frequency of unselected mutations among the total Lac^- population (which now consists only of cells that never entered the hypermutable state). Thus mutation appears directed.

Line 2 shows a case in which the same number of cells undergoes ~5-fold less intense mutagenesis. The yield of revertants falls only a little, but now there are sufficient Lac^- survivors of mutagenesis that the frequency of unselected mutations increases in the surviving nonrevertant population. This approximates the frequency actually observed in nonrevertant cells by Bull *et al.* (2001). For both lines 1 and 2, mutation appears directed since the total Lac^- population (mutagenized plus unmutagenized) shows little or no mutagenesis. However, the intensity of mutagenesis required to produce ~20 revertants (a 10^5-fold increase in mutation rate) is beyond that achievable by chemical mutagenesis of nongrowing cells (due to extensive killing) and is still insufficient to produce the number of Lac^+ revertants (100) that are observed experimentally in the Cairns experiment.

In line 3, a more reasonable level of mutagenesis is applied and the mutagenized population is set so as to predict the level of mutagenesis observed for the Lac^- population (5×). Under these conditions very few Lac^+ revertants are predicted.

Lines 4–8 show various combinations of mutation rate and size of the mutagenized population, all chosen to yield about the number of Lac^+ revertants seen experimentally (100). In all of these cases, the required level of mutagenesis is very high and the Lac^- population shows a higher increase in associated mutations than is observed experimentally.

In lines 9 and 10, the intensity of mutagenesis is set at or near that observed experimentally and the mutagenized population is adjusted to give the maximum number of Lac^+ revertants. This requires mutagenizing the entire plated population and results in loss of all appearance of directed mutation; that is, Lac^+ and Lac^- cells have been mutagenized to the same extent.

We were unable to find any set of parameters for which our implementation of HSM II agrees even approximately with the experimental data.

The load of unselected null mutations predicted by the hypermutable state model: Above we considered only the effect of lethal mutations on the yield of revertants. Another cost of increasing general mutation rates is accumulation of nonlethal mutations that reduce fitness in the long term since the affected genes were maintained under selection. To estimate this cost of general mutagenesis, we calculated the distribution of nonlethal mutations among Lac^+ revertants arising under various conditions.

At the outset (time $t = 0$), we assumed that no cells

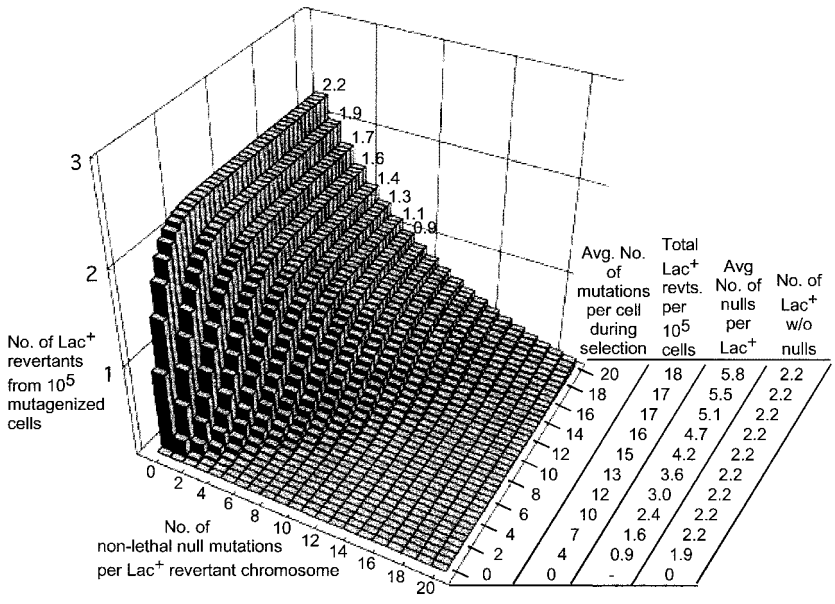

FIGURE 5.—Distribution of nonlethal null mutations among Lac$^+$ revertants. For each intensity of mutagenesis given (z-axis), a histogram presents the distribution of unselected mutations (y-axis) among the predicted Lac$^+$ revertants (x-axis). As the maximum number of 20 revertants is approached (at high levels of mutagenesis), the average number of associated unselected mutations approaches eight. Only ~2 of these revertants are free of associated unselected mutations.

have nonlethal mutations and all cells are Lac$^-$. Let Lac$_i^-$ refer to cells that are phenotypically Lac$^-$ with exactly i null mutations. Let Lac$_i^+$ refer to cells that are phenotypically Lac$^+$ with exactly i associated nonlethal mutations. Clearly, the Lac$_i^-$ cell population must decrease with every Lac$^+$ reversion (resulting in a corresponding increase in Lac$_i^+$), with every nonlethal mutation (resulting in a corresponding increase in Lac$_{i+1}^-$ cells), and with every lethal mutation. The number of Lac$_i^-$ cells will increase with every nonlethal mutation that occurs in a Lac$_{i-1}^-$ cell. A summary of the paths by which cells can move between the Lac$_i^-$ and Lac$_i^+$ states is shown in Figure 3. Once a cell becomes Lac$^+$, HSM II states that mutagenesis stops and the mutation rate drops to that characteristic for nonselective growth (see Figure 1 above). All transitions between Lac$_i^+$ states and all mutational killing of Lac$^+$ cells in Figure 3 are eliminated because the unselected mutation rates following reversion are assumed to be negligible compared with the induced mutation rates required to produce the observed Lac$^+$ revertant cells.

Given these assumptions, the numbers of Lac$^-$ cells with i null mutations (x_i) obey

$$dx_0/dt = -mx_0$$
$$dx_i/dt = -mx_i + cmx_{i-1} \quad \text{for } i = 1, 2, 3, \ldots, \quad (6)$$

and the numbers of Lac$^+$ cells with various numbers of null mutations (y_i) obey

$$dy_i/dt = amx_i \quad \text{for } i = 0, 1, 2, 3 \ldots . \quad (7)$$

The solution to this system is

$$x_i(t) = N_m[(cmt)^i/i!]e^{-mt} \quad (8)$$

and

$$y_i(t) = N_m ac^i\left(1 - e^{-mt}\sum_{j=0}^{i}\frac{(mt)^j}{j!}\right). \quad (9)$$

The simpler model (Equations 4 and 5 above) that ignored nonlethal null mutations is recovered by adding all Lac$_i^-$ (x_i) and Lac$_i^+$ (y_i) over all i null states. For large values of mt, the time-dependent terms in Equations 5 and 9 contribute little and the probability that a Lac$^+$ revertant carries i null mutations is simply

$$y_i(t)/Y(t) = (a + b)c^i, \quad (10)$$

where Y is the total number of Lac$^+$ revertants regardless of number of associated nonlethal nulls. This asymptotic result, which is independent of the mutation rate, is valid also for the model with a constant flow of hypermutable cells (see below).

Visualizing the distribution of nonlethal null mutations: We solved the system above for the numbers of Lac$^+$ revertants with various numbers of nonlethal null mutations (Figure 5). The graph in Figure 5 can be viewed as a series of histograms giving the number of Lac$^+$ revertants (x-axis) with various numbers of associated null mutations (y-axis); a series of such histograms are shown, for various intensities of mutagenesis (z-axis). Viewed another way, the graph shows how the number of Lac$^+$ revertants with various numbers of nonlethal null mutations increases with the intensity of mutagenesis. The table at the right of the graph shows results for some selected cases. As mutagenic intensity increases, the total number of revertants approaches 20 and the average number of null mutations in each revertant approaches 8; the distribution of number of nulls is given by the histograms. With high mutagenesis, only 2 of the Lac$^+$ revertants escape associated null mutations. At a low intensity of mutagenesis (0.5 mutations/cell/6 days) most revertants have no associated null

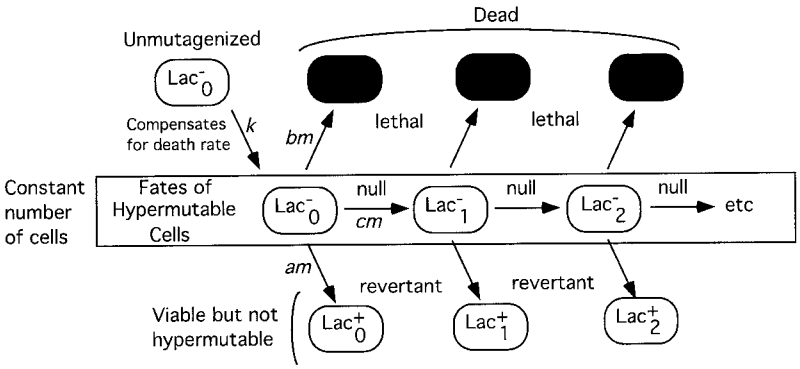

FIGURE 6.—A steady-state version of the hypermutable state model (HSM III). This version of the model predicts that revertants accumulate at a nearly constant rate as seen experimentally. The model assumes that starvation causes cells to enter the hypermutable state at a constant rate k. Once there, they are mutagenized with rate m and either die or revert. At appropriate values of k and m, the number of cells in the hypermutable state approaches a steady state and revertants accumulate nearly linearly with time.

mutations, but only ~1 Lac$^+$ revertant will be generated from 10^5 cells. Thus the number of Lac$^+$ revertants free of associated mutations generated by mutagenesis of 10^5 cells is about that expected to arise in the total population with no mutagenesis. The process of mutagenesis by HSM II provided essentially no increase in revertants free of deleterious mutations.

Verifying the analysis of HSM II by Monte Carlo simulations: To verify this analysis of HSM II (with mutation shutoff following reversion), we ran a Monte Carlo simulation of the model. As expected, Lac$^+$ revertants accumulated and viable Lac$^-$ cells disappeared as described in Figure 4 and the distribution of nonlethal null mutations was very close to that shown in Figure 5.

A constant-flow version of the hypermutable state model (HSM III): In the foregoing versions of the model (HSM I and HSM II), a single subset of cells was caused to undergo mutagenesis to the point of death or reversion. These previous forms of the model predict that the rate of Lac$^+$ revertant accumulation will decrease with increasing mutagenesis intensity or with time (see Figure 3). Experimentally, revertants are seen to accumulate at a constant or slightly increasing rate. We therefore considered a further modification of the model (HSM III) in which parental Lac$^-$ cells continuously enter the hypermutable state at some constant rate as long as selection (starvation) continues. Cells that enter this state accumulate nonlethal mutations and leave the state by either lethal mutation (death) or reversion to Lac$^+$. By this process, the population in the hypermutable state will increase to a nearly steady-state level (when cells enter and leave the hypermutable state at approximately equal rates). Once this state is reached, Lac$^+$ revertants should accumulate at a nearly constant rate and continue to do so until the parent population becomes limiting. The process is diagrammed in Figure 6. A similar model was recently presented by GOLDING *et al.* (2001). Their model differs in some details from the HSM III presented here and does not discuss the distribution of deleterious or lethal mutations among the revertants.

If cells enter the hypermutable state at rate k, an additional term ($+kN_0e^{-kt}$), describing the influx of new cells into the hypermutable state, must be added to Equation 2 above. The modified versions of Equations 2 and 3 were solved for $X(t)$ and $Y(t)$, giving

$$X(t) = \frac{kN_0}{(a+b)m-k}\,(e^{-kt} - e^{-(a+b)mt}) \tag{11}$$

$$Y(t) = \frac{amN_0}{(a+b)m-k}\left(1 - e^{-kt} - \frac{k}{(a+b)m}(1 - e^{-(a+b)mt})\right). \tag{12}$$

After a short initial transient period, the number of hypermutable cells reaches an approximately steady-state level (see Figure 7B), which lasts until a significant fraction of plated cells have entered the hypermutable state. In this limit, where $kt \ll 1$ and $mt \gg 1$, Equations 11 and 12 can be approximated as

$$X = kN_0/[(a+b)m] \tag{13}$$

$$Y(t) = ktN_0a/(a+b) = amtX. \tag{14}$$

At this approximate steady state, the accumulation of revertants, as given by the first equality in Equation 14, is formally independent of the mutation rate m. This happens because the number of cells in the hypermutable state is inversely proportional to m (Equation 13), while the appearance of revertants in this population is proportional to m. Figure 7A shows the accumulation of cells in the hypermutable state [$X(t)$; top surface] and revertants [$Y(t)$; bottom surface], as a function of added mutations (mt). This was calculated for various values of the birth rate of hypermutable cells (k). This figure gives a general view of the process and the values of kt and mt that produce significant numbers of Lac$^+$ revertants. The number of Lac$^+$ revertants predicted is not appreciable without extremely intense mutagenesis, between 10^4 and 10^5 times the normal unselected rate.

The original intent of HSM III was to predict the linear accumulation of revertants with time. Therefore we plotted (Figure 7B) the size of the hypermutable population and the number of Lac$^+$ revertants *vs.* time (Equation 12). The size of the hypermutable population (approaching steady state) is set by the ratio of m and k (reversions/hypermutable cell/unit time and hypermutable cells created/parent cell/unit time). We tested ratios of these values that predict a steady state of 10^5

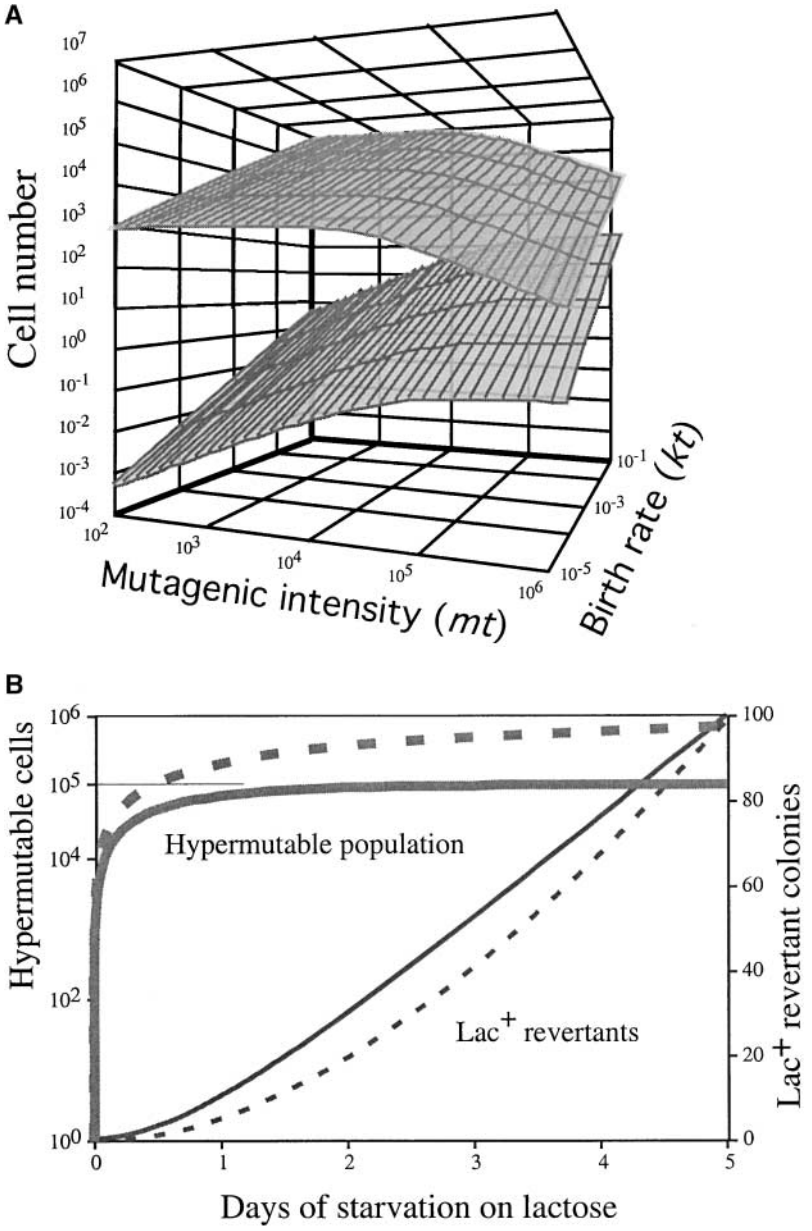

FIGURE 7.—Behavior predicted by the steady-state model (HSM III). (A) The accumulation of Lac^+ revertants (bottom sheet) and number of cells in the hypermutable state (top sheet) as a function of mutational dose (mt) and rate of entry into the hypermutable state (kt). (B) Top curves using log scale at left, changes in the size of the hypermutable population with time; lower curves using the linear scale at right, the accumulation of Lac^+ revertants with time. A ratio of k and m that makes the hypermutable population approach a steady-state value of either 10^5 (solid line) or 10^6 (dashed line) was used. Values of m and k that predict formation of 100 Lac^+ revertants over 5 days were chosen. For the solid lines, $m = 10.8$ mutations/mutagenized genome/day and $k = 0.0012$ hypermutable cells/parent cell/day. For the dashed lines, $m = 2.1$ mutations/genome/day and $k = 0.0012$ hypermutable cells/parent cell/day. Some key predictions of the model are in Table 2.

or 10^6 cells and for each ratio used the unique value of k that predicts formation of 100 Lac^+ revertants within 5 days (as seen experimentally in the Cairns experiment). Several implications emerge from this exercise.

Mutations appear to be directed to *lac* when selection increases the frequency of Lac^+ revertants more than it increases the frequency of unselected nonlethal mutations in the entire viable Lac^- population. This requires that the number of mutagenized cells be small and that almost all of these cells except the Lac^+ revertants are rapidly killed by mutagenesis. That is, the remaining viable Lac^- cells must be largely unmutagenized. The small steady-state values of 10^5 and 10^6 were chosen in the hope that the model might predict the apparent direction of mutation to *lac* seen in the actual experiment.

In Figure 7B, the number of cells in the hypermutable state approaches a steady state and Lac^+ revertants accumulate at a nearly constant rate as observed experimentally. At much later times under these conditions, the number of Lac^+ revertants will approach 20,000 as the entire plated population (10^8 cells) passes through the hypermutable state. As this point approaches, the plated population is mostly dead and the majority of surviving cells are Lac^+ revertants. In trying to account for the Cairns phenomenology using this form of the hypermutable state model, several points should be noted. These are listed in Table 2 and mentioned below.

Very intense mutagenesis is required (a 10^4- to 10^5-fold increase). This contrasts to the 35-fold increase in mutagenesis experimentally observed for the Lac^+ revertant population. Regardless of the intensity or duration of selection, each Lac^+ revertant carries an average of eight nonlethal null mutations—~80 times the

TABLE 2

Behavior of the constant-flow version of the hypermutable state model (HSM III)

Line in Figure 7B	No. of hypermutable cells at steady state	Birth rate of hypermutable (HM) cells (/parent cell/day)	Mutation rate m (mutations/HM cell/day)	Fold increase in mutation rate caused by selection	Nonlethal nulls (average) per Lac^+ revertant (any day)	Nonlethal nulls on day 5 (average) per viable Lac^- cell (mutagenized plus unmutagenized)	Fold increase in frequency of nonlethal null mutations in nonrevertant (Lac^-) cells
Solid	10^5	0.0012	10.8	1.2×10^5	8	8.0×10^{-3}	18×
Dashed	10^6	0.0024	2.2	2.4×10^4	8	5.6×10^{-2}	125×

number expected without mutagenesis. The nonrevertant population (unmutagenized parent cells plus unreverted viable cells in the hypermutable state) shows a significant increase in the frequency of unselected mutations—that is, mutability does not appear to be directed to *lac*. Under the two conditions tested, HSM III predicts an 18- or 125-fold increase in nonlethal mutations in the Lac^- population after 5 days under selection. This greatly exceeds the 4-fold increase observed experimentally (Bull *et al.* 2001). Table 3 provides a glossary of notations used.

DISCUSSION

The hypermutable state model cannot account for important quantitative features of the Cairns experiment. None of the three versions of the HSM tested fit with all of the critical experimental observations—number of Lac^+ revertants, intensity of associated mutagenesis, and apparent direction of mutation to *lac*. With 10^5 mutagenized cells, unrealistically intense mutagenesis can produce a maximum of 20 revertants and give the appearance of directed mutation in that most mutagenized cells are killed and the viable Lac^- population (mostly unmutagenized) shows little or no increase in the frequency of unselected mutations. However, the intensity of mutagenesis required to form these 20 revertants leaves each revertant with an average of eight associated mutations (deleterious in the long term) and only 2 of the 20 Lac^+ revertants escape without any associated null mutation. This approximates the number of Lac^+ revertants that would occur in the parent population without mutagenesis.

If the mutagenized population is increased 10-fold (to 10^6 cells), then a somewhat lower mutation rate can explain 100 revertants, but 10% of the mutagenized Lac^- cells survive and therefore the total nonrevertant population shows a 60-fold increase in associated mutation (much higher than actually observed). The experimentally observed intensity of general mutagenesis (<100-fold the unselected rate) cannot explain formation of even one Lac^+ revertant assuming mutagenesis of 10^5 nongrowing cells. At this low intensity of mutagenesis, 100 revertants can be explained only by exposure of the entire plated population (10^8 cells). With this low dose of mutagenesis, there is very little killing and therefore the entire viable population (both Lac^+ and Lac^-) is predicted to show nearly a 100-fold increase in the frequency of associated mutations; that is, the appearance of directed mutation is lost.

In short, there is no set of conditions under which the hypermutable state model can predict formation of 100 revertants with a 35-fold increase in associated mutations and also show the apparent directed mutation (which depends on killing of the entire nonrevertant mutagenized population). The model comes

TABLE 3

Glossary of notation used

N_0	Total number of cells plated (generally 10^8)
N_m	Total number of starting cells in the hypermutable state
k	Rate of entry into the hypermutable state
m	Mutation rate (−1 mutations)
a	Fraction of −1 mutations that result in reversion to Lac^+
b	Fraction of −1 mutations that are lethal
c	Fraction of −1 mutations that result in nonlethal null mutation $a + b + c = 1$
X	Total number of live Lac^- cells in the hypermutable state
Y	Total number of live Lac^+ cells
Lac_i^-	Live cells that are Lac^- with exactly i null mutations
Lac_i^+	Live cells that are Lac^+ with exactly i null mutations
x_i	Number of Lac^- cells in the hypermutable state with exactly i null mutations
y_i	Number of Lac^+ cells with exactly i null mutations

closest to explaining the observations when a small population (10^5–10^6 cells) is subjected to very intense mutagenesis far higher than that observed. The required intensity is higher than can be attained in resting cells by any known mutagenesis protocol. If this mutagenesis could somehow be realized, the HSM predicts a heavy burden of associated nonlethal null mutations, making it unlikely that the revertants (or the hypothesized regulatory mechanism) would survive in the long term. To our knowledge no one has tested the fitness of Lac^+ revertants obtained in the Cairns experiment. The HSM predicts large losses in fitness; the low and uneven mutagenesis intensity actually observed in the Cairns system (Rosche and Foster 1999) suggests that impaired revertants will be rare.

The difficulty in explaining the Cairns phenomenon by induced general mutagenesis lies mainly in the fact that only a small subset of cells can be mutagenized. This constraint is required because the model proposes no growth during the selection period and because selection causes very little killing and very little mutagenesis in the nonrevertant population as a whole. To even approach generation of the observed revertants by general mutagenesis of such a small population requires implausibly intense mutagenesis (10^5-fold the unselected rate) and greatly increases the load of null mutations carried by each Lac^+ revertant.

We suggest that the behavior of the Cairns system can better be explained by the amplification model in which the mutant *lac* operon is amplified during growth within clones developing under selection (Andersson *et al.* 1998; Hendrickson *et al.* 2002). This growth in isolated colonies can occur even though the lawn of parental cells shows little or no growth (Foster 1994). Once growth is allowed, selection favors cells within developing microclones that amplify the (leaky) mutant *lac* allele and thereby acquire additional copies of the mutant *lac* region. The increase in mutant *lac* alleles in each clone enhances the probability of reversion without requiring any change in mutation rate. The observed low mutagenesis is attributed to a minor side effect of growth with a *lac* amplification, rather than to an evolved mechanism. That is, the SOS system and its error-prone polymerase IV (DinB) are induced by DNA fragments released by recombination between repeated sequences in the amplified array. This SOS induction is expected to occur whenever cells grow with a large amplification (Hendrickson *et al.* 2002). With *lac* amplification, the low level of mutagenesis can be brought to bear more heavily on the *lac* operon (and proportionately away from deleterious targets) simply by increasing the number of *lac* targets per mutagenized cell and the number of cells in the developing colony. This gives the appearance of directed mutation and allows a low level of mutagenesis to make a small (albeit nonessential) contribution to reversion. Whether or not the amplification model is correct, it is an alternative to the HSM that explains the behavior of the Cairns system without requiring regulated mutability (Slechta *et al.* 2002b).

The question of growth is central to the adaptive mutation controversy. If growth is occurring, it becomes difficult to know whether selection creates new mutations or favors the growth of particular preexisting mutant types. Both directed mutation and the HSM were devised to explain the appearance of Lac^+ revertant clones that were thought to be arising in a nongrowing cell population. Pains were taken to assure that the parent lawn (between visible colonies) did not grow during the experiment (Cairns and Foster 1991). If revertants in fact arise in this nongrowing population, then regulated mutability is hard to escape. The amplification model proposes that revertants do not arise in this nongrowing parental population but rather in a population that is actively growing within developing microclones. The original growth tests deliberately avoided visible colonies and thus did not assess the growth under selection that led to the visible revertant clones. These tests would detect growth in colonies that had not yet become visible ($<10^5$ cells), but this growth would contribute very little above the parental popula-

tion (10^8). Furthermore these tests could not account for the *lac* copies added by amplification. Theoretical objections to directed mutation stressed the possibility of nonapparent growth (Lenski and Mittler 1993). The idea of growth within microclones of preexisting, partially reverted cells was explicitly suggested by Lenski *et al.* (1989) and is consistent with the idea that preexisting cells with a *lac* duplication initiate colonies of growing cells within which revertants ultimately occur in the Cairns system. Once the possibility of unappreciated growth is open, there is no need to postulate either directed mutation or the HSM.

For reasons beyond those presented above, the behavior of the Cairns system should not be taken as evidence for an evolved mechanism causing stress-induced general mutability. We know of no other system in which stress increases general mutation rate. The weak general mutagenesis observed in the Cairns system is peculiar to the particular strains used and is not a general property of stressed cells (Hughes and Andersson 1997), of cells growing with a selected amplification (Slechta *et al.* 2002a; E. S. Slechta, unpublished results), or even of cells growing with an induced SOS system (Bunny *et al.* 2002). The weak induced mutagenesis seen in the Cairns system requires that selection, *lac* amplification, and SOS induction all affect a strain carrying *lac* on the particular F'_{128} *lac* plasmid; it is not seen in strains whose *lac* operon is either in the chromosome or on other conjugative plasmids (E. S. Slechta, unpublished results). In the particular strains carrying F'_{128}, mutagenesis occurs and makes a small contribution to *lac* reversion (McKenzie *et al.* 2000), but is not essential to the accumulation of revertants under selection (Slechta *et al.* 2002b).

Any model that proposes a long-term benefit derived from a temporary, stress-induced increase in general mutation rate is likely to encounter the same problems as those encountered by the hypermutable state model. The assumptions on which our estimates were made are quite generous to the HSM in that the ratio of selected Lac^+ revertants to lethals (1/5000) and to deleterious mutations (1/40,000) considered only null mutations caused by −1 frameshifts. By ignoring +1 frameshifts and base substitutions, our analysis underestimates the number of deleterious mutations by at least a factor of 4 (assuming base substitutions are one-half of spontaneous mutations and +1 and −1 mutations are equally likely). In addition, the target for mutations that improve fitness under natural conditions is likely to be smaller than the 100-bp target in the Cairns system. More realistic estimates suggest that the ratio of beneficial to deleterious mutations in natural situations is likely to be much smaller than 1/45,000 and could approach 10^{-6} (Gerrish and Lenski 1998; Funchain *et al.* 2000). As this ratio drops, so does the likelihood that general mutagenesis could provide beneficial mutations without an unacceptable cost in deleterious mutations.

Mutations that appear neutral during selection for reversion to Lac^+ may be severely deleterious or even lethal under other growth conditions. Bacteria in natural populations grow under a variety of conditions and many of their genes are only occasionally useful. However, long-term survival requires the ability to prosper under all the conditions that are often encountered. Most genes in a genome are likely to make a contribution to fitness under some conditions that are at least occasionally encountered. Genes are maintained in a genome by positive selection that eliminates impaired mutants and would eliminate Lac^+ revertants that carried associated deleterious mutations. This later selection against deleterious mutations makes it unlikely that Lac^+ revertants formed by mutagenesis would survive in the long term. The average of eight nonlethal mutations predicted by HSM II or III is a very heavy mutational burden and would make the hypothetical underlying mechanism difficult to evolve or maintain under natural conditions. The fate of beneficial mutations in asexual populations in the face of preponderant deleterious mutations has previously been treated mathematically (Peck 1994; Orr 2000).

For larger populations, less mutagenesis is required to create a rare beneficial mutation (and there is a lower cost in associated deleterious mutations). Conversely, in larger populations, there is an increased likelihood that the selected mutation will arise without any mutagenesis (or associated cost). That is, in very large populations, the mutation rate no longer limits genetic adaptation. As an example, if a population of 100 bacteria includes one mutator cell with a 100-fold increased mutation rate, then half of the new mutations will arise in the majority population (and have no extra cost problems to solve) and half will arise in the mutator cell and have major problems of long-term survival. In the larger populations typical of bacteria, mutagenesis is not needed to assure the presence of new diversity. The relationship between population size and mutation rate has previously been modeled mathematically (Gerrish and Lenski 1998) and shown experimentally (de Visser *et al.* 1999). While an increased recombination rate might reduce the cost of mutagenesis by allowing beneficial mutations to escape their mutational load (Muller 1964; Felsenstein 1974), it would also separate the selected beneficial mutations from the determinants of the hypothetical mutagenic mechanism and thereby limit the likelihood of maintaining that mechanism under selection.

The estimates made here on the basis of the Cairns system understate the cost of a mechanism to induce mutations during stress in natural populations. Many stressful situations would induce the mechanism when no mutation could possibly relieve the stress (for example, the absence of any carbon source). Whenever this occurs, cells with a stress-induced mutagenesis mechanism would futilely reduce their own competitiveness

or kill themselves with no chance of producing fitter progeny. Similarly, many stresses are likely to be temporary and cells have no way of knowing whether mutation is the only alternative to death. If stress were soon to be relieved by environmental improvement, mutagenesis would be a disastrous decision.

The above considerations do not argue against the possibility that mutation rates might increase under particular growth circumstances—we know that they do (*e.g.*, during X-ray irradiation). Such rates might also increase in cells with insufficient resources to repair damage. Such effects are not at issue here. Rather, we argue that a genetic mechanism to increase general mutation rates suddenly and purposefully in response to stress in hopes of creating a beneficial mutation cannot explain the behavior of the Cairns system and seems unlikely to provide the long-term benefit needed to support its evolution or maintenance by natural selection.

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