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The Role of Single-Mutant Intermediates in the Generation of *trpAB* Double Revertants during Prolonged Selection

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Selection-induced mutations are nonrandom mutations that occur as specific, direct responses to environmental challenges and that occur more often when they are selectively advantageous than when they are selectively neutral. One of the most puzzling examples of selection-induced mutations involved the simultaneous reversions of two mutations, one in *trpA* and the other in *trpB*, at rates that were several orders of magnitude greater than would have been predicted if the two mutations had occurred as independent events (B. G. Hall, Proc. Natl. Acad. Sci. USA 88:5882–5886, 1991). Here I examine the possibility that the double mutations might be accounted for by sequential mutations with intervening growth.

Selection-induced mutations (nonrandom mutations that occur as specific, direct responses to environmental challenges and that occur more often when they are selectively advantageous than when they are selectively neutral) occur both in bacteria (1, 2, 4–10, 12, 13, 16) and in *Saccharomyces cerevisiae* (11, 15). I previously (8, 9) reported that when *Escherichia coli* K-12 strains carrying either *trpA46* or *trpB9578* (both of which are missense mutations that confer a Trp^- phenotype) are allowed to form small colonies on medium that contains a limiting supply of tryptophan (5 μM), they produce Trp^+ revertant papillae during prolonged tryptophan starvation over a period of several weeks. These papillae arise as the result of selection-induced reversion mutations (8) that occur in a time-dependent, rather than in a replication-dependent, fashion. The process that produces selection-induced mutations in nondividing cells is different from the process that produces random mutations in growing cells, as is evident from the observation that the two processes produce different mutational spectra (10). Selection-induced mutation rates are expressed as mutations per viable cell per day. When strains carrying *trpA46* and those carrying *trpB9578* were tryptophan starved for a month, the mean selection-induced reversion rates over the period from day 20 to day 30 were 5.2×10^{-10} and 6.4×10^{-10} per cell per day, respectively (9). When a strain that carried both mutations was starved for the same period, the average selection-induced double-reversion rate was 4.5×10^{-11}, rather than 3.3×10^{-19}, as would be expected if the two reversion events were independent (9). The observed double-reversion rate was so high, compared with the single-reversion rates, that it could not be explained by any of the hypotheses that had been advanced to explain selection-induced mutations (9).

Because neither the *trpA46* nor the *trpB9578* mutant can grow in the absence of tryptophan, and indeed each dies exponentially during prolonged tryptophan starvation (8, 9), I had ruled out sequential mutations with intervening growth as an explanation for the observed rate at which Trp^+ revertant papillae occurred during starvation of the *trpAB* double-mutant strain. Nevertheless, Roberto Kolter suggested that intervening growth might occur and offered the following scenario (12a). The *trpA* gene product converts indole-glycerol-phosphate to indole, which is then converted to tryptophan by the *trpB* gene product. The *trpAB* double mutant accumulates indole-glycerol-phosphate, some of which might break down spontaneously to generate indole, which would then be excreted. Thus, indole might accumulate within a *trpAB* colony. If a reversion mutation to $trpB^+$ occurred, then the revertant cell might be able to grow at the expense of the indole and thus be able to generate a large population of $trpB^+$ *trpA* cells within which a second mutation to $trpA^+$ could occur, thus producing a Trp^+ papilla.

To test this hypothesis, I constructed isogenic *trpA46*, *trpB9578*, and *trpA46-trpB9578* (double-mutant) strains and used them to carry out reconstruction experiments that permitted me to measure the growth rates of single-mutant cells within double-mutant colonies. The results of these measurements were used to analyze the previously reported data (9) to determine whether those results could be accounted for by sequential mutations with intervening growth.

MATERIALS AND METHODS

***E. coli* strains.** The following strains were used: FCY2 (*trpA46*), FCY2003 (*trpA46* Tn*10*::*zch-506*), FCY21 (*trpB9578*), FCY2103 (*trpB9578* Tn*10*::*zch-506*), FCY6 (*trpA46 trpB9578*), FCY6B (*trpA46 trpB9578 tna*::Tn*10*), W3110 (wild type), FCY6C (wild type) ($trpA^+$ $trpB^+$ was transduced from W3110 into FCY6), FCY6D (*trpA46* Tn*10*::*zch-506*) (*trpA46* Tn*10*::*zch-506* was transduced from FCY2003 into strain FCY6C), FCY6E (*trpB9578* Tn*10*::*zch-506*) (*trpB9578* Tn*10*::*zch-506* was transduced from FCY2103 into strain FCY6C).

Media. Minimal complete glucose (MCD) medium consisted of 423 mg of sodium citrate, 100 mg of $MgSO_4 \cdot 7H_2O$, 1 g of $(NH_4)_2SO_4$, 540 μg of $FeCl_3$, 1 mg of thiamine, 3 g of KH_2PO_4, 7 g of K_2HPO_4, 20 mg of adenosine, 30 mg of cytosine, 20 mg of guanosine, 20 mg of uridine, 30 mg of thymidine, 20 mg of $MnSO_4$, 200 μg of pantothenic acid, 400 μg of pyridoxine, 400 μg of riboflavin, 200 μg of *p*-aminobenzoic acid, 200 μg of niacin, 1 μg of biotin, 100 mg of each of the 20 common amino acids, and 2 g of glucose per liter. MCD−trp medium was identical to MCD medium except that it lacked tryptophan. MCD+tet medium was identical to MCD medium except that it contained 20 μg of tetracycline per ml. Solid media contained 15 g of Sigma purified agar per liter.

Reconstruction experiments. Cultures of strains FCY6, FCY6D, and FCY6E were grown in MCD medium, washed,

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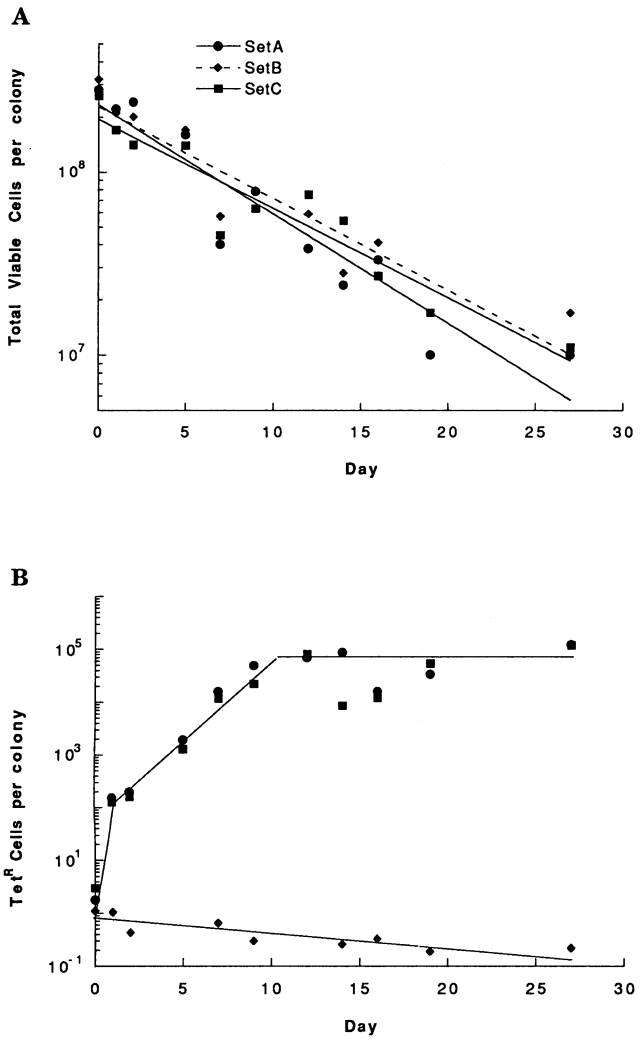

FIG. 1. (A) Total viable cells per colony in three reconstruction experiments. Viable cells were estimated as CFU when dilutions of cell suspensions were plated onto MCD medium. (B) Growth of *trpA* or *trpB* mutant cells within *trpAB* colonies. Instant colony reconstruction experiments were conducted as described in Materials and Methods. Symbols: ●, colonies initially contained, on average, 1.8 *trpA trpB*$^+$ Tet^r cells; ◆, colonies initially contained, on average, 1.1 *trpA*$^+$ *trpB* *Tet*^{r} cells; ■, colonies initially contained, on average, 3.0 Tet^r cells, i.e., about 1.8 *trpA trpB*$^+$ Tet^r cells and 1.1 *trpA*$^+$ *trpB* Tet^r cells.

and concentrated. Suspensions that contained 2.8×10^8 FCY6 cells per μl and either approximately 1 FCY6D cell per μl (set A), 1 FCY6E cell per μl (set B), or both 1 FCY6D and 1 FCY6E cell per μl (set C) were prepared. Ten 1-μl drops of these suspensions were applied to MCD−trp plates (15 ml in 60-mm plates). Each drop formed an "instant" visible colony. To determine the actual number of Tet^r (FCY6D and FCY6E) cells per instant colony, 100 1-μl drops of each suspension were placed onto MCD+tet plates. The Tet^r cells produced papillae on the surface of the resulting instant colonies, and these papillae were approximately Poisson distributed. In set A, the average number of FCY6D cells was 1.8; in set B, the average number of FCY6E cells was 1.1; and, in set C, the average number of FCY6D and FCY6E cells was 3.0. On the days indicated (Fig. 1), two plates from each set were resuspended and appropriately diluted samples were spread onto MCD medium to estimate the total number of viable cells and onto MCD+tet medium to estimate the number of FCY6D or FCY6E cells.

RESULTS

Figure 1A shows that, in each set, the number of viable cells per colony decreased exponentially. For set A, the death rate (±95% confidence limits) was 0.14 ± 0.04 day^{-1}; for set B, it was 0.12 ± 0.03 day^{-1}; and, for set C, it was 0.11 ± 0.03 day^{-1}. Figure 1B shows that FCY6E (*trpA*$^+$ *trpB*) cells were unable to grow in the absence of tryptophan, and, indeed, they died exponentially at the rate of 0.07 ± 0.03 day^{-1}, a rate that was not significantly different from the rate at which the predominant FCY6 double-mutant population died. In contrast, FCY6D (*trpA trpB*$^+$) cells increased 88-fold during the first 24 h and then grew exponentially at 0.64 day^{-1} until day 10, after which no further growth occurred. Since the number of Tet^r cells per colony was about the same whether FCY6D was present alone or together with strain FCY6E, it seems unlikely that *trpA trpB*$^+$ cells could cross-feed *trpA*$^+$ *trpB* cells. On day 16, 100 tetracycline-resistant colonies from a set C plate were tested for the ability to grow on MCD−trp medium to which indole had been added. The observation that all 100 were able to grow, i.e., were *trpA trpB*$^+$, indicates that even if *trpA trpB*$^+$ cells do cross-feed *trpA*$^+$ *trpB* cells to some extent, the contribution of *trpA*$^+$ *trpB* cells to the population of single revertants is insignificant (<1%). It seems likely that there is an initial high concentration of indole present within colonies that permits rapid growth of *trpA trpB*$^+$ cells on the first day. Continued excretion of indole apparently permits growth to continue for several days, but after 10 days of tryptophan starvation the rate at which indole is produced is apparently insufficient to permit any further growth of *trpA trpB*$^+$ cells.

Could the growth of *trpA trpB*$^+$ cells have accounted for the previously published results (9), in which 37 *trpA*$^+$ *trpB*$^+$ double revertants were recovered when *trpA trpB* colonies were tryptophan starved for 4 weeks? The number of *trpA*$^+$ and *trpB*$^+$ single revertants that occurred each day in that experiment was calculated from the number of viable cells on that day and the rate (mutations per viable cell) at which the single-mutant strains produced Trp$^+$ revertants on that day in a parallel experiment (9). It was then assumed that each new *trpA trpB*$^+$ mutant gave rise to 125 descendants during the first 24 h following its occurrence and thereafter grew at 0.64 day^{-1} for the next 9 days (Fig. 2). It was further assumed that newly arisen *trpA*$^+$ *trpB* cells neither grew nor died during the course of the experiment. The number of double revertants expected each day is based on the number of single revertants of each type that were expected to be present each day and the observed rate of mutation to Trp$^+$ for that class of single revertant. This model predicts that the final number of Trp$^+$ double revertants in that experiment would have been 0.29, <1% of the observed number (Fig. 2).

The above-described model (model 1) probably overestimates the number of *trpA trpB*$^+$ cells present each day because (i) it assumes no death of the *trpA trpB*$^+$ cells after growth ceased and (ii) it assumes that as much indole was available to late-arising *trpA trpB*$^+$ mutants as was available to early-arising mutants. It seems likely that diffusion would reduce the amount of indole within colonies, thus limiting the growth of late-arising *trpA trpB*$^+$ mutants more than that of early-arising mutants. If, instead, it is assumed that newly arisen *trpA trpB*$^+$ mutants were able to undergo the initial 125-fold burst of growth but that after the 13th day of that experiment *trpA*

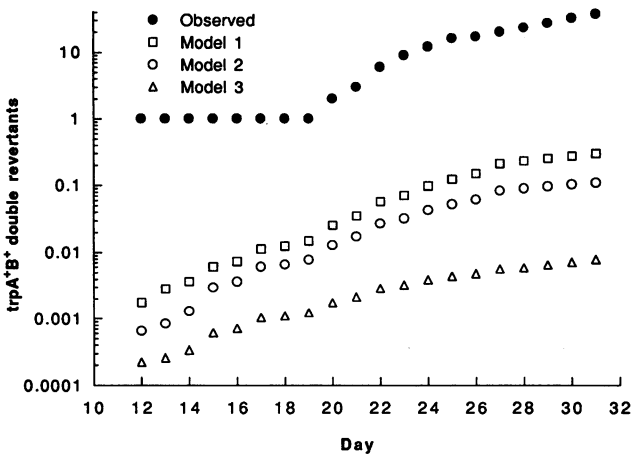

FIG. 2. The estimated and observed numbers of Trp^+ double revertants in an experiment in which *trpA trpB*$^+$ colonies were tryptophan starved for 4 weeks. The observed numbers of double revertants are from the previously published experiment (9). The expected numbers are calculated according to three models for growth as described in the text.

trpB$^+$ mutants could not continue to grow (because indole had diffused away from the colonies by that time, for instance) (model 2), then the final number of Trp^+ double revertants expected in that experiment would be only 0.11, about 0.3% of the observed number.

In general, the rate at which revertant papillae appear remained fairly constant over the course of the experiment (8, 9). Because, during those experiments, cells in the population died exponentially, the selection-induced mutation rates, expressed as mutations per viable cell per day, increased exponentially during the experiment. This acceleration was particularly pronounced in studies with the yeast *S. cerevisiae* (11). Mutation rates have been expressed as mutations per viable cell per day on the reasonable grounds that dead cells do not form revertant colonies. Viable cells are determined from the number of CFU when cells from plates are resuspended and dilutions of those suspensions are spread onto nonselective MCD plates. On the other hand, if rates were expressed as mutations per colony, or mutations per viable cell at the beginning of the experiment, the mutation rate would be fairly constant. If the calculations in model 1 were based on mutation rates expressed as mutations per initial cell per day, then the final number of Trp^+ double revertants expected in that experiment would be only 0.0078, about 2×10^{-4} of the observed number (model 3). This calculation of mutation rates assumes that colonies contain cells that cannot, upon plating, form colonies but that these cells can mutate and subsequently grow to form revertant papillae. Such cells might be called "zombies" or the living dead. It must be emphasized that there is no direct evidence for the existence of zombies and that their existence has been hypothesized only as an alternative to having mutation rates that increase with time.

DISCUSSION

The results of the reconstruction experiments presented here show that Kolter's proposed scenario for growth of *trpA trpB*$^+$ cells within *trpA trpB* colonies during prolonged tryptophan starvation is probably correct.

There are three possible ways in which the two mutations might occur independently: they might occur (i) simultaneously, (ii) sequentially without intervening growth, or (iii) sequentially with intervening growth. The observed number of double revertants is about 10^8-fold higher than expected if the mutations occur independently and simultaneously and about 10^5-fold higher than expected if they occur independently and sequentially without intervening growth (9). The present results suggest that, under the most conservative model for mutations that occur independently and sequentially with intervening growth (model 1, which generates the greatest number of *trpA trpB*$^+$ cells), the observed number of double revertants is more than 100-fold higher than expected. By convention, results that fall outside of the 99% confidence limits of expectation are said to be highly significantly different from the expectation and the model that generated the expectation is firmly rejected. In this case, however, a more cautious interpretation may be warranted. The above calculations assume that the cells in the original double-reversion experiment (9) behaved exactly as did the cells in the reconstruction experiments presented here. There are some reasons why that might not be the case. (i) In the original experiment, the colonies were formed by growth on medium containing a limiting concentration of tryptophan, whereas, in the reconstruction experiment, instant colonies were deposited on medium containing no tryptophan. It is possible that, under those circumstances, the initial increase in *trpA trpB*$^+$ cells might have been greater than the increase reported here for the reconstruction experiment. (ii) The *trpA trpB*$^+$ cells might have grown faster than in the reconstruction experiment. For instance, if the initial increase had been 500-fold, rather than 125-fold, and the growth rate were 0.704 day^{-1}, 10% higher than the rate of 0.64 day^{-1} observed in the reconstruction experiment, then, in the double-reversion experiment, 1.9 mutants would be expected. In that case, one would have less than 95% confidence that the results could not be explained by independent events with intervening growth. Given the uncertainties associated with extrapolating from the reconstruction experiment to the double-reversion experiment, one must consider that the appearance of *trpA*$^+$ *trpB*$^+$ revertants from the *trpA46-trpB9578* parent could have resulted from independent mutations.

Three major models have been proposed to account for selection-induced mutations. The "transcription-induced mutations" model (3) is inconsistent with the results of several experiments (2, 5). The "slow mismatch repair" model (14) is inconsistent with the results of other experiments (5, 12). At this time, there is not a robust model to account for selection-induced mutations. On the basis of the conclusion that the *trpA46-trpB9578* double-reversion mutations were not independent events (9), I considered whether they were consistent with the "hypermutable state" model for selection-induced mutations (8), concluded that they were not (see reference 9 for the detailed argument), and therefore rejected the hypermutable state model. At this time, those considerations remain the sole reason for rejecting that model, and the results of these reconstruction experiments suggest that the rejection of the hypermutable state model may have been premature. It must be emphasized that the observed number of double revertants is still considerably more than would be expected if the mutations were independent and that, if the two mutations were indeed the result of independent events, then the arguments for rejecting the hypermutable state model remain valid. These results do not provide support for the hypermutable state model; they only indicate that it cannot be rejected with strong confidence.

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REFERENCES

1. **Boe, L.** 1990. Mechanisms for induction of adaptive mutations in *Escherichia coli*. Mol. Microbiol. **4:**597–601.
2. **Cairns, J., and P. L. Foster.** 1991. Adaptive reversion of a frameshift mutation in *Escherichia coli*. Genetics **128:**695–701.
3. **Davis, B. D.** 1989. Transcription bias: a non-Lamarkian mechanism for substrate-induced mutations. Proc. Natl. Acad. Sci. USA **86:**5005–5009.
4. **Foster, P.** 1991. Directed mutations in *Escherichia coli*, p. 213–234. *In* A. Tauber (ed.), Organism and the origins of self. Kluwer Academic Publishers, Dordrecht, The Netherlands.
5. **Foster, P. L., and J. Cairns.** 1992. Mechanisms of directed mutation. Genetics **131:**783–789.
6. **Hall, B. G.** 1988. Adaptive evolution that requires multiple spontaneous mutations. I. Mutations involving an insertion sequence. Genetics **120:**887–897.
7. **Hall, B. G.** 1989. Selection, adaptation, and bacterial operons. Genome **31:**265–271.
8. **Hall, B. G.** 1990. Spontaneous point mutations that occur more often when they are advantageous than when they are neutral. Genetics **126:**5–16.
9. **Hall, B. G.** 1991. Adaptive evolution that requires multiple spontaneous mutations: mutations involving base substitutions. Proc. Natl. Acad. Sci. USA **88:**5882–5886.
10. **Hall, B. G.** 1991. Spectrum of mutations that occur under selective and non-selective conditions in *E. coli*. Genetica **84:**73–76.
11. **Hall, B. G.** 1992. Selection-induced mutations occur in yeast. Proc. Natl. Acad. Sci. USA **89:**4300–4303.
12. **Jayaraman, R.** 1992. Cairnsian mutagenesis in *Escherichia coli*: genetic evidence for two pathways regulated by *mutS* and *mutL* genes. J. Genet. **71:**23–41.

12a. **Kolter, R.** Personal communication.

13. **Prival, M. J., and T. A. Cebula.** 1992. Sequence analysis of mutations arising during prolonged starvation of *Salmonella typhimurium*. Genetics **132:**303–310.
14. **Stahl, F. W.** 1988. A unicorn in the garden? Nature (London) **335:**112–113.
15. **Steele, D. F., and S. Jinks-Robertson.** 1992. An examination of adaptive reversion in *Saccharomyces cerevisiae*. Genetics **132:**9–21.
16. **Thomas, A. W., J. Lewington, S. Hope, A. W. Topping, A. J. Weightman, and J. H. Slater.** 1992. Environmentally directed mutations in the dehalogenase system of Pseudomonas putida strain PP3. Arch. Microbiol. **158:**176–182.