Proc. Natl. Acad. Sci. USA
Vol. 89, pp. 4300–4303, May 1992
Genetics

Selection-induced mutations occur in yeast

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Communicated by Stanley M. Gartler, February 10, 1992 (received for review October 1, 1991)

ABSTRACT **Selection-induced mutations are nonrandom mutations that occur as specific and direct responses to environmental challenges and primarily in nondividing cells under conditions of intense prolonged selection. Selection-induced mutations have been shown to occur at six loci in *Escherichia coli*, but their existence has not previously been demonstrated in any eukaryotic organism. Here it is shown that selection-induced mutations occur at the *HIS4* locus in the eukaryotic microorganism *Saccharomyces cerevisiae*.**

Selection-induced mutations, first reported by Cairns *et al.* (1), are nonrandom mutations that occur as specific and direct responses to environmental challenges (2, 3). Such mutations have been variously termed directed mutations (1, 4), Cairnsian mutations (2, 5), and adaptive mutations (3). The evidence that some mutations arise as specific responses to selection is now sufficiently strong to warrant use of the term selection induced. The existence of selection-induced mutations represents a direct challenge to the dogma that mutations occur purely randomly with respect to their eventual effect on fitness of the organism, and as a consequence they have generated considerable controversy (6–11). Selection-induced mutations occur under conditions of prolonged, intense selection in nondividing cells and are therefore time dependent rather than DNA replication dependent (2, 12). Both time dependence and the observation that the spectrum of base substitutions is different from that found in growing cells (5) argue that selection-induced mutations arise by mechanisms substantially different from those responsible for mutations in growing cells under nonselective conditions. Selection-induced mutations can involve base substitutions (1, 2), frameshifts (5, 13), and excision of mobile elements from within genes (4), and their occurrence has been demonstrated in at least six loci (*lacZ, metB, trpA, trpB, cysB,* and *bglF*) in *Escherichia coli*. In a typical experiment, cells are allowed to form colonies on a medium containing some limiting resource under conditions in which a specific mutation will permit the mutant cell to grow when the limiting resource becomes exhausted. Mutations that occur after colony growth has ceased result in the appearance of outgrowths, called papillae, on the surface of the colonies. The continued appearance of papillae for several days or weeks after colony growth has ended suggests the occurrence of selection-induced mutations but does not, by itself, constitute definitive evidence for selection-induced mutations. Convincing evidence for selection-induced mutations requires that several elements be demonstrated: (*i*) the appearance of papillae can not be accounted for by slow growth of preexisting mutants that were present in the colonies at the time when colony growth ceased, (*ii*) the mutations actually occur in nondividing cells—i.e., they cannot be accounted for by ordinary replication-dependent mutations, and (*iii*) the mutations occur in the gene that is under selection but not in other genes that are not under selection—i.e., their occurrence is specific to the environmental challenge or selection pressure. Those elements were explicitly demonstrated for the *trpA* and *trpB* loci (2) in *E. coli*, and the study reported here is an identical experimental design to provide a similar demonstration for the *HIS4* locus in the yeast *Saccharomyces cerevisiae*.

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MATERIALS AND METHODS

S. cerevisiae* strains.** Strain 117-1a is *MATa** *his4-303 ino1-13 ura3-52*::*his4*(AUU)–*lacZ* (Ura$^+$); strain 105-3a is *MAT*α Δ*his4-401 leu2-3 leu2-112 ino1-13, ura3-52*::*HIS4*(AUG)–*lacZ* (Ura$^+$) (14). Strain GT160-34B is *MAT***a** *ade1 leu2 his6 met14 lys9* (15).

Media and Culture Conditions. Synthetic glucose minimal medium (SD) and rich broth medium (YPD; yeast extract/peptone/dextrose) were prepared as described by Sherman (16). To detect β-galactosidase synthesis, the chromogenic substrate 5-bromo-4-chloro-3-indolyl β-D-galactoside (X-Gal) (20 μg/ml) was added to SD medium that had been buffered to pH 7.0. Inositol-free synthetic glucose minimal medium (IFD) was prepared according to Lawrence (17). Liquid cultures were vigorously shaken at 30°C. Plates were incubated in humidified chambers at 30°C.

Fluctuation Tests. Sixty independent cultures were grown from small innocula to midlogarithmic phase in SD medium containing all required supplements. Appropriate dilutions of 10 cultures were plated onto YPD medium to estimate the number of viable cells per culture. The cultures were washed and resuspended in a small volume of saline; each entire culture was plated onto selective medium. The mutation rates were estimated as described (2).

RESULTS

Strain 117-1a carries a *his4* allele in which the translation initiation codon AUG has been mutated to AUU, and it cannot, therefore, synthesize the multifunctional *HIS4* gene product for biosynthesis of histidine. Strain 117-1a can revert to histidine prototrophy either by direct reversion of the initiator codon from AUU to AUG or by mutation in any one of three suppressor loci (14, 18). The suppressor mutations can be distinguished from the true AUU → AUG reversions because the suppressors also act on the *his4*(AUU)–*lacZ* fusion that is present and thus permit synthesis of β-galactosidase, which can be detected on plates containing the chromogenic substrate X-Gal (14).

A fluctuation test (19) was used determine the rate at which *his4-303* reverts to *HIS4* in growing cells under nonselective conditions. Cultures were plated on SD medium, and all revertants obtained were patched onto SD/X-Gal medium to determine whether the reversions were at the initiation codon of *HIS4* or were suppressor mutations. The reversion rate to *HIS4* was $8.7 \pm 6.4 \times 10^{-11}$ per cell division, and none of the reversions that occurred in the fluctuation test was the result of suppressor mutations. (This is, perhaps, not surprising

Abbreviation: X-Gal, 5-bromo-4-chloro-3-indolyl β-D-galactoside.

since the suppressed revertants typically grow much more slowly than do the true revertants.)

To measure the mutation rate under selective conditions, a culture of strain 117-1a was diluted and plated onto SD medium containing 2.5 μM histidine to give $\approx$125 colonies per plate, and the plates were incubated in humidified chambers at 30°C. Each day, two plates (upon which no His^+ papillae were present) were resuspended, diluted, and plated onto YPD medium to estimate the number of viable cells per plate. The maximum number of viable cells per plate, 4.4×10^7, was reached on day 3 of incubation, equivalent to 3.5×10^5 cells per colony. His^+ revertant papillae began to appear on day 6 and continued to appear for the next 17 days (Fig. 1*A*). Each revertant was isolated by streaking onto SD medium lacking histidine, and each was tested for synthesis of β-galactosidase by patching onto SD/X-Gal plates. Because we are interested in the mutation rate at a single locus, *his4*, only true AUU → AUG revertants, which are white on the X-Gal plates, are considered here. (Suppressor mutations did arise at $\approx$40% of the rate of true reversions, but no effort was made to determine which of the three suppressor loci had mutated.) The growth rates of several of the white revertants in SD medium were indistinguishable from the growth rate of the parent strain in SD medium containing histidine, confirming that they were, indeed true revertants.

Estimating Mutation Rates. Reconstruction tests were used to determine whether the papillae could have resulted from slowly growing revertants that arose during the growth of the colonies. Two *HIS4* revertants were tested. Dense suspensions were prepared that contained 9.5×10^5 cells of the Δ*his4* strain 105-3a and approximately one 117-1a *HIS4* revertant cell per μl 100 1-μl drops were placed onto SD medium to form instant colonies. The *HIS4* revertant cultures were grown in the presence of histidine to repress histidine biosynthesis and thus to mimic as much as possible the state of a newly arisen *HIS4* revertant mutant. As a control, 100 drops containing only the 105-3a cells were similarly plated. To determine the actual number of 117-1a *HIS4* revertant cells per μl, parallel suspensions were prepared that contained only the *HIS4* revertant cells, and 1-μl drops were plated onto YPD medium. At 24 hr, no papillae were visible on any of the instant colonies, but, in each case, at 48 hr the number and distribution of papillae on the Δ*his4* colonies were the same as the number and distribution of *HIS4* microcolonies from the drops on YPD plates where the Δ*his4* cells were absent. No papillae were observed on the instant colonies from the Δ*his4* cells alone. It is concluded that *HIS4* revertants form papillae within 48 hr of the occurrence of a *HIS4* reversion mutation. Because the frequency distribution of *HIS4* papillae was indistinguishable from the frequency distribution of the *HIS4* microcolonies on YPD plates, it appears unlikely that preexisting *HIS4* revertants grow slowly and therefore appear as late papillae. In contrast to these results with the true *HIS4* revertants, suppressor mutants required 3–4 days to form papillae on instant colonies. Together with the observation that suppressor mutants grow more slowly than wild-type or true revertants in liquid cultures, this observation suggests that it would not be possible to estimate when a suppressor mutation had occurred from the time at which a papilla appeared, which is another reason why suppressor mutations are not considered here.

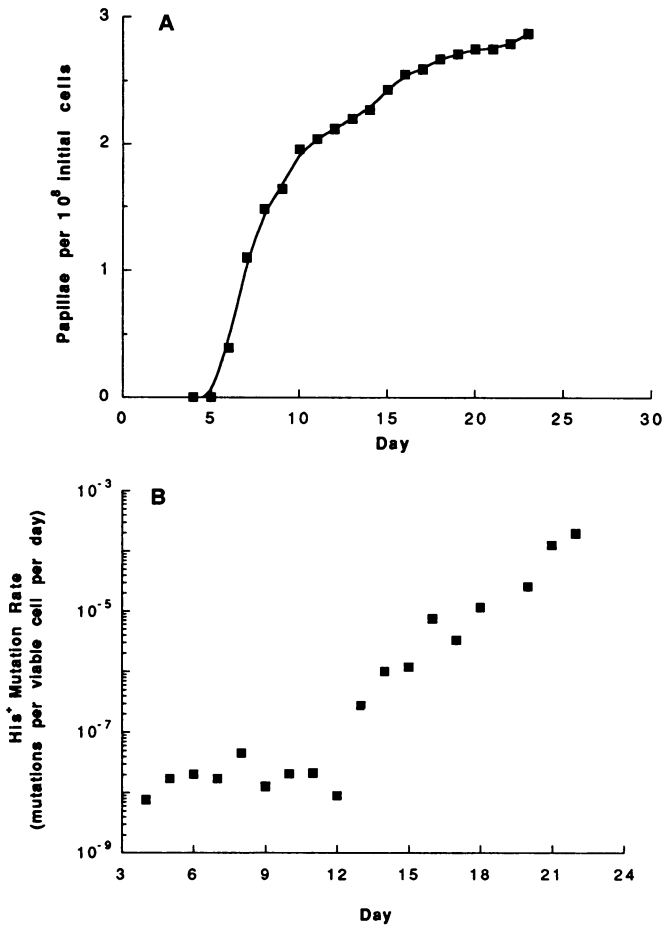

FIG. 1. Mutations from *his4-303* to *HIS4* in strain 117-1a. (*A*) Accumulation of *HIS4* papillae vs. time. (*B*) Mutation rate vs. time. If, during the last half of the experiment, a significant proportion of cells can revert to HIS4 on plates but cannot form viable colonies when resuspended and plated onto rich medium, then those points after day 12 may not represent the true mutation rate.

From the time required to form papillae, and from the number of viable cells present, it was possible to calculate the mutation rate from *his4-303* to *HIS4* each day. The mutation rate remained at $\approx 2 \times 10^{-8}$ per cell per day through day 12, at which time the mutation rate increased exponentially so that by day 22 the rate was 2×10^{-4} per cell per day (Fig. 1*B*).

During the course of this experiment, the cells died exponentially with a first-order rate constant of -0.69 day^{-1}. Because the mutation rates are calculated on the basis of viable cells, as the number of viable cells decreases the estimated mutation rate increases dramatically. It must be recognized that the number of viable cells that are able to mutate to *HIS4* and resume growth on the starvation plates is estimated from the number of cells that are able to survive resuspension and plating onto rich YPD plates. Although the plating efficiencies of cells from starved colonies plated onto YPD and plated onto SD medium containing histidine appear to be the same, we cannot eliminate the possibility that colonies older than 14 days might contain cells that will mutate and grow *in situ* but that will not form colonies upon being plated. Despite these considerations, in order to make comparisons between experiments it is necessary to consider mutation rates because the experiment-to-experiment variation in the death rate of cells makes direct comparisons of the rate of papilla formation (as in Fig. 1*A*) meaningless.

Evidence That the *HIS4* Reversions Were Selection Induced. It is formally possible that the calculated death rate was actually the result of a faster death rate combined with some growth at the expense of the dead cells—i.e., that deaths plus births were actually being measured (2). Were that the case, rather than resulting from selection-induced mutations, the papillae might result from ordinary replication-dependent mutations that were powerfully selected from a slowly growing minority population. The *ino1-13* mutation present in strain 117-1a causes inositol-less death—i.e., cells die if they grow in the absence of inositol (20, 21). If the measured death rate actually concealed some cell replication, then the observed death rate should be faster in the absence than in the

presence of inositol. Strain 117-1a was grown on filters on SD medium containing 5 μM histidine for 4 days, then half of the filters were transferred to SD medium, and the other half were transferred to IFD medium. The death rates were monitored by resuspending filters that had no HIS^+ papillae present, diluting the cell suspensions, and plating onto YPD medium to estimate the number of viable cells. The death rates were indistinguishable: -0.81 ± 0.16 (confidence limits, ±95%) per day on SD medium, and -0.80 ± 0.17 per day on IFD medium (Fig. 2). Unless dying cells provided sufficient inositol to prevent inositol-less death of growing cells (which, while it seems unlikely, cannot be explicitly ruled out), no evidence for growing cells could be detected. The number of new cells (births) that could possibly have arisen during the experiment can be estimated directly from the observed death rate in the presence of inositol (births plus deaths) and the true death rate in the absence of inositol (deaths) (2). If the true death rate was actually -1.24 per day (twice the 95% confidence limits), the number of births would have been 0.55 times the initial number of cells. In the experiment shown in Fig. 1, the observed death rate was -0.70 ± 0.16. Applying the same reasoning, and assuming that the actual death rate could have been as high as -1.02 per day, the maximum number of cell divisions (births) would have been 0.43 times the maximum number of cells in the experiment—i.e., no more than 1.4×10^9 cell divisions. At the observed rate of 8.7×10^{-11} mutations per cell division, no more than 0.13 of the 73 HIS4 revertants could have been accounted for by cell division-dependent mutations. It is concluded that cell replication could not have accounted for the observed number of HIS4 reversions and that the mutations were therefore time, not cell replication, dependent. These experiments do not rule out repair synthesis of DNA, but they argue strongly against genome replication.

The most crucial aspect of selection-induced mutations is that they do not represent a general increase in the genome-wide mutation rate under selective conditions (prolonged histidine starvation in this case); instead, they represent a specific response to the particular selective pressure applied by the current environmental challenge. To measure the specificity of the *HIS4* reversions, mutations at an outside locus that was not under selection, *ino1-13*, were measured on plates during prolonged histidine starvation. Ten-day-old colonies were resuspended and plated onto IFD medium containing histidine to select any *INO1* revertants that might have arisen during histidine starvation. Such measurements are valid only if the locus being tested is not subject to phenotypic lag; i.e., mutants that might be present must not be killed by the selective medium. The *INO1* locus satisfies that criterion because even *ino1-13* mutant cells can grow for one generation before they are killed by the absence of inositol (21). During that growth period, newly arisen *INO1* revertants become phenotypically, as well as genotypically, *INO1* and thus are not killed.

To establish a baseline for the comparisons, the reversion rate of *ino1-13* in growing cells was determined from a fluctuation test by plating cultures onto IFD medium containing histidine. (The one generation of growth on selective plates was taken into account in calculating the mutation rate.) The mutation rate from *ino1-13* to *INO1* in growing cultures was $1.5 \pm 0.7 \times 10^{-10}$ per cell division, very similar to the rate from *his4-303* to *HIS4*.

Strain 117-1a cells were spread onto SD medium containing 5 μM histidine. On the 4th day, 40 plates, each containing $\approx 6.2 \times 10^6$ cells, were individually resuspended and plated onto IFD medium containing histidine. Two of the suspensions produced a single *INO1* revertant; thus, the frequency of *INO1* revertants was 8×10^{-9}. The appearance of His^+ papillae was monitored, and between day 4 and day 10 the mutation rate to *HIS4* remained $\approx 2 \times 10^{-8}$ per cell per day. On the 10th day, 200 plates without papillae were resuspended and plated onto IFD medium containing histidine. No *INO1* revertants were found.

The death rate during that experiment was -0.5 day^{-1}. Since *INO1* revertants could not grow on the limiting histidine plates, they would be expected to die at the same rate as their *ino1-13* parent. (When an *INO1* revertant was mixed with its *ino1-13* parent, and the cell mixture was incubated on SD plates, the death rates of the two strains were indistinguishable.) If the 117-1a cells reverted to *INO1* at about the

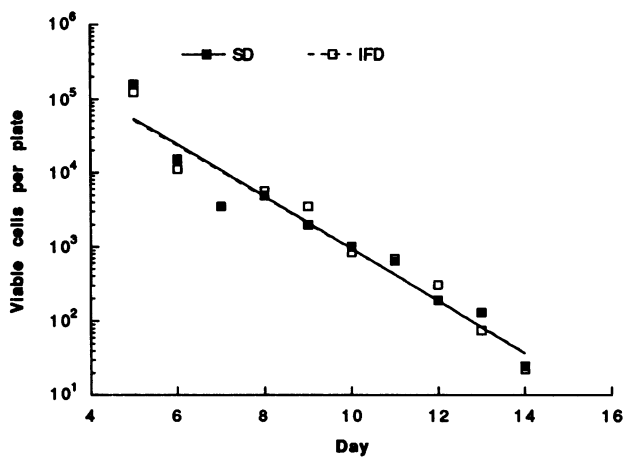

FIG. 2. Death rate of strain 117-1a during histidine starvation in the presence and absence of inositol.

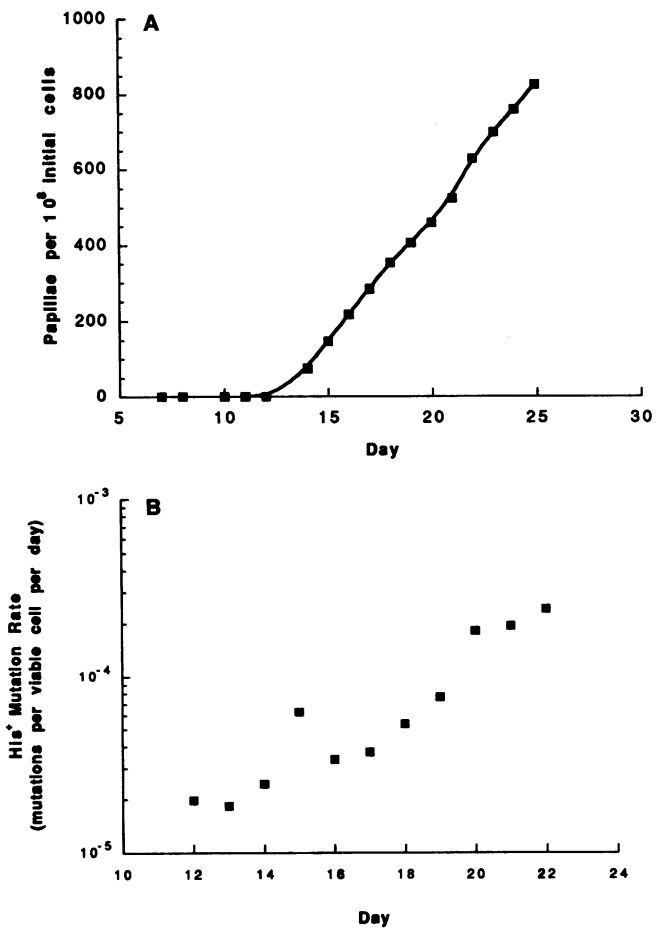

FIG. 3. Mutations from *his6* to *HIS6* in strain GT160-34b. (*A*) Accumulation of *HIS6* papillae vs. time. (*B*) Mutation rate vs. time.

same rate as they did to *HIS4* (as they did during exponential growth) then 15 surviving *INO1* revertants would still have been expected on day 10. The failure to find any *INO1* revertants on day 10 argues that the mutation rate to *INO1* under conditions of histidine starvation is at least an order of magnitude lower than the rate of mutation to *HIS4*. The high exponential death rate during histidine starvation makes it technically impossible to measure the specificity of the mutation process with any greater precision, but there is no reason to believe that the mutation rate to *INO1* is intrinsically much lower than that to *HIS4*. Within the limits of the experiment, it appears that the reversions to *HIS4* are, indeed, specific to the environmental challenge.

Because the issue of specificity with respect to environmental challenge is so critical to this issue, it would be desirable to conduct control experiments that are beyond the limitations of the present system. One very desirable control would be to use a strain with two amino acid auxotrophic mutations and to show that each reverts only when that particular amino acid is absent from the medium [as was done in *E. coli* (22)]. However, because reversion mutations are limited to base substitutions at a very small number of sites, such experiments do not address the issue of whether other kinds of mutations (frameshifts, insertions, deletions, etc.) might occur in genes that are not under selection. Additional experiments designed to further approach the specificity issue on both of these levels will be reported elsewhere.

To be sure that the results were not somehow peculiar to the locus being studied, reversion of a *his6* mutation in strain GT160-34B was also studied. The results were similar to those reported above with two exceptions: there was a 2-week delay before papillae began to appear (Fig. 3*A*), and the mutation rate was much higher, starting in the range of 10^{-5} per cell per day, and accelerating to 4×10^{-4} per cell per day. The death rate in that experiment was -0.35 per day. The rate at which papillae appeared was so high that after day 20 there were no plates without papillae from which to estimate the number of viable cells; thus, those estimates after day 20 were based on extrapolation of the death rate before that time. As a result, the mutation rates after day 20 (Fig. 3*B*) should not be considered to be as reliable as those before day 20.

CONCLUSIONS

These experiments provide no new insights into possible mechanisms of selection-induced mutations beyond those that have been discussed with respect to *E. coli* (1–3, 23, 24). They do provide convincing evidence, however, that selection-induced mutations occur in yeast and therefore are not limited to prokaryotes.

I am grateful to Jef Boeke for suggesting the use of the AUU-*his4* alleles, to T. F. Donahue and D. Campbell for the gift of strains, and to S. Bayoumy for his expert technical assistance. I particularly want to thank reviewer 2 for his comments on earlier versions of this paper. This work was supported by National Science Foundation Grant DMB 8903311.

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