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Direct Estimate of the Mutation Rate and the Distribution of Fitness Effects in the Yeast *Saccharomyces cerevisiae*

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ABSTRACT

Estimates of the rate and frequency distribution of deleterious effects were obtained for the first time by direct scoring and characterization of individual mutations. This was achieved by applying tetrad analysis to a large number of yeast clones. The genomic rate of spontaneous mutation deleterious to a basic fitness-related trait, that of growth rate, was $U = 1.1 \times 10^{-3}$ per diploid cell division. Extrapolated to the fruit fly and humans, the per generation rate would be 0.074 and 0.92, respectively. This is likely to be an underestimate because single mutations with selection coefficients $s < 0.01$ could not be detected. The distribution of $s \geq 0.01$ was studied both for spontaneous and induced mutations. The latter were induced by ethyl methanesulfonate (EMS) or resulted from defective mismatch repair. Lethal changes accounted for ∼30–40% of the scored mutations. The mean s of nonlethal mutations was fairly high, but most frequently its value was between 0.01 and 0.05. Although the rate and distribution of very small effects could not be determined, the joint share of such mutations in decreasing average fitness was probably no larger than ∼1%.

SPONTANEOUS mutation has been invoked to explain several phenomena. Some of these phenomena, such as inbreeding depression (Morton *et al.* 1956) or gradual meltdown of population fitness (Muller 1964; Gabriel *et al.* 1993; Lande 1994), are obvious consequences of the harmful nature of random mutations. It has also been proposed that deleterious mutations can be responsible for evolution of some major features of living organisms such as ploidy cycles (Kondrashov and Crow 1991; Perrot *et al.* 1991; Jenkins and Kirkpatrick 1995), senescence (Medawar 1952), genetic recombination (Kimura and Maruyama 1966; Feldman *et al.* 1980; Barton 1995; Gessler and Xu 2000), sexual reproduction (Kondrashov 1988), and several other features (Charlesworth and Charlesworth 1998). These and other theoretical studies usually find that the actual significance of spontaneous mutations depends critically on the rate at which they arise and the magnitude of effects they exert on fitness. It is thus not surprising that numerous experimental studies sought to estimate these parameters (reviewed in Drake *et al.* 1998; Keightley and Eyre-Walker 1999; Lynch *et al.* 1999). However, this research has been compromised by a serious methodological difficulty, that is, the necessity to use the same data to infer both a number and selection coefficients of mutations (Bateman 1959; Mukai *et al.* 1972; Charlesworth *et al.* 1990; Keightley 1994; Deng and Lynch 1996). A general principle has been to compare changes in mean fitness *vs.* changes in variance. A few mutations with conspicuous effects are inferred when a relatively small decrease in the mean population fitness is accompanied by a large increase in the variance. In contrast, many mutations with weak effects are invoked when a considerable decline in fitness is found but the variance increases only moderately. Unfortunately, the impact of deleterious mutations on fitness is difficult to measure both when laboratory populations have to be maintained for a long time (Keightley 1996) and when samples are taken from natural populations (Charlesworth and Hughes 2000). Additional complications arise while making assumptions about the deleterious effect of mutations. The effect may be fixed or, alternatively, variable and follow distributions with one or more modal values (Davies *et al.* 1999; Deng *et al.* 1999). In effect, the experimental estimates of deleterious mutation rate can vary by more than two orders of magnitude (selected examples of invertebrates: Mukai 1964; Keightley and Caballero 1997; Lynch *et al.* 1998; Garcia-Dorado *et al.* 1999) and it is unclear whether such a discrepancy reflects differences in genetics of the studied species and populations or methodological difficulties.

We aimed at overcoming these obstacles by applying direct scoring and characterization of individual mutations instead of using population-based inferences. Such an approach is not feasible with most organisms when rare mutants of quantitative traits are considered, but the yeast *Saccharomyces cerevisiae* provides some exceptional opportunities in this respect. Its major advantage

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is the ease of recovering all four meiotic products. The four spores produced from a single diploid cell develop into stable vegetative haploid clones whose gene expression pattern is, with few exceptions, the same as in diploids (Galitski *et al.* 1999). A homozygous diploid clone yields four haploid ones that are similar to each other and to their parental diploid. Our goal was to identify diploid clones that acquired a deleterious mutation in one locus only. In such cases, the four haploids showed a 2:2 segregation pattern: two haploids were wild type for the rate of growth and the remaining two grew slower. In our assays, the rate of growth was judged from the size of a colony. Development of a colony from a single-cell spore involved tens of cell-doubling generations and this was another important feature of our study. Growth from a single spore allowed for a natural amplification of the studied trait, growth rate, because even relatively small differences in the rate of growth amounted to considerable differences in size of the resulting colonies. Only small growth rate effects, <1%, would have been missed. The main reasons for using growth rate as a measure of fitness were not technical, however. The rate of growth is affected by more genes than any other phenotype that has been characterized in yeast (Hampsey 1997) and therefore it offers the widest "mutational target." In fact, our measure of fitness accounted not only for the potential to grow fast, but also for the ability to rapidly initiate metabolism when resources become available.

A major aspect of this study is the estimation of the rate and selection coefficients of spontaneous mutation. However, such mutations are infrequent and their molecular basis remains largely unknown. Therefore, in some experiments we introduced relatively numerous mutations of known nature so that the distribution of their selection coefficients could be studied in greater detail. These mutations were induced chemically or generated in strains in which an important system of post-replicational DNA repair, mismatch repair (*mmr*), was missing. The chemical mutagen, ethyl methanesulfonate (EMS), is known to cause primarily GC → AT transitions (Sega 1984) while loss of *mmr* leads to the accumulation of both substitutions and one-base deletions resulting in frameshifts (Kolodner 1996; Marsischky *et al.* 1996; Crouse 1998). This type of repair is found in many organisms, from bacteria to mammals, and therefore the spectrum of defects resulting from its malfunctioning probably represents a common class of mutations as they must arise even in repair-proficient cells, although at much lower rates. A major reason for working with point mutations from two different sources was to be independent of a single mutational spectrum. Both EMS and the absence of mismatch repair are known to have preferential sequence targets on a very small scale, such as GC pairs for EMS and tandem repeats for *mmr*. On a large scale, such as whole genes or genomes, the overall effect should be random. However, there is no systematic research that would ensure that no biases are present.

We started the experiments described below by establishing large populations of diploid cells. These cells were initially isogenic and homozygous at every locus, that is, identical to each other. Control populations accumulated only spontaneous mutations while experimental ones went through one of the mutagenic treatments. The newly arising mutations appeared in heterozygotes in which the wild-type alleles generally protected them from selection. The propagation of the mutated diploids was short and terminated with meiosis and production of spores. The spores developed into haploid strains in which any masking effect of the wild-type genes was absent and therefore fitness effects of both the spontaneous and induced mutations could be evaluated and compared.

MATERIALS AND METHODS

Strains: Two haploid strains that descended from the natural isolate Y55 (McCusker *et al.* 1987) were constructed. One of them was "wild type," *MAT***a** *MSH2* Δ*ho ura3*, and the other was a "mutator" strain, *MAT*α *msh2:KanMX4* Δ*ho ura3*. The latter strain mutated at a higher rate because its mismatch repair system was nonfunctional due to deletion of the *MSH2* gene. The *KanMX4* insert not only inactivated the repair function but also provided the cell with resistance to geneticin, which is a marker that is easy to screen and has no effect on fitness (Baganz *et al.* 1997). The Δ*ho* allele ensures that the mating types *MAT***a** and *MAT*α remain unchanged during propagation. The wild-type mutation rate is restored in the mutator strain when a functional *MSH2* allele is provided. This was accomplished by transforming cells with the pII-2 *MSH2 URA3* plasmid (Reenan and Kolodner 1992). Because the synthesis of uracil was possible only with the functional *URA3* allele, selection for uracil prototrophy or auxotrophy resulted in a presence or absence of the *MSH2* plasmid and thus in a low or high mutation rate.

Although the mutator strain did not mutate at an enhanced rate after receiving the plasmid, it could have accumulated some mutations during the preceding propagation. To begin with an essentially wild-type genotype we removed these mutations by 10 successive backcrosses to the nonmutator strain. In each cross, the two haploids were mated and formed a diploid, which was then induced to divide by meiosis and produce haploids. A haploid with the genotype *MAT*α *msh2:KanMX4 ura3*/pII-2 *MSH2 URA3* was isolated and used in the next back cross with the original *MAT***a**. The haploid products of the 10th sporulation were used to obtain three different diploid clones: *MAT***a** *MSH2*/*MAT*α *MSH2* without pII-2, *MAT***a** *MSH2*/*MAT*α *msh2:KanMX4* with pII-2, and *MAT***a** *msh2:KanMX4*/*MAT*α *msh2:KanMX4* with pII-2. The properties of these three clones and their applicability for the following experiments are explained below. Except for the genetic markers, they are considered to be homozygous and identical to each other because there was little chance that any unidentified variation remained after (or arose during) the serial backcrossing.

The EMS experiment: We started with a single diploid cell, *MAT***a** *MSH2 ura3*/*MAT*α *MSH2 ura3*. One-half of the population derived from this cell was treated with EMS while the other half was maintained as a control. Single clones were subsequently sampled at random from the two populations

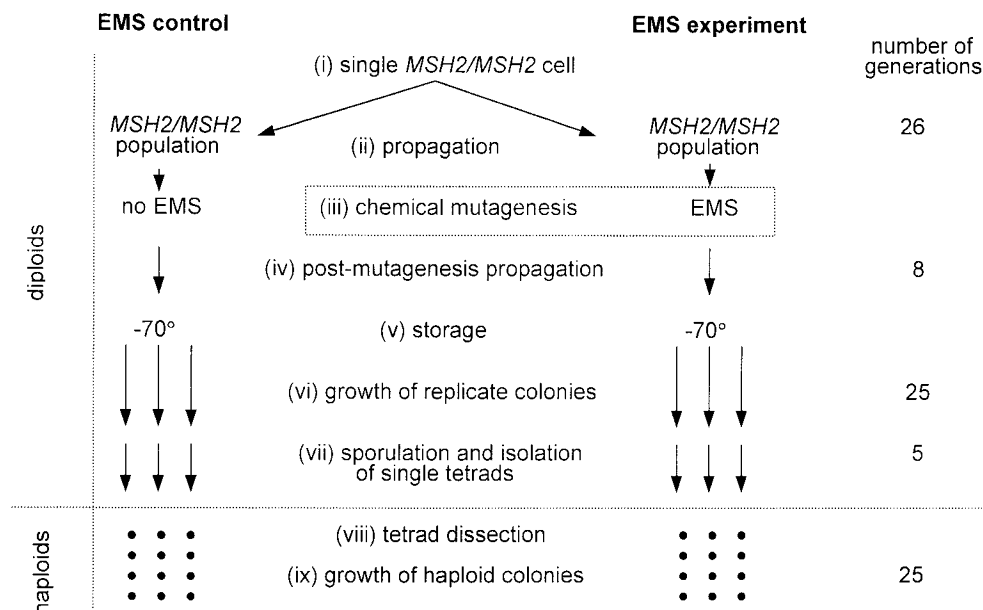

Figure 1.—Mutation accumulation in the EMS experiment. A single diploid cell (i) was used to initiate a liquid culture (ii). All propagation was done on the rich YPD medium. Two aliquots of the stationary phase culture were sampled and to one of them 2 μl of EMS was added for 1 hr (iii). Both aliquots were then washed to terminate mutagenesis and samples of them were used to inoculate fresh medium in which eight generations of growth were completed (iv). These cultures were stored at −70° (v). From these, replicate clones were obtained. This was done by diluting the samples and spreading them to single cells on an agar surface (vi). The resulting colonies were replica plated onto sporulation medium where, after some additional growth, diploid cells underwent meiosis and developed into tetrads of haploid spores (vii). A single tetrad was chosen at random from each colony, and the four spores were separated on a fresh agar (viii) and left to develop into haploid colonies (ix).

and meiosis and sporulation were induced. The effects of acquired mutations were detected by monitoring growth of the resulting haploid strains. Details of the procedure are presented in Figure 1.

The *mmr* experiment: In this experiment, our goal was to have two initially identical populations, but to gentically manipulate them such that in one of them the mutation rate was increased when requested. We used single cells of genotypes *MAT***a** *MSH2 ura3/MAT*α *msh2:KanMX4 ura3* with pII-2 to initiate a control and *MAT***a** *msh2:KanMX4 ura3/MAT*α *msh2:KanMX4 ura3* with pII-2 to initiate an experimental population. The control and experimental population were propagated in the same way but in the latter the mutation rate was elevated by forcing the loss of the *MSH2* containing pII-2 plasmid. (A single chromosomal copy of *MSH2* is sufficient to maintain a wild-type mutation rate.) Randomly chosen clones from both populations were then sporulated and the resulting haploid clones were assayed in the same way as in the EMS experiment. The procedure is fully described in Figure 2.

Measurements of mutational effects: Sporulation of diploid clones was effective; at least three-quarters, and usually many more, of the cells within a clonal colony underwent meiosis and generated four ascospores (tetrads). Tetrads were partially digested with glucuronidase, which prepared them for spore separation. Samples of digests from six sporulated colonies, three experimental and three control, were placed onto a fresh YPD plate in a random order. A single tetrad from each colony was dissected and the spores were placed at 1-cm intervals using a micromanipulator. Plates were incubated at 30° for 48 hr. Diameters of the resulting colonies were measured. An example of such a plate is shown in Figure 3.

The size of colonies could have been affected by an unavoidable variation among plates in thickness of agar, position in an incubator, slight differences in medium composition and preparation, and other factors. Therefore, every plate with 24 haploid colonies was considered an experimental block. The upper quartile of the 24 measurements per plate was chosen as a measure of plate quality because it was significantly less variable than the mean, median, or maximum measurement (data not shown). Every measurement was first divided by its plate's quartile and then multiplied by the average quartile of the respective experiment, EMS or *mmr*. Such a standardized diameter was used to calculate colony volume (assuming hemispheric shape for simplicity). This was then divided by 1.10×10^{-7} μl, the volume occupied by a typical haploid cell (Sherman 1997). A typical colony (~2.5 mm diameter) contained 3.32×10^7 cells. This means that ~25 generations of cell divisions were completed during 48 hr of incubation. The growth rate of a colony was calculated by taking the natural logarithm of the estimated number of cells and dividing it by 48. This is an average rate comprising both the possible differences in the germination time (~4 hr in the wild type) and the rate of subsequent growth that was steadily decelerating, although far from ceasing.

RESULTS

Occurrence of new phenotypes: Figure 4 and Table 1 show that most haploid clones derived from the mutation-accumulating diploids were apparently unaffected and the mutated clones formed only a thin tail of the distribution. This was intended. We wished to have a relatively low frequency of mutations because this ensured that most of the mutations would occur singly in separate diploids and show a 2:2 segregation pattern. Of course, not all of the small colonies, or inviable spores, appeared in pairs within tetrads. Rarely, there were three or even four affected haploids among the four derived from a single tetrad. This happened most often, although still relatively infrequently, in the *mmr* experiment. For example, for the lethal phenotype, there were 11 tetrads with three and 7 with all four haploid clones missing among 531 tetrads analyzed.

Table 2 shows how many tetrads there were with one or two spore clones with aberrant phenotypes. The data

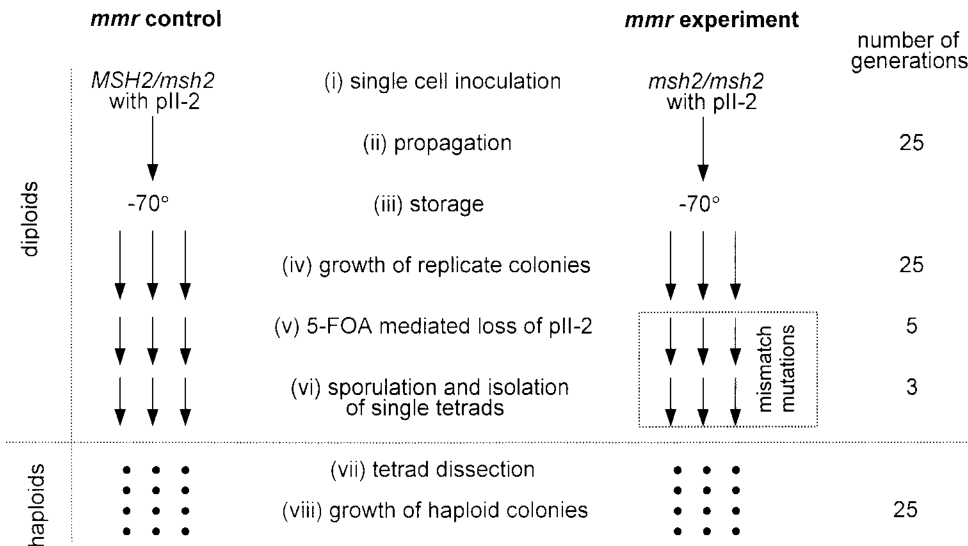

Figure 2.—Mutation accumulation in the *mmr* experiment. Both the control and experimental population were initiated from single cells (i). The two cultures were propagated (ii) on synthetic medium lacking uracil (SC-ura), which maintained the pII-2 plasmid. Samples of the stationary phase cultures were frozen (iii) and then used to obtain replicate clones. This was done by spreading the frozen cultures of single cells onto the same SC-ura medium solidified with agar (iv). The resulting colonies were replica plated onto synthetic medium containing uracil and 5-fluoroorotic acid (5-FOA; v). The latter compound killed effectively all cells containing the plasmid and thus a functional pathway of uracil synthesis (Boeke *et al.* 1984). The cells that happened to lose the plasmid before the exposure to 5-FOA were able to grow. Those clones that did not contain a functional copy of *MSH2*, *i.e.*, the experimental ones, began to mutate at a high rate. The control and experimental clones continued to mutate at different rates after replica plating on the sporulation medium (vi). A single tetrad from each clone was dissected (vii) and the spores allowed to grow and form colonies on the YPD plates. Note that all haploid clones in the experimental populations were repair deficient (*msh2*), while half of the control ones were repair proficient (*MSH2*). These two types were identified by replica plating of each plate with haploid clones onto YPD plates with geneticin.

are separated into lethals and nonlethals. The latter were defined as colonies whose diameter was 90% or less than the plate's standard (*i.e.*, its upper quartile; see materials and methods). This data can be used to assess how many of the observed abnormal phenotypes resulted from environmental effects and how many were likely to have a genetic basis. We conservatively assume that for the tetrads with one abnormal colony only environmental effects need be invoked. Using a Poisson distribution one can calculate the frequency of 2:2 segregation that might have resulted from accidental co-occurrence of two environmentally affected clones. For example, there were 21 tetrads with one dead colony among 508 tetrads of the EMS control, $p_1 = 21/508 = 0.041$. Assuming that environmental effects were randomly distributed over all tetrads and applying a Poisson formula, $p_1 = \mu/e^{\mu}$, the mean of this distribution was $\mu = 0.043$. The expected frequency of tetrads with two dead colonies was $p_2 = 0.0009$ and their predicted number was $0.0009 \times 508 = 0.45$. The observed number was 11. Table 2 shows that in the other experimental treatments the accidental co-occurrence of two environmental effects can account for at most only a few percent of all cases.

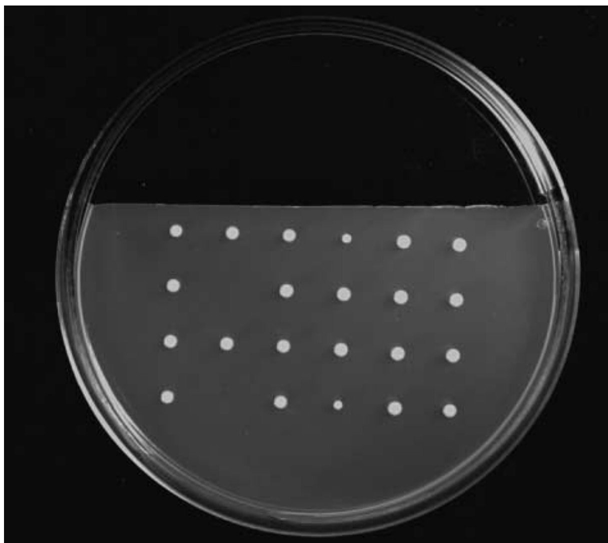

Figure 3.—A photo of a plate with dissected tetrads. Each column represents four haploid clones (a tetrad) derived from a single diploid clone after meiosis and sporulation. Three different experimental and three different control tetrads were located on one plate in a random sequence. An example of a lethal mutation is visible in the second column and of a nonlethal one in the fourth column.

The above analysis demonstrates that abnormal phenotypes tended to occur in pairs, but does not prove that they result from a mutation. The principle of 2:2 segregation is not only that two clones are altered but also that they resemble each other. Lethal phenotypes are unmistakable in this respect. Nonlethal ones are more problematic. For example, there can be a tetrad with two normal colonies, the third 10 times smaller (in volume), and the fourth 20 times smaller. As discussed below, repeated tetrad dissection indicates that these are true 2:2 segregations. However, it is very difficult to set simple quantitative criteria for the 2:2 segregation pattern of nonlethal phenotypes. Therefore we resorted

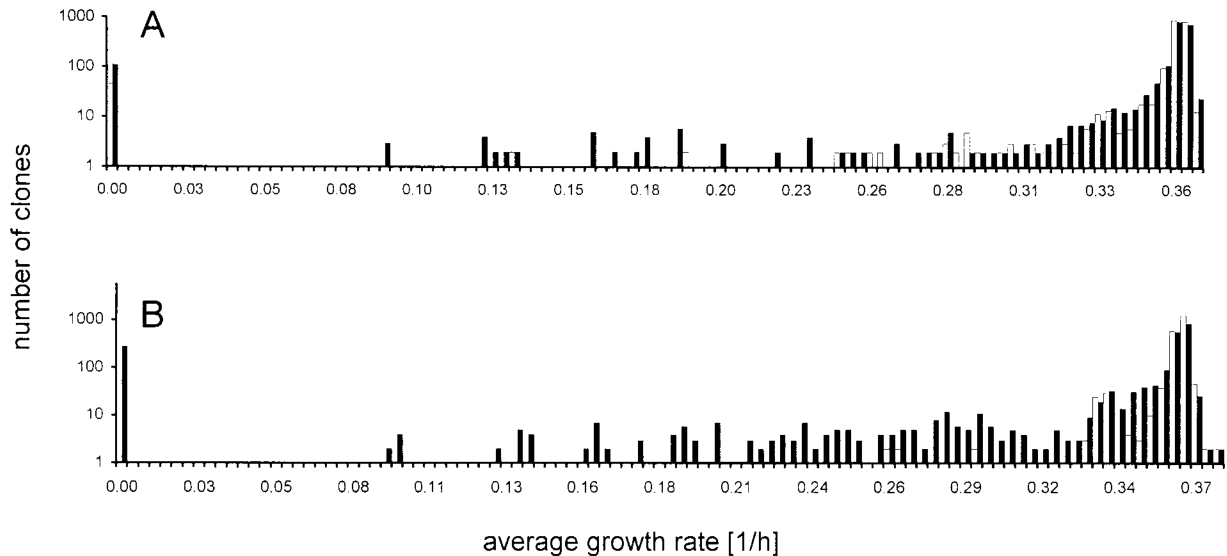

FIGURE 4.—Average growth rates. All clones derived from the mutation-accumulating diploids are included. The whole range of growth rates, from zero to maximum, was divided into 100 equal intervals. Because the frequencies are very variable, the vertical axis is scaled as the $\log_{10}$ of 1 plus the number of clones. (A) Haploid clones from the control (open bars) and EMS-treated (solid bars) populations. (B) Haploid clones from the control (open bars) and mismatch-repair-deficient (solid bars) populations.

to a qualitative method. To identify the cases of 2:2 segregation, two observers independently inspected the plates visually and identified tetrads containing two normal and two smaller (but generally similar to each other) colonies. Immediately after that, the diameters of all colonies were measured and their growth rates calculated (see MATERIALS AND METHODS). The two independent observers identified the same tetrad as a case of 2:2 size segregation when the volume of the mutant colonies differed from the wild-type ones by more than a quarter (equivalent to 8 to 9% of the diameter). Because the haploid colonies completed ~25 generations during 48 hr of growth it can be calculated that such a difference in volume arises when the mutant grows at ~99% of the wild type's rate, $0.99^{25} = 0.778$. Therefore, the method allowed detection of single mutational effects with selection coefficients of 0.01 or greater.

The reliability of our qualitative method could be tested by dissection of several tetrads from the same colony. One can expect that all tetrads derived from a colony would show the same 2:2 segregation pattern when mutations happened before separation of replicate clones (step vi in Figure 1 and step iv in Figure 2). Cases where the mutation arises after replicate clones are generated will give rise to a colony in which some tetrads will not exhibit abnormal phenotypes. Indeed,

TABLE 1

Average growth rates

	EMS			Mismatch repair deficiency		
Comparisons between	Experiment (1/hr)	Control (1/hr)	Difference (1/hr) (%)	Experiment (1/hr)	Control (1/hr)	Difference (1/hr) (%)
Means of all clones[a]	0.334768	0.349094	−0.014326 (100)	0.304496	0.350745	−0.046250 (100)
	$n = 2043$	$n = 2031$	$t = 6.582$***	$n = 2122$	$n = 2098$	$t = 15.933$***
Means of viable clones[a]	0.353453	0.356824	−0.003371 (23.5)	0.349265	0.359484	−0.010219 (22.1)
	$n = 1935$	$n = 1987$	$t = 5.467$***	$n = 1850$	$n = 2047$	$t = 12.207$***
Medians of viable clones[b]	0.358582	0.358631	−0.000049 (0.35)	0.359830	0.360637	−0.000806 (1.75)
	$n = 1935$	$n = 1987$	d.f. = 1, $\chi^2 = 9.95$**	$n = 1850$	$n = 2047$	d.f. = 1, $\chi^2 = 87.29$

Comparisons are made between the experimental and control populations presented in Figure 4. The percentages in parentheses show how much of the difference between the whole populations (set to 100%) remains when lethals are excluded (all viable clones left) and then the means or medians are compared. $^{**}P = 0.0016$; $^{***}P < 0.0001$.

[a] Student's t-test based on n observations and an assumption of unequal sample variances.

[b] Mood's test for comparisons between medians (ZAR 1999).

TABLE 2

The number of tetrads with one or two aberrant phenotypes

	EMS		*mmr*	
	Control	Experiment	Control	Experiment
Total no. of tetrads	508	831[a]	528	531
No. of tetrads with missing colonies				
One missing	21	37	22	35
Two missing—obtained	11	76	9	66
Two missing—predicted[b]	0.45	0.86	0.48	1.24
No. of tetrads with smaller colonies				
One smaller	24	43	9	63
Two smaller—obtained	38	126	7	109
Two smaller—predicted[b]	0.59	1.16	0.08	4.27

[a] The number of tetrads in the EMS experiment was considerably higher than that in the control. This was because some plates contained fewer control and more experimental clones (but still six in total). The additional experimental clones were included only in the analyses concerning single mutations, such as presented in this Table and Figure 5B. The comparisons of growth rates between the control and experimental clones, such as those presented in Table 1 and Figure 4A, always employed the same number of the control and experimental clones per plate.

[b] The "predicted" number of tetrads with two aberrant phenotypes is the expected number of such tetrads if two aberrant phenotypes co-occurred in a single tetrad due to random events as described in the text.

we found colonies of both types, as reported below in the section on spontaneous mutation rate. However, the EMS mutagenesis was done before separation of single clones. Therefore, we chose this experiment to redissect tetrads from stored samples of colonies that had previously given rise to tetrads containing size variants. Of 181 clones reanalyzed, there were only 7 in which no abnormal colonies were found and 2 in which the pattern of segregation was clearly different from that originally found (K. Szafraniec, D. M. Wloch, R. H. Borts and R. Korona, unpublished data). This clearly indicates that only a minor fraction (∼5%) of the abnormal colonies are not due to genetic mutation.

Spontaneous mutation rate: To estimate the spontaneous mutation frequency, we used the counts of mutations obtained from the control clones and not the scores of abnormal phenotypes listed in Table 2. The numbers are similar but not identical because the two methods applied different criteria. To summarize the procedures described in detail above, abnormal phenotypes were defined as those smaller by 10% or more than a typical colony on a plate, while mutations were determined on a basis of a 2:2 pattern of colony sizes within one tetrad. There were 48 single mutations, lethal and nonlethal, among the $n = 508$ control clones in the EMS experiment. Therefore, the fraction of clones with one mutation was $p_1 = 48/508 = 0.0945 \pm 0.0254$. The error term is a 95% confidence interval calculated from the formula $t_{0.05,\, n-1}[p_1(1 - p_1)/(n - 1)]^{1/2}$. We assume that the number of mutations per genome is randomly distributed; that is, it follows a Poisson distribution. From the frequency of the single mutations determined here, p_1, the Poisson distribution allows us to calculate the frequency of clones with zero mutants. This is estimated at $p_0 = 0.9004$. Since there were 64 generations of growth during the mutation accumulation experiment (Figure 1), the probability that no mutation happened in a tested clone is $p_0 = (1 - U_E)^{64}$, where U_E denotes the genomic mutation rate per diploid cell division in the EMS control. Solving the equation results in $U_E = 0.00164$. The lower and upper 95% confidence limits of U_E are 0.00116 and 0.00216, respectively; they were calculated using the confidence interval of p_1. In the control of the *mmr* experiment, 19 mutants occurred among 528 clones during an estimated 58 generations of growth. This yielded a mutation rate in diploid cells of the *mmr* control $U_m = 0.00064$ with 95% confidence limits at 0.00035 and 0.00094. Averaged over the two controls, the rate of spontaneous mutation is $U = 0.00114$ or $\sim 1.1 \times 10^{-3}$.

The estimates of mutation rate might have been biased because the experiments were started from single cells. Therefore, both the number and phenotypic composition of mutants might have been heavily affected by some early mutations. We believe that further analysis of the relatively numerous mutations in the EMS control can be used to confirm or dispel such doubts. We dissected six more tetrads from each colony in which a mutant was found. There were 20 monomorphic colonies in which all six tetrads showed an expected effect: the absence of 2 colonies or their smaller sizes resembled the originally dissected tetrad. These mutations probably arose during the ∼34 generations before sampling replicate clones (Figure 1). In the remaining 28 colonies, the expected phenotype was detected in only some tetrads while the others had no mutation. Such a

polymorphism was likely to arise during the 30 generations of growth after sampling replicate clones. The proportion of the monomorphic colonies, 0.417 = 20/48, does not differ significantly from the proportion of time spent in a common culture, 0.531 = 34/64 (t = 1.593, d.f. = 47, P = 0.234). This means that the mutations from the first phase of accumulation were about as abundant as they should be. Therefore, no jackpot mutation happened in the initial phase of mutation accumulation.

The calculation of mutation rates was based on an assumption that the frequency of deleterious mutations was not affected by natural selection during the accumulation phase. To estimate how strong the masking effect of a wild-type allele could have been, we measured maximum growth rate of the cultures of diploid clones initiated from colonies on master plates in which a mutation was later found (10 lethals, 10 severe, and 10 small growth defects) and compared them with the maximum growth rate of 10 diploid clones in which no mutation was detected. The average maximum growth rates for the lethal, severe, mild, and control clones were 0.53, 0.50, 0.48, and 0.52 (1/hr), respectively. A one-way ANOVA test showed no significant differences (d.f.'s = 3, 36; F = 1.397; P = 0.259), which suggests that the frequencies of mutants could not have been reduced considerably by selection.

Selection coefficients of single mutations: Relative fitness, w, of a single mutant was calculated as the mean growth rate of the two mutant colonies divided by the mean of the two wild-type colonies within a tetrad. Selection coefficient of a mutation was calculated as $s = 1 - w$. The wild-type colonies were defined as the two that were closer to the plate's wild type, *i.e.*, its upper quartile. In only one case in the EMS and one in the *mmr* experimental populations did the mutants grow faster than the wild type (w equal to 1.015 and 1.026, respectively); these two mutants were excluded from the analyses described in this section.

To begin the analysis of selection coefficients, we had to account for the fact that some of those found in the experimental treatments were likely to be spontaneous and therefore had to be excluded. This was done separately for the EMS and *mmr* experiments. We first matched every mutation from a control with a mutation from an experiment so that their selection coefficients were identical or as close as possible, and then we eliminated the latter from the set of experimental mutations. (In case of the EMS experiment, for every control mutation, 1.6 experimental mutations were removed to compensate for a higher number of experimental clones; see Table 2.) For the purpose of comparisons, we pooled the spontaneous mutations from both the EMS and *mmr* controls because their sample sizes were considerably lower than those of induced mutations and the two controls were expected to be equivalent.

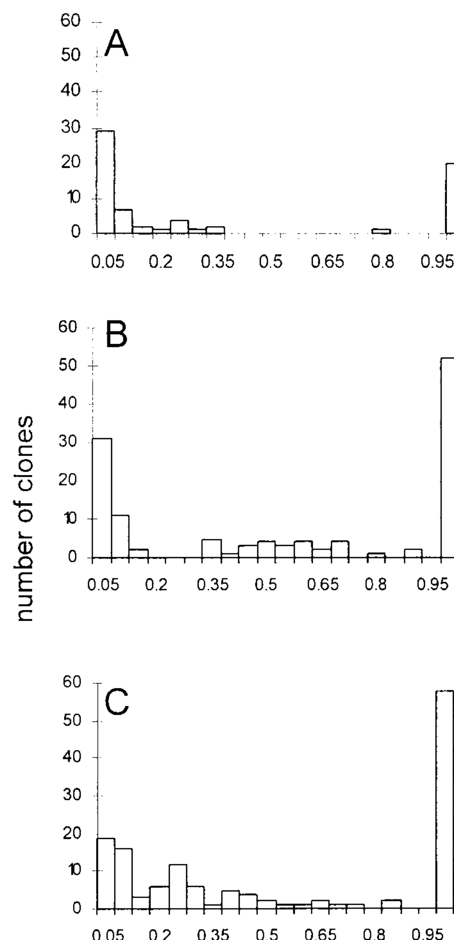

Figure 5.—Selection coefficients of single mutations. (A) Spontaneous mutations collected from controls of both the EMS and mismatch-repair-defective experiments. (B) The net distribution of the EMS mutations. (C) The net distribution of the mismatch repair mutations.

Figure 5 presents the distributions of deleterious mutations graphically and Table 3 summarizes some of the statistical analysis. Comparisons between the selection coefficients of nonlethal mutations suggest that the spontaneous ones are less harmful than both the EMS-induced (Kolgomorov-Smirnov two sample test; D = 0.351, n_1 = 49, n_2 = 74, P = 0.0014) and those obtained in the *mmr* clones (D = 0.443, n_1 = 49, n_3 = 83, P < 0.0001). The distributions of the EMS and *mmr* mutations show some striking similarities. The proportions of lethals to nonlethals are practically identical, as are the average selection coefficients of the nonlethals (Table 3). However, the shapes of the distributions are different (D = 0.255, n_2 = 74, n_3 = 83, P = 0.0124) with a notable bimodality of the EMS distribution.

The single mutations included in the above analyses were tested for two characteristics to avoid potential biases. First, they were verified to grow on the nonfermentable glycerol plates (YPG), which ascertained that they were not *petites, i.e.*, cells with nonfunctional mitochondria. The petites were generally rare. In the *mmr*

TABLE 3

Distribution of the selection coefficient *s* of the individually scored mutations

Parameters of nonlethal mutations	Spontaneous	EMS induced	*mmr* induced
Number (*vs.* no. of lethals)	49 (20)	73 (52)	82 (58)
Average *s*	0.086	0.235	0.214
Coefficient of variation of *s*	1.58	1.11	0.93

experiment, 2.8% of tetrads contained one or more petite haploid clones. These form a small peak in both the control and experimental populations at a growth rate of ~0.335 h^{-1} (Figure 4B). We cannot provide similar counts for the whole EMS experiment, but our incomplete observations suggest that the petites were considerably less frequent. The second trait of interest was the mutator phenotype. Two of the four haploids derived from each control *mmr* tetrad were mismatch repair deficient. The mutator and nonmutator haploid clones grew at a very similar rate ($t = 0.023$, d.f. = 2045, $P = 0.981$), indicating that neither the kanMX4 marker nor the absence of mismatch repair affected growth rate. While it was expected that kanMX4 would be neutral (Baganz *et al.* 1997), one might have expected an absence of mismatch repair to affect growth rate. A possible explanation is that the initial cells had some repair protein provided by the diploid cell. This reserve became insufficient after some divisions, but the developing colonies were already large enough that newly arising mutants composed a small fraction of its volume. To summarize, neither the petite nor mutator phenotypes were likely to interfere with our estimates of the rate and distribution of mutational effects.

DISCUSSION

Classical genetic analysis calls for understanding the segregation pattern of an examined trait. Application of this Mendelian approach to yeast is especially straightforward because it can be realized in the one-step procedure of tetrad analysis. This study shows that even fitness-related traits and rare alleles can be studied effectively in this way.

Spontaneous mutation to visibly harmful effects: Calculations of the rate of deleterious mutation with $s \geq 0.01$ carried out separately for the EMS and *mmr* controls have yielded two estimates, 0.00164 and 0.00064 per diploid cell division, respectively. This inconsistency can possibly be explained by the difference in growth conditions during the mutation accumulation phase. The EMS experiment was carried out in a medium rich in nutrients (YPD) while a synthetic complete medium (SC-ura) had to be used in the *mmr* experiment to maintain the plasmid complementing the repair function. The strain of yeast used in this study grows ~30–40% faster in the rich medium (Korona 1999a). Of course, the two media modify metabolism of a cell in many ways other than simply shortening the time between divisions. Whatever the reason for the difference between the two results, they are not disparate. They are definitely on the same order of magnitude and therefore we decided to use their average, $U = 1.1 \times 10^{-3}$, as a single grand estimate.

We have been able to draw three major conclusions about the selection coefficients of spontaneous mutations. Lethals are relatively frequent, ~30% of the visibly deleterious mutations. Among the nonlethal mutants, the most frequent are those with effects of a few percent. Such small effects were probably more frequent among spontaneous mutations than among induced ones. Some previous studies suggested that the fraction of lethals among spontaneous mutations should be much lower. For example, Mukai *et al.* (1972) suggested that only ~5% of spontaneous mutations were lethal in the fruit fly. On the other hand, our data seem to support the expectation that mutations with effects on the order of 1% should be the most common among the nonlethal ones (reviewed by Charlesworth and Hughes 2000). Some recent studies suggested that more severe mutations may be relatively common (Fernandez and Lopez-Fanjul 1996; Keightley and Caballero 1997; Fry *et al.* 1999; Vassilieva *et al.* 2000). However, these experiments have relied on statistical approaches in which large effects tend to overshadow small ones (Crow and Simmons 1983). A better interpretation of the cited results is probably that large mutational effects are indeed present but this does not mean that the small ones are infrequent.

Our data enable us to test the impact of variation in selection coefficients on the estimates of mutation rate. We applied the Bateman-Mukai method (Mukai *et al.* 1972) to our data by comparing distributions of the live clones in the EMS experiment and its control as well as in the *mmr* experiment and its control. Using the estimate of coefficient of variation of *s* (Table 3) we found that for the EMS data the estimated number of mutants was 41 and the average selection coefficient 0.19. The analogous estimates for the *mmr* experiments were 86 and 0.18. These estimates are not very different from the results obtained by direct scoring (Table 3), although the Bateman-Mukai method tends to underestimate the number of mutations when the difference

in means is as small as in the case of the EMS experiment (Table 1). If the variation of s could not be estimated, however, one could set only a lower limit for the number of mutants and an upper limit for the average s (Mukai *et al.* 1972). These would be, respectively, 19 and 0.42 for the EMS experiment and 46 and 0.33 for the *mmr* experiment. Thus, the error would increase considerably.

Fitness effects of induced substitutions and frameshifts: The distributions of selection coefficients in the EMS and *mmr* experimental treatments were generally similar. The proportion of lethals was 42% for both. Among the nonlethals, those having effects of a few percent were the most common. More pronounced differences between the distributions of effects might have been expected, given the different nature of the mutation generated in the two experiments. EMS treatment causes almost exclusively 1-bp substitutions while *MSH2* defects lead to predominantly 1-bp insertion or deletion mutations (Marsischky *et al.* 1996; Crouse 1998). These cause frameshifts and therefore do more harm to the genes than substitutions. However, as our data show, this need not translate to more severe fitness effects. Comparison of our experiments with studies where genes were silenced completely by deletions or large inserts supports the conclusion that the nature of the mutation may be less important than originally thought. Entian *et al.* (1999) deleted 150 different genes in a diploid yeast and looked for the 2:2 segregation of growth phenotypes by tetrad analysis. Lethals composed 54% of deletants showing any degree of growth defects, although almost two-thirds of all deletants were not visibly affected. Smith *et al.* (1996) inserted a marked Ty1 element to 268 genes. They found that about one-half of the inserts had no visible effect. Among the remaining, 33% were lethal while the rest grew slower when tested on YPD. Thus, the proportion of lethal mutations among all visibly deleterious ones is similar for substitutions, frameshifts, and large deletions or insertions. Of course, we do not know whether the small phenotypic effects found in our study resulted from serious damage to less important genes or minor alterations to important genes. It is also possible that the proportion of substitutions or frameshifts that happened to be neutral or nearly neutral, and thus escaped our attention, was much higher than in the case of the large damages.

Partitioning of mutational load: The difference in the mean growth rates of the whole experimental and control populations is about three times higher than when only viable clones are compared (Table 1). This means that, both in the case of EMS and *mmr*, the lethal mutations contributed about three-quarters of the mutational load of experimental populations. The rest of the load must have been contributed by relatively rare mutations with large nonlethal effects. This conclusion is drawn from the comparisons summarized in Table 1 where the difference between the medians of viable clones is only about one-twentieth of the difference between the means. The very small differences between the experimental and control medians are very informative. The small difference shows how important for the mean fitness the left tails of the distributions are. For example, the difference between the experimental and control median in the EMS experiment divided by the control median was extremely small, −0.00014. Suppose that there were frequent but very small effects that escaped our attention. If the difference between the mean and median was primarily the effect of such mutations, they would be very slightly deleterious indeed. In principle, one could devise a procedure, such as maximum likelihood estimation (Keightley 1994), that would find what distribution of genetic variation was most likely to produce the observed difference in the distribution of the experimental and control phenotypes and thus reveal the presence of slightly deleterious mutations. Unfortunately, although we are confident that that environment was on average the same for the control and experimental populations, the presence of genotype × environment interactions can be neither *a priori* neglected nor reliably assessed. Such influences may be nuances that do not alter significantly the estimates of genetic variation. However, the impact of the slightly deleterious mutations is probably also a "nuance" as it is evidenced by the very small shifts of medians observed.

Confronted with such uncertainties, we did not attempt to assess the parameters of the slightly deleterious mutations. Neither did we neglect their presence. We conclude, however, that whatever their number and selection coefficients are, their joint impact is not larger than about one-hundredth of the total mutational load and one-twentieth of the load of nonlethal mutations (compare the percentages in Table 1).

Comparison with other estimates: Zeyl and DeVisser (2001) have reported in a recent study that the spontaneous mutation rate in yeast is $U = 9 \times 10^{-5}$, while the average effect is $hs = 0.21$. The parameter h stands for the dominance coefficient of a mutation and, unless the mutation is dominant, its value is between 0 and 0.5. U is much lower and hs much higher than the values obtained in this study. Zeyl and deVisser (2001) measured mutations in a diploid yeast. The major class of mutations affecting fitness that they obtained (19/20) were those that lost mitochondrial function as measured by inability to grow on a nonfermentable carbon source. The maximum likelihood analysis that they performed was based on the one remaining mutant and the 30 unaffected clones and therefore the U and hs should be treated with caution. We suggest that the discrepancy between our estimates and those of Zeyl and DeVisser reflects the fact that they studied diploids and therefore many, and probably most, mutations were largely masked by wild-type alleles.

Our experiment introduces several novelties into the research on fitness effects of spontaneous mutation. One of its strengths is the considerable sample size, 67 mutants among over 1000 accumulation lines. Calculations were based on a simple model derived from a Poisson distribution and experimentally verified assumptions about a constant rate of mutation and the strong masking effect of the wild-type alleles. The latter was confirmed not only by the data presented here on compensation of the EMS mutants, but was also evident in our former study in which hundreds of diploid clones were transiently deprived of mismatch repair and their fitness was found mostly unaffected under standard laboratory conditions (Szafraniec *et al.* 2001). The primary advantage of this study was the opportunity to score individual mutations manifesting their chromosomal origin by Mendelian 2:2 segregation. Such an analysis is not possible in other organisms; thus, studies attempting to establish these parameters for other organisms have had to rely on indirect approaches. Baker's yeast contains 6200 genes of an average length of 1450 bp (Sherman 1997). A fruit fly has 13,600 genes of an average length of 1770 bp (Adams *et al.* 2000), which are replicated ~25 times in the germline of an individual. Therefore extrapolation of our estimate for the fly would yield $U = 0.074$. In humans, there should be no more than 39,000 genes of an average length of 1340 bp (Genome Sequencing Consortium 2001; Venter *et al.* 2001). The average number of germ-cell divisions in a 25-year-old is 144 (Crow 2000) and therefore an extrapolation of our rate to humans would yield $U = 0.92$ per generation. The reader can easily find in extensive reviews on the subject that our estimate of the deleterious mutation rate is neither extremely high nor extremely low when compared to those reported in studies applying approximate methods (Drake *et al.* 1998; Keightley and Eyre-Walker 1999; Lynch *et al.* 1999).

The experimental investigation of spontaneous mutation has been recently challenged by studies based on comparisons of DNA sequences between different species (Eyre-Walker and Keightley 1999; Keightley and Eyre-Walker 2000). An especially attractive feature of such an approach is that it promises to uncover all mutations, including the smallest. In fact, the distribution of s is ignored as any non-neutral allele would be necessarily lost over time spans on a phylogenetic scale. The estimates obtained in this way—$U = 0.066$ for fruit flies and 1.31 for humans—are very similar to our extrapolations. [Keightley and Eyre-Walker (2000) report $U = 3.0$ for humans, but we have scaled it down according to the above cited newer findings about the number and average length of genes.] This concordance between the new phylogenetic data and the first direct experimental estimate is interesting and potentially important. Some cautionary notes should be made, however. We could not score mutations of s below 0.01. Yet they must have been there, perhaps more numerous than those individually discernible. From this perspective our estimate could potentially be higher than those obtained by the phylogenetic approach. Such comparisons are not straightforward, however. First of all, we do not know how many mutations analyzed in this experiment were substitutions, the kind of mutations studied by Eyre-Walker and Keightley, and how many were of different types. Linear extrapolations based on the genome size could be inappropriate because of differences between genetic systems. The larger genomes may be, for example, more "redundant" and thus less susceptible to mutations affecting the fitness-related traits. Furthermore, we have studied mutations appearing on an isogenic background in a stable environment while the natural populations are genetically heterogeneous and confronted with a wide array of habitats. It is still largely unknown how often mutations are only conditionally deleterious and how often the direction of selection may change across different environments and genetic backgrounds (Rose 1982; Curtsinger *et al.* 1994). We do find evidence that the selective value of a mutation depends both on the environment and genetic background (Korona 1999a,b; Wloch *et al.* 2001). We also expect that masking of mutations by the wild-type alleles often may be very strong if not complete (Szafraniec *et al.* 2001). Mutational load of the fruit fly is also strongly dependent on environment (Shabalina *et al.* 1997; Yang *et al.* 2001). It is possible that comparing the DNA sequences will be useful while assessing a "grand" deleterious mutation rate averaged over large populations and many generations, as well as all encountered habitats and genetic backgrounds, although this method has its own uncertainties (Keightley and Eyre-Walker 1999; McVean and Vieira 2001). Such estimates may help in answering questions about major biological phenomena (Keightley and Eyre-Walker 2000). However, mutational load of extant populations depends both on the current rate and distribution of mutational effects that are influenced by genetic, population, and environmental factors. Understanding of many issues of evolutionary biology, and especially those of conservation biology and human health, will rely on further careful experimental work.

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