Recently obtained data on the influence of microorganism cultivation conditions on frequencies of spontaneous mutations (Ilyina et al., 1986; Cairns et al., 1988) have revived interest in the origin of spontaneous mutants. There subsequently appeared more and more new experimental data, as well as models, that try to explain such observations (Ilyina et al., 1987; Stahl, 1988; Hall, 1988, 1990; Lenski et al., 1989; Korogodin et al., 1989, 1990; Davis, 1989; Boe, 1990). Existence of a large number of models bears evidence that the laws of this phenomenon are not yet studied properly. In this paper we report data that add new features to the known laws of this phenomenon.

Materials and methods

Strains

The haploid strains *NA3-24* (*a leu2-1 lys1-1 can1-23 RAD*) and prototroph *S288Cα* (Chepurnoy and Mikhova-Tsenova, 1988) of the yeast *Saccharomyces cerevisiae* were used in this study.

Experimental procedures

Using the method of ordered plating (Von Borstel, 1978), we suspended the yeasts in water and plated them by an inoculator with 220 pins on a lavsan nuclear filters (Fleroff, 1984) covering a solid medium in the Petri dishes. Thus in every Petri dish about 220 macrocolonies emerged. After growing the cells at 30°C for a given time interval, a few tens of colonies were resuspended in water, and the number of cells per colony was determined by microscopy. Buds and parental cells were counted as separate cells. Other colonies were transferred, together with the filters, to selective media for selection of *leu2* and *lys1* revertants (in strain *NA3-24*) or *LYS2* mutants (in strain *S288C*), which are clearly indentifiable in these media as colonies of secondary growth.

Media

The growth medium for *NA3-24* strain cells was minimal medium *M3* (Zakharov et al., 1984) containing lysine (30 mg/l) and leucine (0.3 - 300 mg/l). For the *S288C* strain cells, the media *M3*, and *M3* with 10 mM of 3-Amino-1,2,4-Triazole (*3AT*), were used. The selective media included *M3* with lysine for detection of *leu2* revertants, *M3* with leucine for detection of *lys1* revertants, and medium with α-aminoadipate as a nitrogen source (Chattoo et al., 1979) for detection of *LYS2* mutants.

Quantitative estimation of mutants

The dynamics of appearance of revertants on selective media is usually characterized by a curve with two peaks: the first population corresponds to the revertants originating before the transfer of the cultures on the selective medium, whereas the second one corresponds to those originating after the transfer, during residual growth (Chepurnoy and Mikhova-Tsenova, 1988). Quantitative estimation of mutants in the culture at the moment of transferring the cells to the selective medium was based on their number in the first wave of the "appearance curve".

Mutation rate was calculated from the proportion of colonies without revertants:

$$R = 1/n \cdot \ln(N/N_0) \qquad (1)$$

where n is the number of cells in a colony, N is the total number of colonies, $N_0 = (N-M)$ is the number of colonies without mutants, M is the number of colonies with mutants.

To determine the mutation spectrum of *NA3-24* cells, all selected revertants were checked for ability to grow in *M3*. The *leu2-1* and *lys1-1* alleles are ochre-suppressible, so a revertant able to grow in medium without leucine and lysine was classified among suppressor (S) reversions while a revertant unable to grow in *M3* was classified among locus (L) reversions.

Synchronization of the cells

Synchronization of the cells was carried out by the method of Williamson and Scopes (1962). For strain *NA3-24* this method gives the very high degree of synchrony. Only 3% of cells had the buds after three cycles of synchronization.

Results

After determining the *leu2* revertant contents in the *Saccharomyces cerevisiae* culture by the earlier described technique (Chepurnoy and Mikhova-Tsenova, 1988), the number of revertants (per cell per generation) was found to depend on the leucine concentration in the medium. On the other hand, the spontaneous L-type *lys1* revertant content remained constant in the investigated leucine concentration range (Fig.1).

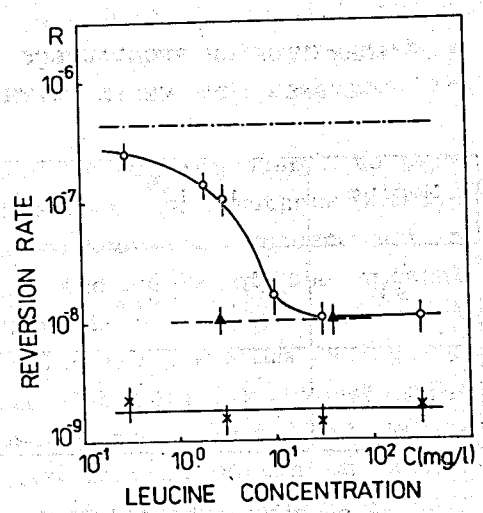
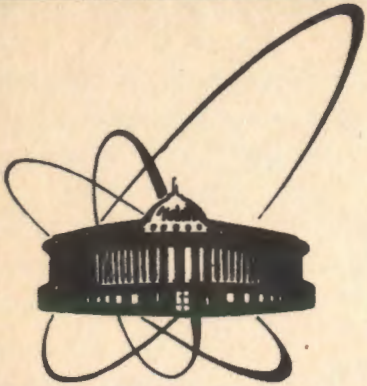


Fig.1. Rate of occurrence of locus-type revertants in the exponential phase of growth of strain *NA3-24* cultures as a function of the leucine content in the medium: locus revertants to leucine prototrophy (o); locus revertants to lysine prototrophy (x); locus revertants to leucine prototrophy in the cells of subline (*sub1*) of strain *NA3-24* (▲); hypothetical dependence of reversion rate on leucine contents (–·–).

The results shown in Fig.1 are consistent with hypothesis of increased levels of mutagenesis in actively transcribed DNA (Ilyina et al., 1987; Davis, 1989) since *LEU2* is activated at the level of transcription when the leucine content in the medium decreases (Andreadis et al., 1984). Simi-



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larly, the constant formation rate of L-type *lys1* revertants may be due to the fact that transcriptional activation of *LYS1* does not depend on the lysine contents in the medium.

To check this hypothesis, experiments were carried out to determine direct mutation rates in *LYS2* gene in *S288C* cultured in media with and without *3AT*. Wolfner et al. (1975) described a change in the activity of gene *LYS2* by a factor of 7 after *3AT* was added to the medium. As seen from the data in Table 1, the mutation rate of this gene does not change in *3AT*-containing medium. The assumption that the mutation rate of genes depends on their activity does not fit with this result. A similar result was obtained by Cairns et al. (1988) with activation of the lac-operon of *E.coli* by isopropyl-thiogalactoside (*IPTG*). Activation of the operon did not change the spontaneous reversion rate of the *lacZ*-gene.

Table 1

FORMATION OF SPONTANEOUS *LYS2* MUTANTS IN STRAIN *S288Cα* ON MEDIA WITH AND WITHOUT *3AT*

Medium	$n, \cdot 10^6$	N	M	$R, \cdot 10^{-7}$
M3	4,5 ± 0,5	240	158	2,4 ± 0,4
M3 + *3AT*	3,0 ± 0,3	240	127	2,5 ± 0,4

n, number of cells in a colony;
N, number of colonies;
M, number of colonies with mutants among *N* colonies;
R, mutation rate per cell per generation.

Concerning the data shown in Fig.1, it should be mentioned that we interpreted these functions as an increase in the spontaneous reversion rate of the *leu2* gene with decreasing

leucine concentration in the medium (Chepurnoy et al., 1989; Korogodin et al., 1989; 1990). These results allow another interpretation as well. One may assume that on all media, whatever the leucine content, revertants form with the same rate (hypothetical line in Fig.1). Yet, the major fraction of revertants appearing in the culture under non-selective conditions (30 mg/l of leucine) cannot compete with the cells of the initial strain and therefore die. As the leucine content decreases, fewer revertants die because their selective preferences increase as compared with the initial strain cells. The increase in the number of detected mutants is interpreted as an increase in the spontaneous mutation rate of the gene.

This possibility was considered in the paper of Korogodin et al. (1988), where this assumption was checked by testing a part of *ade2* revertants, which formed on a medium with a low adenine content and had a selective advantage while growing, for their ability to compete with the initial strain cells when they are cultured together on a medium with a higher (30 mg/l) adenine content. All revertants had competitive ability.

Since we used another system, it was reasonable to perform a similar reconstruction experiment. All mutants detected on medium with a low leucine content were resuspended in water in equal proportions, and the cells of the initial strain *NA3-24* were added, so that there was no more than 1 mutant per $\approx 10^5$ initial strain cells per inoculum during the ordered plating. If the competitive ability of revertants is low, one should expect a decrease in their number per colony during joint cultivation with the initial strain on medium with a normal (30 mg/l) leucine content. The results of this cultivation are shown in Table 2. The revertants selected on medium with 3 mg/l of leucine do not die when cultured jointly with the initial strain on medium with 30 mg/l of leucine.

Since we investigated mutants that had formed many generations after their origin, we were not sure of the competitive ability of mutants in the first hours following their

Table 2

JOINT CULTIVATION OF *leu2* REVERTANTS AND STRAIN *NA3-24* CELLS ON A MEDIUM WITH 30 mg/l OF LEUCINE

t	0	2	4	6
n	$1,4 \cdot 10^5$	$1,6 \cdot 10^5$	$3,6 \cdot 10^5$	$6,0 \cdot 10^5$
M	404	431	452	393
m	0,62 ± 0,03	0,67 ± 0,04	0,72 ± 0,04	0,59 ± 0,03

t, cultivation time, hours;
n, number of initial strain cells in a colony;
M, number of colonies with mutants in samples of $N = 880$;
m, mean number of mutant cells in a colony.

formation. To address this issue, one must be able to distinquish mutants that have just been formed and never divided, as well as those that have divided one, two, or more times. Evidently, there is a set of these spontaneous mutants in the culture in the exponential phase of growth. So, we carried out a series of experiments according to the following scheme.

Cells cultured on a medium with low leucine content (3 mg/l) were transferred to a medium with normal leucine content (30 mg/l) in the exponential phase of the culture growth. The revertant content in the culture was determined over a 8-hour period on medium where mutants had no selective advantage. The results are shown in Fig.2. Cultivation of cells on a medium with a low leucine content is characterized by reduced dividing time (8÷10 hours per generation), and by higher leucine revertant frequency. The L-type revertant rate

calculated by formula (1) is $\approx 1.5 \cdot 10^{-7}$ per cell per generation. After transfer of cells to a medium with 30 mg/l of leucine a two-hour lag-period was observed, after which the cells start dividing at a rate of 2÷3 hours per generation. At the same time a reduction of *leu2* revertant content was observed in the culture. The number of revertants in the culture was observed to decrease for the first 4 hours after transfer. Then the number of L-revertants grew at a rate $R \simeq 1 \cdot 10^{-8}$ per cell per generation which is typical of cell cultivation on medium with 30 mg/l of leucine (see Fig.1).

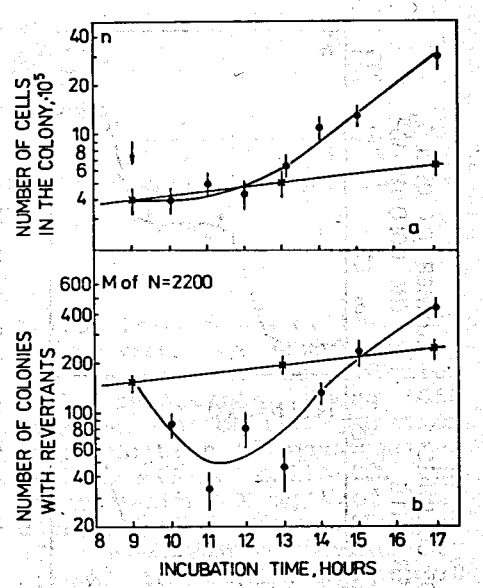


Fig.2. The reduction of number of revertants colonies after transfering the culture of strain *NA3-24* cells from the medium with 3 mg/l of leucine (x) to that with 30 mg/l of leucine (●).

In different experiments, the reduction in the mutant content varied from 30% to 70% of the number of mutants at the moment of transfer, i.e. practically half of the revertants were not found after several hours of cultivation on a medium with normal leucine content. The reduction in the number of revertants was evidently due to a reduction of "newborn" mutants.

It should be mentioned that accidentally we picked up a strain *NA3-24* subline (*sub1*) with *R* independent of leucine content (Fig.1) . On the other hand, in this subline no disappearance of revertants was observed after transfer from medium with low leucine content to medium with normal leucine content. Therefore, the data shown in Fig.1 and Fig.2 may be the consequences of the same process.

If revertants are formed with the same rate on different media, and the difference in mutant yield is caused by their different competitive ability on media with different leucine content, one may expect that there is a time interval between formation and disappearance of mutants. Then, in a synchronous culture growing on the medium with 30 mg/l of leucine, one may observe many more revertants in the period of theirformation at the S-phase of the cell cycle (Chepurnoy et al., 1989) than, for example, at the beginning of the S-phase of the next cell cycle. However, the experiments with the synchronous culture did not show that the number of revertants in the culture decreased from one S-phase to another (Fig.3). The observed number of both types of revertants appearing in S-phase corresponds to a rate $2 \cdot 10^{-8}$ per cell per generation.

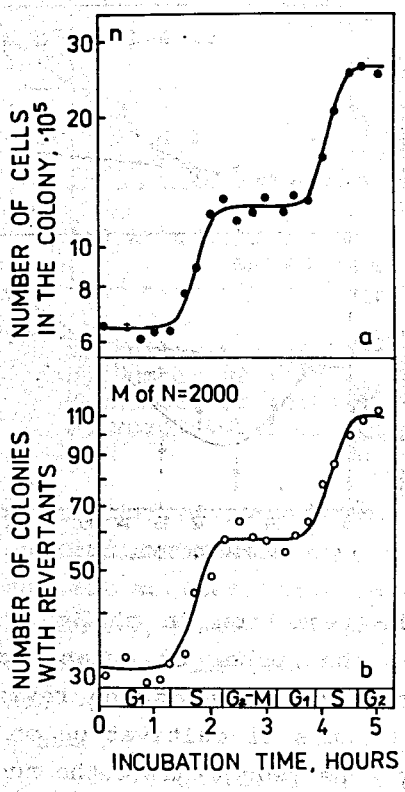
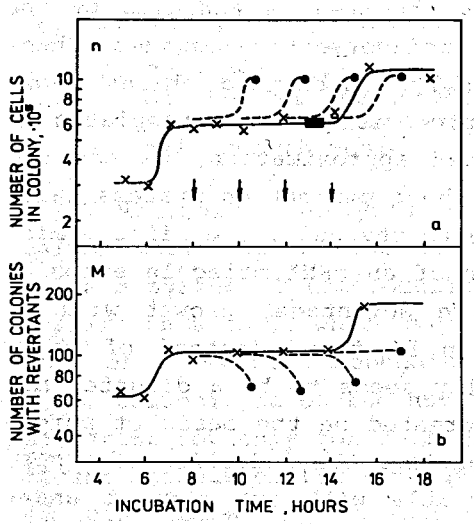


Fig.3. The content of colonies with revertants in the synchronizing culture of strain *NA3-24* cells during two cell cycles.

Therefore if the different revertant frequency of the culture can be explained by death of revertants, it follows that there is a very short time interval between formation to death which cannot be detected by our technique.

Further experiments with the synchronous culture (Fig.4) showed that disappearance of revertants after culture transfer takes place at the end of the G_1-phase of the cell cycle, and this phenomenon was observed up to the end of the G_1-phase of the next cell cycle (Fig.5).

Fig.4. Generation of revertants during the S-phase on the medium with 3 mg/l of leucine (x) and reduction of its number at the end of G_1-phase of the next cell cycle on the medium with 30 mg/l of leucine (●). The arrow shows the transfer of the culture from the first medium to the second one.



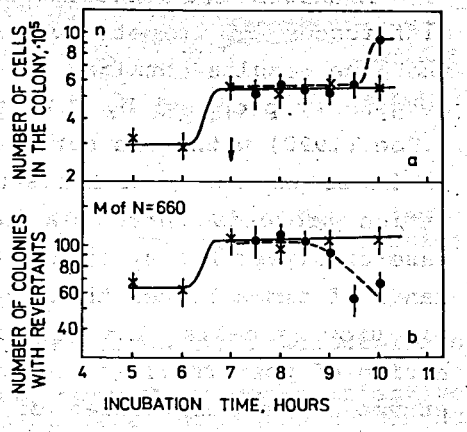


Fig.5. Dependence of the number of cells in a colony (a) and the number of colonies with revertants among 660 colonies (b) on the duration of incubation of synchronized strain *NA3-24* cells on the medium with 3 mg/l of leucine (x) and after transfer of the cultures (marked by arrows) to the medium with 30 mg/l of leucine (●).

Discussion

All results presented here are based on the assumption that spontaneous mutations in genes are formed randomly and with a constant rate for the given gene. In the future the survivability of a mutant depends on its competitive ability under given conditions. If mutations are advantageous for cell growth, the mutants survive. If mutations make cells less competitive, the mutants have more chance to die than the initial strain cells. This leads to an increase in the yield of advantaged mutants, which has been interpreted as an increase in the rate of "adaptive" mutations.

Although the experiments described here are based on differences in competitive ability of mutants, we believe that the results obtained can also be explained by the model, originally proposed by F.W.Stahl (1988) and complemented by L.Boe (1990) with some our additional assumptions. This model is based on the post-Luria-Delbruck demonstration that mutation proceeds through a reversible intermediate. Polymerase-catalysed DNA synthesis has a mistake rate that is thousands of times higher than the observed mutation rate of freely growing cells. The fidelity observed is achieved by the action of post-replicative mismatch-correction enzymes. These enzymes remove stretches of newly synthesized chains whose sequences fail to be fully complementary to the templates on which they were made. To a good approximation, we can say that mutation result only when these correction systems fail. If the mismatch is of advantage to the cell, i.e. if the mismatch results in the production of an mRNA molecule encoding a product providing a selective advantage, growth will be stimulated and the mutation can be fixed instead of being repaired. What phenomenologically seems to be a directed mutational event can thereby be created on the basis of purely random processes. In this explanation the induction of a specific mutation is not incompatible with our current understanding of molecular biology and genetics. In fact, this model involves only known enzymatic activities and processes known to take place in the cell.

Our first assumption actually is that not only post-replicative, but also pre-replicative, correction of heteroduplexes occurs (Fig.6). The second assumption states that pre-replicative and post-replicative corrections work effectively on the coding strand of *leu2* when cells are cultured on medium with 30 mg/l of leucine, and do not work (or work ineffectively) when cells are cultured on the medium with 3 mg/l of leucine.

In the case of the first assumption, it should be mentioned that a similar hypothesis was put forward by N.V.Luch-

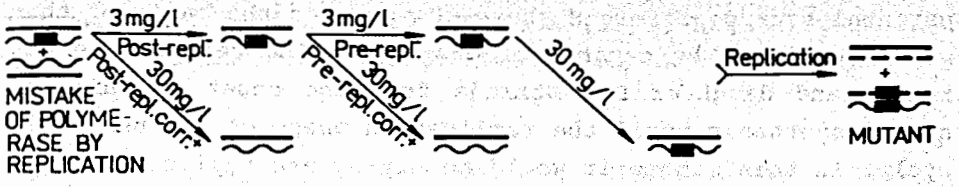


Fig.6. The model for generation of spontaneous *leu*$^+$-reversions on the cell growth media with different leucine contents (3 and 30 mg/l).

Major assumption of the model:

1. The pre-replicative correction of heteroduplexes exists in the cell.
2. Pre- and post-replicative correction works effective on the DNA strand encoding *leu2*- gene product at 30 mg/l of leucine but not at 3 mg/l of leucine in a cell growth media.

nik (1971) after analysis of chromosome aberrations in mammalian cells. As to the second assumption, it actually implies a system of heteroduplex correction regulation (or DNA repair) in a cell, which may increase or decrease the efficiency of correction depending on the cultivation conditions (e.g. composition of the nutrient medium). This effect can be different not only for different genes, but also for two different strands of the same gene. Regulation of the efficiency of this process may be similar to the work of the general or specific system of gene activity regulation being at the same time independent of it. Post-replicative correction cannot be separated from replication in our system. Pre-replicative correction (if it exists) works at the end of the G_1-phase or at the beginning of the S-phase of the cell cycle (the black rectangle on the growth curve of the synchronized culture, Fig.5a) and can be temporally separated from replication by our technique. Transferring cells from medium with low leucine content to medium with normal or high (≥30 mg/l) leucine contents before pre-replicative correction results in correction of heteroduplexes by daughter strand repair. It is not known how a cell can distinguish between the daughter and

parental strands before S-phase. However on the basis of this model, cell may be capable of distinguishing between the parental and daughter DNA strands from the onset of daughter strand synthesis until the replication phase of the next cell cycle. In this scheme it would be during pre-replicative correction that the "status" of the daughter strand and that of the parental strand are determined. Transferring cells to medium with high leucine content after pre-replicative correction would not lead to a decrease in the number of revertants, as we have observed.

In conclusion, we think there are two possible explanations for "adaptive" mutagenesis. One is based on dependence of competitive ability of mutants as determined by cultivation conditions, and the other is the hypothesis of intermediate heteroduplex correction proposed by F.W.Stahl (1988).

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