JOURNAL OF BACTERIOLOGY, Mar. 1973, p. 1307-1310

Vol. 113, No. 3
Printed in U.S.A.

# Evidence for an Extrakaryotic Mutation Affecting the Maintenance of the rho Factor in Yeast

A. HANDWERKER, R. J. SCHWEYEN, K. WOLF, AND F. KAUDEWITZ
*Institut für Genetik der Universität München, 8 Munich 19, Germany*

Received for publication 14 November 1972

A newly isolated, temperature-sensitive mutant of a haploid strain of *Saccharomyces cerevisiae* is described. Its shift to nonpermissive temperature (35 C) resulted in an irreversible change to rho$^-$, causing, within four to six generations, more than 90% of the cells to form petite colonies. Genetic analysis revealed extrakaryotic inheritance of this temperature-sensitive mutation. Data presented indicate mutation of a gene in the mitochondrial deoxyribonucleic acid affecting the maintenance of the rho factor.

In yeast, two main classes of mitochondrial mutants have been described: (i) rho$^-$ mutants and (ii) antibiotic-resistant mutants. The first class consists of two groups, namely, neutral rho$^-$ mutants which have lost the genetic information of mitochondrial deoxyribonucleic acid (DNA), therefore called rho$^0$ (6, 8, 9), and suppressive rho$^-$ which retain at least some mitochondrial DNA with meaningful genetic information (9). The presence of this genetic information was demonstrated by experiments on recombination and hybridization of mitochondrial DNA (2, 3). Expression of the residual information has not been observed, because all rho$^-$ mutants tested so far are deficient in mitochondrial protein synthesis (10). Their usefulness in uncovering the information content of the mitochondrial DNA, therefore, seems to be limited. The second class of mitochondrial mutants comprises types resistant to different antibiotics inhibiting mitochondrial protein synthesis (4, 13) or oxidative phosphorylation (5, 11). These mutants have proved to be useful in experiments on mitochondrial recombination and promise to be helpful in the identification of products coded by the mitochondrial DNA (2). A new mutant, belonging to a third class, is described in this paper. At 35 C, this mutant is unable to form colonies on media with the nonfermentable carbon source glycerol. Data are presented which prove the extrakaryotic, presumably mitochondrial, inheritance of this temperature-sensitive instability of the rho factor.

## MATERIALS AND METHODS

**Strains.** The temperature-sensitive mutant is derived from the haploid strain of *Saccharomyces cerevisiae* M 12 *a ilv5, trp2, ura3,* originating from S. N. Kakar. The following strains were used in crosses: 1761 *α gal1, his4, trp1, ade2, leu2, lys2, tyr6* (Seattle Stock Culture); 1422 *α his2, trp5, ura3, met2, ade1, leu1, lys7* (originating from O. C. Hawthorne); 26III *α lys7, met2, trp* (derived from M12 and 1422); H243 *α ade1, lys2, trp1* (spore isolate from D 243, kindly provided by F. Sherman); and IL 126-2A/52 *α ura, rho°,* (analyzed and kindly provided by P. P. Slonimski).

**Media.** The media used were essentially those described earlier in detail (1, 7). Complete media are based on Wickerham minimal medium supplemented with yeast extract (1%) and glucose (3%) or glycerol (3%). The selection of diploids and the analysis of auxotrophic markers were performed on Wickerham minimal medium supplemented with amino acids and bases as needed and with glucose (5%). Sporulation was obtained on acetate agar plates (sodium acetate, 0.4%; agar, 1.5%).

**Isolation of temperature-sensitive mutants.** Cells of strain M 12, previously grown in glycerol, were incubated with *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine at doses leading to 80 to 90% inactivation. Cells were then cultured for three to five generations in glucose media and were subsequently plated on glucose media at 23 C. Colonies arising on these plates were replica plated to test temperature-sensitive growth on glycerol.

**Mitotic segregation.** Cells of both mating types were grown for at least four generations in glycerol liquid media (respiratory-deficient cells in glucose), mixed (2 ml of each culture), and grown for about six generations in static culture with glucose (5%) as

carbon source. After dilution, the mixture was plated on Wickerham minimal medium to form single colonies. These colonies were differentially replica plated and grown, i.e., on glycerol at 23 and 35 C, and on glucose at 35 C. After 3 to 4 days, the plates were scored for temperature-sensitive growth on glycerol and for petite colonies (no growth on glycerol).

**Tetrad analysis.** Diploids were sporulated on acetate agar. Asci were digested by use of glucuronidase (Boehringer, Mannheim) and dissected by use of a micromanipulator (Leitz, Wetzlar). Spores were grown to colonies and tested by replica plating on different media.

## RESULTS

**Growth characteristics and temperature-dependent, irreversible change to rho$^-$.** The study of mutations affecting mitochondrial functions is complicated by the high mutation rate of most strains to rho$^-$. To avoid these difficulties as far as possible, we started induction and isolation of mutants with strain M 12, which is known to have a low spontaneous rho$^-$ mutation rate of about 0.05% (1). Cultures grown in glucose at 23 or 35 C contained less than 3% rho$^-$ cells.

The mutant M 12/ts-8 exhibited growth on glucose media at 23 and 35 C. On glycerol, growth of the mutant was slightly reduced at 23 C, as compared to the parental strain. At 35 C on glycerol media, no growth was observed after 4 to 6 days, when plating single cells, or after replica-plating colonies previously grown at 23 C. At 23 C, the mutation rate to rho$^-$ was low. Cultures grown on liquid glucose media for 10 generations, contained less than 5% rho$^-$ cells. When grown at 35 C, the mutant M 12/ts-8 showed a high instability of the rho factor. With an increasing number of generations, there was an increase in the percentage of cells forming petite colonies (Fig. 1). After four to six cell generations at 35 C, more than 90% of the temperature-sensitive cells had changed to cells forming petite colonies. The genetic analysis of these petite colonies (complementation to rho$^+$ and to suppressive and neutral rho$^-$) revealed that the petite cells were rho$^-$. This implies that growth of the temperature-sensitive mutant M 12/ts-8 at 35 C results in mutation of the mitochondria to rho$^-$. The curve shown in Fig. 1 does not represent the kinetics of manifestation of rho$^-$ cells at 35 C. Its slope is affected by several factors resulting in the formation of mixed colonies composed of rho$^-$ and rho$^+$ cells. Only pure colonies showing no growth on glycerol after replica-plating were taken as petite.

**Tetrad analysis.** The mutant M 12/ts-8 was crossed to three wild-type strains of different origin (Table 1). Tetrads were analyzed for temperature-sensitive growth on glycerol media. In crosses 1 and 3, all spores analyzed showed wild phenotype. No temperature sensitivity was recovered after meiosis. At least three auxotrophic markers were tested and segregated 2:2. In cross 2, spores exhibited a wide and varying range in their ability to grow on glycerol at 23 C. A few spores showing reduced growth on glycerol at 23 C were unable to grow at 35 C on glycerol. These spores, however, did not carry the temperature-sensitive mutation of the parent strain M 12/ts-8, as demonstrated by their failure to induce rho$^-$ mutation at 35 C. No meiotic segregation of the temperature-sensitive mutation studied occurred, thus indicating an extrakaryotic inheritance of this mutation in strain M 12/ts-8.

**Mitotic segregation.** To prove the occurrence of mitotic segregation, cells of the mutant M 12/ts-8 were diploidized with cells of several wild-type strains. The diploids were grown for about six generations in fermentable substrate and then plated on glucose medium; the colonies arising were tested for the occurrence of

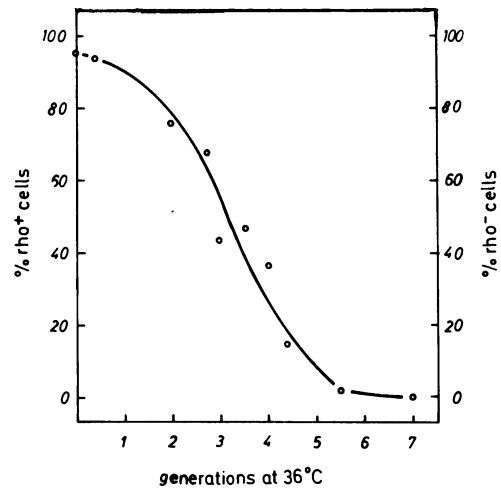


FIG. 1. *Cells of the mutant M 12/ts-8 were grown at 23 C in 3% glucose medium to early stationary phase. After dilution, the cells were grown at 35 C in an aerated liquid culture containing 3% glucose. At different times, cells were diluted and plated on glucose media at 23 C to form single colonies. The number of generations was calculated from the number of colonies arising. These colonies were differentially replica plated. Colonies which failed to grow on glycerol plates, both at 23 and 35 C, are petite. A more direct estimation of petite cells, by differential plating, was not useful because of varying colony-forming ability of the temperature-sensitive cells on glycerol at 23 C.*

TABLE 1. *Analysis of tetrads from crosses of the temperature-sensitive mutant M 12/ts-8 with wild-type strains showing normal growth on glycerol*[a]

| Cross | No. of tetrads analyzed and ratio | | | | | |
|---|---|---|---|---|---|---|
| | 4-spore | RC-ts | 3-spore | RC-ts | 2-spore | RC-ts |
| M 12/ts-8 × 243 RC | 12 | 4:0 | 53 | 3:0 | 55 | 2:0 |
| M 12/ts-8 × 1422 RC | 17<br>2 | 4:0<br>3:1 (?) | 68<br>2 | 3:0<br>2:1 (?) | 19 | 2:0 |
| M 12/ts-8 × 1761 RC | 7 | 4:0 | 6 | 3:0 | | |

[a] In the second cross, and partly in other crosses, spore clones were isolated, plated to form single colonies, and differentiated by replica plating. The question marks indicate that within this tetrad the phenotype of all spores could not be analyzed beyond doubt. RC, respiratory competent; ts, temperature-sensitive growth on glycerol media.

TABLE 2. *Mitotic segregation of the temperature-sensitive mutation in crosses with rho⁺ strains*[a]

| Cross | Diploids tested | Percent RC | Percent ts | Percent RD |
|---|---|---|---|---|
| M 12/ts-8 × 1761 RC | 1,280 | 70 | 29 | 1 |
| M 12 RC × 1761 RC | 230 | 99 | 0 | 1 |
| M 12/ts-8 × 26 III RC | 274 | 65 | 34 | 1 |
| M 12 RC × 26 III RC | 325 | 98 | 0 | 2 |
| M 12/ts-8 × H 243 RC | 560 | 57 | 41 | 2 |
| M 12 RC × H 243 RC | 185 | 99 | 0 | 1 |

[a] The experiments were repeated at least two times, with similar results. RC, respiratory competent; ts, temperature-sensitive growth on glycerol media; RD, respiratory deficient.

TABLE 3. *Mitotic segregation of the temperature-sensitive mutation in crosses with rho⁰ or rho⁻ strains*

| Cross | Percent RC | Percent ts | Percent RD |
|---|---|---|---|
| M 12/ts-8 × IL 126—2A-52 rho⁰ | 0 | 96 | 4 |
| M 12 RC × IL 126—2A/52 rho⁰ | 100 | 0 | 0 |
| M 12/ts-8 × H 243 EB-1 $rho_n^-$ | 0 | 100 | 0 |
| M 12 RC × H 243 EB-1 $rho_n^-$ | 100 | 0 | 0 |

[a] RC, respiratory competent; ts, temperature-sensitive growth on glycerol media; RD, respiratory deficient; rho⁰, loss of mitochondrial DNA.

the temperature-sensitive mutation by differential replica-plating. The results of experiments with three different strains are presented in Table 2. The transmission of the temperature-sensitive phenotype among the diploids was about 30 to 40% in these crosses. A small number of the diploid colonies were mixed, consisting of wild-type and temperature-sensitive cells. After prolonged growth of the diploid clones, up to 20 generations before plating, the mixed colonies disappeared. The transmission rate, however, decreased as a result of a reduced growth rate of the temperature-sensitive diploid clones in the media used. In crosses with other wild-type strains, transmission of the temperature-sensitive mutation was low. The occurrence of mitotic segregation proves extrakaryotic inheritance and indicates that the temperature-sensitive mutation is localized in the mitochondrion (2).

**Localization of the mutation in the mitochondrial DNA.** To prove the mitochondrial type of extrakaryotic inheritance, cells of the mutant strain M 12/ts-8 were diploidized with a strain shown to be rho⁰ (Slonimski, *personal communication*) and with a petite mutant of strain H 243 used in the experiment reported in Table 2. This petite mutant 243-EB-1 was isolated after treatment of wild-type cells with high ethidium bromide concentrations and growth of the treated cells for more than 50 generations in fermentable substrate. As this petite mutant proved to be neutral, it also is likely to be rho⁰ (9). The diploids of these crosses were temperature-sensitive (Table 3). Elimination of the rho⁺ information in the temperature-independent parental strain resulted in loss of temperature-independent diploids (respiratory competent) in the mitotic segregation experiment. From this, it is concluded that the temperature-sensitive muta-

tion in strain M 12/ts-8 is localized in the mitochondrial DNA.

## DISCUSSION

Three lines of evidence are presented which prove the mitochondrial inheritance of the temperature-sensitive mutation in strain M 12/ts-8: (i) absence of meiotic segregation in crosses with rho$^+$ wild-type strains (the spore ratio in the tetrads is 4:0, wild type-mutant); (ii) presence of mitotic segregation in crosses with rho$^+$ wild-type strains (the transmission of the mutant phenotype amounts to 30 to 40%); and (iii) loss of mitotic segregation in crosses with rho$^0$ strains (100% of the diploids exhibit the temperature-sensitive phenotype). The inheritance of the temperature-sensitive mutation follows the rules of mitochondrial genetics as proposed by Bolotin et al. (2). The mutation is assumed to be localized in the mitochondrial DNA.

A "cold-sensitive" mutant showing a phenotype comparable to that of the mutant presented here was reported by Weislogel and Butow (12). However, preliminary genetic analysis of this mutant indicates karyotic inheritance (12; *personal communication*). Growth at nonpermissive temperature leads, within four to six generations, to more than 90% rho$^-$ cells in both mutants. Respiration decreases during this process at a rate which seems to reflect a dilution of functional mitochondria at nonpermissive temperature (data not presented here).

Inhibition of mitochondrial protein synthesis by antibiotics, for 8 to 10 generations, was shown to affect the maintenance of the rho factor (12, 14). Preliminary experiments, however, indicate mitochondrial protein synthesis in the mutant M 12/ts-8 to show no temperature sensitivity. It thus may be supposed that in M 12/ts-8 cells the information coded by a mitochondrial gene is changed, which affects the replication of mitochondrial DNA in a more direct way.

The number of mitochondrial genes detectable by mutations leading to antibiotic resistance is limited. Therefore, isolation of mutants with temperature-sensitive phenotype may be a promising approach for detecting new mitochondrial genes. Such mutants will be useful both in recombination experiments and in the identification of products coded by the mitochondrial DNA, thus widening the field of mitochondrial genetics.

### ACKNOWLEDGMENTS

We thank B. Heinerich for excellent technical assistance.
This investigation was supported by the Deutsche Forschungsgemeinschaft.

### LITERATURE CITED

1. Bachofen, V., R. J. Schweyen, K. Wolf, and F. Kaudewitz. 1972. Quantitative selection of respiratory deficient mutants in yeasts by triphenyltetrazolium chloride. Z. Naturforsch. **27b**:252-256.
2. Bolotin, M., D. Coen, J. Deutsch, B. Dujon, P. Netter, E. Petrochilo, and P. P. Slonimski. 1971. La récombinaison des mitochondries chez Saccharomyces cerevisiae. Bull. Inst. Pasteur (Paris) **69**:215-239.
3. Casey, J., M. Cohen, M. Rabinowitz, H. Fukuhara, and G. S. Getz. 1972. Hybridization of mitochondrial transfer RNA's with mitochondrial and nuclear DNA of grande (wild type) yeast. J. Mol. Biol. **63**:431-440.
4. Coen, D., J. Deutsch, P. Netter, E. Petrochilo, and P. P. Slonimski. 1970. Mitochondrial genetics, methodology and phenomenology, p. 449-496. *In* P. L. Miller (ed.), Control of organelle development. Cambridge University Press, New York.
5. Lancashire, W. E., and D. E. Griffiths. 1971. Biocide resistance in yeast: isolation and general properties of trialkyl tin resistant mutants. FEBS Lett. **17**:209-214.
6. Michaelis, G., S. Douglass, M. J. Tsai, and R. S. Criddle. 1971. Mitochondrial DNA and suppressiveness of petite mutants in Saccharomyces cerevisiae. Biochem. Genet. **5**:487-495.
7. Michel, R., R. J. Schweyen, and F. Kaudewitz. 1971. Inhibition of mitochondrial protein synthesis in vivo by erythromycin in Schizosaccharomyces pombe and Saccharomyces cerevisiae. Mol. Gen. Genet. **111**:235-241.
8. Nagley, P., and A. W. Linnane. 1970. Mitochondrial DNA deficient petite mutants of yeast. Biochem. Biophys. Res. Commun. **39**:989-996.
9. Nagley, P., and A. W. Linnane. 1972. Biogenesis of mitochondria. J. Mol. Biol. **66**:181-793.
10. Schatz, G., and J. Saltzgaber. 1969. Protein synthesis by yeast promitochondria in vivo. Biochem. Biophys. Res. Commun. **37**:996-1001.
11. Wakabayashi, K., and N. Gunge, 1970. Extrachromosomal inheritance of oligomycin resistance in yeast. Fed. Eur. Biol. Soc. Lett. **6**:302-304.
12. Weislogel, P. O., and R. A. Butow. 1970. Low temperature and chloramphenicol induction of respiratory deficiency in a cold-sensitive mutant of Saccharomyces cerevisiae. Proc. Nat. Acad. Sci. U.S.A. **67**:52-58.
13. Wilkie, D., G. W. Saunders, and A. W. Linnane. 1967. Inhibition of respiratory enzyme synthesis in yeast by chloramphenicol: relationship between chloramphenicol tolerance and resistance to other antibacterial antibiotics. Genet. Res. **10**:199-203.
14. Williamson, D. H., N. G. Maroudas, and D. Wilkie. 1971. Induction of the cytoplasmic petite mutation in Saccharomyces cerevisiae by the antibacterial antibiotics erythromycin and chloramphenicol. Mol. Gen. Genet. **111**:209-223.