A MUTANT OF YEAST WITH TEMPERATURE SENSITIVE RESPIRATION

AKIRA UCHIDA AND KOHTA SUDA

Biology Division, Faculty of General Education, Kobe University, Kobe 657
and

Department of Botany, Faculty of Science, Kyoto University Kyoto 606

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Most vegetative (cytoplasmic) and segregational (nuclear) petite mutants of yeast which are unable to utilize nonfermentable substrates for growth show simultaneous deficiencies in certain mitochondrial enzymes including cytochromes *a* and *b* (Sherman and Slonimski 1964). It is well known that temperature sensitive and suppressor susceptible mutants have been powerful tools in recent work in microbial genetics. Under appropriate conditions, such mutations provide useful techniques for the study of gene-protein relationships. Thus, these mutants would be most desirable in the study of the respiratory system of yeast.

Temperature sensitive mutants of yeast have been reported with respect to growth (Hartwell 1967), an amino acid activating enzyme (Hartwell and McLaughlin 1968) and protoporphyrin synthesis (Gunge *et al.* 1967). This report describes our preliminary experiments on the isolation and characterization as well as the genetic analysis of a mutant of *Saccharomyces cerevisiae* with temperature sensitive respiration.

MATERIALS AND METHODS

Strain: A diploid strain $a \left(\begin{smallmatrix} a, gal, pan \\ @, gal, pan \end{smallmatrix} \right)$ of *Saccharomyces cerevisiae* (Seno 1962) was used to isolate temperature sensitive mutants. Haploid strains used for genetic analysis were *a*-1C (*a, gal, pan*) which was obtained by ascus dissection from the parental strain *a*, and C814-50B (*a, ade 1, thr 4*) which was kindly provided by Dr. T. Takahashi, Asahi Brewery Co.

Media: PY contained 10 g each of peptone and yeast extract per 1000 ml of deionized water. PYS and PYL contained 50 g of sucrose and 50 ml of 70% Na-lactate, respectively, in 1000 ml of PY. Liquid cultures were grown on a shaker unless stated otherwise. For anaerobiosis, cells were grown by bubbling nitrogen gas through liquid PYS enriched with ergosterol solution (Morpurgo *et al.* 1964). Nitrogen gas to be used was purified by passing through Fieser's solution. Spore forming medium contained 4 g of Na-acetate and 0.5 g of Na-glyoxalate in 1000 ml of deionized water. To distinguish respiratory-deficient cells from respiratory-competent cells, two methods were applied. One was to see whether or not could cells grow in PYL. The other was color plate method (Nagai 1965). Respiratory-deficient colonies formed on the color plate turned dark purple whereas respiratory-competent ones turned faint purple.

Isolation of temperature sensitive mutants: Cells of the strain *a*, precultured in liquid PYS for 2 days at 20°C, were inoculated at a 1/100 dilution in liquid PYL to which *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine (Nitrosoguanidine) was added at a final concentration of 10 µg/ml. The culture was incubated at 20°C with bubbling air until stationary phase. Cells were then spread on PYS agar plate after appropriate dilutions and incubated for 3 days at 20°C. The resulting colonies were replicated onto PYS and PYL agar plates. Among the colonies which formed on PYS but not on PYL plates at 30°C, a mutant that showed temperature sensitivity with respect to respiration was isolated. The frequency was about 2×10^{-5} . This mutant has been designated as *a*-ts1830.

Spectrophotometric analysis of cytochrome and measurement of oxygen uptake: Spectrophotometric analysis was carried out with a *Hitachi* recording spectrophotometer according to the method of Huang *et al.* (1966). Oxygen uptake was measured by means of an oxygen electrode. For this purpose, cells were sampled at time intervals and washed with M/15 potassium phosphate buffer (pH 6.8). The washed cells (3 to 15 mg dry weight) were suspended in 3ml of the same buffer supplemented with a final concentration of 0.6% of glucose and incubated at 30°C in the presence of the electrode.

Genetic techniques: Diploid cells were cultured in PYS for 2 days at 20°C and transferred to spore forming medium. After 3 to 4 days of incubation at 20°C four-spored asci were dissected under a microscope by means of a *De-Fonbrune* type micromanipulator. A random spore analysis was performed according to the method of Hartwell (1967). For hybridization, cells of opposite mating type were grown separately in PYS for 2 days at 20°C. A small amount of each culture was mixed on PYS plate with a platinum loop and incubated for 3 to 4 days. The diploidy was checked by the spore forming ability.

RESULTS

1. Respiratory properties of *a*-ts1830

A. Respiratory activity

As shown in Table 1, the respiratory activities of the parental strain *a* and the mutant *a*-ts1830 were comparable when both strains were grown in PYS at 20°C. In contrast the respiratory activity of the mutant was reduced to approximately 1/4 of that of the parental strain after grown in the same medium at 30°C. The effect of temperature on the respiratory activity of the mutant was further investigated. The mutant cells were grown in PYS for 24 hours at both 20°C and 30°C, then transferred to PYS and to M/15 potassium phosphate buffer (pH 6.8). Oxygen uptake of each culture was measured at the time of transfer (0 hour) and after 27 hours of incubation at both 20°C and 30°C. The results shown in Table 2 indicate that the respiratory activity of the mutant depends on the growth temperature and that the temperature shift itself has no effect on it when no growth occurred.

The mutant cells, previously grown at 20°C or 30°C, were streaked on the color plates (See MATERIALS AND METHODS), followed by incubation at 20°C and 30°C. It was observed that all the colonies formed at 20°C were faint purple but those formed at 30°C were dark purple, independent of the temperatures of the precultures. This

Table 1. Respiratory activities of *a*-ts1830 and its parental strain *a*

Strain	cultural condition		QO ₂
	temperature (C)	time (h)	(μ l O ₂ uptake/mg dry weight/h)
<i>a</i> -ts1830	30	25	42.4
<i>a</i> -ts1830	20	38	143.2
<i>a</i>	30	25	160.4
<i>a</i>	20	52	149.8

Table 2. Effect of temperature shift on QO₂ of *a*-ts1830

(a) precultured for 24h at 20°C				(b) precultured for 24h at 30°C			
time (h)	medium	temperature (C)	QO ₂	time (h)	medium	temperature (C)	QO ₂
0	—	—	142.2	0	—	—	34.5
27	PYS	20	151.6	27	PYS	20	137.7
27	PYS	30	39.1	27	PYS	30	34.5
27	buffer	20	158.6	27	buffer	20	37.7
27	buffer	30	151.5	27	buffer	30	39.6

observation leads to a conclusion that the reduced respiratory activity of the mutant would not result from the induction of petite mutants during growth at 30°C.

B. Absorption spectra of cytochromes

The respiratory-deficient mutants ordinarily have altered patterns of cytochromes (Sherman and Slonimski 1964). To see the cytochrome patterns of *a*-ts1830, the mutant cells were grown in PYS for 17 hours at both 20°C and 30°C. They were collected by centrifugation and subjected to spectrophotometry. As shown in Fig. 1, cytochrome *c* (550 m μ) is apparent in the culture grown at either 20°C or 30°C. On the other hand, it can be seen that smaller amounts of cytochromes *b* (562 m μ) and *a* (602 m μ) are present in the culture at 30°C than those at 20°C.

C. Respiratory adaptation

Yeast cells grown under an anaerobic condition are known to have little respiratory activity. The cells are transferred to an aerobic condition, then the respiratory activity improves gradually. This phenomenon is called respiratory adaptation (Slonimski 1953). The effect of temperature on the respiratory adaptation of *a*-ts1830 was examined. The mutant cells were anaerobically grown for 44 hours at 20°C or for 24 hours at 30°C, and then respiratory adaptation was commenced at 20°C and 30°C by replacing nitrogen gas by air. At time intervals samples were withdrawn to determine oxygen uptake. The results are shown in Fig. 2. Although the values of QO₂ were rather high for anaerobic cultures, the respiratory activity increased during the aerobic incubation at either 30°C or 20°C after grown anaerobically at 20°C. It increased little, however, after grown anaerobically at 30°C.

The progressive alterations of absorption spectra during the respiratory adaptation

Fig. 1. Absorption spectra of *a-ts1830* cells grown at 20°C and 30°C. Cells, aerobically grown for 17 hours at 20°C (A) and at 30°C (B), were collected by centrifugation and spectrophotometric analysis was carried out with a *Hitachi* recording spectrophotometer according to the method of Huang *et al.* (1966).

Fig. 2. Development of respiratory activities of *a-ts1830* cells during aerobic adaptation. Cells were anaerobically grown for 44 hours at 20°C (○) and for 24 hours at 30°C (●) and then aerated at 20°C (—) and at 30°C (---). Samples were taken at time intervals, and QO_2 (μ l O_2 uptake/mg dry weight/hour) was measured polarographically.

are presented in Figs. 3 and 4. The mutant cells were anaerobically precultured for 20 hours at 20°C (Fig. 3) and for 15 hours at 30°C (Fig. 4), and introduced to air. It is seen that cytochrome *c* (550 m μ) was synthesized by the cells precultured at both 20°C and 30°C; cytochromes *b* (562 m μ) and *a* (602 m μ) seemed to be synthesized only after grown at 20°C.

2. Genetic analyses of *a-ts1830*

To see the genetic properties of the mutant *a-ts1830*, 3 tetrads were analyzed. All the four-segregants from each ascus showed the temperature sensitivity with respect to respiration, the character of which has been designated as RC^{ts} . This indicates that the mutant *a-ts1830* would be a homozygous diploid for the gene concerned.

One of the segregants, *ats1830-1A* (@), obtained above, was crossed with the respiratory-competent strain, C814-50B (*a*), the character of which has been designated as RC^+ . The cross gave rise to respiratory-competent diploid cells.

The diploid, derived from the cross *ats1830-1A* (@, *ade*+) $\times$ C814-50B (*a*, *ade*1), was subjected to tetrad analysis. The result is shown in Table 3. The markers of mating

Fig. 3. Absorption spectra before and after aeration of anaerobic *a-ts1830* cells grown at 20°C. Cells were anaerobically grown at 20°C for 20 hours and then aerated at 20°C and 30°C. A; before aerobic adaptation, B; after 3 hours' aeration at 20°C, C; after 3 hours' aeration at 30°C.

Fig. 4. Absorption spectra before and after aeration of anaerobic *a-ts1830* cells grown at 30°C. Cells were anaerobically grown at 30°C for 15 hours and then aerated at 20°C and 30°C. A; before aerobic adaptation, B; after 3 hours' aeration at 20°C, C; after 3 hours' aeration at 30°C.

type and adenine requirement showed 2 to 2 segregation. However, the temperature sensitivity with respect to respiration segregated irregularly. Namely, in addition to RC^+ and RC^{ts} there brought forth a new type of segregants, designated as RC^- . These new segregants could grow well in PYS but not at all in PYL at both 20°C and 30°C. One of the RC^- segregants, 2A (*a*) shown in Table 3, was backcrossed to *ats1830-1A* (@). The phenotype of the resulting diploids was RC^{ts} . The tetrad analysis of 5 complete asci showed that the segregation ratio of RC^{ts} to RC^- was 2 to 2, and the ratio was 20 to 17 by the random spore analysis. These results suggest that two nuclear genes are responsible for the irregular segregation for the respiratory activity.

A question arose, then, as to whether the two mutations which were closely related with each other were induced simultaneously by the treatment with nitrosoguanidine. To see this point the following analyses were performed. The strain *a-1C* (*a*) which was obtained by ascus dissection from the parental strain *a* was crossed with the segregant *a-ts1830-1A* (@), and 6 complete asci from the resulting diploids which were RC^+

Table 3. Tetrad analysis of cross, *ats1830-1A* (@, *ade*⁺) × *C814-50B* (*a*, *ade* 1)

genotype				genotype			
mating type	adenine	phenotype concerning respiration		mating type	adenine	phenotype concerning respiration	
1A	@	—	RC ^{ts}	5A	@	+	RC ⁺
1B	<i>a</i>	+	RC ⁺	5B	<i>a</i>	+	RC ⁺
1C	@	—	RC ^{ts}	5C	@	—	RC [—]
1D	<i>a</i>	+	RC ⁺	5D	<i>a</i>	—	RC ^{ts}
2A	<i>a</i>	—	RC [—]	6A	@	—	RC ⁺
2B	@	+	RC ⁺	6B	@	—	RC ^{ts}
2C	@	+	RC ⁺	6C	<i>a</i>	+	RC ⁺
2D	<i>a</i>	—	RC ^{ts}	6D	<i>a</i>	+	RC ^{ts}
3A	<i>a</i>	+	RC ⁺	7A	<i>a</i>	+	RC ⁺
3B	@	—	RC ^{ts}	7B	@	—	RC [—]
3C	<i>a</i>	—	RC ^{ts}	7C	<i>a</i>	+	RC ⁺
3D	@	+	RC ⁺	7D	@	—	RC ^{ts}
4A	@	+	RC ⁺	8A	@	—	RC ⁺
4B	@	+	RC ^{ts}	8B	<i>a</i>	—	RC [—]
4C	<i>a</i>	—	RC ⁺	8C	<i>a</i>	+	RC ^{ts}
4D	<i>a</i>	—	RC ^{ts}	8D	@	+	RC ⁺

were dissected. It was observed that all the segregation ratios of RC⁺ : RC^{ts} were 2:2. Moreover, the ratio was 21 : 23 by the random spore analysis. The cross of *a-1C* (*a*) with an RC[—] segregant 5C (@) shown in Table 3, produced also RC⁺ diploids. The random spore analysis of the resulting diploids showed the segregation ratio of RC⁺ : RC^{ts} : RC[—] to be 36 : 14 : 22. These results suggest that the parental strain had been endured with one mutation (*mod* gene for temperature sensitivity; see DISCUSSION) of the two before the treatment with nitrosoguanidine.

DISCUSSION

It could be possible to assume that the temperature sensitivity with respect to respiration of yeast would result from one of the following changes of a certain protein; (1) an enzyme or structure which participates in the replication or retention of cytoplasmic gene (*rho*) could neither have its function nor be synthesized at high temperature, (2) an enzyme concerning respiration could not have its catalytic activity at high temperature and (3) one of the proteins requisite to the respiratory assembly of mitochondrial enzymes could not be synthesized or even if synthesized at high temperature it would be abnormally formed having lost its function.

The case of *a-ts1830* would not be (1) because of the fact that the colonies formed at 20°C after grown at 30°C showed respiratory-competent phenotype. The mutant cells, grown at 20°C under an aerobic condition, retained their respiratory activity

during a longer period of incubation in nongrowing medium at 30°C (Table 2a). One may say, therefore, that (2) would be also inadequate to elucidate the mutant characteristics. From the observations described here the following explanation seems to be most likely, *i. e.*, a protein concerning respiration could be normally synthesized at low temperature but once it was synthesized at high temperature, it would have partially lost the ability to make normal assembly of respiratory system unless growth so occurred at low temperature as to dilute out the protein synthesized at high temperature.

The genetic analyses suggest a two-gene segregation for the respiratory activity of the mutant *a*-ts1830. As for the two mutations, it might be possible to assume that one would be a segregational petite mutation (*pet*) and the other a "modifier gene" mutation (*mod*) which causes the temperature sensitive respiration when the *pet* mutation was present in the genome. Assuming the above hypothetical genes, the genotypes of the strains used in the cross could be postulated as follows; *a*-1C (*a*, *pet*+, *mod*), *ats*1830-1A (@, *pet*, *mod*), C814-50B (*a*, *pet*+, *mod*+) and 2A & 5C (*a*, *pet*, *mod*+) & (@, *pet*, *mod*), respectively.

The tetrad analysis of the mutant *a*-ts1830 revealed that all the segregants were RC^{ts}. Hence the genotype of *a*-ts1830 would be postulated to be $\left(\frac{a, pet, mod}{@, pet, mod}\right)$. Since nitrosoamides were reported to enhance the frequency of mitotic recombination in yeast (Zimmerman *et al.* 1966), one could further assume that the *pet*+ to *pet* mutation was first induced, followed by mitotic recombination in the presence of nitrosoguanidine, producing a homozygous diploid.

On the basis of the biochemical and genetic data described here, the gene-protein relationship might be such that a protein which is requisite to make assembly of respiratory system could not be synthesized due to the *pet* mutation. But the modified protein which is functional at low temperature but not at high temperature could be synthesized by the action of the *mod* gene.

SUMMARY

A temperature sensitive mutant of *Saccharomyces cerevisiae* was isolated with partially defective respiratory activity at high temperature. The respiratory activity of the mutant was reduced to approximately 1/4 of the parental strain after grown at 30°C, and it could be improved to be normal after grown at 20°C. Spectrophotometric analysis showed that the mutant could synthesize only small amounts of cytochromes *b* and *a* at 30°C. Genetic analyses revealed that two gene mutations for the respiratory activity of the mutant were involved.

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