Isolation of Uracil Auxotrophic Mutants of *Saccharomyces cerevisiae* Unable to Reduce 2,3,5-Triphenyl-tetrazolium Chloride

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Received August 11, 1983

A new type of mutant unable to reduce 2,3,5-triphenyltetrazolium chloride (TTC) was isolated from mutagenized cells of *Saccharomyces cerevisiae*. These TTC⁻ mutants were respiration-competent although most TTC⁻ mutants reported so far are respiration-deficient. The isolated mutants were also uracil auxotrophic, and genetic analysis indicated that a recessive mutation took place on a single gene tightly linked to the *ura2* gene or in the *ura2* gene in all these mutants. Moreover, we found that authentic *ura* mutants (*ura1* to *ura5*) also exhibited the TTC⁻ phenotype while other auxotrophic mutants tested were of the TTC⁺ phenotype. These findings suggest that yeast cells have a TTC reduction system related to uracil metabolism besides that related to respiratory potential.

Normal (respiratory competent) strains of *Saccharomyces cerevisiae* reduce 2,3,5-triphenyltetrazolium chloride (TTC) to TTC-formazan, a water insoluble red pigment, but respiratory deficient (RD) mutants do not. Therefore, when colonies grown on a nutrient medium are overlaid with TTC agar, normal cells are stained red on a few hours incubation while RD cells remain white.¹⁾ This difference in TTC reduction has been efficiently used for diagnosis of RD mutants since Ogur *et al.* reported the TTC overlay method.¹⁾ Later, Furukawa and Akiyama²⁾ utilized this TTC overlay method for classification of yeast strains in terms of color intensity of colonies stained with TTC. For example, colonies of industrial *sake* yeasts are stained deeply red while those of wild yeasts are mostly stained lightly red or pink. Thus, the TTC overlay method could be used for detection of wild yeast contaminants in the fermentation process of *sake* production.

However, the mechanism of TTC reduction

in yeast cells is still unknown. Nagai *et al.*³⁾ assumed that succinate dehydrogenase which catalyzes the succinate-to-fumarate conversion at a redox potential near that of TTC is absent in RD cells. Trevors⁴⁾ described that deposition of formazan granules in yeast cells was related to the respiratory potential or electron transport system. In this work, we isolated a new type of respiration-competent mutant unable to reduce TTC. They are neither mitochondrial RD mutants nor nuclear RD mutants. Genetic analysis revealed that the mutation took place on a single nuclear gene. This finding strongly suggests that the TTC reduction involves another system besides the respiratory system. In this paper, isolation of the new mutants and their genetical characteristics are described.

MATERIALS AND METHODS

Yeast strains. Strains of *Saccharomyces cerevisiae* used in this study are listed in Table I.

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TABLE I. STRAINS OF *Saccharomyces cerevisiae*

Strain	Genotype	Origin
X2108-1A	<i>a SUC2 CUP1 mal gal2</i>	R. K. Mortimer
X2180-1B	<i>α SUC2 CUP1 mal gal2</i>	R. K. Mortimer
427	<i>α ura1 ade1 thr1 kex2</i>	R. B. Wickner
X1069-2D	<i>α ura1 ade1 leu2 his4 thr4 met2 trp5</i>	R. K. Mortimer
A364A	<i>a ura1 ade1 ade2 his7 lys2 tyr1 gal1</i>	R. K. Mortimer
5095B	<i>α ura2</i>	This work
C4414-4C	<i>a ura2</i>	T. Takahashi
D160-2C	<i>α ura3 arg4 hom3 his1 ilv1 trp2 ade1</i>	R. K. Mortimer
D160-4D	<i>a ura3 arg4 hom3 his1 ilv1 trp2 ade1</i>	R. K. Mortimer
C5076-2D	<i>α ura4 his2 lys9</i>	T. Takahashi

Media. The complete medium for growth was YPAD which contained 1% yeast extract, 2% peptone, 0.04% adenine sulfate and 2% glucose. The defined medium for counterselecting RD cells was YPG which contained 1% yeast extract, 2% peptone, 0.04% adenine sulfate and 2% glycerol. The defined minimal medium was SD which contained 0.67% Yeast Nitrogen Base w/o amino acids (Difco) and 2% glucose. For the determination of nutritional requirements, the defined complete medium (CMM) and omission media (OM) were used. Supplementation and omission of the nutrients in these media were the same as described by Wickner and Leibowitz.⁵ The sporulation medium was composed of 1% potassium acetate, 0.1% yeast extract and 0.05% glucose. The poor nutrient medium contained 0.15% yeast extract, 0.2% peptone, 0.04% adenine sulfate, 1% glucose, 0.1% KH_2PO_4 , 0.04% $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ and 2% agar. The TTC agar contained 0.05% TTC (Wako Junyaku-kogyo Co., Osaka, Japan), 0.5% glucose and 1.5% agar. The pH of each medium was adjusted to around 6, and 2% agar was added for the solid media.

Assay of the TTC reduction. Colonies grown on the poor nutrient medium were overlaid with melted TTC agar and incubated at 30°C for 3 hr. In this incubation, the colonies capable of reducing TTC were stained red and those lacking the ability remained white.

Isolation of TTC non-reducing mutants. Stationary phase cells grown in YPAD medium were washed 3 times with distilled water and resuspended in 10 ml of distilled water at a cell density of 1.5×10^8 /ml. The suspension was poured into a 9-cm glass petri dish and irradiated for 1 min with a 15 W germicidal lamp at a distance of 30 cm. Fifty ml of YPG medium was inoculated with 1 ml of the UV-irradiated suspension in which the cell survival rate was 14.9%, and the mixture incubated at 30°C for 2 days. The culture was plated on the poor nutrient medium after appropriate dilution to give about 1,000 colonies per plate, and the colonies grown at 30°C for 2 days were

overlaid with TTC agar as described above. The colonies remaining white at 3 hr incubation were selected and characterized.

Genetic analyses. Diploid hybrids were isolated using the complementary nutritional requirements of their parents, or by pairing two cells of both parents on YPAD medium with a micromanipulator. Sporulation and dissection of asci were performed according to Mortimer and Hawthorne.⁶

Notation. $\text{TTC}^+/\text{TTC}^-$, $\text{GLY}^+/\text{GLY}^-$ and $\text{URA}^+/\text{URA}^-$ denote phenotypes for ability/inability to reduce TTC, growth/no growth on YPG medium (or respiration competent/deficient) and growth/no growth on the uracil free medium, respectively.

RESULTS

Isolation of TTC^- mutants with respiratory competence

Isolation of TTC^- mutants was carried out using mutagenized cells of strain X2180-1A. From about 12,000 colonies overlaid with TTC agar, 227 white colonies were selected, and 6 of these were confirmed to be GLY^+ or respiration-competent. It is notable that all these mutants were uracil auxotrophs.

Meiotic segregation

Each of the 6 TTC^- mutants isolated (T1 to T6) was crossed with the wild type strain X2180-1B and the resultant hybrids were subjected to tetrad analysis (Table II). Phenotypes of the diploid hybrids were all TTC^+ and URA^+ , indicating that the mutations were

TABLE II. MEIOTIC SEGREGATION OF THE TTC REDUCTION AND URACIL REQUIREMENT TRAITS

Strains ^a crossed with X2180-1B	Phenotypes ^b of the hybrid	Number of asci exhibiting									
		TTC ⁺ :TTC ⁻					URA ⁺ :URA ⁻				
		4:0	3:1	2:2	1:3	0:4	4:0	3:1	2:2	1:3	0:4
X2180-1A	TTC ⁺ URA ⁺	16	0	0	0	0	16	0	0	0	0
T1	TTC ⁺ URA ⁺	0	0	24	0	0	0	0	24	0	0
T2	TTC ⁺ URA ⁺	0	0	24	0	0	0	0	24	0	0
T3	TTC ⁺ URA ⁺	0	0	20	0	0	0	0	20	0	0
T4	TTC ⁺ URA ⁺	0	0	23	0	0	0	0	23	0	0
T5	TTC ⁺ URA ⁺	0	0	24	0	0	0	0	24	0	0
T6	TTC ⁺ URA ⁺	0	0	22	0	0	0	0	22	0	0

^a Strains T1 to T6 were the TTC⁻ mutants and X2180-1A was their parent.

^b Phenotypes TTC⁺/TTC⁻ and URA⁺/URA⁻ represent ability/inability to reduce TTC and growth/no growth on the uracil minus medium, respectively.

TABLE III. COMPLEMENTATION TEST OF TTC⁻ MUTATION BETWEEN THE ISOLATED TTC⁻ MUTANTS

Crosses were made between the TTC⁻ mutants and the meiotic derivatives of the α mating type of the mutants, and the phenotype of the diploids was determined. The sign “—” represents TTC⁻.

Mutants	α Mating type derivatives of the mutants					
	T1	T2	T3	T4	T5	T6
T1	—	—	—	—	—	—
T2		—	—	—	—	—
T3			—	—	—	—
T4				—	—	—
T5					—	—
T6						—

recessive to the wild types. On tetrad analysis, the TTC reduction trait was segregated into 2 TTC⁺:2 TTC⁻, and the uracil requirement trait also segregated into 2 URA⁺:2 URA⁻. In all asci, the TTC⁻ segregants were always URA⁻, suggesting that each TTC⁻ mutation took place on a single nuclear gene very close to the *ura* gene or within the *ura* gene.

Complementation analysis

A complementation test was carried out among the 6 TTC⁻ mutants (Table III). Meiotic TTC⁻ derivatives of the α mating type

were obtained from the meiotic products of crosses in Table II and used for the complementation test. The results showed that none of the mutants could complement each other. This finding suggests that the mutation occurred on the same locus in all these mutants.

Identification of the *ura* gene in TTC⁻ mutants

To identify the *ura* gene which occurred simultaneously in the TTC⁻ mutants, one of the mutants (T1) was crossed with each of the authentic URA⁻ mutants for the complementation and allelism test (Table IV). The results showed that the *ura* mutation complemented *ura1*, *ura3* and *ura4*, but not *ura2*. The cross between T1 and the *ura2* mutants exhibited 0 URA⁺:4 URA⁻ segregation. Therefore, the *ura* mutation in the TTC⁻ mutation was confirmed to take place on the *ura2* locus in the chromosome X.⁷⁾ In the course of these analyses, we found that the *ura1*, *ura2*, *ura3*, and *ura4* mutants were also TTC⁻, and that URA⁻ segregants were always TTC⁻ in every ascus dissected.

Mutations affecting the TTC reduction

To determine whether or not only the *ura* mutants exhibited TTC⁻, we investigated various authentic mutants for the TTC reduc-

TABLE IV. COMPLEMENTATION AND ALLELISM TEST OF THE *ura* GENE IN THE TTC⁻ MUTANTS

Strains (gene) crossed with T1	Phenotypes ^a of the hybrid	Meiotic segregation pattern		
		Number of asci exhibiting URA ⁺ :URA ⁻		
		2:2	1:3	0:4
427 (<i>ura1</i>)	URA ⁺ TTC ⁺	6	29	9
5095B (<i>ura2</i>)	URA ⁻ TTC ⁻	0	0	23
D160-2C (<i>ura3</i>)	URA ⁺ TTC ⁺	3	13	4
C5076-2D (<i>ura4</i>)	URA ⁺ TTC ⁺	6	18	7

^a See Table II.

tion. The results showed that none of the mutations⁷⁾ tested (*ade1*, *ade2*, *arg1*, *arg4*, *asp5*, *can1*, *gal1*, *his4*, *his5*, *his7*, *ilv3*, *lys1*, *lys2*, *lys10*, *leu1*, *leu2*, *mal*, *pho81*, *thr1*, *trp1*, *trp3*, *tyr1*, *kar1*, *kex1*, *mak1*, *mak3* to *mak13*, *mak15* to *mak17*, *mak19* to *mak21*, *skil* to *ski4*) exhibited TTC⁻ while *ura5* also exhibited TTC⁻ like the other *ura* genes. This finding showed that the *ura* mutations seemed to be specifically related to TTC⁻.

DISCUSSION

The TTC reduction system in yeast cells has been discussed so far on the basis of the fact that RD mutants are unable to reduce TTC.^{3,4)} However, the findings obtained in this work strongly suggest that a different system from the respiration potential is involved in TTC reduction as well. As the TTC⁻ mutation was found to take place on a single nuclear gene tightly linked to the *ura2* gene, and moreover, as authentic *ura* mutants (*ura1* to *ura5*) were found to pleiotropically exhibit TTC⁻, it is reasonable to consider that this TTC reduction system must be related with uracil metabolism. Uracil (UMP) biosynthesis in yeast cells is catalyzed by carbamoylphosphate synthetase (coded by *ura2-C*), aspartic transcarbamylase (*ura2-B*), dihydroorotase (*ura4*), dihydroorotic

dehydrogenase (*ura1*), orotidylate pyrophosphatase (*ura5*) and orotidine-5-phosphate decarboxylase (*ura3*) in succession.^{8,9)} However, the exact mechanism of the TTC reduction was not clear in this work. Further study on the relationship between the TTC reduction and uracil metabolism will be reported later.

Acknowledgments. We wish to thank Dr. R. K. Mortimer, University of California, Dr. R. B. Wickner, NIH, and Dr. T. Takahashi, Asahi Brewery Co., for their kind gifts of yeast strains.

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