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Effect of Methanol on the Induction of Respiration-Deficient Mutants in Yeast by Acriflavine

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The effect of methanol on the induction of cytoplasmic respiration-deficient (RD) mutants of *Saccharomyces cerevisiae* by acriflavine (AF) was investigated. After 24 h incubation, AF at above 0.5 μg/ml induced approximately 100% RD mutants in surviving cells. Methanol prevented the RD mutation by AF at concentrations less than 1.5 μg/ml. The RD mutation by 0.15 μg/ml AF was completely repressed by the addition of 4.0% methanol to the culture fluid. The induction frequency of RD mutants by 0.5 μg/ml AF was reduced from 97 to 15% by the addition of 8.0% methanol.

The AF uptake by the yeast cells was decreased in the presence of methanol. However, this methanol-induced repression in the RD mutation could not be explained simply in terms of the decrease of AF content in the yeast cells.

Keywords——acriflavine; methanol-repression of mutation; respiration-deficient mutation; mitochondrial DNA; yeast; *Saccharomyces cerevisiae*

We have reported[1] that methanol almost completely prevented the cytoplasmic respiration-deficient (RD or petite) mutation in yeast, *Saccharomyces cerevisiae*, induced by some aromatic alcohols such as α-phenethyl alcohol and 1-phenyl-1-propanol. However, the mechanism of methanol-repression in RD mutation remains to be elucidated.

In the present paper, it is shown that methanol can also repress the RD mutation in yeast induced by acriflavine (AF). Acriflavine is known to induce RD mutants in yeast under normal growth conditions at high frequency.[2] The mitochondrial deoxyribonucleic acids (mtDNAs) are considered to be largely deleted or completely lost in the majority of the AF-induced RD mutants.[3] However, the induction mechanism of these abnormalities in mtDNA molecules by AF is not fully understood.

The RD mutant induction by AF was also repressed by basic dyes structurally related to AF.[4] In this case, the basic dyes may specifically block the access of AF to a receptor site such as mtDNA. However, methanol seems to be a different type of antimutagen from the basic dyes and may be a nonspecific inhibitor of RD mutation.

In the present paper, the effect of methanol on the RD mutation in yeast, *Saccharomyces cerevisiae*, was examined. A preliminary experiment on the inhibitory effect of methanol on AF uptake by the yeast cells was also carried out.

Materials and Methods

Strain——*Saccharomyces cerevisiae* ATCC 26422 (*S. sakė* Kyokai no. 7) was used.

Chemicals——Acriflavine (trypaflavine, guaranteed reagent grade) and methanol (specially prepared reagent grade) were purchased from Nakarai Chemicals Co., Ltd., Kyoto, Japan.

Cultivation and Detection of RD Mutants——The yeast cells were grown in Ogur's medium[5] containing 2%

glucose as a carbon source at 30 °C for 24 h on a Monod shaker and inoculated into the same medium at 1.0×10^5 cells/ml. Acriflavine was added to this medium with or without methanol. After incubation for 24 h, cells were diluted with sterilized water and spread onto Ogur agar plates to give about 200 colonies. After incubation at 30 °C for 2 d, colonies were scored for survival and RD mutant induction by using Nagai's tetrazolium salt overlay method.[6] Only colonies remaining completely white after tetrazolium salt overlay were scored as RD mutants. An average of 5 plates was used in each experiment. The cell number was determined with a hemocytometer.

Determination of AF Content in Yeast Cells——Cells were inoculated into Ogur's medium at 2.0×10^8 cells/ml in the presence of 1.0 μg/ml AF with or without 8.0% methanol, incubated at 30 °C for 30 min and harvested by centrifugation at $14500 \times g$ at 5 °C for 5 min. The amount of AF in the supernatant was determined spectrophotometrically to estimate AF content in the yeast cells indirectly as the amount of AF lost from the culture medium according to the method of Nakamura,[7] except that 454 nm was used as the absorption maximum. On the other hand, the precipitated cells were extracted 3 times with 3 ml each of methanol for the estimation of AF extractable from the cells. The amount of eluted AF was determined spectrophotometrically at 460 nm.

Results

Effect of Methanol on the Induction of RD Mutants by Acriflavine

As shown in Fig. 1, the spontaneous induction frequency of RD mutants in *Saccharomyces cerevisiae* was 0.7% after 24 h incubation. Methanol was not mutagenic in the concentration range (up to 8.0%) that was used in this experiment. The induction frequency of RD mutants reached almost 100% in the presence of AF at more than 0.5 μg/ml. Methanol repressed the induction of RD mutants by AF at concentrations less than 1.5 μg/ml. The induction by 0.15 μg/ml AF was prevented almost completely by the addition of 4.0%

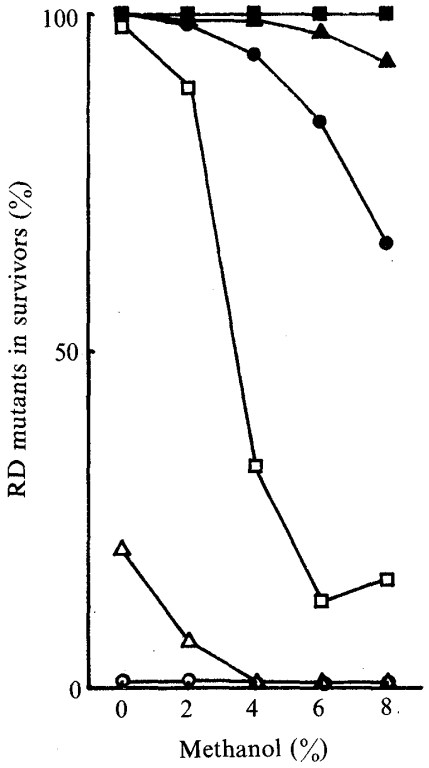

Fig. 1. Effect of Methanol Concentration on the Induction Frequency of RD Mutants in Yeast by AF

Yeast cells were inoculated at 1.0×10^5 cells/ml and incubated at 30 °C for 24 h in the presence of AF and methanol. AF was added at various concentrations (μg/ml); none (○), 0.15 (△), 0.5 (□), 1.0 (●), 1.5 (▲) and 2.0 (■). The percentage of RD mutants among surviving cells is shown.

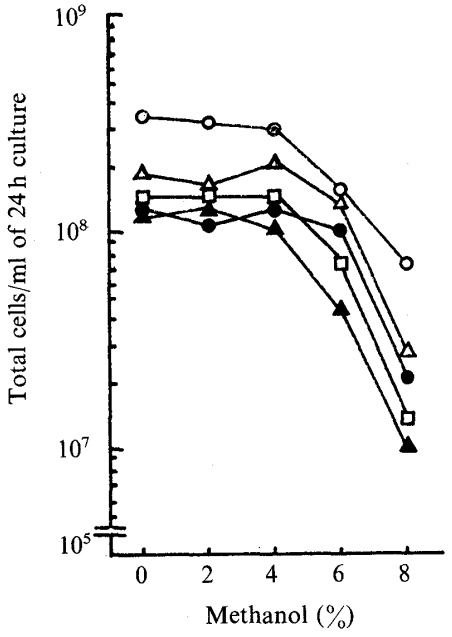

Fig. 2. Effects of AF and Methanol on the Yeast Cell Growth

Cell numbers in each medium were scored with a hemocytometer after 24 h incubation and are expressed as total cells in 1 ml of culture. Other conditions and symbols are the same as in Fig. 1.

methanol.

As shown in Fig. 2, the cell population after 24 h incubation reached 3.3×10^8 cells/ml (control) and decreased in the presence of AF. However, cell growth was essentially not inhibited by the addition of methanol up to 4.0%, regardless of the presence of AF. Actual colony-forming cells (survivors) amounted to more than 80% of cell numbers counted by the hemocytometer in each case.

Time Course of Induction of Respiration-Deficient Mutants

A time course experiment was carried out to determine in detail the effect of 8.0% methanol on the growth of yeast and on the RD mutant induction by 1.0 μg/ml AF.

As shown in Fig. 3A, the growth rate of yeast was almost unchanged in the presence of AF and reached about 1.0×10^7 cells/ml after 10—12 h incubation. However, the final cell population in the presence of 1.0 μg/ml AF was only half (1.5×10^8 cells/ml) that in the absence of AF. The addition of 8.0% methanol caused a reduction of the growth rate. An incubation time of 18—20 h was required for the cell population to reach 1.0×10^7 cells/ml, and it reached 1.5×10^8 cells/ml after 28—48 h.

As shown in Fig. 3B, the induction frequency of RD mutants by 1.0 μg/ml AF reached

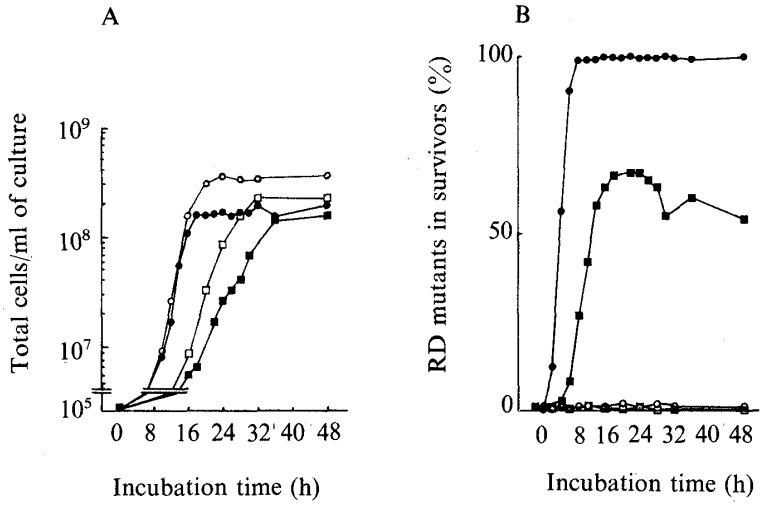

Fig. 3. Time Courses of Yeast Cell Growth and RD Mutant Induction in the Presence of 8.0% Methanol

Yeast cells were inoculated at 1.0×10^5 cells/ml and incubated at 30 °C in the absence (○) or presence (□) of 8.0% methanol, 1.0 μg/ml AF (●) or both (■). A: Cell numbers in each medium were scored with a hemocytometer and are expressed as total cells in 1 ml of culture. B: The percentage of RD mutants among surviving cells is shown.

TABLE I. Effect of Methanol on the AF Content in Yeast Cells

Methanol (%)	AF content in yeast cells (μg/2.0×10^9 cells)	
	(a)	(b)
None	2.3±0.33	2.2±0.14
8.0	1.7±0.21	1.6±0.07

Yeast cells (2.0×10^9) in 10 ml of Ogur's medium containing 1.0 μg/ml AF were incubated at 30 °C for 30 min with or without 8.0% methanol. AF content in the yeast cells was estimated on the basis of the amount of AF lost from the medium (a) and that of AF extracted from yeast cells (b). Each value represents the mean ± standard deviation of six determinations.

almost 100% after 10 h incubation. In the presence of 8.0% methanol, the induction frequency was only 67% after 20 h incubation. Thereafter, the induction frequency did not increase, though cell growth continued up to 1.5×10^8 cells/ml.

Acriflavine Content in Yeast Cells

Freshly harvested yeast cells were inoculated into 10 ml of Ogur's medium containing 1.0 μg/ml AF at 2.0×10^8 cells/ml and incubated at 30 °C for 30 min. As shown in Table I, AF contents in yeast cells as estimated on the basis of the amount of AF lost from the medium and the contents obtained by extraction from the cells agreed well. The yeast cells absorbed 22—23% of AF in the medium under the experimental conditions used. The addition of 8.0% methanol to the medium caused a decrease of AF uptake by yeast cells to about 73% of that in methanol-free medium. The weight of cells was practically unchanged during incubation for 30 min.

Discussion

Acriflavine could induce RD mutants in *Saccharomyces cerevisiae* at a frequency of 99% after 10 to 12 h incubation, during which the cell population increased by approximately 100 times from the starting level (1.0×10^5 cells/ml). This increase in the cell population corresponds to about 7 generations. There are 50—100 mtDNA molecules per yeast cell,[8] and if mtDNA molecules in daughter cells are completely damaged by AF, the RD mutants in the population may be expected to exceed 99% after 6—7 generations.

Methanol, which did not induce any RD mutants in *S. cerevisiae*, could repress the RD mutation by AF. We have already reported[1] that methanol almost completely prevented RD mutant induction in yeast by some aromatic alcohols. Thus, the methanol repression of RD mutation is considered to be nonspecific. The RD mutant induction by AF was also prevented by basic dyes structurally related to AF.[4] In that case, dyes may block the access of AF to receptor sites such as mtDNA or its replication system by competition. However, this is not the case with methanol, though methanol may block the access of AF to the receptor site through a nonspecific interaction with the site.

The simplest interpretation is that methanol inhibits the uptake of RD mutant inducers nonspecifically. In the present experiment, the repression of RD mutant induction by methanol could be explained partly by the decrease of AF uptake by the yeast cells. In a preliminary experiment, the cellular distribution of AF could not be clearly defined. It seems likely that a large amount of AF may bind to the cell wall. This ambiguity may be eliminated by using the protoplasmic cells for the experiment. The RD mutant induction by 0.5 μg/ml AF reached approximately 100% after 12 h incubation (data not shown). However, the induction by 1.0 μg/ml AF did not exceed 67% in the presence of 8.0% methanol. These results suggest that the methanol-induced decrease of the induction frequency could not be explained simply by the decrease of AF content in the yeast cells.

It seems to be probable that methanol affects physiological factors such as mtDNA polymerase activity, mtDNase activity or the mtDNA repair system which may be involved in the RD mutation. Further experiments are required to elucidate the mechanism of methanol-repression in the RD mutation.

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