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Effect of Methanol on the Frequency of Respiration-Deficient Mutation Induced by Ethidium Bromide in Yeast under Growing and Non-growing Conditions

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The effect of methanol on the frequency of cytoplasmic respiration-deficient (RD) mutation induced by ethidium bromide was investigated in *Saccharomyces cerevisiae*. When growing cells were treated with ethidium bromide at concentrations higher than 1.0 μg/ml, 95 to 100% of surviving cells were RD mutants as judged by the tetrazolium overlay method. The RD mutants induced by 1.0 μg/ml ethidium bromide were drastically decreased by addition of 6—8% methanol in both growing and non-growing conditions. Methanol reduced uptake of ethidium bromide by yeast cells, but it did not modify the inhibitory effect of ethidium bromide on *in vitro* yeast mitochondrial deoxyribonucleic acid synthesis.

Keywords——yeast; *Saccharomyces cerevisiae*; ethidium bromide; respiration-deficient mutation; methanol effect; growing condition; non-growing condition

Methanol is one of the aliphatic alcohols which decrease the frequency of respiration-deficient (RD) mutation of *Saccharomyces cerevisiae* induced by aromatic alcohols.[1] In a previous paper,[2] we have demonstrated that the frequency of RD mutation induced by 0.5 μg/ml acriflavine was decreased from 97% to 15% by the addition of methanol to a growing culture of yeast cells. Since the RD mutation by acriflavine is induced only in daughter cells,[3] growing conditions were thought to be essential for the induction of RD mutation. In acriflavine-induced RD mutants, mitochondrial deoxyribonucleic acid (DNA) was largely deleted or completely lost,[4] and it was suggested that methanol either counteracts inhibition of mitochondrial DNA replication by acriflavine or simply inhibits the permeation of acriflavine through the cell membrane and/or mitochondrial membrane.

It is known that ethidium bromide inhibits replication of mitochondrial DNA and destroys pre-existing mitochondrial DNA, and then induces RD mutants in both mother and daughter cells.[5] In the present paper, we report that methanol reduces the frequency of RD mutants induced by ethidium bromide under growing and non-growing cell conditions, and the uptake of ethidium bromide by yeast cells is decreased in the presence of methanol. The effects of ethidium bromide and methanol on the activity of mitochondrial DNA polymerase are also reported.

Experimental

Yeast Strain——*Saccharomyces cerevisiae* ATCC 26422 was used.

Chemicals——Ethidium bromide (reagent grade) and methanol (reagent grade) were purchased from Nakarai Chemicals.

Induction of RD Mutation by Ethidium Bromide under Growing Conditions——As described previously,[2] yeast cells of an overnight culture were inoculated into Ogur's liquid medium[6] containing 2% glucose so as to give 1×10^5 cells/ml. Ethidium bromide was added to the medium with or without methanol. After shaking at 30 °C, cells were diluted with distilled water and spread onto Ogur's agar plates so as to give about 200 colonies per plate. After incubation at 30 °C for 2 d, colonies of RD mutants were scored by the tetrazolium overlay method,[7] and completely white colonies were counted as RD mutants. The frequency of RD mutation was represented as the percentage of RD mutants among survivors. Results were expressed as averages of two to three independent experiments, each of which was done with 5 plates. Total cell number in a liquid culture was counted using a hemocytometer. Colony-forming ability on Ogur's agar plates was used as the criterion for survivors.

Induction of RD Mutation by Ethidium Bromide under Non-growing Conditions——Cells grown in Ogur's liquid medium at 30 °C for 24 h were harvested, washed three times with distilled water and suspended in 0.1 M potassium phosphate buffer (pH 5.7) so as to give 1×10^6 cells/ml. Ethidium bromide was added to the cell suspension in the presence or absence of methanol, and the suspension was shaken at 30 °C. The induction frequency of RD mutation was examined by plating the treated cells on Ogur's agar plates as described above.

Content of Ethidium Bromide in Yeast——Cells of an overnight culture were suspended in 0.1 M potassium phosphate buffer (pH 5.7) so as to give 2.0×10^8 cells/ml in the presence of 1.5 μg/ml ethidium bromide with or without methanol, and shaken at 30 °C for 30 min. Cells were harvested and washed three times with the buffer by centrifugation. The precipitated cells were extracted twice with 3 ml each of methanol according to the previous paper.[2] The amounts of ethidium bromide in the methanol extract and in the supernatant were determined by measuring the absorption at 520 and 480 nm (the absorption maxima in each solution, respectively).

Assay for Mitochondrial DNA Polymerase Activity——Mitochondrial DNA polymerase was extracted from *Saccharomyces cerevisiae* D273-10B/A1 followed by the method of Wintersberger and Blutsch.[8] Its activity was measured by counting radioactivity of [methyl-^{3}H]dTTP incorporated into denatured salmon sperm DNA during incubation for 10 min at 37 °C in the presence of 50 μg/ml aphidicolin.[8,9] The activity was expressed in pmol of dTTP incorporated into DNA per mg of protein.

Results

Effect of Methanol on Cell Growth and on the Frequency of RD Mutation Induced by Ethidium Bromide

The cell growth and the frequency of RD mutation induced by ethidium bromide were determined after 24 h incubation in Ogur's medium supplemented with 0—5.0 μg/ml ethidium bromide and 0—8% methanol. As shown in Fig. 1A, cell density of the control culture reached a level of about 2×10^8 cells/ml. Although methanol up to 6% did not affect the final

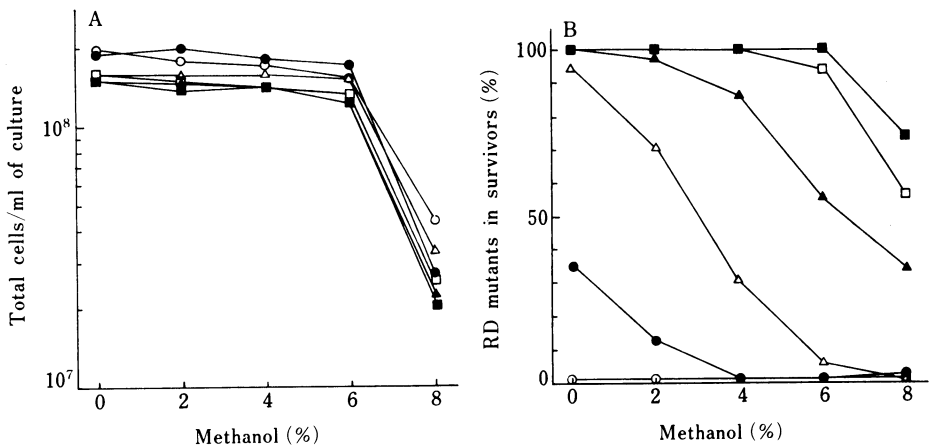

Fig. 1. Effect of Methanol Concentration on the Cell Growth (A) and on the Frequency of RD Mutation (B) Induced by Ethidium Bromide

Yeast cells were incubated at 30 °C for 24 h in the presence or absence of methanol. Ethidium bromide was added at the following concentrations (μg/ml); 0.0 (○), 0.5 (●), 1.0 (△), 1.5 (▲), 2.0 (□) and 5.0 (■). A: Cell densities in each medium were scored with a hemocytometer and were expressed as total cells per ml of culture. B: Percent of RD mutants among survivors.

cell density, 8% methanol lowered the cell yield to one-fifth of the control. The cell yield was not significantly affected by 0.5 to 5.0 μg/ml ethidium bromide.

As shown in Fig. 1B, the frequency of RD mutation in the control culture was less than 0.7% and was not affected by the presence of methanol at concentrations up to 8%. The frequencies of RD mutation were about 100, 95 and 35% in the presence of 1.5, 1.0 and 0.5 μg/ml ethidium bromide.

Addition of methanol to the culture medium markedly decreased these frequencies. In particular, the frequency of RD mutation in the culture containing 1.0 μg/ml ethidium bromide was decreased almost to the control level by the addition of 6 or 8% methanol. As seen in Fig. 1B, the larger the ethidium bromide content in the culture, the larger the amount of methanol was required to cancel the mutagenic effect. More than 80% of cells survived at all concentrations of methanol and ethidium bromide tested in the present experiments. Growth of respiration-deficient cells was not more inhibited by the addition of methanol than that of respiration-competent cells (data not shown).

Time Course of the Effect of Methanol on the Frequency of RD Mutation Induced by Ethidium Bromide

A time course experiment was carried out to determine the effect of methanol on cell growth and on the frequency of RD mutation induced by ethidium bromide.

As shown in Fig. 2A, the cell number in the culture reached a level of 2×10^7 cells/ml after a 12-h incubation with or without 1.5 μg/ml ethidium bromide. In the presence of 6 and 8% methanol with 1.5 μg/ml ethidium bromide, it took 16 to 20 h and 24 to 28 h for full growth, respectively.

As shown in Fig. 2B, in the presence of 1.5 μg/ml ethidium bromide, the frequency of RD mutation reached almost 100% after 12 h. The frequencies were reduced to levels of 55 and 30% by addition of 6 and 8% methanol to the culture, respectively, though cell numbers in these cultures attained the maximum level in 32 h. Thus methanol inhibited RD mutant induction by ethidium bromide and not cell growth.

Effect of Methanol on the Frequency of RD Mutation Induced by Ethidium Bromide in Non-growing Yeast Cells

As shown in Fig. 3, the frequency of spontaneous RD mutation in the non-growing cell

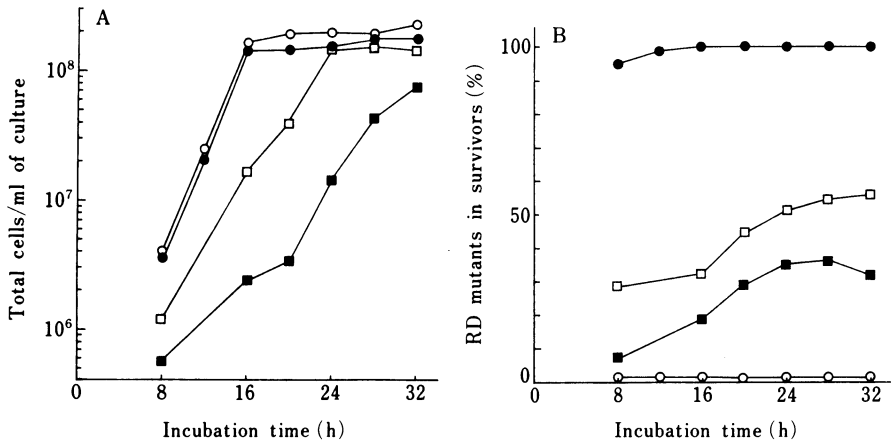

Fig. 2. Time Course of the Cell Growth (A) and Frequency of RD Mutation (B) Induced by Ethidium Bromide in the Presence of Methanol

Yeast cells were incubated at 30 °C in the presence (●) or absence (○) of 1.5 μg/ml ethidium bromide. Methanol was added to the culture containing ethidium bromide at a concentration of 6% (□) or 8% (■).

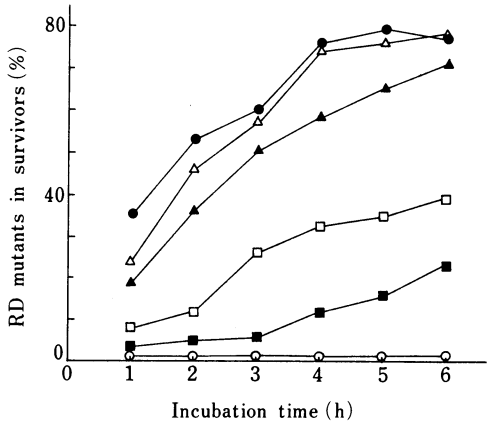

Fig. 3. Effect of Methanol on the Frequency of RD Mutation Induced by Ethidium Bromide in Non-growing Cells

Cells were washed and suspended in 0.1 M potassium phosphate buffer (pH 5.7) with (●) or without (○) 1.5 μg/ml ethidium bromide and shaken at 30 C. Methanol was added to the suspension supplemented with 1.5 μg/ml ethidium bromide at the following concentrations (%); 2 (△), 4 (▲), 6 (□) and 8 (■).

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

Methanol (%)	Amount of ethidium bromide (μg) in	
	Yeast cells	Supernatant
None	3.6±0.1	10.4±0.2
8.0	2.5±0.2	12.2±0.1

Yeast cells (2×10^9) in 10 ml of 0.1 M potassium phosphate buffer (pH 5.7) with 15 μg of ethidium bromide were incubated at 30 °C for 30 min with or without 8% methanol. Each value represents the mean ± standard deviation of six experiments.

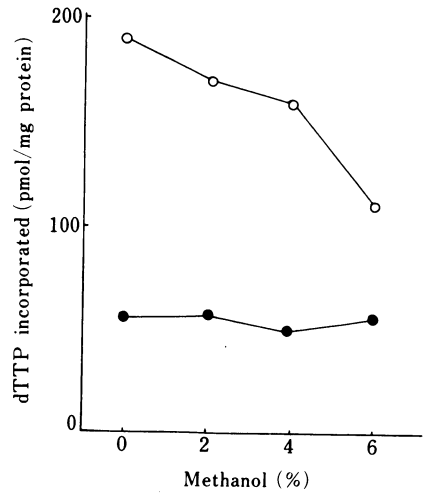

Fig. 4. Effect of Methanol on dTTP Incorporation by Mitochondrial DNA Polymerase

The incorporation of dTTP was assayed in the presence (●) or absence (○) of 1.5 μg/ml ethidium bromide and expressed in pmol of dTTP incorporated into DNA per mg of protein.

suspension in 0.1 M potassium phosphate buffer (pH 5.7) was less than 0.7% throughout the incubation time. Methanol at the concentration range of 2 to 8% did not show any marked effect on the formation of RD mutants in the cell suspension (data not shown).

In the presence of 1.5 μg/ml ethidium bromide, the frequency of RD mutation reached about 80% after a 4-h incubation. The frequency was decreased to 31 and 12% by the addition of 6 and 8% methanol, respectively. Addition of 2% methanol, however, did not affect the frequency of RD mutation. In these experiments, ethidium bromide and methanol did not show any killing effect (data not shown).

Ethidium Bromide Content in Yeast Cells

Cells were suspended in 0.1 M potassium phosphate buffer (pH 5.7) with 1.5 μg/ml ethidium bromide and shaken for 30 min. As shown in Table I, 24% of ethidium bromide added to the suspension was taken up by the cells. Addition of 8% methanol decreased the ethidium bromide uptake to a level of about 70% of that found in the methanol-free suspension. The amount of ethidium bromide remaining in the supernatant was higher in the presence of methanol than in its absence.

Effect of Methanol on Mitochondrial DNA Polymerase Activity

As shown in Fig. 4, *in vitro* dTTP incorporation induced by mitochondrial DNA polymerase was strongly inhibited in the presence of 1.5 μg/ml ethidium bromide; only about 30% of the incorporation was observed under these conditions. Methanol also inhibited the incorporation, but did not modify the inhibitory effect of ethidium bromide on the incorporation. Methanol at 6% lowered the dTTP incorporation to 60% of that of the control, but this concentration of methanol did not induce any RD mutant under growing conditions (Fig. 1B) or non-growing conditions (data not shown).

Discussion

It has been reported that ethidium bromide induced RD mutation in *Saccharomyces cerevisiae* cells under growing and non-growing conditions.[5] The induction frequency was decreased by addition of methanol to the incubation medium. As shown in Fig. 2A and 2B, the frequency of RD mutation induced by ethidium bromide increased when cells were growing. Methanol inhibited the cell growth and retarded the increase of the frequency of RD mutation induced by ethidium bromide. However, this decrease of the frequency of RD mutation can not be explained only by the inhibitory effect of methanol on cell growth, because even when cells were incubated for long time to permit full growth, the frequency of RD mutation in the presence of ethidium bromide and methanol was clearly lower than in the absence of methanol. This result can be rationalized in terms of the fact that methanol decreases the frequency of RD mutation among yeast cells treated with ethidium bromide in potassium phosphate buffer, where cell growth is not allowed.

As shown in Table I, uptake of ethidium bromide into yeast cells was decreased by addition of methanol. Thus, the decrease of the frequency of RD mutation can be explained partly by the decrease of ethidium bromide uptake. It has been reported that ethidium bromide is taken up into yeast cells through the transport system for potassium ion.[10] In addition, it has been shown that ethidium-resistant mutants of *Kluyveromyces lactis* have lost the ability to transport both potassium ion and ethidium.[11] We have previously reported that the uptake of acriflavine into yeast cells is decreased by the addition of methanol to the culture medium.[2] It is known that acriflavine is also taken up at least partially through the potassium ion transport system, though the uptake by other route(s) could not be sensitive to potassium ion.[10b] Therefore, it is possible that the potassium ion transport system of yeast is affected by the addition of methanol.

It is thought that the direct mechanism of RD mutant induction by ethidium bromide in growing cells is to inhibit replication of mitochondrial DNA and to promote subsequent degradation of the DNA.[12] In non-growing cells, pre-existing mitochondrial DNA is broken into large segments, but growing conditions are required for further degradation of these segments.[13] In this report, we show that the frequency of RD mutation induced by ethidium bromide in non-growing conditions, where mitochondrial DNA synthesis is low or even absent, was decreased by the addition of methanol. This fact suggested that methanol did not counteract the inhibitory effect of ethidium bromide on mitochondrial DNA synthesis. This suggestion was further supported by the experiment carried out with mitochondrial DNA polymerase. Ethidium bromide inhibited the dTTP incorporation induced by the polymerase, and the inhibition was not reversed by the addition of methanol to the reaction mixture.

It is not clear from the present experiment whether the inhibition of dTTP incorporation is caused by a direct inhibitory effect of ethidium bromide on mitochondrial DNA polymerase or by an indirect effect due to intercalation of ethidium bromide into template DNA. Since the inhibitory effect of methanol on dTTP incorporation is dependent on the concentration of methanol, it seem to be a direct effect on mitochondrial DNA polymerase.

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