Leakage of 5'-Guanosine Monophosphate from *Candida lipolytica* (IFO, 0746) and Its Mutants

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Received February 23, 1977

5'-Guanosine monophosphate (5'-GMP) was found to leak from *Candida lipolytica* (IFO, 0746) and its mutant Y and Sm.

Mutant Sm obtained from the parent strain by treatment with N-methyl-N'-nitro-N-nitrosoguanidine (NTG) and ultraviolet irradiation was found to leak 5'-GMP at 5 times the amount of the parent strain. The properties of mutant Y and Sm were compared with the parent strain, and differences were present in growth, amount of 5'-GMP leakage and ribonuclease activity.

Since leakage of nucleic acid-related substances from yeast was first reported by Challinor and Rose,¹⁾ Higuchi and Uemura²⁾ also reported that 2'- or 3'-nucleotides leaked from cells of *Saccharomyces cerevisiae* by shaking in citrate buffer or ethylenediamine tetraacetic acid (EDTA) solution.

In continuation to these reports, screenings of yeasts that leaked nucleotides were carried out by Nakao *et al.*³⁾ It was concluded by them that yeasts were divided into four groups according to their ability to leak nucleotides: first, those that leaked 3'-nucleotides at acid pH; second, those that leaked 5'-nucleotides at alkaline pH; third, those that leaked 5'-nucleotides at both acidic and alkaline pHs; and fourth, those that leaked little nucleotides at acidic or alkaline pH.

There has, however, not yet been a report on *Candida* yeasts.

Recently, *C. lipolytica* has been recognized as a citric acid producing yeast.⁴⁾ In connection with the report by Higuchi and Uemura²⁾ in which some nucleotides were leaked into medium by shaking *S. cerevisiae* cells in citrate buffer, the authors thought that there might be some connection between leakage of nucleotides and citric acid production of *C. lipolytica*. Thus, the authors first tested whether citric acid is produced, and next

tested whether nucleotides are leaked by *C. lipolytica*. Then, isolation of mutants that leaked nucleotides much more than the parent strain was carried out by mutagenesis with NTG and by ultraviolet irradiation. Finally, comparisons of properties of the parent strain and mutants were carried out in growth, amount of 5'-GMP leakage and ribonuclease (RNase) activity.

MATERIALS AND METHODS

Materials. Nucleotides, DEAE-cellulose and Sephadex G-10 were purchased from Nakarai Chemicals Co.

Microorganisms. *Candida lipolytica* (IFO, 0746) mutant Y was isolated from the parent strain by NTG treatment, and mutant Sm was isolated from mutant Y by UV-irradiation as described under *Isolation of mutants*

Cultural conditions. Medium A (100 ml) contained 10 g of D-glucose, 0.5 g of $\text{NH}_4\text{H}_2\text{PO}_4$, 0.05 g of KH_2PO_4 , 0.05 g of $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 1 mg of inositol, 50 μg each of nicotinamide, riboflavin, pyridoxal and Ca-pantothenate, 10 μg of thiamine and 12 μg of biotin, adjusted pH to 6.0, after sterilization at 120°C for 15 min. Agar slant medium (100 ml) contained 10 g of D-glucose, 0.5 g each of Polypepton, NaCl and yeast extract and 2 g of agar, adjusted pH to 6.0, after sterilization at 120°C for 15 min. A loopful of agar slant culture was inoculated to 100 ml of medium A and incubated at 30°C for 4 days by shaking (7 cm in

stroke, 140 reciprocation/min). The cells were harvested by centrifugation at 10,000 rpm for 15 min. The cells thus obtained were washed with distilled water until the washings did not show absorbance at 260 nm. The cells (10 g wet weight) were shaken in 50 ml of 0.1 M acetate buffer (pH 6.0) in a 500 ml shaking flask.

Measurement of 260 nm-absorbing substances. The culture fluid was measured on absorbance at 260 nm with a Shimadzu-UV-200 spectrophotometer.

Isolation of mutants.⁵⁾ When cell growth of *C. lipolytica* (IFO, 0746) reached the log phase (cells, 10^8 /ml), NTG was added to the culture to give a final concentration of 30 μ g per ml, and the cultivation was continued for a further 24 hr at 30°C with shaking. After treatment with NTG, 0.1 ml of the culture was pipetted up and spreaded over a plate of agar medium A and incubated at 30°C for 24 hr. Several colonies appeared on the plate. Among these colonies, a yellow colony which leaked the largest amounts of UV-absorbing substance in the cultivation test was designated as mutant Y and used for further mutation. Mutant Y was cultivated in medium A at 30°C for 24 hr. The cells were harvested by centrifugation and were suspended into 0.1 M Tris-malate buffer (pH 6.0) to give a final cell count about 10^8 per ml. Ten milliliters of the cell suspension were poured into a petri dish (diameter, 9 cm) and irradiated by a sterilizing lamp (Toshiba, 15W) from a distance of 40 cm for 1 min. Then, 0.1 ml of the UV-treated suspension was spreaded over a plate of agar medium A and incubated at 30°C for 24 hr. Among colonies on the plate, a smooth colony which leaked the largest amounts of UV-absorbing substance in the cultivation test was chosen as mutant Sm.

Identification of leaked substance. For identification of organic acids in broth, to the culture fluid was added one-fifth volume of charcoal to absorb the residual sugar and coloring impurities. After the mixture was filtered, the charcoal portion was washed with water. To the filtrate and washings was added one-fifth volume of Amberlite IR-120 to remove the cationic impurities. After filtration, to the filtrate was added one-fifth volume of Amberlite 4B to absorb the organic acids on the resin. After filtering and washing the resin with water, one-fifth volume of 2% NaOH was added to elute the organic acids from the resin. Finally, Amberlite IR-120 was added to convert the sodium salt of organic acid to free acid. Then, the concentrate was kept in a refrigerator for crystallization. The crystals were recrystallized from MeOH, and the melting point of the crystals was determined. But, crystals of organic acid were not obtained in large quantity by this method. To obtain crystals of organic acid in bulk, an experiment was carried out to convert organic acid to derivative of its organic acid in culture

fluid. That is, to 200 ml of culture fluid adjusted to pH 6.0 with 1 N NaOH, 0.3 g of *p*-bromophenacyl-bromide in 90% EtOH was added, and the reaction mixture was refluxed. Then, the crystals formed were separated by filtration from the reaction mixture and dissolved in 4 ml of 99% EtOH. Crystals were formed by cooling in a refrigerator.

Identification of nucleotide leaked from the yeast. Incubation mixture samples (5 ml) were occasionally taken from the shaking mixture and centrifuged at 10,000 rpm for 10 min. The supernatant solution thus obtained was used for measuring the UV-spectrum. Next, the scale up experiment was carried out, that is, 100 ml of supernatant solution were taken from the broth and dialyzed against distilled water. The dialyzed outer solution was concentrated *in vacuo* at 40°C, and the concentrated solution was subjected on a Sephadex G-10 column (2 $\times$ 60 cm) and eluted by distilled water. The flow rate was 5.0 ml per hr, and 5.5 ml each fraction was collected. The UV-absorbing fractions were pooled, adsorbed on a Dowex 1 $\times$ 8 AG column (0.8 $\times$ 10 cm) equilibrated with 0.005 N formic acid, and eluted stepwise by 0.005 N-formic acid, 0.02 N-formic acid, 0.17 N-formic acid and 0.17 N-formic acid containing 0.05 M Na-formate, respectively. Next, the eluate with 0.17 N formic acid-0.05 M Na-formate was concentrated and applied to paper chromatography using two kinds of solvents: (1) MeOH-EtOH-conc.HCl-H₂O (50:25:6:19, v/v) and (2) isoPrOH-conc.HCl-H₂O (65:16.7:18.3, v/v). For detection of nucleotides, Super light (Nikko Seiki Works) was used. Thin layer chromatography (TLC) was also conducted on an Avicel Gel plate with the same solvent systems as paper chromatography.

Identification of 5'-nucleotide. To detect whether phosphate bonds of the nucleotide was 5'- or 3'-linkage, enzymatic cleavage of phosphate bonds of the nucleotide with 5'-nucleotidase (Sigma) was tested on the nucleotide fraction that had been extracted from the UV-absorbing spot on thin-layer chromatogram with phosphate buffer (pH 9.0). Then, inorganic phosphate released was measured by the Fiske-Subbarow's method.⁶⁾

Intracellular RNase activity. Unless otherwise stated, RNase activity was determined as in the previous paper⁷⁾ except that 0.1 M acetate buffer (pH 6.0) was used as buffer for the enzyme assay.

RESULTS

Identification of citric acid

As for organic acids formed by *C. lipolytica*, Tabuchi *et al.*⁴⁾ reported that much citric acid and some isocitric acid were formed. Thus,

the first experiment was carried out to collect these organic acids from yeast. The result was that only citric acid was identified as the derivative of *p*-bromophenacyl ester (mp, 110°C).

Relationship of UV-absorbing substance leaked from growing cells or from washed cells with cultivation time

An aliquot of incubation mixture was taken, concentrated and the supernatant solution thus obtained was used for measurement of UV-absorption spectrum to make clear whether the UV-absorbing substance was nucleotide or not. The UV-absorption spectrum showed maximal absorption at about 260 nm and minimal absorption at about 240 nm.

This means that the UV-absorbing substance is a nucleic acid-related compound. Next, an experiment was carried out using washed cells to confirm this finding. The harvested cells after 4 day-incubation were suspended in 0.1 M acetate buffer, pH 6.0 and shook. The relationship of the UV-absorbing substance

FIG. 1. Leakage of Ultraviolet-absorbing Substance with Incubation Time.

with incubation time is shown in Fig. 1. The amount of UV-absorbing substance increased with incubation time. Changes in pH, growth and 260 nm UV-absorbing substance with

FIG. 2. Changes of pH, Optical Density at 260 nm and Growth of the Parent Strain. Growth (○), pH (×) and OD₂₆₀ (▲).

incubation time are shown in Fig. 2. The amount of UV-absorbing substance increased with cell growth.

UV-absorbing substance

Result of paper chromatography and TLC with filtrate by washed cells is shown in Table I. Moreover, the following findings clarified that the UV-absorbing substance was 5'-GMP: first, inorganic phosphate was released from the nucleotide by 5'-nucleotidase; second, the elution pattern of the nucleotide being eluted with 0.17 N-formic acid-0.05 M Na-formate on Dowex 1 × 8 AG column coincided with that of authentic 5'-GMP; and third, ratios of extinction coefficients (pH 7.0) of the nucleotide ($\epsilon_{250}/\epsilon_{260} = 1.16$, $\epsilon_{280}/\epsilon_{260} = 0.65$ and $\epsilon_{290}/\epsilon_{260} = 0.30$) coincided with those of authentic 5'-GMP.

Isolation of mutants

Mutant Y was isolated from *C. lipolytica* (IFO, 0746) after treatment with NTG, and mutant Sm was obtained from mutant Y by further UV-irradiation.

Properties of mutant Y and Sm

a) Effect of amino acids: Each amino acid of the standard 20 amino acids in concentration of 50 mM was added to medium A, and the effect of the amino acids on growth was

TABLE I. *R_f*-VALUES OF AUTHENTIC NUCLEOTIDES AND ULTRAVIOLET ABSORBING SUBSTANCE

Paper chromatography			Thin-layer chromatography		
Solvent system	A (<i>R_f</i>)	B (<i>R_f</i>)	Solvent system	A (<i>R_f</i>)	B (<i>R_f</i>)
5'-AMP	0.40, DV	0.38, DV	5'-AMP	0.38, DV	0.00, DV
5'-CMP	0.00, DV	0.45, DV	5'-CMP	0.45, DV	0.49, DV
5'-GMP	0.32, W	0.30, W	5'-GMP	0.30, W	0.28, W
5'-UMP	0.61, DV	0.67, DV	2'(3')-GMP	0.39, W	0.37, W
			5'-UMP	0.67, DV	0.00, DV
UV-absorbing substance	0.32, W	0.30, W	UV-absorbing substance	0.30, W	0.28, W

Note: A system: MeOH-EtOH-conc. HCl-H₂O (50: 25: 6: 19, v/v).

B system: iso-PrOH-conc. HCl-H₂O (65: 16.7: 18.3, v/v).

W, white color by UV-lamp detector.

DV, deep violet color by UV-lamp detector.

checked. Among these amino acids, serine, aspartic acid, methionine and arginine produced stimulative effects on growth of mutant Y and Sm as well as the parent strain, whereas cysteine inhibited growth at the initial stage of the two mutants. These effects, however, decreased with a lapse of incubation time. b) Effect of vitamins: The effect of thiamine and other vitamins was checked. As expected, mutant Y and Sm as well as the parent strain required thiamine for growth. As Rose⁸⁾ reported that the permeability of *C. albicans* increased by the absence of biotin in medium, the effect of biotin on the parent strain and mutant Y and Sm was tested. There was no effect with biotin on permeability of mutant

Y and Sm as well as the parent strain. c) About growth: A comparison of the growth curve of the parent strain with mutant Y and Sm is shown in Fig. 3.

A similar growth was found in all strains up to 2 days but after 2 days, the growth of mutant Y and Sm decreased compared with the parent strain. d) Effect of glucose: A comparison of glucose consumption in the parent strain with mutant Y and Sm is shown in Fig. 4. Mutant Sm showed a similar pattern to the parent strain, but mutant Y showed a different pattern compared with the two other strains. e) Leakage of UV-absorbing substance: A comparison of the amount of UV-absorbing substance from the parent strain with mutant Y and Sm is shown in Fig. 5. There was about

FIG. 3. Growth with Cultivation Time.

Parent strain (○), mutant Y (▲) and mutant Sm (×).

FIG. 4. Consumption of Glucose with Incubation Time.

Parent strain (○), mutant Y (▲) and mutant Sm (×).

FIG. 5. Leakage of Ultraviolet-Absorbing Substance with Incubation Time.

Parent strain (○), mutant Y (▲) and mutant Sm (×).

5 times more UV-absorbing substance by mutant Sm than by the parent strain. f) About intracellular RNase: Cells (15 g wet weight) from the parent strain and mutant Y or Sm, were disrupted by a Braun cell homogenizer, and the respective cell-free extracts were obtained after centrifugation. After dialysis against distilled water overnight, the RNase activities from the three preparations were compared. Mutant Y and Sm both showed about one-ninth the RNase activity compared with the parent strain. Comparison of some properties, cell growth, consumption of glucose, RNase activity and UV-absorbing substance of the parent strain with mutant Y and Sm is shown in Table II.

TABLE II. COMPARISONS OF PROPERTIES OF THE PARENT STRAIN WITH MUTANT Y AND Sm

Strain	Cells (g)/ 100 ml of culture	RNase (Relative activity)	Consumption of glucose (%)	OD ₂₈₀ / g.D.W
Parent	4.08	100.0	65.2	1.30
Y	2.19	11.6	48.2	3.11
Sm	1.80	11.9	37.8	6.44

DISCUSSION

As described in the introduction for leakage of nucleotides from yeasts, Higuchi and Uemura²⁾ reported that 3'- or 2'-nucleotides

were leaked from washed cells of *S. cerevisiae* by shaking in 0.1 M acetate buffer (pH 6.0). Nakao *et al.*³⁾ later reported in detail that the yeasts of *Rhodotorula*, *Schizosaccharomyces* and *Saccharomyces* leaked 5'-nucleotides. They concluded that 3'-nucleotides were leaked at acid pH, but 5'-nucleotides were ordinarily leaked at alkaline pH. However, in *Rhodotorula pakkida*, 5'-nucleotides leaked both at acidic and alkaline pHs. In our experiments with *C. lipolytica*, only 5'-GMP leaked at acidic pH. It means that this yeast belongs to another type. Moreover, from the finding that degradative type of intracellular RNase of this yeast belongs to 3'-former, 5'-GMP must be produced by another enzyme than intracellular RNase, but on this point, it is necessary to examine the problem further in more detail.

As to the relationship between nucleotides leakage and mutation, Tsukada and Sugimori⁹⁾ have already reported that a mutant obtained from *Zygosaccharomyces soja* by cycloheximide treatment leaked 5'-nucleotides in larger amounts than the parent strain. In addition, it has been reported by Ikeno *et al.*,¹⁰⁾ that a mutant obtained from *C. lipolytica* by NTG treatment leaked citric acid in larger amounts than the parent strain. By combining the above procedures, we tried to get mutants with the capacity to leak 5'-GMP by treatment similar to the above methods and got mutant Y which leaked 5'-GMP 2.5 times more than the parent strain. Next, we obtained another mutant which leaked 5'-GMP at 5 times more than the parent strain by irradiating mutant Y with UV lamp. These results suggest that by mutation with UV-irradiation or NTG, some changes may occur in the structure of the cell membrane, and 5'-GMP was leaked in larger amounts.

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