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Isolation of Cytochrome *c* Abundant Mutant of *Candida utilis*

Yoshihisa Iwamoto and Ichiji Mifuchi

Shizuoka College of Pharmacy[1]

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A mutant of *Candida utilis* containing about five fold amount of cytochrome *c* than that of a parental strain was induced by N-methyl-N′-nitro-N-nitrosoguanidine.

In this mutant (I-35), repression of cytochrome *c* synthesis by glucose was not observed, while the parental strain was repressed cytochrome *c* content by glucose in medium.

The amount of cytochrome *c* in the mutant varied from phase to phase in its growth stage.

Iso-2-cytochrome *c* was found both in the parent and the cytochrome *c* abundant mutant, and was about 10% of total amount of cytochrome *c* in the both strains.

Introduction

It was reported by De Deken[2] that some kinds of yeast including *Candida utilis* did not appear to be inducible to its respiratory-deficient mutant by euflavine. We have also attempted the induction of the respiratory-deficient mutant of *C. utilis* by using acriflavine,[3] ethidium bromide[4] and pinacyanole[5] which are known as "petite inducing reagents," and confirmed the results reported by De Deken. When a potent mutagen N-methyl-N′-nitro-N-nitrosoguanidine (NG) was used, however, several mutants of *C. utilis* which could not reduce 2,3,5-triphenyl tetrazolium chloride (TTC) were obtained. Followed analysis of cytochrome pattern and measurement of respiration of the mutants revealed that most of the mutants were respiration-sufficient, in spite of lack of ability to reduce TTC.

One of the mutants (I-35) was clearly distinguished from the others by a characteristic difference which indicated a remarkable absorption at 520 and 550 mμ in the cytochrome pattern. In the present study, the cytochrome *c* content and the properties about respiratory metabolisms of this mutant were examined.

Experimental

Strain——*Candida utilis* 0619 was kindly provided by Dr. T. Sugimura, Biochemistry division, National Cancer Center Research Institute, Tokyo.

Culture Media——Cells were cultured aerobically under shaking at 30° in a medium containing: 3.5 g polypeptone (Wako), 3.0 g yeast extract (Wako), 2.0 g KH_2PO_4, 1.0 g $(NH_4)_2SO_4$, 1.0 g $MgSO_4 \cdot 7H_2O$, 20 g glucose, per litre of distilled water.

Isolation of Cytochrome *c* Abundant Mutant——*C. utilis* 0619 was inoculated in the L-shape tube containing 3 ml of the medium supplemented with NG(50 μg/ml), and shaked moderately for 12 hr at 30°. Cells were plated on glucose medium after washed and diluted adequately and were cultured for 48 hr at 30°. The mutants incapable of reducing TTC were detected by Nagai's method.[6]

The cytochrome *c* abundant mutant was found among these mutants.

Cytochrome Pattern——Cytochrome absorption spectra of the intact cells (20% w/v) were measured by opal glass method with Carry 14 model spectrophotometer at room temperature.

1) Location: *2-2-1, Oshika, Shizuoka-shi, 420, Japan.*
2) R.H.De Deken, *Exp. Cell Res.*, **24**, 145 (1961); R.H.De Deken, *J. Gen. Microbiol.*, **44**, 157 (1966).
3) S. Nagai, N. Yanagishima, and H. Nagai, *Bact. Rev.*, **25**, 404 (1961).
4) P.P. Slonimski, G. Perrodin, and J.H. Croft, *Biochem. Biophys. Res. Commun.*, **30**, 232 (1968).
5) T. Sugimura, K. Okabe, and M. Kodama, *J. Bacteriol.*, **97**, 964 (1969).
6) S. Nagai, *Science*, **133**, 1188 (1959).

Respiration——Respiration was measured with an oxygen meter (Yanagimoto).

Estimation of Cytochrome *c*——The amount of cytochrome *c* in the autolyzate by ethyl acetate was determined spectrophotometrically after separation through ion exchange column of Amberlite CG-50 as reported by Sels, *et al.*[7] Namely the autolyzates of the yeast cells by ethyl acetate were diluted with 0.5M NaCl, and centrifuged for 10 min at $3000 \times g$ to remove cell debris. The supernatant was dialysed against 10 mM ammonium phosphate buffer (pH 7.0), and followed to pass the Amberlite CG-50 resin column. Cytochrome *c* fractions adsorbed to the upper parts of the resin were eluted by 0.1M ammonium phosphate buffer (pH 7.0) containing 2M NaCl.

The amounts of cytochrome *c* were estimated from difference of between 550 (peak) and 534 mμ (trough), by using a calibration curve, after reduced by addition of an aliquot of sodium hydrosulfite.

Separation of iso-2-Cytochrome *c*——The concentrated cytochrome *c* fraction obtained was dialysed against 20 mM ammonium phosphate buffer (pH 7.0) and passed through Amberlite CG-50 resin column (1×10 cm) after oxidized by addition of an aliquot of pottasium ferricyanide. The adsorbed cytochrome *c* was eluted with 0 to 0.5M gradient concentration of ammonium phosphate buffer (pH 7.0).

Result

By the examination of the biochemical properties, including reduction of TTC and utilization of non fermentable carbon source, the mutant (I-35) of *C. utilis* indicated the similar properties to those of the petite mutants induced from the other strains which do not reduce TTC, nor utilize non fermentable carbon source, but the newly found mutant exhibited a high respiratory activity.

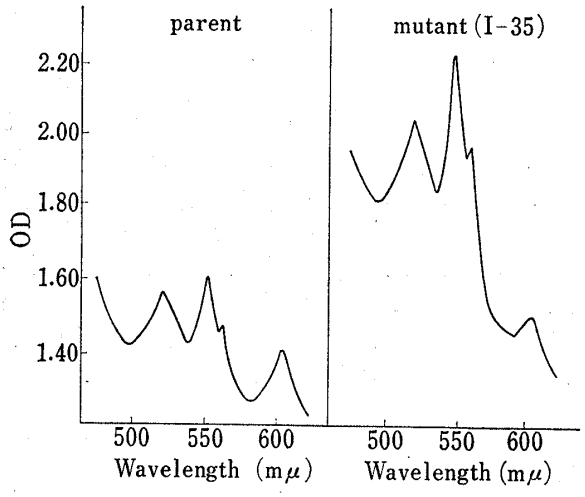

Fig. 1. Cytochrome Absorption Spectrum of *C. utilis* 0619 Parental Strain and the Mutant (I-35)

Cells were cultured aerobically for 48 hours at 30° in the medium described before, washed three times by centrifugation and 1g (wet weight) of packed cells were suspended in 4 ml of 0.1M phosphate buffer. Absorption spectrum of cell suspension were measured by opal glass methods.

The cytochrome absorption spectrum of the mutant showed the same absorption maximum at 603, 562, 550, and 520 mμ as those of the parent, corresponding to cytochrome *a*, *b*(α-band), *c*(α-band), *b* and *c*(β-band) respectively, however the mutant showed the prominent absorption at 550 and 520 mμ comparing to the parent (Fig. 1).

Therefore the cytochrome *c* content of the mutant was quantitatively compared with those of the parent and some other yeast strains.

As shown in Table I, the parental strain of *C. utilis* showed about the same level of the cytochrome *c* content as those of

Table I. The Amount of Cytochrome *c*

Strain		Amount of cytochrome *c*
Candida utilis 0619 Parent	2% glucose 24 hr	3.17 ± 0.18 (mμ mole/g wet weight)
	2% glucose 48 hr	3.46 ± 0.16
Mutant (I-35)	2% glucose 24 hr	22.68 ± 1.69
	2% glucose 48 hr	19.24 ± 2.75
	10% glucose 48 hr	30.69 ± 1.21
Pressed yeast	purchased	6.89 ± 0.84
Saccharomyces cerevisiae	$R_2$01B × R_3AlE 48 hr	3.85
	R_2Q2B ρ^+ 45 hr	5.70
	R_2Q2B ρ^- 45 hr	4.14

7) A.A. Sels, H. Fukuhara, G. Péré, and P.P. Slonimski, *Biochim. Biophys. Acta*, **95**, 486 (1965).

the other yeast strains. On the contrary, the mutant contained much higher amount of the cytochrome *c*.

Particularly it is of interest that the mutant had more cytochrome *c* when grown in the medium containing higher concentration of glucose, in which synthesis of the cytochrome in the normal strain was repressed. We examined the effect of the various concentration of glucose on the cytochrome *c* content of the parent and the mutant.

As indicated in Fig. 2, the difference of cytochrome *c* level of the both strains was clear. The cytochrome *c* of the parent evidently decreased as the glucose concentration was increased, whereas, the amount of the mutant was several times higher than that of the parent at lower concentration of glucose, and increased to the maximum level at about 7% of glucose. This high level of the cytochrome *c* was maintained at higher than 10% of glucose.

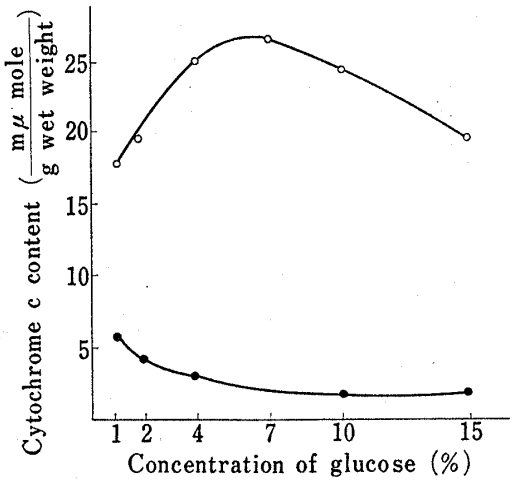

Fig. 2. The Variation of Cytochrome *c* Content by Glucose Concentration in the Medium

Cells were cultured aerobically for 48 hours at 30° in the medium containing each concentration of glucose, washed three times by centrifugation and the content of cytochrome *c* was estimated by the method described at "Material and Method".
—●— : parent —○— : mutant (I-35)

Correlation between the growth and the cytochrome *c* contents of the parent and the mutant were examined and the results were shown in Fig. 3.

The parent showed the typical growth curve, and the cytochrome *c* content was the highest at early logarithmic phase, and followed by gradual reduction. While the mutant showed a singular growth curve which had two steps of logarithmic phase, and the cytochrome *c* content increased with its growth then reached to the maximum at interphase between the first and the second logarithmic growth.

It was already known that the yeasts contain iso-2-cytochrome *c*,[7] the separation of iso-2-cytochrome *c* of *C. utilis* was

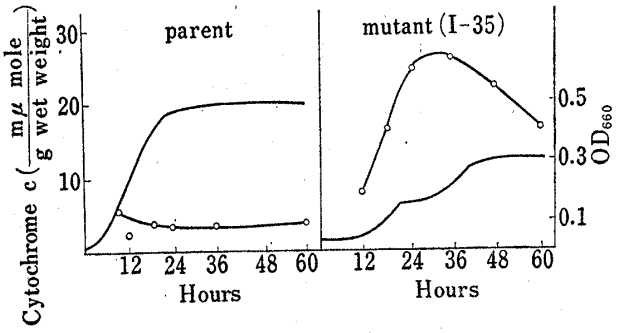

Fig. 3. The Variation of Cytochrome *c* Content by Growth

Cells wee cultured aerobically each time at 30°. Optical density at 660 mμ of one tenth diluted culture medium was measured by Hitachi EPO-B model photoelectric photometer. The content of cytochrome *c* was estimated by the method described at "Material and Method".
—○— : cytochrome *c* content ——— : growth curve

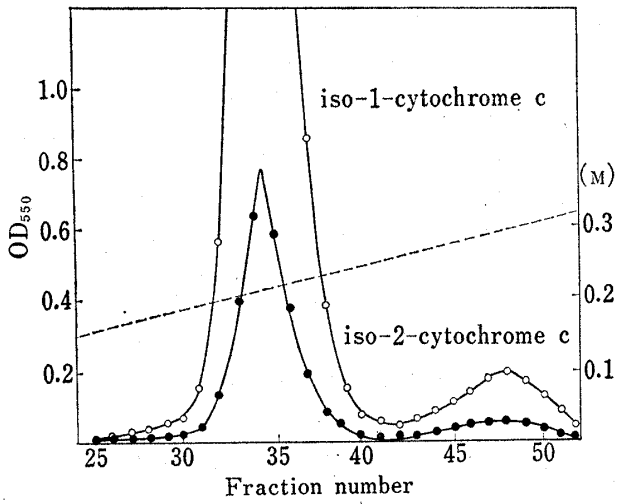

Fig. 4. Elution Pattern of iso-Cytochrome *c* of *C. utilis* 0619 Parental Strain and the Mutant (I-35)

The concentrated cytochrome *c* fraction obtained from yeasts were dialyzed against 20 mM ammonium phosphate buffer (pH 7.0) and passed through Amberlite CG-50 resin column (1 × 10 cm) after oxidized by addition of an aliquot of potassium ferricyanide. The mixture of iso-cytochrome *c* adsorbed to the upper parts of the resin was eluted with 0 to 0.5M linear gradient concentration of ammonium phosphate buffer (pH 7.0).
—●—: parent —○—: mutant (I-35)
------: concentration of buffer

attempted to examine whether *C. utilis* contains iso-2-cytochrome *c* and the iso-2-cytochrome *c* content increase or not.

As shown in Fig. 4, the iso-1-cytochrome *c* was eluted first by 0.21 M buffer and followed by 0.3 M buffer to obtain the iso-2-cytochrome *c*. The ratio of the amount of iso-2-cytochrome *c* to the total cytochrome *c* was about 10% in both the parent and the mutant, and there was no significant difference under the condition examined (Table II).

Table II. The Amount of Total Cytochrome *c* and iso-2-Cytochrome *c*

Strain	Medium	Time of culture (hr)	Amount of cytochrome *c* (mμ mole/g wet weight)	Percentage of iso-2-cytochrome *c* (%)
Parent	2% glucose Ogur	13	4.02	8.7
Mutant (I-35)	2% glucose Ogur	24	20.33	8.3
	2% glucose Ogur	48	21.86	10.0
	2% glucose Ogur	48	18.03	11.3
	10% glucose Ogur	48	29.75	10.5
	10% glucose Ogur	48	32.17	8.5

Discussion

A mutant was newly found among the small colonies of *C. utilis* by the treatment with NG.

This mutant could not utilize non fermentable carbon source and was in defect of ability to reduce TTC, however possessed all the types of cytochrome and exhibited the higher respiratory activity than that of the parental strain. Such a TTC-negative and respiratory-sufficient mutant of *C. utilis* was also resulted by ultraviolet (UV) irradiation.[8] These informations may suggest that the mutation which lack of ability of TTC reduction may not be always accompanied by the mutation of the respiratory deficiency.

By the cytochrome absorption spectrum at 520 and 550 mμ, the mutant was expected to contain the higher amount of cytochrome *c*.

In fact, the estimation of cytochrome *c* of the mutant revealed about five-fold amount of the cytochrome *c* compared to that of the parent. It may be said therefore the mutant seemed to be a particular mutant in its biochemical characteristics. The mutant showed increased in the amount of cytochrome *c* when cultured in the medium containing higher concentrations of glucose which usually represses the cytochrome *c* synthesis of the parent.

Sherman[9] reported that the cytochrome *c* synthesis of S. cerevisiae D273-10B was repressed by the glucose concentration in the medium. Accordingly, it is suggestive that a part of the regulatory system for the cytochrome *c* synthesis was altered and the cytochrome *c* was synthesized enormously in the mutant.

This suggestion is also supported by the result that the cytochrome *c* content of the mutant indicated the notable change during the growing time while the content of cytochrome *c* of the parent was almost maintained constantly.

This mutant showed the singular growth curve which had two exponential growth phases, and the cytochrome *c* content and the respiratory activity reached to the maximum at interphase between the first and the second exponential growth. It was suggested that the notable metabolic change by mutation might occur at this phase.

iso-2-Cytochrome *c* was found in the parent and this mutant of *C. utilis*, and the content of the iso-2-cytochrome *c* was about 10% of total cytochrome *c*, regardless to growth condition. This value coincides with that in the report of Matoon.[10]

8) I. Mifuchi, Y. Iwamoto, and T. Sugimura, *Medicine and Biology*, **80**, 161 (1970).
9) F. Sherman, H. Taber, and W. Campbell, *J. Mol. Biol.*, **13**, 21 (1965).
10) J.R. Matoon and F. Sherman, *J. Biol. Chem.*, **241**, 4330 (1966).

According to Sherman,[9] the content of iso-2-cytochrome *c* of S. cerevisiae D273-10B was 4.3% and this amount varied in several cytochrome *c* mutants.

Sels[7] proporsed the hypothesis that iso-2-cytochrome *c* was repressor factor to the synthesis of functionally important iso-1-cytochrome *c*. On the other hand, it was reported[10] that iso-2-cytochrome *c* was functional as iso-1-cytochrome *c* because it could restore the respiration and the oxidative phosphorylation of the cytochrome *c* deficient mutant of S. cerevisiae.

Further investigations are required to elucidate the biological roles of iso-2-cytochrome *c*.

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