Comparative Studies on Respiratory Activity and Cytochrome Content of *Candida tropicalis* pK 233 Grown on Hydrocarbon and on Glucose[†]

Yutaka TERANISHI, Shin SHIMIZU, Atsuo TANAKA and Saburo FUKUI*

Laboratory of Industrial Biochemistry, Department of Industrial Chemistry,
Faculty of Engineering, Kyoto University, Kyoto

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Some differences in the respiratory activity as well as in cytochrome content were observed between glucose-grown (G-cells) and hydrocarbon-grown cells (H-cells) of *Candida tropicalis* pK 233. The Q_{O_2} values of H-cells measured with *n*-alkanes were markedly high when compared with those measured with glucose during the whole growth phase. On the other hand, the values of G-cells measured with glucose were significantly higher than those measured with *n*-alkanes in the exponential growth phase, but there was little difference between both values in the stationary phase. Although a significant difference in quality was not recognized in the cytochrome patterns between G-cells and H-cells, the contents of *a*-, *b*- and *c*-type of cytochromes in H-cells were twice as much as those in G-cells.

In general, H-cells were more sensitive to respiratory inhibitors than G-cells. When glucose was used as oxidation substrate, KCN did not exert any inhibitory effect, but rather stimulated the respiration of G-cells. On the other hand, respiratory activity of H-cells measured with *n*-alkanes was remarkably lowered by KCN.

During the course of investigations on *n*-alkane-assimilating microorganisms, some interesting features on the physiology and metabolism of *Candida tropicalis* were observed. As reported in the preceding paper of this series,¹⁾ we observed a remarkable morphological change of *C. tropicalis* pK 233 depending upon the kind of carbon source. Despite the fact that *n*-alkane-grown cells of *C. tropicalis* (especially well-developed filamentous-form cells) showed a significantly higher *n*-alkane-oxidizing capacity, the cell yield on *n*-alkanes (based on the amount of the substrate consumed) was relatively low compared with those of other *n*-alkane-assimilating microorganisms.

Although many investigators have reported on the respiratory activities of microorganisms grown on hydrocarbons,^{2~8)} very little is actually known of the respiratory system of *n*-

alkane-assimilating microorganisms. The present study was undertaken to obtain further information on the respiratory system and the cytochrome content of cells of *C. tropicalis* grown on glucose and on *n*-alkanes.

MATERIALS AND METHODS

Cultivation of the yeast. The yeast used in this study, *Candida tropicalis* (Castellani) Berkhout pK233,⁹⁾ was kindly supplied by the National Fermentation Research Institute, Chiba, Japan. The organism was cultured in the same manner as that described in our previous paper.¹⁾ *n*-Alkane used throughout this experiment was "hydrocarbon mixture 6," consisting of *n*-decane 23.9, *n*-undecane 46.8, *n*-dodecane 26.3 and *n*-tridecane 3.0% by weight.

Measurement of oxygen uptake. Oxygen uptake of intact cells was measured by the conventional Warburg manometric technique as described in the previous paper.¹⁾ In the inhibition experiments, various concentrations of inhibitors dissolved in water or dimethyl sulfoxide were added to the main chamber.

Spectrophotometric measurement. Spectra of the intact yeast cells were obtained by the opal-glass method¹⁰⁾ on a Shimadzu Multi-purpose Spectrophoto-

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* To whom correspondence should be addressed.

meter Model 50L. The difference spectra (reduced minus oxidized) were measured according to the procedure of Chance¹¹ after reduction by sodium dithionite or oxidation by hydrogen peroxide of the cell suspension. The extinction coefficients used for the estimation of *a*-, *b*- and *c*-type cytochromes were 16.0, 21.0 and 19.0 $\text{cm}^{-1}\text{mM}^{-1}$,¹¹ respectively.

Enzyme assay. Yeast cells were disrupted by sonication at 20 kc for 20 min below 4°C. After centrifugation at $1,000 \times g$ for 10 min, the resulting supernatant was used as the enzyme source. NADH oxidase activity was measured according to the method of Green and Ziegler,¹² by following the reduction of NADH at 340 nm. Cytochrome *c* oxidase activity was assayed by the method of Polakis *et al.*¹³ by following the decreased peak of reduced cytochrome *c* at 550 nm.

RESULTS

1. Growth and respiratory activity

Cells of *Candida tropicalis* pK 233 growing on *n*-alkanes or glucose were harvested at intervals of several hours and washed twice with distilled water. The washed cell suspensions thus obtained (about 1 mg-dry cells/ml) were employed for the measurement of oxygen uptake by use of Warburg manometric method. The results are shown in Figs. 1 and 2. Figure 1 illustrates the change of respiratory activity

FIG. 1. Relationship between Growth and Respiratory Activity of *Candida tropicalis* pK 233 Grown on *n*-Alkanes.

Growth (□—□), respiratory activity measured with *n*-alkanes (○---○) and measured with glucose (●—●).

of *n*-alkane-grown cells on *n*-alkanes in comparison with that measured with glucose. As shown in this figure, an appreciable difference

was observed between the time-course change of respiratory activities measured with each substrate. The respiratory activity measured with *n*-alkanes increased linearly with the growth and the development of the filamentous-form cells until the middle exponential growth phase; it decreased gradually toward the stationary phase and then remained at almost the same level (Q_{O_2} = about 200 $\mu\text{l/hr/mg-dry cells}$) for further 20 hr. On the other hand, when measured with glucose, the activity reached the maximum at an earlier period than with *n*-alkanes, then decreased slightly and kept at the constant level (Q_{O_2} = about 70 $\mu\text{l/hr/mg-dry cells}$). During the whole growth phase, the respiratory activity of *n*-alkane-grown cells measured with *n*-alkanes was significantly higher than that measured with glucose.

In the case of glucose-grown cells, the activity on glucose attained the maximum (Q_{O_2} = about 300 $\mu\text{l/hr/mg-dry cells}$) in the middle exponential growth phase, then declined rapidly toward the stationary phase, and thereafter decreased gradually to a lower level (Q_{O_2} = about 80~90 $\mu\text{l/hr/mg-dry cells}$). When me-

FIG. 2. Relationship between Growth and Respiratory Activity of *Candida tropicalis* Grown on Glucose.

Growth (□—□), respiratory activity measured with *n*-alkanes (○---○) and measured with glucose (●—●).

asured with *n*-alkanes, the activity reached maximum earlier than with glucose and decreased gradually in the stationary phase. The respiratory activity of glucose-grown cells on glucose was always higher than that measured with *n*-alkanes, but there was only a little dif-

ference at the stationary phase, although a large difference was present at the exponential phase (Fig. 2).

2. Difference spectra of intact cells

In order to obtain data on the respiratory pigments of *C. tropicalis*, difference spectra (reduced minus oxidized) of the intact cells were measured. The spectra obtained are shown in Fig. 3. Both glucose- and *n*-alkane-

FIG. 3. Difference Spectra(Reduced Minus Oxidized) of Cells of *Candida tropicalis* Grown on Glucose and *n*-Alkanes.

The yeast cells used were cultured on glucose for 22 hr and on *n*-alkanes for 44 hr.

grown cells showed essentially similar difference spectra. In the case of glucose-grown cells, the peaks at 605 and 445 nm indicated the presence of *a*-type cytochrome; the peaks at 553 and 523 nm were ascribable to *c*-type cytochrome though the absorption peak at the Soret band was not obviously observed; and the shoulders at 562 and 532 nm as well as the peak at 425 nm revealed the presence of *b*-type cytochrome. On the other hand, in the case of *n*-alkane-grown cells the peaks of *a*- and *c*-type cytochromes were shifted 2~3 nm to shorter wave-length. The reason why such shifts occurred remains to be elucidated.

3. Relationship between growth and cytochrome content

As mentioned above, the change of respiratory activities of *n*-alkane-grown cells was slightly different from that of glucose-grown cells, although the cytochromes of both cells were very similar. Figures 4 and 5 show the time-course changes in the contents of three types of cytochromes during growth on *n*-alkanes and on glucose.

FIG. 4. Time-course Change of Cytochrome Content of *Candida tropicalis* during Growth on *n*-Alkanes.

Growth (●-●), content of *a*-type (□-□), *b*-type (○-○) and *c*-type cytochrome (△-△).

Despite the fact that maximal respiratory activity was clearly observed at the middle exponential growth phase of glucose- and *n*-alkane-grown cells (Figs. 1 and 2), not only were distinct peaks not observed in time-course changes in the content of cytochromes, but also the content increased gradually even in the stationary phase. In the case of *n*-alkane-grown cells, however, the *c*-type cytochrome content showed dual peaks at the late and the middle periods of the exponential growth phase, although the respiratory activity of the cells was almost constant during this period. This time-course change was analogous to those of coenzyme Q and riboflavin contents of this yeast grown on *n*-alkanes, as reported previ-

FIG. 5. Time-course Change of Cytochrome Content of *Candida tropicalis* during Growth on Glucose. Growth (●—●), content of *a*-type (□---□), *b*-type (○—○) and *c*-type cytochrome (△---△).

ously.^{14,15)}

4. Effect of inhibitors on respiration

Although a significant difference in quality was not observed in difference spectra of intact cells and in the respiratory activities (measured with growth substrates), the cytochrome contents of *n*-alkane-grown cells were far higher than those of glucose-grown cells.

Hence, the resistance of both cells against respiratory inhibitors was compared. Inhibitors used in this experiment were KCN, NaN₃, antimycin A, rotenone and amytal. Since antimycin A and rotenone were insoluble in water, dimethyl sulfoxide was employed as the solvent. This solvent did not inhibit the respiration of this yeast under the conditions employed. The results obtained are shown in Table I.

With rotenone, the best known inhibitor of electron transport between NADH and co-

TABLE I. EFFECTS OF VARIOUS INHIBITORS ON RESPIRATION OF *Candida tropicalis* pK 233 GROWN ON GLUCOSE AND ON *n*-ALKANES

Growth substrate	Oxidation substrate	Inhibitor	Conc. (mM)	Relative activity (%)
Glucose ^{a)}	Glucose ^{c)}	None	—	100
		KCN	1.0	129
			3.3	117
		NaN ₃	1.0	97
			3.3	47
		Rotenone ^{b)}	1.0	107
			3.3	105
		Antimycin A ^{b)}	1.0	58
			3.3	14
		Amytal	3.3	110
<i>n</i> -Alkanes ^{a)}	<i>n</i> -Alkanes ^{c)}	None	—	100
		KCN	1.0	65
			3.3	31
		NaN ₃	1.0	11
			3.3	4
		Rotenone ^{b)}	1.0	28
			3.3	18
		Antimycin A ^{b)}	1.0	11
			3.3	14
		Amytal	3.3	104

^{a)} Cells used in this experiment were of 24 hr-culture on glucose and 46 hr-culture on *n*-alkanes.

^{b)} Dimethyl sulfoxide was used as the solvent for rotenone and antimycin A. In these cases, the reaction mixture containing only dimethyl sulfoxide was employed as control.

^{c)} Respiratory activities of glucose-grown cells on glucose and *n*-alkane-grown cells on *n*-alkanes in the absence of any inhibitor were 89 and 225 μl/hr/mg-dry cells, respectively.

enzyme Q or cytochrome b, an interesting result was obtained. The respiration of glucose-grown cells (oxidation substrate, glucose) was not affected by this compound, while that of *n*-alkane-grown cells (oxidation substrate, *n*-alkanes) was inhibited by 70~80%. Cyanide, the inhibitor of cytochrome *c* oxidase, rather stimulated the respiration of glucose-grown cells measured with glucose at concentrations of 1.0 and 3.3 mM, respectively. On the other hand, the respiration of *n*-alkane-grown cells on *n*-alkanes was seriously inhibited at these concentrations of cyanide. The respiration of *Saccharomyces cerevisiae* on glucose was completely inhibited by 1.0 mM of cyanide under our experimental conditions. Both filamentous-form and yeast-form cells of *C. tropicalis*, separated from the *n*-alkane-grown culture by filtration¹⁾ showed a similar susceptibility to cyanide. NaN₃, an inhibitor at the same site as cyanide, and antimycin A showed relatively strong inhibitory effects on the respiration of both glucose- and *n*-alkane-grown cells. Amytal exerted no inhibitory effect on the respiration of both cells (Table I).

5. Effects of cyanide and azide on the activities of NADH oxidase and cytochrome *c* oxidase

As shown in Table II, both cyanide and azide inhibited strongly the oxidation of reduced

cytochrome *c* and of NADH by cell-free extracts obtained from *n*-alkane-grown *C. tropicalis*. In the case of glucose-grown cells, cytochrome *c* oxidase was inhibited completely by both inhibitors, whereas NADH oxidase seemed to be rather tolerant to azide. This fact may be consistent with the observation that the respiration of *n*-alkane-grown cells was more sensitive to this inhibitor than that of glucose-grown cells (See Table I).

The inhibitory effects of cyanide and azide on both enzyme systems mentioned above suggested that a cytochrome-linked respiratory chain was surely present in both glucose- and *n*-alkane-grown cells, even though cyanide stimulated the respiratory activity of glucose-grown cells. More detailed studies on the respiratory systems of these cells are now under way.

DISCUSSION

The spectrophotometric data presented here indicated the presence of *a*-, *b*-, and *c*-type cytochromes in the cells of *C. tropicalis* pK 233 grown on *n*-alkanes and glucose (Fig. 3). The activities of NADH oxidase and cytochrome *c* oxidase were strongly inhibited by cyanide and azide (Table II). These results are consistent with the existence of a cyanide-

TABLE II. EFFECTS OF INHIBITORS ON NADH OXIDASE AND CYTOCHROME C OXIDASE OF *n*-ALKANE-GROWN CELLS AND OF GLUCOSE-GROWN CELLS

Enzyme	Cells	Inhibitor (3.3 mM)	% Inhibition
NADH oxidase	Glucose-grown cells ^{a)}	None	0
		KCN	92
		NaN ₃	47
	<i>n</i> -Alkane-grown cells ^{a)}	None	0
		KCN	94
		NaN ₃	87
Cytochrome <i>c</i> oxidase	Glucose-grown cells	None	0
		KCN	92
		NaN ₃	91
	<i>n</i> -Alkane-grown cells	None	0
		KCN	98
		NaN ₃	96

^{a)} The cells used were 20 hr-culture on glucose and 44 hr-culture on *n*-alkanes.

sensitive cytochrome-linked respiratory chain in this yeast as is found in various kinds of organisms.¹⁶⁾ Nevertheless, the respiration of intact *Candida* cells was not inhibited but stimulated by some kinds of respiratory inhibitors when glucose was used as an oxidation substrate. The respiration of glucose-grown cells was enhanced about 1.3 times by cyanide, in spite of the fact that the activities of NADH and cytochrome *c* oxidases in the cell-free extracts were inhibited by cyanide.

Although the role of cyanide-insensitive respiration of this yeast is not clear, such phenomena have been observed in many organisms,^{17,18)} including yeasts.¹⁹⁾ In mature silkworm, it was suggested that an alternative pathway was operating, in which cytochrome *b₅* could be acting as the inhibitor-insensitive terminal oxidase.²⁰⁾ A strain of *Achromobacter* has been demonstrated to contain an enhanced concentration of the terminal oxidase when adapted to cyanide during the growth.^{21~23)} The enhancement of the oxidase may correlate to the cyanide-insensitivity of the respiration of this bacterium. But this would not be the case of *Candida tropicalis* pK 233.

Furthermore, studies on a mold, *Myrothecium verrucaria*, suggested the presence of two types of cytochrome *c* oxidase, one of which was resistant to some respiratory inhibitors.²⁴⁾ The transport of electron in *C. tropicalis* appears to be normally linked at site II²⁵⁾ (antimycin-sensitive site of the respiration chain between cytochrome *b* and *c₁*), since the inhibitory effect of antimycin A on the respiration was drastic. However, the terminal electron transport system in *n*-alkane oxidation would differ from that in glucose oxidation at the steps before and after site II. The different susceptibilities of *n*-alkane-grown cells and of glucose-grown cells toward rotenone and cyanide (See Table I) seem to support the above-mentioned assumption. In addition, the respirations of both *n*-alkane- and glucose-grown cells were stimulated by cyanide when glucose was used as the oxidation substrate, whereas those of both cells were inhibited by cyanide when *n*-alkane was used as oxidation

substrate (The detailed data will be presented in a subsequent paper). The sensitivity to cyanide of *n*-alkane-oxidizing ability of *C. tropicalis* may be partly ascribable to the inhibition at the initial oxidation step of *n*-alkanes as described by Liu and Johnson²⁶⁾ by use of *Candida intermedia*.

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