Mutants of *Hansenula polymorpha* and *Candida boidinii* Impaired in their Ability to Grow on Methanol

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Five mutants of *Hansenula polymorpha* and *Candida boidinii* have been isolated which were impaired in their ability to grow on methanol. Two of these mutants lacked the enzyme triokinase, one contained lowered activities of fructose 1,6-bisphosphatase and two had unknown lesions. Revertants isolated with wild-type phenotype had regained the missing enzyme activities. The relationship of the mutant phenotypes to the proposed dihydroxyacetone pathway for methanol assimilation is discussed.

INTRODUCTION

Recent studies (van Dijken *et al.*, 1978) have provided evidence which suggests that a novel pathway for the assimilation of methanol operates in methanol-utilizing yeasts. This metabolic scheme, called the dihydroxyacetone (DHA) pathway, involves three key enzymes: triokinase, fructose 1,6-bisphosphatase (FBPase) and a novel and as yet uncharacterized formaldehyde-condensing enzyme leading to the formation of DHA from formaldehyde plus pentose phosphate.

Mutant evidence is presented in this paper which implicates the first two enzymes in the utilization of methanol by *Hansenula polymorpha* and *Candida boidinii*, thus providing support for the operation of the dihydroxyacetone pathway in these organisms.

METHODS

Organisms and growth. *Hansenula polymorpha* CBS 4732 and *Candida boidinii* CBS 5777 were grown in shake flasks at 37 °C (for *H. polymorpha*) or 30 °C (for *C. boidinii*) in the mineral salts medium used by van Dijken *et al.* (1976). Filter-sterilized methanol (0.5 %, w/v), ethanol (0.2 %, w/v) or DHA (0.1 %, w/v) and autoclaved glucose or glycerol (0.2 %, w/v) were used as carbon and energy sources. For all enzyme studies, cultures were harvested in late-exponential phase. For growth curve studies, cells were grown in test tubes containing 5 ml medium and the turbidity was estimated using an EEL colorimeter. For studies involving mutants, wild-type and mutant cells were grown on ethanol to late-exponential phase, harvested, washed and resuspended in methanol medium for 12 to 14 h (for *H. polymorpha*) or 20 to 24 h (for *C. boidinii*) to allow induction of enzymes involved in methanol metabolism.

Preparation of extracts. Cell-free extracts were prepared by ultrasonication (MSE model 150 W) for 7 × 1 min (*H. polymorpha*) or 5 × 1 min (*C. boidinii*) at 1 min intervals in 4 ml 20 mM-KH₂PO₄/NaOH buffer, pH 7.1. The supernatant obtained after centrifugation at 38000 g for 20 min was used for all enzyme studies.

Enzyme assays. All assays were carried out in 1 ml volume at 37 °C (*H. polymorpha*) or 30 °C (*C. boidinii*) using a Pye Unicam SP1800 double-beam recording spectrophotometer.

Triokinase (EC 2.7.1.28) was assayed by following triose-phosphate production by the method of van Dijken *et al.* (1978) using DL-glyceraldehyde as the substrate in extracts of *H. polymorpha* and DHA as the substrate in all other cases except where noted. Unless otherwise noted, activities reported in *H. polymorpha* have been adjusted to those for DHA as the substrate, as described by van Dijken *et al.* (1978).

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Fructose 1,6-bisphosphatase (EC 3.1.3.11) was assayed by the method of van Dijken & Quayle (1977) and methanol oxidase (EC 1.1.3.13) as described by van Dijken *et al.* (1976).

Survival curves. (1) Ethyl methanesulphonate (EMS). An exponential phase ethanol-grown culture (15 ml) was harvested, washed and resuspended in 15 ml fresh medium. The cells were divided among five test tubes (3 ml each) containing different amounts of EMS and incubated with shaking for 3 h. After incubation, the cultures were washed once with sterile medium, once with sterile medium containing 6 % (w/v) sodium thiosulphate and twice more with sterile medium. Samples were then plated on to ethanol plates and incubated.

(2) Nystatin. An early-exponential phase methanol-grown culture (15 ml) was divided among five test tubes (3 ml each) and 0.3 ml nystatin (1 mg in 1 ml methanol, diluted to 10 ml with distilled water) was added to each (final concentration $10 \mu\text{g ml}^{-1}$). The cultures were incubated for various periods, and samples were then plated on to methanol plates and incubated.

Mutant isolation. (1) Mutagenesis and nystatin selection. An exponential phase ethanol-grown culture (5 ml) was harvested, resuspended in fresh medium containing 1 % (w/v) EMS and incubated with shaking for 3 h. The culture was washed four times as described above, resuspended in fresh ethanol medium and grown overnight. This culture was washed twice in sterile nitrogen-free medium, resuspended in nitrogen-free medium and incubated with shaking for 12 h. The cells were then transferred to medium containing nitrogen plus the non-permissive substrate and incubated with shaking for 8 h; then nystatin was added (final concentration $10 \mu\text{g ml}^{-1}$) and incubation was continued for 90 min. The culture was washed twice, resuspended in fresh ethanol medium, and grown for 24 h. The nystatin selection procedure was then repeated, and after the cells were grown for a final time on ethanol medium, samples were plated on to ethanol plates and incubated. [It may be noted that Sanchez & Demain (1977) have previously used nystatin for enrichment of auxotrophs of *H. polymorpha*.]

(2) Mutant selection. Two types of mutants were selected: those impaired in their ability to grow on methanol but growing normally on ethanol, and those impaired in their ability to grow on DHA, but growing normally on ethanol. The master plates from the nystatin selections were replica-plated on to either methanol or DHA plates and ethanol plates, and mutants were picked and streaked for isolation. All mutant strains were purified by further restreaking and tested for growth on ethanol, methanol, glycerol, DHA and glucose.

RESULTS

Mutants of Hansenula polymorpha

The sensitivity of *H. polymorpha* to EMS and nystatin is shown in Fig. 1(a). For mutagenesis, an EMS concentration of 1 % (w/v) was chosen (giving 4 % survival); for selection, nystatin ($10 \mu\text{g ml}^{-1}$) was incubated with the cells for 90 min (giving 0.015 % survival). Several mutants were isolated from *H. polymorpha* using this procedure, and their phenotypes are shown in Table 1.

Isolate M24 was unable to grow on methanol or DHA and grew slowly on glycerol (doubling time increased 4.3-fold). This mutant had a low activity of what appeared to be triokinase in crude extract, but ammonium sulphate fractionation revealed no DHA-dependent triokinase activity in any fraction. Although the specific activity was quite low in crude extract, sufficient units of enzyme activity were fractionated such that a detectable activity with DHA was expected in the 70 to 90 % saturation fraction (van Dijken *et al.*, 1978). However, no activity was observed. It is possible that the low activity detected in crude extract was due to a combination of other enzymes. A revertant of M24 (M24r), isolated using EMS, showed wild-type growth on methanol, DHA and glycerol and had wild-type triokinase activity.

Isolate M23 was impaired in its ability to grow on both methanol and DHA (doubling times increased 6- and 7-fold, respectively). It had lowered FB Pase activity. A revertant (M23r), which grew normally on methanol and DHA, contained normal FB Pase activity.

Isolate M29 was unable to grow on methanol and showed slower growth on DHA (doubling time increased 3.6-fold). However, it had normal activities of triokinase, FB Pase and methanol oxidase. A revertant of M29 (M29r) was isolated which grew normally on methanol.

Fig. 1. Viable units remaining after treatment of cultures of *H. polymorpha* (a) or *C. boidinii* (b) with EMS or nystatin: ○, nystatin (10 $\mu\text{g ml}^{-1}$); ●, EMS (3 h incubation).

Mutants of *Candida boidinii*

Several mutants of *C. boidinii* were isolated using EMS and nystatin. The sensitivity of *C. boidinii* to these two compounds is shown in Fig. 1(b). For mutagenesis, an EMS concentration of 1% (w/v) was chosen (giving 1.3% survival); for selection, nystatin (10 $\mu\text{g ml}^{-1}$) was incubated with the cells for 90 min (giving 0.07% survival).

The phenotypes of the mutants are shown in Table 1. Isolate m6 was unable to grow on either methanol or DHA and lacked trikinase activity. It also contained low activities of FBPase and methanol oxidase.

Mutant m2 was unable to grow on methanol but had normal activities of trikinase, FBPase and methanol oxidase. A revertant of m2 (m2r) was isolated which grew normally on methanol.

The specific activities of the methanol-induced enzymes found in *C. boidinii* grown on ethanol and transferred to methanol for 20 to 24 h were lower than in cells grown on methanol (see van Dijken *et al.*, 1978). This was due to incomplete induction. However, longer induction times were not feasible due to takeover of the mutant cultures by wild-type cells.

DISCUSSION

The phenotypes of the mutants described in this paper lend further support to the operation of the proposed DHA pathway for methanol assimilation in two yeasts, *H. polymorpha* and *C. boidinii* (see Fig. 2). In *H. polymorpha*, three different types of methanol mutants are described. All show little or no growth on methanol, and all three are revertible.

Isolate M24 lacked trikinase and its revertant M24r in regaining the ability to grow on

Table 1. *Phenotypes of mutant and wild-type H. polymorpha and C. boidinii*

Cultures for enzyme studies were grown on ethanol and induced on methanol as described in Methods. Enzyme activities are expressed as $\mu\text{mol min}^{-1} (\text{mg protein})^{-1}$ and are the average of two or more experiments; values agreed within $\pm 15\%$. Values in parentheses are doubling times (h generation^{-1}).

Phenotype	<i>H. polymorpha</i>							<i>C. boidinii</i>			
	Wild-type	M24	M24r	M23	M23r	M29	M29r	Wild-type	m6	m2	m2r
Growth on:											
Methanol	++ (5.3)	—	++	++ (30)	++	—	++	++	—	—	++
Dihydroxyacetone	++ (3.2)	—	++	++ (22)	++	++ (11.5)	++	++	—	++	++
Glycerol	++ (1.5)	++ (6.5)	++	++	++	++	++	++	++	++	++
Ethanol	++	++	++	++	++	++	++	++	++	++	++
Glucose	++	++	++	++	++	++	++	++	++	++	++
Enzyme activities:											
Triokinase	0.12	0.03*	0.10	0.12	0.12	0.09	0.10	0.073	0	0.086	0.090
Fructose 1,6-bisphosphatase	0.30	0.34	0.32	0.08	0.33	0.29	0.25	0.055	0.027	0.065	0.056
Methanol oxidase	0.30	0.19	0.25	0.18	0.22	0.18	0.25	0.18	0.024	0.12	0.15

—, No growth; +, detectable growth, but less than wild-type; ++, good growth.

* No activity detected upon ammonium sulphate fractionation (see text).

Fig. 2. The postulated dihydroxyacetone pathway (van Dijken *et al.*, 1978), showing the sites blocked in methanol⁻ mutants of *H. polymorpha* (M numbers) and *C. boidinii* (m numbers): Xu5P, xylulose 5-phosphate; DHA, dihydroxyacetone; DHAP, dihydroxyacetone phosphate; GAP, glyceraldehyde 3-phosphate; FBP, fructose 1,6-bisphosphate; F6P, fructose 6-phosphate.

methanol also regained the ability to synthesize wild-type levels of triokinase. This points to a necessary involvement of triokinase in methanol metabolism by this organism. It is also interesting to note that M24 could not grow on DHA, while revertant M24r could. This indicates that growth of *H. polymorpha* on DHA proceeds via initial phosphorylation to DHA phosphate using the same triokinase as is used during growth on methanol. The lack of triokinase was also accompanied by a 4-fold reduction in the growth rate on glycerol, suggesting that triokinase may also be involved in growth on glycerol. Since glycerol is, relative to DHA, an extremely poor substrate for this triokinase (van Dijken *et al.*, 1978), it seems likely that the major route of glycerol metabolism in this organism is by dehydrogenation to DHA followed by phosphorylation of the latter. Such glycerol metabolism would contrast with that shown by some other yeasts, e.g. *Candida utilis*, *Candida mycodema* and *Saccharomyces cerevisiae*, which synthesize a glycerol kinase during growth on glycerol (Gancedo *et al.*, 1968). Both routes of glycerol metabolism are found in bacteria (see Weinhouse & Benziman, 1976). The slow growth on glycerol by mutant M24 suggests that a slower, alternative route to that involving triokinase exists in *H. polymorpha*.

Isolate M23, the mutant having lowered FBPase activity, showed impaired growth on methanol and DHA. Since FBPase is needed to provide hexose phosphate during growth on all compounds except hexoses, it is not surprising that a totally negative phenotype was not found. The FBPase activity required for growth on methanol should be higher than that required for growth on other compounds, since during growth on methanol, the enzyme catalyses a major step in primary carbon assimilation, whereas during growth on other compounds it performs a gluconeogenic function only. As shown by van Dijken *et al.* (1978), FBPase activity is quite low in cells grown on all compounds tested except methanol and, of course, it is possible that two isoenzymes of FBPase might occur in this organism, one which is constitutive and one which is induced in response to methanol. The impaired growth of this mutant on DHA is curious, but a similar phenotype was also found in isolate M29. Indeed, of the five methanol mutants isolated, only those which showed growth deficiencies for methanol alone were methanol oxidase mutants (M. L. O'Connor, unpublished results). It is possible that controls involved in methanol utilization also affect the utilization of DHA, such that efficient utilization of DHA is dependent on a functional methanol assimilation pathway.

Isolate M29 had normal activities of the three enzymes tested, but it could not grow on methanol. This suggests that M29 lacks an unknown C₁ function. In view of the proposed DHA pathway (Fig. 2), the most likely lesion is the formaldehyde-condensing enzyme. This possibility cannot be tested until this enzyme is characterized and a reliable assay

developed. Isolate M29 was also impaired in its growth on DHA, for unknown reasons (as discussed above).

In *C. boidinii*, mutants were also isolated which had deficiencies in triokinase and an unknown function. Mutant m6 contained lower activities of FBPase and methanol oxidase than the wild-type but had no detectable triokinase activity. As in the *H. polymorpha* mutant M24, this was accompanied by inability to grow on methanol or DHA; however, it was not possible to demonstrate the simultaneous regaining of wild-type growth and enzyme activities by reversion since the mutant was not revertible. Unlike the *H. polymorpha* mutant M24, growth of m6 on glycerol was unaffected by lack of triokinase, suggesting that metabolism of glycerol by *C. boidinii* may proceed via initial phosphorylation of glycerol.

Mutant m2 resembles the *H. polymorpha* mutant M29 and hence may possibly lack the formaldehyde-condensing enzyme – a possibility that again must await characterization of the enzyme system.

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