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Glycerol Metabolism and Osmoregulation in the Salt-Tolerant Yeast *Debaryomyces hansenii*

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A glycerol-nonutilizing mutant of the salt-tolerant yeast *Debaryomyces hansenii* was isolated. When subjected to salt stress the mutant produced glycerol, and the internal level of glycerol increased linearly in proportion to increases of external salinity as in the wild-type strain. However, at increased salinity the mutant showed a more pronounced decrease of growth rate and growth yield and lost more glycerol to the surrounding medium than did the wild type. Uptake experiments showed glycerol to be accumulated against a strong concentration gradient, and both strains displayed similar kinetic parameters for the uptake of glycerol. An examination of enzyme activities of the glycerol metabolism revealed that the apparent K_m of the *sn*-glycerol 3-phosphate dehydrogenase (EC 1.1.99.5) was increased 330-fold for *sn*-glycerol 3-phosphate in the mutant. Based on the findings, a scheme for the pathways of glycerol metabolism is suggested.

Debaryomyces hansenii is a xerotolerant yeast with the capacity for growth in an unusually broad range of salt concentrations (17). Under salt stress, the intracellular salt level is not sufficient to balance the osmolarity of the medium (18), which is compensated by the accumulation of two polyhydroxyalcohols, glycerol (8) and arabinitol (1). Exponentially growing cells osmoregulate chiefly by adjustment of the glycerol level, whereas in stationary-phase cells arabinitol becomes the predominant solute (1). Since glycerol is the major osmoticum used by growing cells and since the glycerol concentrations may reach molar levels under strongly saline conditions (8), the enzymatic pathways and the controls of the glycerol metabolism are of particular interest.

Previous studies on the metabolism of glycerol in yeasts have led to the description of a catabolic pathway involving glycerol kinase (EC 2.7.1.30) and a mitochondrial *sn*-glycerol 3-phosphate (G3P) dehydrogenase (EC 1.1.99.5) (7, 19). In *Candida utilis*, these enzymes were found to be inducible (7), although catabolite repression was the main factor controlling the activities in *Saccharomyces cerevisiae* (19). In yeasts lacking glycerol kinase, an alternative pathway is indicated by the presence of an NAD-dependent glycerol dehydrogenase (EC 1.1.1.6) and a dihydroxyacetone kinase (2, 14).

This report presents a probable scheme for the glycerol metabolism in *D. hansenii* based on studies of enzyme activities in a glycerol-nonutilizing mutant and the parental strain. It is also shown that glycerol is transported into the cells against a strong concentration gradient.

MATERIALS AND METHODS

Yeast strains. *D. hansenii* (Zopf) van Rij strain 26 (17) and a mutant strain, 26-3, isolated as described below, were maintained on nutrient Wickerham agar (21).

Media and growth conditions. The defined medium described by Adler and Gustafsson (1) was used. Carbon sources (glucose, succinate, glycerol, pyruvate, or ethanol) were autoclaved separately and added to 0.5% (wt/vol) in liquid media and to 1% in media solidified by 1.5% Bacto-Agar (Difco Laboratories, Detroit, Mich.). Tests for growth on the various carbon sources were performed in 500-ml Erlenmeyer flasks containing 50 ml of medium. Otherwise, cells were grown in 2.8-liter Fernbach flasks containing 500 ml of medium. Unless specifically noted, glucose was used as the carbon source. The flasks were incubated at 27°C on a rotatory shaker. Growth was followed at 610 nm in a Beckman B spectrophotometer. Dry weight was determined as previously described (1).

Mutagenesis. Cells were grown in glucose medium to mid-log phase and treated with *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine (1) for 2 h, washed by centrifugation, resuspended in growth medium containing 0.1 M KCl, and plated onto solid glucose medium after dilution to give ca. 100 colonies per petri plate. After 4 days of incubation, colonies were replica plated to glycerol and glucose media. Colonies showing no growth on glycerol medium were purified by single-colony isolation on glucose medium. Isolates capable of growth on pyruvate medium were characterized further.

Preparation of cell extract. Cultures of 500 ml were harvested by centrifugation, washed twice in 50 mM Tris-hydrochloride buffer (pH 7.5), resuspended in 5 ml of the same buffer, and then disrupted by passing three times through a pre-cooled French pressure cell (Amicon Corp., Scientific Systems Div., Lexington, Mass.). The homogenate was centrifuged at 8,000 × *g* for 15 min at 4°C, and the resulting supernatant fluid was used for enzyme assays.

Assays. The spectrophotometric assays were performed in a recording Shimadzu UV-240 double-beam spectrophotometer at room temperature in 1-ml cuvettes. Blank cuvettes contained all constituents, except that water replaced the substrate. The reactions were started by simultaneously adding crude extract to both cuvettes to a final protein concentration of 0.2 to 0.6 mg/ml. Specific activities are expressed as nanomoles of substrate utilized per minute per milligram of protein and represent averages of at least three assays. Protein was determined by the method of Lowry as modified by Dunn and Maddy (5), with bovine serum albumin as the standard. The production of oxidized or reduced

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NAD was followed at 340 nm. An absorption coefficient for reduced NAD of $6.2 \times 10^3\ M^{-1}\ cm^{-1}$ was used to calculate enzyme activity.

When indicated, the crude extract was desalted by passage through a Sephadex G-25 column (1 by 5 cm; Pharmacia Fine Chemicals, Uppsala, Sweden). NAD-dependent G3P dehydrogenase (EC 1.1.1.8) was assayed in 20 mM imidazole-hydrochloride buffer (pH 7.5)–1 mM dithioerythritol–1 mM $MgCl_2$–200 mM NaCl–1 mM dihydroxyacetone (DHA) phosphate–0.09 mM NADH. The addition of dithioerythritol, $MgCl_2$, and NaCl increased enzyme activity and improved reproducibility of the assay.

Mitochondrial G3P dehydrogenase was assayed by phenazine methosulfate-mediated reduction of 3-(4,5-dimethylthiazolyl-2)-2,5-diphenyl tetrazolium bromide (MTT) followed at 550 nm. The reaction mixture contained 25 mM imidazole-hydrochloride (pH 7.5), 21 mM G3P, 0.07 mM MTT, 0.3 mM phenazine methosulfate, 10 mM KCN, and 0.1% (vol/vol) Triton X-100 (12). An absorption coefficient for reduced MTT of $8.1 \times 10^3\ M^{-1}\ cm^{-1}$ was used to calculate enzyme activity (19). The enzyme activity was completely sedimented by centrifugation of crude extract at 33,000 × *g* for 45 min.

NADP-dependent glycerol dehydrogenase was assayed as described by Lin and Magasanik (13), with the following modification of the reaction mixture: 100 mM glycine-NaOH buffer (pH 9.5), 35 mM $(NH_4)_2SO_4$, 550 mM glycerol, and 0.14 mM NADP.

DHA kinase was assayed by a modification of the method of Babel and Hofmann (2). The assay contained 50 mM imidazole-hydrochloride buffer (pH 7.5), 20 mM $MgCl_2$, 0.5 mM DHA, 0.13 mM NADH, and 10 U of rabbit muscle G3P dehydrogenase per ml (Sigma Chemical Co., St. Louis, Mo.). Assays were first performed without and then with 10 mM ATP. The increment of activity caused by the presence of ATP was attributed to the kinase.

Glycerol kinase activity was measured by two assays. The spectrophotometric assay was modified from Lin et al. (12). The reaction mixture contained 100 mM glycine-NaOH buffer, 300 mM hydrazine sulfate, 20 mM $MgCl_2$, 20 mM ATP, 10 mM glycerol, 1.4 mM NAD^+, and 10 U of rabbit muscle G3P dehydrogenase per ml (Sigma Chemical Co.). The final pH was 9.5.

For inhibition studies, a modification of the radioactive assay of Newsholme et al. (16) was used. The reaction mixture, a total volume of 520 μl, contained 80 mM Tris-hydrochloride buffer (pH 7.5), 20 mM NaF, 1 mM EDTA, 20 mM $MgCl_2$, 18 mM ATP, and 1.4 mM [^{14}C]glycerol (uniformly labeled at 0.73 μCi/μmol; Amersham International, Amersham England; 1 Ci = 37 GBq). The crude extract was added to a final concentration of 1.0 mg of protein per ml. The reaction was allowed to proceed for 15 min at 25°C, and triplicate 50-μl samples were removed at time intervals. Each sample was applied to a disk of DEAE filter paper (Whatman DE 82), and the reaction was terminated by soaking the filters in 80% aqueous ethanol at room temperature. The ethanol does not dissociate the [^{14}C]G3P from the filter paper. The disks were washed once with 4% (vol/vol) glycerol and three times with distilled water. After drying, each disk was placed in a vial together with 500 μl of 0.1 M HCl and 8 ml of Aquassure (New England Nuclear Corp., Boston, Mass.). Scintillation counting was performed in a Packard Tri-Carb liquid scintillation spectrometer (model 3255). The disintegrations per minute values were calculated by using the counting efficiency determined by the external standard ratio method.

Uptake of labeled glycerol. Glycerol uptake was assayed by using early-log-phase cells, collected by centrifugation and washed twice with potassium phosphate buffer (pH 6.0; ionic strength = 0.05), containing the same concentration of NaCl as the growth medium. The cells were suspended in the same buffer to a final concentration of 1.5 mg of cells (dry weight) per ml. Portions of 950 μl were incubated with shaking in a 25°C water bath. After preincubation for 5 min, uptake was initiated by the addition of 50 μl of uniformly labeled [^{14}C]glycerol (0.1 μCi/μmol). At time intervals, triplicate samples of 50 μl were withdrawn, and the cells were collected on the center of a filter (0.4-μm pore size; Nuclepore Corp., Pleasanton, Calif.) at reduced pressure. The wet spot on the filter was washed twice with 5 drops of buffer. Each filter was shaken overnight in a vial together with 1 ml of 10% (wt/vol) aqueous sodium dodecyl sulfate. To each vial, 8 ml of Aquassure was added for measurement of radioactivity by scintillation counting as described above. Transport was linear over the 1- to 10-min interval of the assay. The uptake rate was determined for the 1- to 5-min period. The sensitivity to metabolic inhibitors was assayed by preincubating the cells for 10 min with 5 mM *N*-ethylmaleimide or sodium azide before the addition of labeled glycerol to a final concentration of 12.8 mM. Competition experiments were performed by simultaneously adding labeled glycerol to 0.4 mM and various unlabeled polyols to 10 mM concentration.

Extraction and analysis of glycerol. An appropriate amount of cell culture (usually 1.5 ml) was rapidly sampled and centrifuged in an Eppendorf centrifuge. The clear supernatant containing the extracellular glycerol was transferred to a water bath and boiled for 10 min together with a parallel sample of the uncentrifuged cell culture. The latter sample containing extracellular plus intracellular (total) glycerol was cleared by centrifugation, and both samples were frozen until analyzed. Intracellular glycerol was determined by subtracting the extracellular glycerol from that of the total.

Glycerol was determined enzymatically by the glycerol kinase method (Biochimica Test combination; Boehringer Mannheim, Mannheim, Federal Republic of Germany).

RESULTS

Mutant selection. Several mutants were selected for their ability to grow on glucose but not on glycerol. Mutants with enzyme lesions in the pathways for glycerol utilization should fail to grow on glycerol but be capable of growth on glucose and gluconeogenic carbon sources other than glycerol. One of the glycerol-nonutilizing mutants that was able to grow when supplied with glucose, succinate, pyruvate, or ethanol as the carbon and energy sources was selected for further studies. Plated on glycerol medium, the mutant showed a reversion frequency of 10^{-5}.

Salt tolerance. The effect of salinity on two growth parameters of the mutant and the wild-type strains is seen in Fig. 1. In the absence of salt stress, both strains showed the same growth yield and the same generation time. However, as salinity increased, the mutant displayed a progressively larger decrease in growth yield and growth rate (increase in generation time) than did the wild type.

Glycerol levels. Figure 2 shows the intra- and extracellular levels of glycerol during a cultural cycle of the wild-type and the mutant strain in medium containing 8% NaCl. In both strains, the intracellular content of glycerol was maintained at a high and fairly constant level during the early log phase, although in the late log phase there was a decrease of

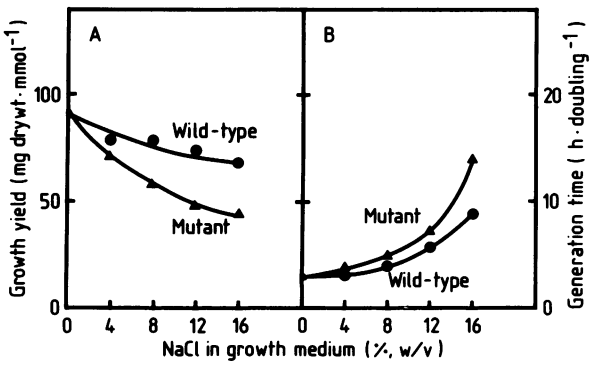

FIG. 1. (A) Growth yield and (B) generation time of wild-type and mutant strains of *D. hansenii* grown in glucose medium containing various concentrations of NaCl. Symbols: (●) wild-type strain and (▲) mutant strain.

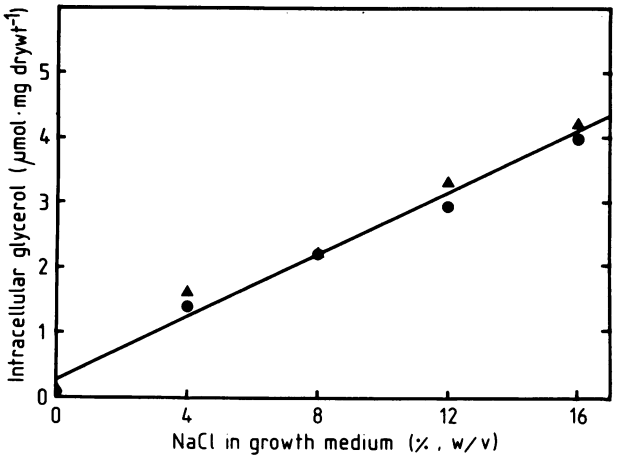

FIG. 3. Intracellular glycerol content of *D. hansenii* grown in glucose medium containing various concentrations of NaCl. The values represent the plateau level of glycerol attained in the log phase. Symbols: (●) wild-type strain and (▲) mutant strain.

intracellular glycerol that was concomitant with a leakage of glycerol into the medium. As growth approached the stationary phase, the extracellular glycerol was rapidly salvaged by the wild type and accompanied by a decrease in intracellular glycerol, also, so that in late stationary phase all glycerol had disappeared from the culture. In the mutant culture, considerably more glycerol was lost to the medium than in the wild-type culture. This glycerol was partly taken up by the cells at the onset of the stationary phase. However, glycerol was gradually lost again in the late stationary phase. Obviously, the mutant is unable to remetabolize the osmotically produced glycerol, and in the stationary culture the total glycerol content was kept fairly constant at 6 to 7 μmol/ml of the culture.

The intracellular accumulation of glycerol by cells grown at various salinities is shown in Fig. 3. These values represent the plateau level of glycerol attained in the log phase before the decline of intracellular glycerol. Both the mutant and the wild-type strain responded similarly, and the glycerol content of the cells increased in linear proportion with increase of external salinity. Thus, the lack of capacity for glycerol dissimilation does not prevent the mutant strain from appropriately regulating its internal level of glycerol in response to changes of external salinity.

Uptake of glycerol. The uptake of glycerol against a steep concentration gradient, as seen for wild-type cells entering the stationary phase in cultures of 8% NaCl (Fig. 2A), reveals a capacity for the active uptake of glycerol. To confirm that glycerol could be taken up by cells containing a high concentration of glycerol, uptake experiments were performed with wild-type cells grown to early log phase with 8% NaCl. The glycerol content of these cells was 2.1 μmol/mg (dry weight). Assuming that 1 mg of cells (dry weight) is equivalent to 2 μl of intracellular water (cf. reference 15), the intracellular glycerol concentration was ca. 1 M in the salt-stressed cells. The uptake experiments were started by the addition of labeled glycerol to final concentrations in the range 0.2 to 12.8 mM; i.e., the concentration gradient of glycerol across the membrane was 75- to 5,000-fold. An Eadie-Hofstee plot of the glycerol uptake

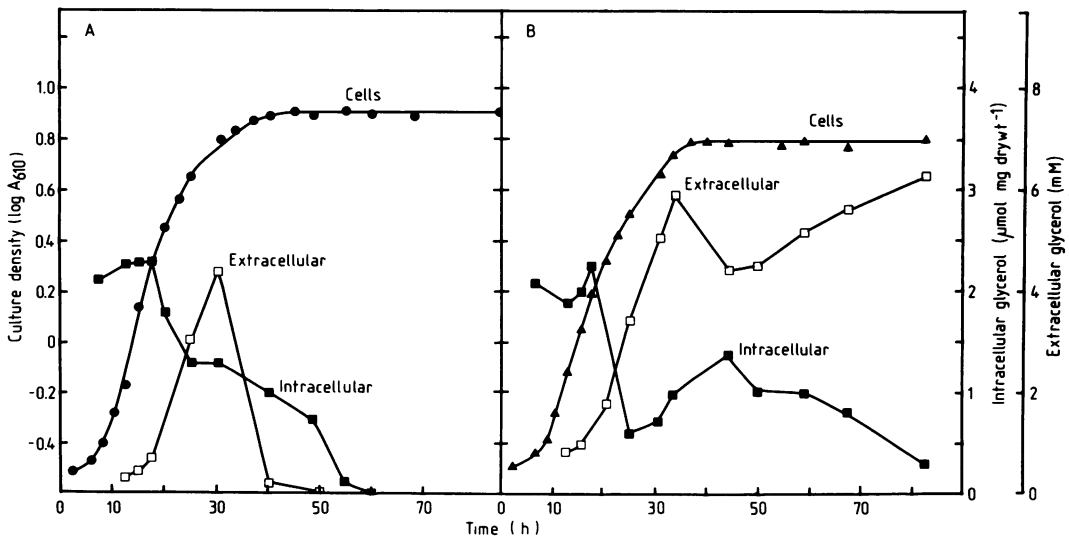

FIG. 2. Changes in the levels of intra- and extracellular glycerol of wild-type (A) and mutant (B) strains of *D. hansenii* during growth in glucose medium containing 8% NaCl.

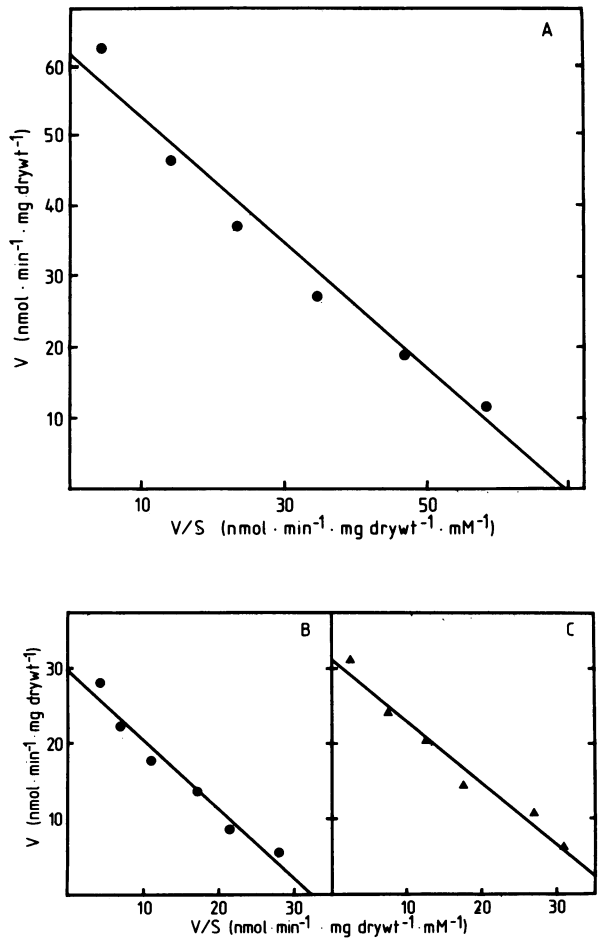

FIG. 4. Eadie-Hofstee plots of glycerol uptake by *D. hansenii* grown in glucose media and harvested in early log phase. (A) Wild-type cells from a culture grown with 8% NaCl. (B) Wild-type and (C) mutant cells from cultures grown without added NaCl. Cells were washed and suspended in buffer containing an appropriate concentration of NaCl and incubated with [^{14}C]glycerol (0.1 μCi/μmol) in the concentration range 0.2 to 12.8 mM.

yielded an apparent K_m of 0.9 mM and a V_{max} of 61 nmol/min per mg (dry weight) (Fig. 4A). The uptake was sensitive to sodium azide and *N*-ethylmaleimide, inhibitors which in 5 mM concentration caused inhibition to 3 and 17%, respectively, of the control value (glycerol concentration, 12.8 mM; control value, 60 nmol/min per mg [dry weight]). Competition experiments showed that the presence of various unlabeled polyols had little effect on the uptake of glycerol. The uptake values obtained with ethylene glycol, erythritol, arabinitol, or mannitol in 25-fold excess over labeled glycerol were 91, 99, 96, and 91%, respectively, of the control. In this study, the glycerol concentration was 0.4 mM (ca. one-half of the apparent K_m value), and the uptake of the control was 18 nmol/min per mg (dry weight). The above results together indicate that glycerol is actively transported into the cells by a carrier-mediated system with a high capacity and a high specificity for glycerol.

The inability of the mutant strain to grow on glycerol led us to compare its capacity for glycerol uptake with that of the wild type. The apparent K_ms obtained from Eadie-Hofstee plots by using cells grown in the absence of salt stress was 1.0 mM for the wild type and 0.9 mM for the mutant; the values for V_{max} were 30 and 32 nmol/min per mg (dry weight), respectively (Fig. 4B and C). The similar kinetic parameters for the two strains make it unlikely that the inability of the mutant to utilize glycerol stems from an impaired transport system for glycerol.

Enzyme levels. In the further search for the enzymatic expression of the mutation, various enzyme activities of the glycerol metabolism were assayed in crude extract obtained from cells grown under various conditions (Table 1).

The NAD-dependent G3P dehydrogenase, which mediates the interconversion of DHA phosphate and G3P, showed under all experimental conditions the highest specific activity of the enzymes assayed. In cells producing glycerol, i.e., mid-log-phase cells from cultures of 8% NaCl, the activity of the enzyme was increased by ca. twofold above the level of log-phase cells not subjected to salt stress; in stationary-phase cells (glucose depleted) and in cells grown on glycerol, the level was reduced to less than half of that activity.

Glycerol kinase, catalyzing the utilization of glycerol through ATP-dependent phosphorylation, showed in extracts from cells grown on glycerol roughly twice as much specific activity as in extracts from cells grown under other conditions. This enzyme was assayed with extracts desalted by gel filtration (Sephadex G-25) since the unfiltered extracts gave low and variable activities.

The mitochondrial G3P dehydrogenase, which mediates the subsequent step in the glycerol catabolism, the oxidation of G3P, also showed an elevated specific activity for cells grown on glycerol. However, unlike the activity of glycerol

TABLE 1. Specific activities of enzymes of glycerol metabolism in cell extracts of wild-type and mutant strains of *D. hansenii*

Strain	Medium	Growth phase	Sp act (nmol min^{-1} mg of protein^{-1}) for[a]: NAD-dependent G3P dehydrogenase	Glycerol kinase[b,c]	Mitochondrial G3P dehydrogenase	Glycerol dehydrogenase[d]	DHA[b] kinase
Wild-type	Glucose	Logarithmic	144 ± 7.0	25.5 ± 1.3	33.6 ± 5.3	2.0 ± 0.6	3.2 ± 0.9
Wild-type	Glucose	Stationary	62.6 ± 9.2	20.2 ± 0.5	24.7 ± 3.0	1.1 ± 0.4	9.0 ± 4.5
Wild-type	Glucose + 8% NaCl	Logarithmic	256 ± 5.6	18.2 ± 0.6	60.0 ± 3.8	0.9 ± 0.2	7.2 ± 0.7
Wild-type	Glycerol	Logarithmic	45.9 ± 9.1	50.3 ± 4.9	50.0 ± 4.7	1.8 ± 0.6	10.5 ± 1.4
Mutant	Glucose	Logarithmic	166 ± 15	27.3 ± 1.7	0.0	1.8 ± 0.7	6.0 ± 1.0
Mutant	Glucose + 8% NaCl	Logarithmic	235 ± 14	23.9 ± 1.5	0.0	1.5 ± 0.1	6.1 ± 2.1

[a] Data are mean ± standard deviation of triplicate measurements.
[b] Extracts desalted by gel filtration.
[c] Enzyme activity measured by the spectrophotometric assay.
[d] Assayed with $NADP^+$

kinase, that of the dehydrogenase was also higher in cells grown under salt stress on glucose. For this enzyme, no activity was detected in extracts of the mutant cells when assayed under standard conditions.

In the search for an alternative route for glycerol catabolism, the enzyme activities of glycerol dehydrogenase and DHA kinase were also assayed. Glycerol dehydrogenase mediates the interconversion of glycerol and DHA coupled with the reduction and oxidation of $NADP^+$ and NADPH, respectively. With crude extract from *D. hansenii*, only negligible activity was obtained by using glycerol and NAD^+ as substrates, though significant activity was found with $NADP^+$ under all experimental conditions. Apparent activity of DHA kinase, catalyzing the phosphorylation of DHA by ATP, was also present. This activity was assayed in extracts desalted by gel filtration to decrease the level of interfering background reactions.

Inhibition of glycerol kinase. Glycerol kinase was exceptional among the enzymes tested in that its activity was increased (2- to 10-fold) after the removal of low-molecular-weight compounds from the crude extract by gel filtration. This behavior suggested the presence of metabolic inhibitor(s) in the extract. The observation that inhibition was less severe with extracts obtained from cells after glucose exhaustion made intermediates of glycolysis likely candidates for exerting the inhibition. Several such compounds, e.g., glucose-1-phosphate, glucose-6-phosphate, fructose-6-phosphate, fructose-1,6-bisphosphate (FBP), 2-phosphoglycerate, and phosphoenolpyruvate, were tested at 5 mM concentration for their effect on the activity of the kinase. In these experiments, the radioactive assay of pH 7.5 was used rather than the usual spectrophotometric assay which is performed at pH 9.5.

Of the compounds used, FBP caused a strong inhibition of the enzyme. The activity of the enzyme as a function of the FBP concentration is shown in Fig. 5. Under the assay conditions, the maximum inhibition obtained was 95%. In showing sensitivity to FBP, the enzyme is similar to the *Escherichia coli* glycerol kinase (20, 22). However, the kinase of the yeast *Candida mycoderma* is not affected by FBP (9), and specific inhibition does not seem to be important in the control of the *S. cerevisiae* enzyme (19).

Kinetic properties of mitochondrial G3P dehydrogenase. As mentioned above, the mutant was devoid of mitochondrial G3P dehydrogenase when assayed under standard conditions. However, enzyme activity was observed when higher substrate concentrations were used and when 1 mM dithioerythritol was present in the crude extract, suggesting that

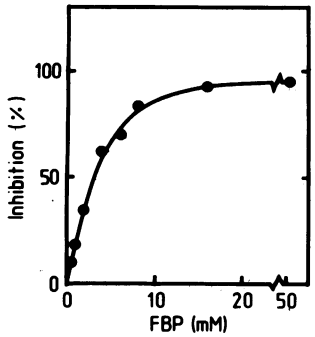

FIG. 5. Inhibition of glycerol kinase by FBP. The enzyme activity was measured at various concentrations of FBP by the radioactive assay.

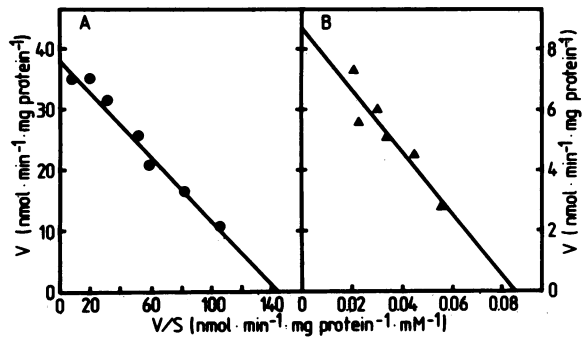

FIG. 6. Eadie-Hofstee plots of the activity of mitochondrial G3P dehydrogenase of the wild-type (A) and the mutant (B) strains. The activity was measured in the 0.1 to 5 mM (A) and the 50 to 350 mM (B) concentration range.

the mutant cells produced an altered enzyme with increased K_m for G3P. The K_m of the mutant enzyme was estimated to be 0.1 M, a value 330 times higher than that (0.3 mM) obtained for the wild-type enzyme (Fig. 6). The results suggest that the mutant enzyme is nonfunctional at physiological substrate levels.

DISCUSSION

The results presented indicate that the failure of the mutant strain to grow on glycerol is associated with a mutationally altered mitochondrial G3P dehydrogenase. The apparent K_m for its substrate, G3P, was increased by a factor of 330. For all other enzyme activities tested, the mutant strain behaved like the wild type. Also, the high reversion frequency of the mutant (10^{-5}) makes the existence of an additional mutation in another function of the glycerol metabolism unlikely.

The scheme in Fig. 7 summarizes our present conception of the glycerol metabolism in *D. hansenii*. The presence of an active transport of glycerol is suggested by uphill accumulation of labeled glycerol against an up to 5,000-fold concentration gradient (Fig. 4A) and by the strong inhibition of the uptake caused by azide and *N*-ethylmaleimide. Furthermore, the insensitivity of the uptake to competition by other polyols suggests a high specificity of the transport

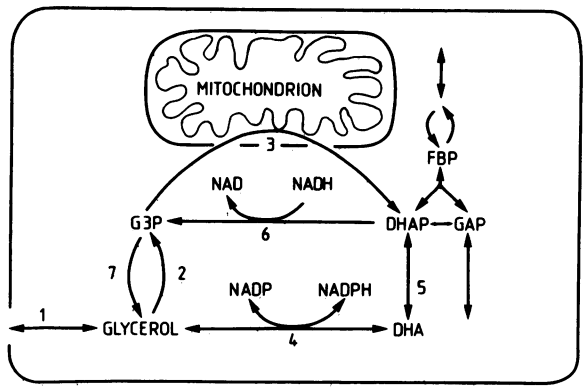

FIG. 7. Scheme for glycerol metabolism in *D. hansenii*. (1) Glycerol transport protein(s); (2) glycerol kinase; (3) mitochondrial G3P dehydrogenase; (4) glycerol dehydrogenase; (5) DHA kinase; (6) NAD-specific G3P dehydrogenase; and (7) phosphatase. DHAP, DHA phosphate; GAP, glyceraldehyde phosphate.

system. It is also noteworthy that the uptake appeared to be unlinked to the dissimilation of glycerol, as indicated by the similar kinetic parameters for the uptake by the mutant and the wild type (Fig. 4B and C). Growth at high salinity did not affect the apparent K_m of the transport system but caused a ca. twofold increase of V_{max}.

The enzymatic activities observed in the extract of *D. hansenii* indicate the existence of two pathways for glycerol dissimilation. One way involves glycerol kinase and mitochondrial G3P dehydrogenase (G3P pathway), and another way involves glycerol dehydrogenase and DHA kinase (DHA pathway). There are reasons to believe that the DHA pathway is of less importance for the dissimilation of glycerol since the specific activity of the glycerol dehydrogenase was low also when glycerol was used as the carbon source (Table 1) and since the chemical equilibrium for the dehydrogenation of glycerol is highly unfavorable (3). More importantly, despite wild-type levels for the enzymes of the DHA pathway, the mutant failed to grow on glycerol. Thus, glycerol seems to be mainly, if not exclusively, dissimilated through the G3P pathway in *D. hansenii*.

The enzymes of the G3P pathway do not need added glycerol for production, but the levels are slightly increased (ca. twofold) when glucose is substituted by glycerol as the carbon source. Glycerol kinase, which mediates the first step in the glycerol catabolism, is subject to inhibition by FBP (Fig. 5). Inhibition of this type may at least partly explain why endogenous glycerol is utilized by salt-stressed cells by the end of the log phase (Fig. 2) when glucose becomes limiting and the glycolytic intermediates can be expected to decline. However, the physiological relevance of the observed inhibition remains to be established by a more detailed study of the inhibition characteristics.

The pathway for the synthesis of glycerol involves the NAD-dependent G3P dehydrogenase and a phosphatase, presumably one that is highly specific for G3P. Since overproduction of G3P may cause growth inhibition (cf. reference 4), the control of glycerol production may require the regulation of both these enzymes. The G3P dehydrogenase is referred to by Lin (11) as the G3P synthase, since the equilibrium strongly favors synthesis of G3P. This is consistent with the finding that the *D. hansenii* mutant does not utilize glycerol despite a functional NAD-dependent G3P dehydrogenase. The specific activity of this enzyme is ca. twofold increased in glycerol-producing, salt-stressed cells (Table 1), and the activity of the enzyme is also strongly salt stimulated (unpublished data), which may be of importance for glycerol production in osmotically dehydrated cells in which the concentration of cytoplasmic constituents is raised.

The *D. hansenii* mutant was capable of accumulating wild-type levels of internal glycerol when subjected to salt stress (Fig. 3), indicating that appropriate osmoregulation can be achieved despite a deficient glycerol catabolism. However, the block in the dissimilatory pathway caused the overproduction of extracellular glycerol (Fig. 2), and this excretion of surplus glycerol may be an alternative to adjustment of the internal level through reutilization. The lesion in the G3P pathway gave the mutant a less competitive growth during salt stress (Fig. 1). Showing similar growth characteristics as the wild type in the absence of salt, the mutant experienced a more severe decrease in growth rate and growth yield as the salinity increased. An obvious reason for the decreased yield is that the osmotically produced glycerol cannot be reutilized by the mutant. This glycerol, which does not contribute to the cell yield since all glycerol (also the intracellular pool) is lost during the washings, corresponds to ca. 13% of the carbon source in cultures of 8% NaCl. Furthermore, the block in the dissimilatory pathway might during salt stress cause metabolite accumulations that exert regulatory disorders. There might also be an increased need for the "G3P shuttle" as salinity increases. This shuttle has been proposed to deliver reducing equivalents from the cytoplasm to the mitochondrion through the combined activities of the mitochondrial G3P dehydrogenase and the NAD-dependent G3P dehydrogenase (6, 10). Both of these enzymes showed an elevated activity in the wild-type strain during salt stress (Table 1). However, the identical growth pattern of the two strains seen in the absence of salt (Fig. 1) points to a minor role for the G3P cycle in unstressed cells.

ACKNOWLEDGMENT

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LITERATURE CITED

1. **Adler, L., and L. Gustafsson.** 1980. Polyhydric alcohol production and intracellular amino acid pool in relation to halotolerance of the yeast *Debaryomyces hansenii*. Arch. Microbiol. **124**:123–130.
2. **Babel, W., and K. H. Hofmann.** 1982. The relation between the assimilation of methanol and glycerol in yeasts. Arch. Microbiol. **132**:179–184.
3. **Burton, R. M.** 1955. Glycerol dehydrogenase from *Aerobacter aerogenes*. Methods Enzymol. **1**:397–400.
4. **Cozzarelli, N. R., J. P. Koch, S. Hayashi, and E. C. C. Lin.** 1965. Growth stasis by accumulated L-α-glycerophosphate in *Escherichia coli*. J. Bacteriol. **90**:1325–1329.
5. **Dunn, M. J., and A. H. Maddy.** 1976. Techniques for the analysis of membrane proteins, p. 242. *In* A. H. Maddy (ed.), Biochemical analysis of membranes. Chapman and Hall, London.
6. **Estabrook, R. W., and B. Sacktor.** 1958. α-glycerophosphate oxidase in flight muscle mitochondria. J. Biol. Chem. **233**:1014–1019.
7. **Gancedo, C., J. M. Gancedo, and A. Sols.** 1968. Glycerol metabolism in yeasts. Pathways of utilization and production. Eur. J. Biochem. **5**:165–172.
8. **Gustafsson, L., and B. Norkrans.** 1976. On the mechanism of salt tolerance. Production of glycerol and heat during growth of *Debaryomyces hansenii*. Arch. Microbiol. **110**:177–183.
9. **Grunnet, N., and F. Lundquist.** 1967. Kinetics of glycerol kinases from mammalian liver and *Candida mycoderma*. Eur. J. Biochem. **3**:78–84.
10. **Klingenberg, M.** 1970. Localization of the glycerol-phosphate dehydrogenase in the outer phase of the mitochondrial inner membrane. Eur. J. Biochem. **13**:247–252.
11. **Lin, E. C. C.** 1976. Glycerol dissimilation and its regulation in bacteria. Annu. Rev. Microbiol. **30**:535–578.
12. **Lin, E. C. C., J. P. Koch, T. M. Chused, and S. E. Jorgensen.** 1962. Utilization of L-α-glycerophosphate by *Escherichia coli* without hydrolysis. Proc. Natl. Acad. Sci. U.S.A. **48**:2145–2150.
13. **Lin, E. C. C., and B. Magasanik.** 1960. The activation of glycerol dehydrogenase from *Aerobacter aerogenes* by monovalent cations. J. Biol. Chem. **235**:1820–1823.
14. **May, J. W., J. H. Marshall, and J. Sloan.** 1982. Glycerol utilization by *Schizosaccharomyces pombe*: phosphorylation of dihydroxyacetone by a specific kinase as the second step. J. Gen. Microbiol. **128**:1763–1766.
15. **Meredith, S. A., and A. H. Romano.** 1977. Uptake and phosphorylation of 2-deoxy-D-glucose by wildtype and respiration-deficient bakers' yeast. Biochim. Biophys. Acta **497**:745–759.
16. **Newsholme, E. A., J. Robinson, and K. Taylor.** 1967. A radiochemical enzymatic activity assay for glycerol kinase and hexokinase. Biochim. Biophys. Acta **132**:338–346.
17. **Norkrans, B.** 1966. Studies on marine occurring yeasts: growth related to pH, NaCl concentration and temperature. Arch.

Mikrobiol. **54**:374–392.
18. **Norkrans, B., and A. Kylin.** 1969. Regulation of the potassium to sodium ratio and the osmotic potential in relation to salt tolerance in yeasts. J. Bacteriol. **100**:836–845.
19. **Sprague, G. F., Jr., and J. E. Cronan, Jr.** 1977. Isolation and characterization of *Saccharomyces cerevisiae* mutants defective in glycerol catabolism. J. Bacteriol. **129**:1335–1342.
20. **Thorner, J. W., and H. Paulus.** 1973. Catalytic and allosteric properties of glycerol kinase from *Escherichia coli*. J. Biol. Chem. **248**:3922–3932.
21. **Wickerham, L. J.** 1951. Taxonomy of yeasts. U.S. Department of Agriculture bulletin no. 1029, p. 1–55. U.S. Department of Agriculture, Washington, D.C.
22. **Zwaig, N., and E. C. C. Lin.** 1966. Feedback inhibition of glycerol kinase, a catabolic enzyme in *Escherichia coli*. Science **153**:755–757.