Glycerol utilization in *Fusarium oxysporum* var. *lini*: regulation of transport and metabolism

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Glycerol was transported in the fungus *Fusarium oxysporum* var. *lini* by a facilitated diffusion transport system with a half-saturation constant, $K_s$, of 0.5 mM and a maximum velocity, $V_{max}$, of 0.9 mmol (g dry wt)$^{-1}$ h$^{-1}$ at pH 5 and 25°C. 1,2-Propanediol was a competitive inhibitor of glycerol transport, but the cells did not actively accumulate 1,2-propanediol. The transport system was partially constitutive. In cells grown in the presence of glucose, glycerol was not transported, indicating that the synthesis of the system was under glucose repression. Glycerol base and NADP+-dependent glycerol dehydrogenase activities were present under all physiological conditions tested. A flavin-dependent glycerol phosphate dehydrogenase was induced only when glycerol was the sole energy source in the medium. This enzyme, together with the transport system, constitute the regulated steps in the glycerol metabolic pathway.

**Introduction**

The utilization of nutrients by micro-organisms involves an initial uptake step and subsequent metabolism. In the case of glycerol, although metabolism has been quite extensively studied in several cases, transport has been characterized only for *Escherichia coli*, in which the existence of a glycerol facilitator was first reported by Sanno et al. (1968). A report on the cloning of the gene (glpF) which codes for this facilitator in *E. coli* was recently published by Sweet et al. (1990). Metabolism of glycerol in *E. coli* involves an initial phosphorylation step by a glycerol kinase (EC 2.7.1.30), which may take place either inside or outside the inner membrane (Koch et al., 1964), and a subsequent dehydrogenation step by glycerol-3-phosphate dehydrogenases (EC 1.1.2.1) (Koch et al., 1964; Kistler et al., 1969; Kistler & Lin, 1972).

For eukaryotic micro-organisms two main routes for glycerol metabolism have been identified. In the first route, discovered in the yeasts *Saccharomyces cerevisiae* and *Candida utilis*, glycerol is first phosphorylated and subsequently dehydrogenated. The performances of the two yeast species were compared by Gancedo et al. (1968), who showed that *C. utilis* could metabolize glycerol much faster than *S. cerevisiae*. The authors did not find enough evidence for the existence of a glycerol transporter in *C. utilis* and proposed that the plasma membrane of this yeast was much more permeable to glycerol than that of *S. cerevisiae*. In *C. utilis* glycerol 3-phosphate is dehydrogenated in the mitochondria by a flavin-dependent glycerophosphate oxidase (EC 1.1.99.5) (Gancedo et al., 1968).

In the second metabolic route found in yeasts, glycerol is first oxidized by a NAD+-dependent dehydrogenase to dihydroxyacetone which is subsequently phosphorylated. This mechanism was described for *Schizosaccharomyces pombe* (May & Sloan, 1981; Kong et al., 1985; Gancedo et al., 1986) and *Candida valida* (Gartner & Kopperschlager, 1984).

There have been a number of studies on glycerol metabolism in filamentous fungi (for a review see Jennings, 1984). In *Aspergillus niger* a metabolic pathway involving glycerol phosphorylation prior to glycerol phosphate dehydrogenation was reported by Witteveen et al. (1990). The existence of a carrier-mediated transport system for glycerol was postulated for *A. nidulans* after characterization of glcC mutants in which no internal glycerol could be detected after incubation of the cells in a medium containing glycerol, in contrast to observations on wild-type strains (Visser et al., 1988). For *Neurospora crassa* the main metabolic pathway also involves phosphorylation of glycerol prior to dehydroge-
nation (Courtright, 1975; Denor & Courtright, 1978). Both enzymes were shown to be inducible and their synthesis was repressed by glucose (Courtright, 1975). A recent genetic analysis revealed a new type of mutant (Glp–), in which a putative glycerol transporter was proposed to be missing (Denor & Courtright, 1982).

Glycerol plays a prominent role in osmoregulation in the yeast Debaryomyces hansenii (Nobre & Costa, 1985; Adler et al., 1985; Andre et al., 1988). A cotransport system for Na⁺ and glycerol that is involved in osmoregulation in this yeast was described by Lucas et al. (1990). Evidence was presented that the concentration gradients of glycerol and Na⁺ are linked through the Na⁺/glycerol symport, using the Na⁺ gradient as a driving force for maintaining the glycerol gradient across the plasma membrane.

In this work we present data on the transport and metabolism of glycerol in Fusarium oxysporum var. lini under different growth conditions. Glycerol is a main constituent of ethanol distillery waste in Brazil, where this waste constitutes a serious pollution problem. F. oxysporum is being utilized tentatively in the processing of the waste for the production of microbial protein. Studies on growth of F. oxysporum on glycerol indicate that the synthesis of a glycerol transport system is a regulatory step in the utilization of this polyol by the fungus. To our knowledge this is the first time that a transport system for glycerol, used in its dissimilation, has been characterized in a eukaryotic micro-organism.

**Methods**

**Organism and growth conditions.** Fusarium oxysporum var. lini ATCC 10960 was maintained on potato dextrose agar at 4 °C after growth for 7 d at 30 °C. The fungus was grown in a medium containing 1% (w/v) peptone, 0.2% yeast extract, 0.05% KH₂PO₄ and 0.075% MgSO₄·7H₂O. Carbon sources (glycerol, ethanol, glucose) were added at a concentration of 2% (w/v). For growth 200 ml of appropriate medium were used in 1 litre shake flasks in an orbital incubator at 25 °C. Cells were harvested in mid-exponential phase by centrifugation at 20000 × g and washed three times with ice-cold water for transport measurements. For enzyme assays, cells were harvested by filtration and washed on the filter.

**Measurement of glycerol uptake rates.** The initial uptake rate of [2-H]glycerol was measured as previously described (Loureiro-Dias & Peinado, 1984). Cell suspension (20 μl; about 0.5 mg dry wt), 20 μl Tris/citrate buffer (pH 5.0, 100 mm) and 10 μl of a solution of [2-H]glycerol were incubated at 26 °C for 10 s (the range of final concentrations of glycerol was 0-20 to 20 mM and the specific activity was about 90 GBq mol⁻¹). Incorporation was stopped by the addition of 5 ml of ice-cold water. Cells were filtered immediately and washed on GF/C Whatman glass-fibre filters and radioactivity was counted in a liquid scintillation system. Controls were prepared by the addition of 5 ml of cold water prior to the addition of labelled glycerol. To check that the measurements were good estimates of initial uptake rates, the linearity of incorporation with time was confirmed for periods up to 20 s. All assays were done in duplicate. [2-H]Glycerol was from Amersham.

**Measurement of intracellular volume.** The intracellular volume was determined using ⁶⁰Co-EDTA as an extracellular marker (Ferreira & Swensson, 1979). Cell suspension (2 ml; 20 mg dry wt) was mixed with 4 ml potassium phosphate buffer (100 mM, pH 5), 60 μl 10 mM-CoCl₂, 60 μl 100 mM-EDTA and 100 μl ⁶⁰CoCl₂ (3 kBq). Samples (5 ml) were filtered on 0.45 μm Sartorius membranes. Pellets were removed with a spatula into tared Eppendorf tubes and counted for 10 min in a γ-counter. As a reference, 120 ml of supernatant was used. Parallel samples were prepared for measuring the amount of fungal material present in each assay. The water volume was taken as the difference between wet and dry weight. External volume was derived from the ratio between counts in the pellet and in the supernatant. Intracellular volume was calculated as the difference between water and external volumes. A value of 2.16 ± 0.22 (n = 8) μl (mg dry wt⁻¹) was obtained.

**Accumulation ratio of 1,2-propanediol.** The ability of cells grown on glycerol and on glucose to accumulate 1,2-propanediol was investigated by measuring the external concentration of 1,2-propanediol in a thick suspension of cells and calculating the internal concentration taking into account the value for internal volume measured above. The accumulation ratio was calculated as the ratio between intracellular and external concentrations. Cells were filtered and the pellet (1 g wet wt) was suspended in 5 ml 1 mM-1,2-propanediol in potassium phosphate buffer (100 mM, pH 5) in a beaker at 26 °C. The suspension was manually stirred with a glass rod and filtered through a GF/C Whatman filter after 10 min; the filtrate was then harvested for subsequent analysis. The concentration of 1,2-propanediol in filtrates was determined according to the method described by Jones & Riddick (1957). A 1 ml volume of the sample was mixed with 5 ml concentrated sulphuric acid, kept at 70 °C for 10 min and cooled to room temperature. Ninhydrin reagent (0.2 ml; 3%; w/v) solution in 5% (w/v) aqueous NaHSO₃ was added and the mixture was allowed to stand for 1 h at 25 °C. The mixture was then diluted to 12.5 ml with concentrated sulphuric acid and the optical density was read at 595 nm after allowing the colour to develop for 10 min. A calibration curve was prepared in the concentration range 5 to 50 μg ml⁻¹. Where indicated (see Results) 50 μM-CCCP or 30 mM-glycerol were added.

**Inactivation of the glycerol transport system by glucose.** A culture growing exponentially on glycerol was divided into aliquots to which glucose was added in the concentration range 10 to 300 mM. Incubations were done as described above under growth conditions. At various times cells were harvested by filtration, washed and the initial uptake rate of 5 mM-glycerol was measured as described above. The reversibility of the effect of glucose was tested in cells that had been inactivated by 100 mM-gluconate by filtering, washing, suspending in 100 mM-Tris/citrate buffer at the original density and measuring glycerol uptake after incubation for periods of 10 and 20 min.

**Monitoring H⁺-uptake.** Changes in the pH value of unbuffered suspensions of glycerol-grown cells upon addition of glycerol were monitored as described previously (Loureiro-Dias & Peinado, 1984).

**Glycerol and glucose concentrations.** Glycerol and glucose concentrations in culture media were estimated enzymically using Boehringer Biochemica Test Combinations for neutral fat and glycerol and for glucose, respectively. Samples were prepared by centrifugation.

**Preparation of cell-free extracts.** Fresh cells were frozen at −20 °C and about 0.3 g (wet wt) was transferred into 10 ml tubes. After addition of 0.5 ml of the appropriate buffer (containing 1 mM-dithiothreitol and 1.2 g 0.5 mm diam. glass beads), extracts were obtained by vigorous shaking for four periods of 1 min on a laboratory tube shaker (Vortex Junior) with 1 min intervals between shaking steps (Funayama et al., 1980). During these intervals the tubes were placed on ice. After addition of 1 ml of buffer, cell homogenates were transferred to micro test-tubes, and centrifuged in an Eppendorf 5414
centrifuge at 6000 r.p.m. for 2 periods of 4 min. The supernatants were used for enzyme assays.

**Enzyme assays and protein determination.** The activities of enzymes were assayed at 30 °C in a Perkin-Elmer spectrophotometer model 552 connected to a Perkin-Elmer absorbance recording system model 561. Except for glycerol phosphate oxidase, enzyme activities were monitored at 340 nm to follow changes in the concentration of NADH or NADPH occurring directly in the reaction mixture or by coupling the reaction to an auxiliary enzyme system. Glycerol kinase (EC 2.7.1.30) activity was assayed as described by Gancedo et al. (1968) by following ADP formation in a system containing 50 mM-Tris/HCl buffer, pH 7.6, 10 mM-MgCl₂, 1 mM-ATP, 1 mM-phosphoenolpyruvate, 0.2 mM-NADH and 1 unit of pyruvate kinase and lactate dehydrogenase ml⁻¹. The reaction was started by addition of 0.1 mM-glycerol. The rate of NADH oxidation prior to substrate addition was taken as the control. Glycerophosphate oxidase (EC 1.99.1.8) activity was measured at 420 nm using ferricyanide as an electron acceptor (Dawson & Thorne, 1975) in a reaction system containing 25 mM-potassium phosphate buffer, pH 7.6, and 1 mM-potassium ferricyanide. The reaction was started by addition of 70 mM-DL-glycerophosphate, after monitoring endogenous reduction of ferricyanide for 3 min.

Glycerol-3-phosphate dehydrogenase (EC 1.1.1.8) was measured in a reaction system containing 50 mM-imidazole/HCl, pH 7.0, 0.2 mM-NADH and 10 mM-EDTA. The reaction was started by the addition of 0.1 mM-dihydroxyacetone phosphate (Gancedo et al., 1968). Glycerol dehydrogenase (glycerol: NAD⁺ oxidoreductase; EC 1.1.1.72) activity was measured in a reaction system containing 50 mM-potassium phosphate buffer, pH 6.5, 0.2 mM-NADPH and 10 mM-MgCl₂. The reaction was started by the addition of 0.1 mM-DL-glyceraldehyde (Flynn & Cromlish, 1982). Glycerol dehydrogenase (glycerol:NAD⁺ oxidoreductase; EC 1.1.1.6) activity was measured in a reaction system containing 50 mM-potassium pyrophosphate buffer, pH 7.0, 10 mM-MgCl₂, and 0.2 mM-NADH. The reaction was started by the addition of 0.1 mM-dihydroxyacetone (Burton, 1955).

Enzyme activities, expressed as international units, were calculated by using the method of the following absorption coefficients: 6.22 mm⁻¹ cm⁻¹ for NADH and 1.04 mm⁻¹ cm⁻¹ for ferricyanide. Protein in crude extracts was estimated by the method of Bradford (1976) at 495 nm, using bovine serum albumin as a standard.

**Chemicals and enzymes.** Enzymes and substrates were obtained from Sigma. Other reagents were all of analytical grade.

**Results**

**Growth on glycerol**

*F. oxysporum* var. *lini* was able to grow on glycerol at 25 °C with a specific growth rate of 0.14 h⁻¹ and a yield coefficient of 44% as compared with values of 0.16 h⁻¹ and 56% for growth on glucose under the same conditions. In culture medium with glycerol plus glucose the fungus exhibited diauxic growth, i.e. two exponential growth phases, with glycerol being utilized only after glucose had been exhausted.

**Glycerol transport**

The kinetics of initial uptake of [³H]glycerol was investigated in glycerol-grown cells over the concentration range 0.02 to 20 mM. The results indicated that glycerol transport followed saturation kinetics. At pH 5 and 25 °C the half-saturation constant (Kₛ) was 0.48 ± 0.25 mM (n = 5) and the maximum velocity (Vₘₐₓ) was 0.87 ± 0.25 mmol (g dry wt)⁻¹ h⁻¹ (n = 5). A number of related compounds were tried as possible competitive inhibitors of glycerol transport (1,2-propanediol, 1,3-propanediol, erythritol, glyceraldehyde, ethylene glycol, dihydroxyacetone and ethanol). Only 1,2-propanediol affected glycerol transport in a competitive way (Kᵢ = 22 mM; results not shown). In order to elucidate the nature of the glycerol transport system, we investigated whether there was any movement of H⁺ upon addition of glycerol to unbuffered cell suspensions of glycerol-grown cells, in order to check whether a H⁺/glycerol symporter was involved. However, no external alkalinization was observed. Also, the ability of the cells to accumulate a nonmetabolizable analogue (which would be an indication of active transport) was examined. The accumulation ratio of 1,2-propanediol was evaluated for cells grown on glycerol and on glucose. Results are summarized in Table 1. Addition of 50 μM-CCCP had no effect and 30 mM-glycerol did not induce outflow of 1,2 propanediol. The results suggest that in *F. oxysporum* var. *lini* glycerol is transported by a facilitated diffusion system, since results were similar for glycerol- and glucose-grown cells.

**Regulation of glycerol transport**

Initial rates of glycerol uptake were measured in cells grown under different physiological conditions in order to study regulation. Glucose-grown cells did not transport glycerol at all. This observation indicates that the cytoplasmic membrane is impermeable to glycerol and is in good agreement with the result stated above concerning the kinetics of glycerol transport in glycerol-grown

<table>
<thead>
<tr>
<th>Growth substrate</th>
<th>Treatment</th>
<th>Accumulation ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glycerol</td>
<td></td>
<td>2.1</td>
</tr>
<tr>
<td>+ 50 μM-CCCP added at time zero</td>
<td>2.5</td>
<td></td>
</tr>
<tr>
<td>+ 30 mM-glycerol added after 10 min; incubation proceeded for a further 3 min</td>
<td>2.3</td>
<td></td>
</tr>
<tr>
<td>Glucose</td>
<td></td>
<td>1.9</td>
</tr>
<tr>
<td>+ 50 μM-CCCP added at time zero</td>
<td>1.0</td>
<td></td>
</tr>
<tr>
<td>+ 30 mM-glycerol added after 10 min; incubation proceeded for a further 3 min</td>
<td>1.5</td>
<td></td>
</tr>
</tbody>
</table>
Table 2. Activities of enzymes involved in glycerol metabolism in F. oxysporum var. lini in cells grown on different carbon sources

<table>
<thead>
<tr>
<th>Carbon source</th>
<th>Glycerol kinase</th>
<th>Glycerol phosphate oxidase</th>
<th>Glycerol dehydrogenase (NADP*-linked)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glycerol</td>
<td>88 ± 62 (n = 34)</td>
<td>47 ± 27 (n = 15)</td>
<td>52 ± 10 (n = 10)</td>
</tr>
<tr>
<td>Glycerol + glucose</td>
<td>78 ± 50 (n = 17)</td>
<td>0</td>
<td>26 ± 8 (n = 10)</td>
</tr>
<tr>
<td>Glucose</td>
<td>98 ± 68 (n = 17)</td>
<td>0</td>
<td>28 ± 12 (n = 10)</td>
</tr>
<tr>
<td>Ethanol</td>
<td>59 ± 13 (n = 7)</td>
<td>0</td>
<td>35 ± 10 (n = 10)</td>
</tr>
</tbody>
</table>

Fig. 1. Inactivation by glucose of the glycerol transport system in F. oxysporum var. lini. Initial uptake rates, \( v \), of 5 mM-[3H]glycerol are shown. ●, Control; ○, 10 mM-glucose; □, 20 mM; △, 100 mM; ■, 300 mM.

cells, in which saturation kinetics described the data without any diffusional component, in the concentration range used. A similar result was obtained for cells grown in the presence of glycerol plus glucose, indicating that synthesis of the glycerol transport system was repressed by glucose.

In ethanol-grown cells glycerol was transported with a \( V_{\text{max}} \) of 0.46 ± 0.15 mmol (g dry wt)\(^{-1}\) h\(^{-1}\) (n = 2), indicating that the transport system is partially constitutive.

Incubation of glycerol-grown cells with glucose resulted in a rapid decrease in the activity of the glycerol transport system, measured as the initial rate of uptake of 5 mM-glycerol (Fig. 1). The rate of inactivation was independent of the glucose concentration in the range examined (10 to 300 mM). Lower concentrations were not suitable for the experiment, since a significant decrease in glucose concentration would occur due to assimilation by the fungus. After 10, 30 and 60 min, samples from the culture that had been incubated with 100 mM-glucose were tested for reversibility of the process, by filtration and incubation in buffer without glucose for periods of 10 and 20 min. Inactivation was irreversible (results not shown). 2-Deoxyglucose (50 mM) produced similar inactivation, while 50 mM-6-deoxyglucose was ineffective.

**Enzyme activities and their regulation**

In order to gain an understanding of glycerol metabolism and enzyme regulation in F. oxysporum var. lini we measured the activities in cell extracts of enzymes that presumably were involved in glycerol metabolism in cells grown under different conditions (Table 2). Our results show that glycerol kinase and NADP*-glycerol dehydrogenase were constitutive while glycerol phosphate oxidase was only found when glycerol was the sole carbon source. NAD*-glycerol dehydrogenase or glycerol-3-phosphate dehydrogenase activities were not detected in extracts of cells grown under any conditions. The growth conditions had no effect on the specific activities of glycerol kinase or NADP*-glycerol dehydrogenase. 1,2-Propanediol, dihydroxyacetone and glyceraldehyde were phosphorylated in the assay of glycerol kinase activity in the crude extracts.

**Discussion**

Our results indicate that glycerol is transported in F. oxysporum var. lini by a facilitated diffusion transporter and that the synthesis of this system is under control of glucose repression and inactivation.

An interesting feature of the kinetics of the initial uptake of glycerol in F. oxysporum was that only saturable kinetics were observed for glycerol concentrations up to 10 mM. Biphasic kinetics for glycerol transport have been reported in other micro-organisms. In Nocardia asteroides saturation kinetics was reported for concentrations up to 80 \( \mu \)M, although a linear
component was detected at higher concentrations and interpreted as simple diffusion through the lipid bilayer (Calmes & Deal, 1972). Uptake by simple diffusion was described for S. cerevisiae. In C. utilis no evidence for mediated transport was found although saturation kinetics were observed (Gancedo et al., 1968). These observations indicate differences in membrane lipid structure between F. oxysporum and the other microorganisms considered.

In spite of the importance in fungal physiology of glycerol as an osmoregulatory solute and in maintaining a proper anabolic reduction charge, no particular studies have focused on the transport system for glycerol. Mutants of N. crassa (Denor & Courtright, 1982) and A. nidulans (Visser et al., 1988) unable to grow on glycerol have been isolated, and in both cases it was assumed that the mutation had occurred in genes for putative transporters. Since these mutations prevented growth it can be concluded that the plasma membranes of these fungi are also quite impermeable to glycerol.

In order to obtain evidence as to whether transporters accumulate solutes actively, measurement of the accumulation ratio of a nonmetabolizable analogue is a common procedure. A value of 1 for the accumulation ratio indicates that the transport system cannot accumulate the solute against a concentration gradient. 1,2-Propanediol was a competitive inhibitor of glycerol transport and an accumulation ratio of about 2 was obtained after uptake for 10 min. CCCP had no effect. The fact that no counterflow was induced by the addition of an excess of glycerol can be explained by the presumed phosphorylation and hence trapping of 1,2-propanediol inside the cells. From the low value obtained for the accumulation ratio we presume that the system is not active. It is significant that in glucose-grown cells (which do not transport glycerol) the results were similar: the accumulation ratios were the same either when the analogue was transported by simple diffusion (in glucose-grown cells) or by facilitated transport (in glycerol-grown cells). Also, the fact that no H⁺ uptake was observed during the initial uptake of glycerol supports the hypothesis that a H⁺-symporter is not involved. Movements of K⁺ and Na⁺ were also investigated using appropriate selective electrodes. No movements of these ions were associated with the initial uptake of glycerol (results not shown).

The glycerol transport system was rapidly inactivated by glucose. Inactivation was independent of the external sugar concentration in the range 10 to 300 mM. This is probably an indication that metabolism of glucose is required in order for inactivation to occur. The high-affinity transporter for glucose is probably operating and is saturated in this concentration range (the half-saturation constant for glucose is about 40 μM) (Brandão & Loureiro-Dias, 1990). Furthermore, inactivation was induced by glucose and 2-deoxyglucose but not by 6-deoxyglucose, indicating that phosphorylation is probably involved in the inactivation process.

The metabolic pathway that we encountered in F. oxysporum is probably the most common among eukaryotic micro-organisms: glycerol is phosphorylated and then oxidized in the mitochondria by a flavin-dependent enzyme. It is significant that this last step is the only one (besides transport) which is regulated. Both the kinase and the NAD⁺-dependent glycerol dehydrogenase are probably involved in anabolic control of reducing power.

Our results indicate that the transport step is a main regulatory step in the utilization of glycerol by F. oxysporum var. lini. The system is certainly highly specific since only one competitive inhibitor was found. Mechanisms of regulation (e.g.) glucose repression and inactivation of the transporter and of the glycerol phosphate oxidase should be taken into consideration when the fungus is grown on complex substrates.

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References


