Isolation and characterization of a *Rhodotorula glutinis* mutant defective in glucose transport

BIRGITTA MILBRADT* and MILAN HÖFER

Botanisches Institut der Universität Bonn, Kirschallee 1, 5300 Bonn, FRG

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Mutants of the obligatorily aerobic yeast *Rhodotorula glutinis*, obtained by treating wild-type cells with *N*-methyl-*N*'-nitro-*N*-nitrosoguanidine, were selected on fructose medium containing 1% 2-deoxy-D-glucose. One mutant, designated R₃₃, displayed a significantly decreased initial rate of uptake of D-glucose, 2-deoxy-D-glucose, D-galactose and D-xylose, and was unable to accumulate the latter three sugars. However, the residual monosaccharide transport was still energy-dependent. The mutant displayed significantly altered transport parameters for D-glucose and D-xylose. These results are evidence for the isolation of a mutant defective in the glucose carrier.

Introduction

The obligatory aerobic yeast *Rhodotorula glutinis* is capable of transporting a wide variety of metabolizable and non-metabolizable monosaccharides by an electrogenic H⁺-symport (Höfer & Misra, 1978; Hauer & Höfer, 1978). Early results suggested that the uptake of monosaccharides was mediated by a common membrane transport system, displaying a high substrate specificity for D-glucose (Kotyk & Höfer, 1965; Höfer & Kotyk, 1968; Höfer, 1970; Höfer & Dahle, 1972). Later results showing different relationships between transport velocity and pH with various substrates, as well as the lack of competition for transport in certain cases, indicated that D-fructose may be taken up by a different transport system from that for D-glucose (Janda *et al.*, 1976; Höfer & Dahle, 1972). D-Glucose seems to have a special position in monosaccharide transport. Its uptake is relatively independent of pH, and it effectively blocks other transport systems, although it does not use all of them for its own uptake. Hence, we attempted to isolate glucose-transport-defective mutants, by using chemical mutagenesis and selecting for resistance to 2-deoxy-D-glucose (2-DG) during growth on a synthetic D-fructose medium. 2-DG-resistant mutants often lack sugar-phosphorylating activities (Megnet, 1965; Maitra, 1970; Mahlberg *et al.*, 1985). To distinguish between sugar-phosphorylating deficiency and transport deficiency of the selected 2-DG-resistant mutants, a special screening

system has been developed. The results presented show that the mutant R₃₃ exhibits a distinct defect of the glucose transport system, whereas its growth on D-fructose + 2-DG was comparable with that of the wild-type strain without 2-DG.

Methods

Organism and growth. The collection strain used, *Rhodotorula glutinis* ATCC 26194 (CBS 6681) (taxonomically *Rhodospiridium toruloides* Banno, mating type a), was cultured in a synthetic medium with 2.5% (w/v) D-glucose as described by Kotyk & Höfer (1965). For mutant selection the medium contained, instead of D-glucose, 2.5% (w/v) D-fructose and 1% (w/v) 2-deoxy-D-glucose (2-DG). For solid media 2.5% (w/v) agar was added. The screening plates contained 2.5% D-glucose or 2% D-galactose or 2% D-xylose as carbon source. The pH of all media was adjusted to 5.4 by adding HCl. For transport experiments, wild-type cells and mutants were cultivated in liquid media on a gyratory shaker at 30 °C for 24 and 26 h respectively. The cells were harvested by centrifugation, washed twice with distilled water, and aerated for 4 h as a 5% aqueous suspension (wet wt/vol.).

Isolation of mutants. Mutagenesis was performed by a modification of the method described by Mahlberg *et al.* (1985). A 1% aqueous wild-type cell suspension (wet wt/vol.) was aerated for 20 to 24 h, then diluted to 0.1, 0.2 and 0.5% and treated for 1 h at 30 °C with 0.17, 1.4 and 1.7 mM-*N*-methyl-*N*'-nitro-*N*-nitrosoguanidine, respectively. The cells were washed three times with distilled water and cultured in liquid selection medium. After 10 d growth on a gyratory shaker at 30 °C, the cells were plated on agar plates of the same composition. The 2-DG-resistant colonies (numbering 10823) were further tested for a potential transport defect phenotype by replica plating on the screening plates.

Transport experiments. The uptake and accumulation of D-[¹⁴C]xylose and 2-deoxy-D-[³H]glucose were measured by analysing hot-water cell extracts as described by Niemietz *et al.* (1981). The uptake of

Abbreviation: 2DG, 2-deoxy-D-glucose.

Table 1. Growth of 2-DG resistant mutants on the screening plates

2-DG-resistant mutants were patched on D-fructose-containing plates (for media and sugar content see Methods) and incubated at 30 °C. After 48 h the colonies were replica plated on plates containing D-xylose (Xyl), D-galactose (Gal), D-glucose (Glc), 2-DG + D-fructose (2-DG + Fru) or D-fructose (Fru). The phenotypes of the 15 mutants potentially transport deficient after 48 h growth are presented: + + + + decreasing to – indicate very good growth to no growth on the screening plates. The glucokinase negative mutant (Glc⁻) was isolated by Mahlberg *et al.* (1985).

Strain	Phenotype on agar plates				
	Xyl	Gal	Glc	2-DG + Fru	Fru
R ₃₅	–	–	+++	+++	+++
R ₂₄	–	–	++	++	++
R ₄₈	+	+	++	+++	+++
R ₄₉	+	+	+++	+++	++++
R _{52,54}	+	+	++	+++	+++
R ₅₃	+	–	++	++	+++
R _{56,47}	+	+	+	+++	+++
R ₁	+	–	++	+++	+++
R ₈₅	–	–	++	++	++
R ₁₀₁	–	–	++	+++	++
R ₁₀₅	–	+	+	+++	++++
R ₁₀₆	+	–	+++	+++	++++
R ₃₃	–	–	++	+++	+++
Glc ⁻	+++	+	++	+++	++++
Wild-type	+++	+	++++	–	++++

D-[¹⁴C]glucose, D-[¹⁴C]galactose and D-[¹⁴C]fructose was measured by determining the sugar concentration in the medium after centrifugation of 1 ml samples taken from the reaction suspension at intervals. The radioactivity of 200 µl of the supernatants in 4 ml of scintillation cocktail was counted in a Packard Tricarb scintillation counter (model He/18/1980). The reaction suspension, incubated at 30 °C, consisted of 2.5% (wet wt/vol.) cells in 0.15 M-KH₂PO₄ buffer, pH 4.5. Transport was started by the addition of the particular sugar at its chosen concentration. All experiments were repeated three to five times; error bars on graphs indicate SEM. Measurements of transport under anaerobic conditions were carried out as described by Höfer & Nassar (1987).

Chemicals. 2-Deoxy-D-glucose was from Calbiochem; *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine was purchased from Boehringer. The radio-labelled monosaccharides were from Amersham-Buchler. All other compounds were of reagent grade, from Merck.

Results and Discussion

Development of media to screen for transport-deficient mutants

In *R. glutinis* 2-DG is transported by the 'glucose carrier' (Taghikhani *et al.*, 1984) but is toxic for cells when phosphorylated and incorporated as D-mannose analogues into the cell wall (Lehle & Schwarz, 1976; Frankas *et al.*, 1979). Resistance to 2-DG could be conferred upon mutants in either the uptake or the phosphorylating step. Earlier attempts to obtain glucose-transport-deficient mutants from *R. glutinis* in this laboratory resulted in the isolation of a glucokinase-negative mutant (Mahlberg *et*

al., 1985). To exclude mutants with phosphorylating deficiency during the screening procedure, the population of cells grown following the mutagenic treatment was replica plated on agar plates containing three different sugars: D-glucose (2.5%), D-galactose (2%) and D-xylose (2%). All three monosaccharides are taken up in *R. glutinis* via the same carrier system; however, each is phosphorylated by a different phosphokinase (Höfer, 1968; Janda *et al.*, 1976). The monosaccharide concentrations used for screening were those which gave the most stable mutant phenotypes on agar plates.

Following mutagenesis and selection, as described in Methods, 15 out of 10823 mutants showed a potential transport-defective phenotype, as manifested by their decreased rate of growth on D-glucose, D-galactose and D-xylose (Table 1). These mutants were chosen for further biochemical characterization.

Biochemical characterization of the mutants

The transport properties of the mutants were analysed first. All mutants showed a significantly decreased initial rate of uptake of D-glucose, 2-DG, D-galactose and D-xylose, as compared with wild-type cells. Fig. 1 illustrates the decreased initial rate of D-glucose uptake in one of the mutants, designated R₃₃. Mutant R₃₃ was also unable to accumulate sugars, as shown for D-xylose in Fig. 2. The same results were obtained with D-galactose

Fig. 1. Uptake of D-glucose into wild-type (●) and mutant R_{33} (▲) cells, measured as consumption from the external medium. After starting the experiments (initial concentration 2 mM-D-glucose), samples were withdrawn at intervals and treated as described in Methods. Error bars represent SEM.

Fig. 2. Uptake and accumulation of D-xylose into wild-type (▲) and mutant R_{33} (●) cells. After starting the experiment (initial concentration 10 mM-D-xylose), samples of 1 ml were withdrawn at intervals and treated as described in Methods. The broken line corresponds to the diffusion equilibrium. Error bars represent SEM.

and 2-DG as substrate (data not shown). Since the capacity for intracellular sugar accumulation is a characteristic property of the glucose carrier in wild-type cells, (Kotyk & Höfer, 1965) the mutant R_{33} was chosen for further studies.

R_{33} exhibited significantly altered transport parameters for D-glucose and D-xylose (Table 2). The half-saturation constant K_T , a measure of the effective

Table 2. Half-saturation constant (K_T) and maximal uptake velocity (V_T) for glucose and xylose in the wild-type strain (WT) and mutant R_{33}

Cell suspensions (about 4 mg dry wt ml⁻¹) were incubated in 0.15 M-KH₂PO₄ buffer, pH 4.5, at 30 °C. The kinetic parameters were estimated from double reciprocal plots. The results are the mean of four measurements; SEM averaged $\pm 3\%$ of the mean.

Monosaccharide	Strain	K_T (mM)	V_T [nmol (mg dry wt) ⁻¹ min ⁻¹]
D-Glucose	WT*	0.04	21
	R_{33}	4	2.8
D-Xylose	WT*	2	25
	R_{33}	40	2

* Data taken from Klöppel & Höfer (1976), recently verified for this paper.

Fig. 3. Transport of D-glucose under anaerobic conditions into wild-type (○) and mutant R_{33} (▲) cells. The experiments were started by the addition of 1.5 mM-D-glucose. Anaerobic conditions were introduced by blowing purified nitrogen into a closed cuvette. Aerobic wild-type (●) and mutant R_{33} (▲) cells were used as controls. Error bars represent SEM.

affinity of the transport system for its substrate, was 100-fold higher for D-glucose, and 20-fold higher for D-xylose, in the mutant as compared with wild-type cells. The maximal velocity V_T of D-glucose and D-xylose uptake in the mutant was also about 10-fold lower than that in wild-type cells. Thus, mutant R_{33} was affected both in the substrate binding and in the translocation step of the glucose transport cycle. The transport parameters for fructose are not presented, since fructose uptake decreased rapidly during cell aeration (by 95% in 1 h). Even

Fig. 4. Competition between D-fructose and D-glucose for entry in *R. glutinis* cells. Uptake of 0.1 mM-D-fructose, measured as substrate consumption from the external medium, into wild-type (open symbols) and mutant R_{33} (filled symbols) cells. D-Glucose concentrations: 0 mM (○, ●); 0.1 mM (△, ▲); 1 mM (□, ■); 4 mM (◇, ◆).

during the harvesting period (15–20 min), the rate of fructose uptake diminished by one-third to one-half of the maximal value (unpublished results).

To test whether or not the altered properties of mutant R_{33} were due to a defect in its energy coupling, the uptake of D-glucose into R_{33} cells under anaerobic conditions was examined. The method used was chosen because anaerobic conditions in the obligatorily aerobic yeast *R. glutinis* have the same effect as addition of metabolic inhibitors or uncouplers, without having any side-effect on cellular membranes (Höfer & Kotyk, 1968; Höfer, 1971). The reduced uptake of D-glucose into R_{33} cells was still inhibited under anaerobic conditions, indicating that uptake was tightly coupled to metabolic energy (Fig. 3). Since D-fructose uptake showed the same energy dependence as D-glucose (data not shown), a defect in the energy coupling of transport in the mutant appeared unlikely.

The present results are interpreted as evidence for the isolation of a mutant impaired in the glucose carrier. Even if the mutagenesis did not result in a loss of the glucose carrier, the special role of D-glucose in monosaccharide transport (Höfer & Dahle, 1972; Janda *et al.*, 1976) was affected. Competition experiments between D-fructose and increasing concentrations of D-glucose were carried out (Fig. 4). Whereas higher concentrations of D-glucose strongly inhibited D-fructose transport in wild-type cells, D-glucose had no effect on D-fructose uptake in the mutant R_{33} cells.

The results of competition experiments, as well as the fact that R_{33} was isolated using D-fructose as the carbon source for selection, have laid the way for investigation of a cryptic fructose transport system. Mutant R_{33} will be a useful tool for examination of cryptic transport systems and for obtaining evidence of the existence of more monosaccharide transport systems in the plasma membrane of *R. glutinis*.

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