Folia Microbiol. 45 (6), 521–529 (2000) http://www.biomed.cas.cz/mbu/folia/

Sugar Repression in the Methylotrophic Yeast *Hansenula polymorpha* Studied by Using Hexokinase-Negative, Glucokinase-Negative and Double Kinase-Negative Mutants

T. KRAMARENKO, H. KARP, A. JÄRVISTE, T. ALAMÄE*

Department of Genetics, Institute of Molecular and Cell Biology, University of Tartu, 51010 Tartu, Estonia

fax +372 7 420286
e-mail talamae@ebc.ee

Received 14 June 2000
Revised version 27 December 2000

ABSTRACT. Two glucose-phosphorylating enzymes, a hexokinase phosphorylating both glucose and fructose, and a glucose-specific glucokinase were electrophoretically separated in the methylotrophic yeast *Hansenula polymorpha*. Hexokinase-negative, glucokinase-negative and double kinase-negative mutants were isolated in *H. polymorpha* by using mutagenesis, selection and genetic crosses. Regulation of synthesis of the sugar-repressed alcohol oxidase, catalase and maltase was studied in different hexose kinase mutants. In the wild type and in mutants possessing either hexokinase or glucokinase, glucose repressed the synthesis of maltase, alcohol oxidase and catalase. Glucose repression of alcohol oxidase and catalase was abolished in mutants lacking both glucose-phosphorylating enzymes (*i.e.* in double kinase-negative mutants). Thus, glucose repression in *H. polymorpha* cells requires a glucose-phosphorylating enzyme, either hexokinase or glucokinase. The presence of fructose-phosphorylating hexokinase in the cell was specifically needed for fructose repression of alcohol oxidase, catalase and maltase. Hence, glucose or fructose has to be phosphorylated in order to cause repression of the synthesis of these enzymes in *H. polymorpha* suggesting that sugar repression in this yeast therefore relies on the catalytic activity of hexose kinases.

In yeasts high concentrations of glucose in the medium repress the transcription of genes encoding enzymes responsible for the utilization of alternative carbon sources. The phenomenon is called glucose repression and its mechanisms have been thoroughly studied in *Saccharomyces cerevisiae* using mostly *MAL*, *SUC* and *GAL* genes as a model (*as review see* Ronne 1995 and Gancedo 1998). In this yeast Mig1 protein is identified as the main transcriptional repressor binding to the promoters of glucose-repressed genes, and hexokinase PII protein is assumed to trigger the repression (Ronne 1995; Gancedo 1998; Klein *et al.* 1998). *S. cerevisiae* has three hexose kinases: the hexokinases PI and PII, which phosphorylate both glucose and fructose, and glucokinase, which is specific for glucose (*as a review see* Entian 1997). However, among the three glucose-phosphorylating enzymes only hexokinase PII seems to be involved in glucose repression under *in vivo* conditions. Mutants of *S. cerevisiae* with reduced glucose phosphorylation and defective glucose repression of invertase and maltase described in Zimmermann and Scheel (1977), revealed allelism with structural gene of hexokinase PII (Entian 1980). Study of mutants carrying different combinations of null alleles of structural genes for hexose kinases also proved the absolute requirement for hexokinase PII in glucose repression (Ma and Botstein 1986; Walsh *et al.* 1991). However, if hexokinase PI was removed in addition to hexokinase PII, glucose repression was further reduced, showing that hexokinase PI can also have some function in glucose repression (Rose *et al.* 1991).

Despite extensive studies the mechanism of glucose repression triggering by hexokinase PII is still the matter of dispute. First, it has been suggested that elevated level of glucose 6-phosphate generated in the hexokinase reaction can be the triggerer of repression. This hypothesis is strongly supported by the inverse correlation between the catalytic activity of different hexokinase PII mutants of *S. cerevisiae* and the extent of glucose repression in these mutants (Ma *et al.* 1989; Rose *et al.* 1991). Hexokinase PII is a major glucose-phosphorylating enzyme in *S. cerevisiae* grown on glucose (Herrero *et al.* 1995) and should therefore be the main producer of glucose 6-phosphate under glucose repression conditions. According to the second hypothesis, hexokinase PII triggers glucose repression due to the presence of a specific regulatory domain in this protein. It is assumed that in the process of glucose binding and/or glucose phosphorylation a conformational change of the hexokinase protein takes place that triggers repression directly or through interaction with other proteins. This possibility is supported by the following data: (*1*) hexokinase PII mutants with unchanged catalytic activity but defective in glucose repression have been described (Entian and Fröhlich 1984);

*Corresponding author.

(*2*) *S. cerevisiae* glucokinase cannot trigger glucose repression even if overepressed (Rose *et al.* 1991); (*3*) hexokinase PII protein has been found in the nucleus of glucose-grown *S. cerevisiae* cells and the nuclear localization was shown to be crucial for glucose repression (Randez-Gil *et al.* 1998; Herrero *et al.* 1998); (*4*) expression of heterologous hexokinases in *S. cerevisiae* hexokinase-deficient mutants always restores sugar-phosphorylating activity, but glucose repression is restored only in some cases (Prior *et al.* 1993; Rose 1995; Petit and Gancedo 1999).

Among non-*Saccharomyces* yeasts, participation of hexokinase in glucose repression has been shown for *Schwanniomyces occidentalis* (Rose 1995), *Pachysolen tannophilus* (Wedlock and Thornton 1989) and *Kluyveromyces lactis* (Goffrini *et al.* 1995). At the same time, hexokinase is probably not involved in glucose repression in the aerobic yeast *Candida utilis* (Espinel *et al.* 1995) or the filamentous fungus *Aspergillus nidulans* (Ruijter *et al.* 1996).

Due to the presence of several glucose-repressed functions, *e.g.*, the synthesis of methanol-specific enzymes and organelles (Eggeling and Sahm 1978; Roggenkamp 1988; Sibirny *et al.* 1988; Parpinello *et al.* 1998), methylotrophic yeasts are convenient for studying glucose repression mechanisms. Moreover, glucose repression mechanisms of methylotrophic yeasts should be studied from the biotechnological standpoint. The extremely strong methanol-induced and glucose-repressed promoter of alcohol oxidase has been widely used to drive the expression of foreign proteins in methylotrophic yeasts (Gellissen and Hollenberg 1997). Different glucose repression mutants have been described in methylotrophic yeasts. In mutants of *Candida boidinii* described by Sakai *et al.* (1987) and in analogous mutants of *Pichia pinus* characterized by Alamäe and Simisker (1994), glucose repression defect was linked to changes in glucose transport system. The *Pichia pinus* mutants insensitive to glucose repression described by Sibirny *et al.* (1988) had a lower activity of phosphofructokinase, and glucose repression mutants of *Hansenula polymorpha* described by Roggenkamp (1988) and Alamäe and Liiv (1998) were both suggested to be deficient in a transcriptional repressor. Data on the participation of glucose-phosphorylating enzymes in glucose repression of methylotrophic yeasts are lacking since no mutants deficient in glucose-phosphorylating enzymes have been characterized in these yeasts.

Here we present data on the pattern and characteristics of hexose kinases in *H. polymorpha*. We also show that hexokinase has no specific role in glucose repression in this yeast, and that sugar repression of maltase and methanol-specific enzymes is dependent on the ability of the cell to phosphorylate the corresponding sugar.

MATERIALS AND METHODS

Strains and cultivation. Derivatives of collection strains of *Hansenula polymorpha* were used (both hexo- and glucokinase positive): strain LR9 (from ATCC 34438, uracil-auxotrophic; Roggenkamp *et al.* 1986), and strain A16 (from CBS 4732, leucine-auxotrophic; Veale *et al.* 1992). Mutant strains (A3, A25-19, A25-26, A31-1, A31-7 and A31-10) were derived from strain LR9 (*see* Table I).

The cells were grown in a liquid medium on a rotary shaker or on agar plates at 37 °C. The growth medium used was 0.67 % Yeast nitrogen base (YNB). Uracil or leucine, if required, were added (20 mg/L). Different carbon sources were added to the medium at concentrations shown in the text.

Preparation of cell extracts and enzyme assay. Cells were harvested by centrifugation at 4 °C, washed twice in distilled water and stored at –65 °C till assay. They were then thawed, suspended in 200–400 μL of 50 mmol/L Tris buffer (pH 7.5) and disrupted with glass beads. The supernatant obtained after the centrifugation (10 000 ***g***, 10 min, 4 °C) was further clarified by centrifugation (12 000 ***g***, 20 min, 4 °C) and used as cell extract. For enzyme activity assays, the cell extract was first diluted with appropriate buffer. Protein concentration of the extract was measured according to Lowry. Hexokinase activity was measured as described previously (Alamäe and Simisker 1994). Concentrations of glucose and fructose in the reaction mixture were 1 and 10 mmol/L, respectively. Eadie–Hofstee plots were used to calculate the K_m values for glucose and fructose. Activities of maltase, alcohol oxidase and catalase were measured as shown by Alamäe and Liiv (1998). Enzyme activities are given in μkat/g protein or nkat/g protein if not stated otherwise.

Glucose and fructose repression assay of maltase and alcohol oxidase. Semiquantitative assay of maltase and alcohol oxidase in mutants, hybrids and random spores was carried out by using permeabilized cell suspensions (Alamäe and Liiv 1998). For maltase activity assay, cells were grown on agar plates with 2 % glucose + 2 % maltose, or 2 % fructose + 2 % maltose. For the assay of alcohol oxidase activity, the cells were grown on 2 % glucose + 0.5 % methanol or 2 % fructose + 0.5 % methanol, respectively. Cells were cultivated at 37 °C for 2 d.

Electrophoretic separation of hexose kinases was conducted in 7.5 % polyacrylamide gel (Angell *et al.* 1992) but with the use of Tris–borate buffer (pH 8.8). Electrophoresis was run for 2–3 h (100 V, 4 °C). Bands with glucose-phosphorylating activity were developed by overlaying the gel with a mixture containing 50 mmol/L Tris buffer (pH 7.5), 0.5 % agarose, 10 mmol/L glucose, 0.2 mmol/L $NADP^+$, 5 mmol/L $MgCl_2$, 2 mmol/L ATP, *N*-methylphenazonium methanesulfonate and nitroblue tetrazolium (both 60 mg/L) and 1 U/mL of glucose-6-phosphate dehydrogenase. Glucose was substituted with 10 mmol/L fructose and 1 U/mL of phosphoglucose isomerase was added to the mixture if fructose-phosphorylating activity was assayed.

Genetic crosses, sporulation, isolation of spores and random spore analysis were performed as described by Alamäe and Liiv (1998).

RESULTS AND DISCUSSION

Electrophoretic separation of glucose-phosphorylating enzymes and isolation of mutants with different spectrum of these enzymes

Study of hexokinase-negative mutants of several yeasts has revealed the key role of hexokinase in glucose repression (Zimmermann and Scheel 1977; Wedlock and Thornton 1989; Rose 1995; Goffrini *et al.* 1995). To study the role of hexokinase in glucose repression in *H. polymorpha,* we first elucidated the isozyme pattern of glucose-phosphorylating enzymes in this yeast. PAGE of extracts from glucose-grown cells revealed the presence of two distinct glucose-phosphorylating enzymes. One of them was specific to glucose, while the other phosphorylated both glucose and fructose (Fig. 1). Hexokinase-negative mutants were obtained

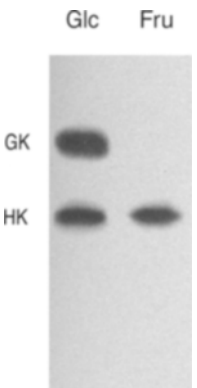

Fig. 1. Electrophoretic separation of glucokinase and hexokinase from *H. polymorpha* LR9 in polyacrylamide gel; cells were grown on 2 % glucose, cell extracts were electrophoresed and the gels were stained for glucose- and fructose-phosphorylating activity; Approximately 50 μg of protein was loaded onto a line. Bromophenol blue marker is seen at the bottom right of the gel; GK – glucokinase, HK – hexokinase.

by us using 2-deoxyglucose (2-dGlc) as follows. *H. polymorpha* strain LR9 was mutagenized with ethyl methanesulfonate (EMS) according to Gleeson *et al.* (1984). Cell survival after the mutagenesis was 21 %. Mutants tolerating 1 g/L 2-dGlc in maltose medium were selected by using periodic transfer of mutagenized cells into a fresh medium containing increasing concentration of 2-dGlc, and subsequent cultivation. The resulting 2-dGlc-resistant culture was plated out onto maltose medium to obtain isolated colonies that were further examined for growth on glucose and fructose. Three glucose-growing mutants unable to grow on fructose were obtained among the 3000 colonies tested. Enzymic and electrophoretic tests of cell extracts revealed the absence of catalytically active hexokinase in the mutants. One of the mutants (T9) was repeatedly back-crossed to the wild type and several hexokinase-negative segregants were obtained. Hexokinase-negative segregant A3 was further analyzed for glucose and fructose repression. In the next step, double kinase-negative mutants lacking both hexokinase and glucokinase activity were obtained from the hexokinase-negative mutant T9. The mutant T9 was treated with EMS (survival of cells 28 %) and selected in the presence of 2-dGlc (as with the isolation of T9 from LR9) but the concentration of 2-dGlc in the final cultivation round was 10 g/L and glycerol was used as the carbon source in the selective medium instead of maltose. 2-dGlc-resistant mutants were plated out onto glycerol medium and replicated onto glucose medium. Out of 3334 colonies, 73 glucose-negative colonies were obtained. Extracts of glycerol-grown glucose-negative mutants were assayed for glucose-phosphorylating activity and two mutants, A25 and A31, lacking both hexokinase and glucokinase activity, were obtained among the 40 clones studied. Mutants A25 and A31 were back-crossed to the wild type and segregants with different pattern of hexose kinases were isolated from the fourth back-cross. The following segregants were picked up for the study of sugar repression: double kinase-negative segregants A31-10 and A25-26, hexokinase-negative segregant A25-19 and

glucokinase-negative segregants A31-7 and A31-1. The auxotrophic requirements of the mutants are presented in Table I, and electrophoretic pattern of hexose kinases in the mutants is shown in Fig. 2.

Table I. Glucose- and fructose-phosphorylating activity[a] (GP and FP, respectively) in hexose kinase mutants and in the wild type *H. polymorpha* LR9

Strain	Auxotrophic marker	Hexose kinases[b] spectrum	GP[c]	FP[c]	FP/GP[c,d]	Growth on[e]			
						Glc	Fru	Glo[f]	Mal
LR9	*ura*	HKGK	13.3	25.0	1.88	+	+	+	±
A3	*leu*	hkGK	4.8	0	–	+	–	–	±
A25-19	*leu*	hkGK	4.7	0	–	+	–	–	±
A31-7	*leu*	HKgk	10.0	28.0	2.80	+	+	+	±
A31-1	*leu*	HKgk	9.3	26.2	2.82	+	+	+	±
A31-10	*leu*	hkgk	0	0	–	–	–	–	–
A25-26	*ura*	hkgk	0	0	–	–	–	–	–

[a]For enzyme analysis double-kinase negative mutants were grown on 2 % glycerol, and other strains on 2 % glucose; mean values of two separate experiments.
[b]HK – presence of hexokinase, GK – presence of glucokinase, hk – absence of hexokinase, gk – absence of glucokinase.
[c]μkat/g protein.
[d]Mutual relation (FP/GP) between specific activities of fructose and glucose phosphorylation in extract.
[e]Growth on sugars (2 %) evaluated on YNB agar plates after 2-d incubation; (+) – good growth, (±) – medium growth, (–) – no growth.
[f]Glucitol.

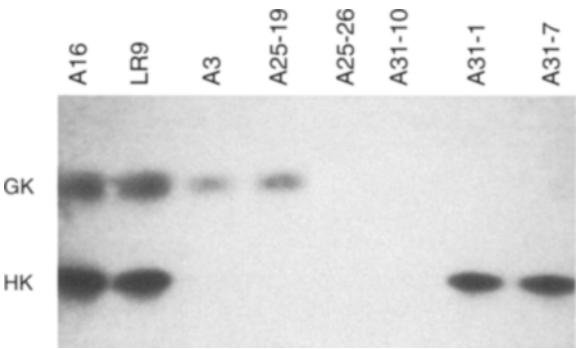

Fig. 2. Isozymic pattern of hexose kinases of *H. polymorpha* wild type strains (LR9 and A16) and different hexose kinase mutants derived from LR9 evaluated by electrophoresis of cell extracts in a polyacrylamide gel; the gels were stained for glucose-phosphorylating activity; all strains were grown on 2 % glucose, except the double-kinase negative mutants which were grown on 2 % glycerol; approximately 30 μg of protein was loaded onto a line; GK – glucokinase, HK – hexokinase.

Glucose and fructose phosphorylation in hexose kinase mutants and their growth properties

Two representatives from each group of hexose kinase mutants (*see* Table I and Fig. 2) were analyzed for sugar-phosphorylating activity and growth properties, with wild type LR9 assayed as a reference. Hexokinase-negative mutants A3 and A25-19 did not phosphorylate fructose and the activity of glucose phosphorylation in their extracts was about 35 % of the respective level in the wild type. These mutants grew well on glucose and maltose but did not grow on either fructose or glucitol. In yeasts, initial katabolism of glucitol yields intracellular fructose (Barnett 1968); this explains the inability of hexokinase-negative mutants to grow on glucitol. Mutants A31-1 and A31-7 possessing only hexokinase activity, phosphorylated both fructose and glucose and the activity of glucose phosphorylation in their cell extracts was about 72 % of the wild-type level. The growth properties of glucokinase-negative mutants on sugars and glucitol did not differ from the wild type. The double kinase-negative mutants A31-10 and A25-26 did not phosphorylate glucose and could not grow on either sugars or glucitol. However, all studied strains grew similarly well on glycerol, methanol and ethanol (*data not shown*).

Kinetic properties of hexokinase and glucokinase in extracts of mutants possessing either hexokinase or glucokinase

To obtain preliminary data on the kinetic properties of *H. polymorpha* glucokinase and hexokinase, we used extracts of hexokinase-negative and glucokinase-negative mutants as crude preparations of these enzymes. Hexose kinases from yeasts are usually characterized by their K_m values towards fructose and glucose and by the fructose–glucose phosphorylation ratio (F/G) (Gancedo *et al.* 1977; Entian 1997). The F/G value in extracts of mutants A31-1 and A31-7 possessing only hexokinase was 2.8 (Table I). A high F/G value is characteristic for hexokinase PI from *S. cerevisiae* (Gancedo *et al.* 1977), hexokinase B from *Pachysolen tannophilus* (Wedlock *et al.* 1989) and for the only hexokinase of *Rhodotorula glutinis* (Mazon *et al.* 1975). The hexokinase from *H. polymorpha* is thus similar to the above-mentioned hexokinases. The K_m of hexokinase for fructose measured in extracts of A31-1 and A31-7 was 1.3 and 1.2 mmol/L, respectively. The K_m of hexokinase for glucose measured in the same extracts was 0.35 and 0.29 mmol/L, respectively. These data are close to the K_m values published for hexokinases from *S. cerevisiae* (Entian 1997), *Rhodotorula glutinis* (Mazon *et al.* 1975), *Candida tropicalis* (Hirai *et al.* 1977) and *Yarrowia lipolytica* (Petit and Gancedo 1999). The K_m of glucokinase for glucose measured in extracts of mutants A3 and A25-19 was 0.05 mmol/L, *i.e.* close to the K_m value of *S. cerevisiae* glucokinase (Maitra 1970).

Glucose and fructose phosphorylation in the wild-type strain LR9 grown on different carbon sources and evaluation of the expression of hexokinase and glucokinase

Glucose- and fructose-phosphorylating activities in cells were clearly dependent on the carbon source in the growth medium. Table II shows that the activities were high when the cells were grown on glycolytic substrates, and much lower if the gluconeogenic substrates ethanol, methanol or glycerol were used as a carbon source. Low activity of glucose phosphorylation in methanol-grown *H. polymorpha* and its up-regulation in glucose-grown cells has also been shown (Parpinello *et al.* 1998).

The ratio of fructose phosphorylating activity to glucose phosphorylating activity (F/G) was high (close to 2.0) if the cells were grown on glucose and fructose, and much lower if gluconeogenic substrates were used as a carbon source (Table II). As glucokinase does not phosphorylate fructose (*see* Fig. 1 and Table I) the F/G value in the cell extract should reflect the relative amounts of hexokinase and glucokinase proteins in the cell. Taking into account glucose- and fructose phosphorylating activities in extracts and F/G ratio of hexokinase (2.8), phosphorylation of glucose due to glucokinase and hexokinase can be calculated as previously done in other yeasts (Mazon *et al.* 1975; Hirai *et al.* 1977). It turned out that during growth of *H. polymorpha* on glucose or fructose, hexokinase accounted for 67–72 % of the total glucose-phosphorylating activity of the cell whereas in cells grown on ethanol, methanol or glycerol glucose-phosphorylating activity due to hexokinase was only 28–33 %. High expression of glucokinase in ethanol- or glycerol-grown cells, and its repression in glucose- or fructose-grown cells has also been shown for *S. cerevisiae* (Herrero *et al.* 1995). Comparison of glucose-phosphorylating capacity of mutants possessing either hexokinase or glucokinase with that of the wild type (Table I) also shows that in glucose-grown cells of *H. polymorpha* hexokinase accounts for about 70 % of total glucose-phosphorylating capacity.

Table II. Activities (μkat/g protein) of glucose phosphorylation (GP) and fructose phosphorylation (FP) in *H. polymorpha* LR9 grown on different carbon sources

Carbon source	%	GP	FP	FP/GP[a]
Glucose	2	13.3	25.0	1.87
Fructose	2	10.8	22.0	2.04
Methanol	0.5	2.2	2.0	0.91
Glycerol	2	3.8	3.0	0.79
Ethanol	0.5	4.8	4.5	0.94

[a]See footnote *d* to Table I; means of three separate experiments.

Sugar repression in mutants with different hexose kinase patterns

Maltase, alcohol oxidase and catalase were used as marker enzymes for studying sugar repression in different hexose kinase mutants, the wild type LR9 being used as a reference. Data on sugar repression of maltase are presented in Table III.

Maltase synthesis in *H. polymorpha* has previously been shown to be maltose-induced and glucose-repressed (Alamäe and Liiv 1998). Table III shows that in all studied strains maltase synthesis was induced in maltose-grown cells, repressed in glucose-grown cells, and also in cells grown on glucose + maltose

mixture. However, if the cells were grown on maltose + fructose mixture, high maltase activity was induced in hexokinase-negative mutants but not in strains possessing hexokinase (in the wild type and in glucokinase-negative mutants). It should be noted that in fructose-grown *H. polymorpha* maltase activity was below 80 nkat/g protein (*not shown*), *i.e.* comparable to the maltase activity in glucose-repressed cells. Thus, Table III shows that the sugar has to be phosphorylated in order to repress maltase synthesis.

Table III. Activity of maltase (nkat/g protein) in the wild type *H. polymorpha* LR9, in hexokinase-negative and glucokinase-negative mutants grown[a] on different carbon sources

Strain and pattern[b] of hexose kinases	Carbon source(s)[c]			
	Mal	Glc	Glc + Mal	Fru + Mal
LR9 (HKGK)	5120	<80	<80	<80
A3 (hkGK)	4630	<80	<80	7750
A25-19 (hkGK)	5630	<80	<80	8020
A31-7 (HKgk)	5900	<80	<80	<80
A31-1 (HKgk)	5870	<80	<80	<80

[a]Cells were harvested from the exponential growth phase.
[b]See footnote *b* to Table I.
[c]In all cases 2 % of each carbon source.

Synthesis of alcohol oxidase and catalase in methylotrophic yeasts is methanol-induced and glucose-repressed (Eggeling and Sahm 1978; Alamäe and Simisker 1994; Alamäe and Liiv 1998; Parpinello *et al.* 1998). To reveal the role of hexose kinases in sugar repression of methanol-specific enzymes, hexokinase-negative, glucokinase-negative and double kinase-negative mutants of *H. polymorpha* were examined for sugar repression of alcohol oxidase and catalase. The wild-type LR9 was analyzed as a reference. Cells were grown on glucose + methanol or fructose + methanol mixtures (*see* Table IV) and harvested before the depletion of the sugar from the medium. Fructose repression was not studied in double kinase-negative mutants. Methanol-grown cells were used as a reference to evaluate the induced level of these enzymes. Table IV shows a high level of alcohol oxidase and catalase in methanol-grown cells in the wild type and in all types of mutants. In the wild type and in glucokinase-negative mutants, induction of alcohol oxidase and catalase by methanol was repressed by both glucose and fructose. In hexokinase-negative mutants these enzymes were glucose-repressed but fructose repression was abolished. Study of the wild-type and mutant cells grown on either 2 % glucose or 2 % fructose (only those capable to grow on these sugars were tested) revealed a strong repression of alcohol oxidase and catalase by these sugars: alcohol oxidase activity in these cells was below detection, and catalase activity was below 1.2 mkat/g protein (*data not shown*). Thus, data presented in Table IV show that glucose repressed alcohol oxidase and catalase synthesis in strains that possess at least one glucose-phosphorylating enzyme. Hexokinase-negative mutants that cannot phosphorylate fructose were deficient in fructose repression of methanol-specific enzymes. Notably, fructose repression of maltase was also abolished in these mutants (*see* Table III). From the whole set of mutants studied, only double kinase-negative mutants lacked glucose repression of alcohol oxidase and catalase. Repression of maltase synthesis by glucose was not studied in double kinase-negative mutants since these mutants cannot grow on glucose + maltose mixture. Results show that for the glucose or fructose repression to take place *H. polymorpha* must possess a kinase that can phosphorylate the corresponding sugar. The presence of either hexokinase or glucokinase is sufficient for glucose repression, and hexokinase is specifically needed for fructose repression. In this aspect *H. polymorpha* differs from *S. cerevisiae*, in which hexokinase PII is mainly responsible for glucose repression, and glucokinase cannot compensate the glucose repression defect in hexokinase PII mutants, even if overexpressed (Rose *et al.* 1991).

Genetic analysis of hexokinase-negative and double kinase-negative mutants

Hexokinase-negative and double kinase-negative mutants were crossed to the wild type strain to study the phenotype of the hybrids. Growth was followed on solid media for 2 d after replicating of glycerol-grown cells onto YNB medium supplemented with different carbon sources. The glucose- and fructose-phosphorylating activity in cell extracts of hybrids and electrophoretic pattern of their hexose kinases were assayed as well (*data not shown*). Hexokinase-negative mutants A3 and A25-19 did not phosphorylate

Table IV. Activity of alcohol oxidase (Aox; μkat/g protein) and catalase (Cat; mkat/g protein) in *H. polymorpha* LR9 and in different hexose kinase mutants grown on different carbon sources[a,b]

Enzyme	LR9 (HKGK)			A3 (hkGK)			A25-19 (hkGK)			A31-7 (HKgk)			A31-1 (HKgk)			A31-10 (hkgk)		A25-26 (hkgk)	
	M	GM	FM	M	GM	FM	M	GM	FM	M	GM	FM	M	GM	FM	M	GM	M	GM
Aox, μkat/g	31.7	0	0	21.7	0	46.7	28.3	0	70.0	30.0	0	0	31.7	0	0	18.3	31.7	25.0	30.0
Cat, mkat/g	30.0	<1.2	<1.2	20.4	<1.2	33.3	32.3	<1.2	27.8	38.5	<1.2	<1.2	22.8	<1.2	<1.2	21.2	7.0	14.8	9.3

[a]M – 0.5 % methanol, GM – 2 % glucose + 0.5 % methanol, FM – 2 % fructose + 0.5 % methanol; cells were harvested before the depletion of sugar from the medium.
[b]See footnote *b* to Table I.

fructose and could not grow on either fructose or glucitol (Table I). Fructose-phosphorylating activity and growth on fructose or glucitol was restored in the hybrids. The double kinase-negative mutants A31-10 and A25-26 did not phosphorylate either glucose or fructose and showed no growth on either glucose, maltose, fructose or glucitol (*see* Table I). Their hybrids with the wild type regained the ability to phosphorylate glucose and fructose and to grow on these substrates. Electrophoresis of cell extracts of hybrids revealed the presence of both hexokinase and glucokinase (*not shown*). Therefore the mutations responsible for the growth phenotype and for the hexose kinase pattern in the mutants were suggested to be recessive.

Random spore analysis of the two hybrids, A25-19 × LR9 and A31-10 × LR9, was carried out to study the segregation of growth and sugar repression properties in the hybrid spore progeny. Sugar repression was tested semiquantitatively in permeabilized suspensions as described in *Materials and Methods*. The mutants A25-19, A31-10 and the wild-type LR9 were analyzed as controls.

The **hybrid A25-19 × LR9** exhibited glucose and fructose repression of alcohol oxidase and maltase synthesis similarly to the wild type while the mutant A25-19 lacked fructose repression of these enzymes. All 106 studied segregants of this hybrid grew on glucose and exhibited glucose repression of alcohol oxidase and maltase. Fifty seven segregants out of 106 did not grow on fructose and glucitol and revealed no fructose repression of alcohol oxidase and maltase. The segregation ratio (≈1 : 1) indicated that a single recessive mutation in the hexokinase-negative mutant A25-19 was responsible for the absence of growth on fructose or glucitol, and lack of fructose repression of alcohol oxidase and maltase.

The **hybrid A31-10 × LR9** exhibited glucose and fructose repression of alcohol oxidase similarly to the wild type while no repression by these sugars was detected in the double kinase-negative mutant A31-10. Glucose growth among the 104 studied random spores of this hybrid co-segregated with glucose repression with a 86 : 18 ratio (≈3 : 1). Mutations in two unlinked recessive genes were therefore responsible for the absence of both glucose growth and glucose repression in the double kinase-negative mutant A31-10. Growth on fructose and glucitol among the segregants co-segregated with fructose repression trait with a ratio of 57 : 47 (close to 1 : 1). Therefore a single recessive mutation in the double kinase-negative mutant caused the inability to grow on fructose and glucitol and absence of fructose repression. We suppose that the mutants studied in this work carry mutations in structural genes of hexokinase and glucokinase. This is supported by the recessive nature of the mutants, and also by the fact that, by using transformation of the genomic library of *H. polymorpha* into double kinase-negative mutant A31-10 we could isolate only plasmids carrying structural genes of hexokinase and glucokinase (*unpublished data*).

Resistance to 2-dGlc in the mutants

Hexokinase-negative and double kinase-negative mutants were initially isolated by using their ability to tolerate 2-dGlc, as shown for several other yeasts (Lobo and Maitra 1977; Wedlock *et al.* 1989). Double kinase-negative mutants A31-10 and A25-26 tolerated 2-dGlc on glycerol medium, but glucokinase-negative mutants A31-1 and A31-7 did not, behaving like the wild type LR9. The hexokinase-negative mutant A3 did not tolerate 2-dGlc, but the hexokinase-negative mutant A25-19 did. As both these mutants behaved similarly in

terms of fructose and glucose repression, we consider that 2-dGlc-resistance of the mutants has no influence on sugar repression. It should be noted that 2-dGlc-resistant mutants with no defects in glucose repression appear frequently in *S. cerevisiae* (Heredia and Heredia 1988).

Hypothetic mechanism of sugar repression in H. polymorpha

Methylotrophic yeasts including *H. polymorpha*, are perfect objects for the study of glucose repression mechanisms. Utilization of methanol in these yeasts depends on a specific set of enzymes (alcohol oxidase, catalase, formaldehyde dehydrogenase, formate dehydrogenase, glycerone* synthase), synthesis of which is highly induced by methanol and strongly repressed by glucose (Sibirny *et al.* 1988; Roggenkamp 1988; Alamäe and Simisker 1994; Parpinello *et al.* 1998).

We have previously shown that in *H. polymorpha* synthesis of maltase is also subject to glucose repression (Alamäe and Liiv 1998). To initiate the study of glucose repression triggering in *H. polymorpha*, we described the pattern of hexose kinases in this yeast and studied glucose and fructose repression of alcohol oxidase, catalase and maltase in different hexose kinase mutants. We could show presence of two distinct hexose kinases: a hexokinase that can phosphorylate both glucose and fructose, and a glucose-specific glucokinase (cf. Fig. 1). Study of hexose kinase mutants revealed that hexokinase was specifically required for fructose repression, while presence of either hexokinase or glucokinase was sufficient for glucose repression of test enzymes (Tables III and IV). The most unexpected result was the ability of glucokinase to mediate glucose repression in H. polymorpha. Therefore, glucose repression mechanisms in this yeast should certainly be different from those in *S. cerevisiae*, *Schwanniomyces occidentalis*, and *Pachysolen tannophilus* in which glucose repression relies on the presence of hexokinase (Zimmermann and Scheel 1977; Wedlock and Thornton 1989; Rose 1995). Specific feature of methylotrophic yeasts is possession of extremely strong methanol-induced promoters that enables synthesis of a huge amount of methanol-specific proteins when cells are grown on methanol. As cited in van Dijk *et al.* (2000) key enzymes of methanol utilization (alcohol oxidase and glycerone synthase) may constitute over 60 % of the total cell protein in methanol-limited continuous cultures of methylotrohic yeasts. If glucose as the most favorable substrate becomes available, synthesis of methanol-specific proteins has to be stopped rapidly. We hypothesize that as glucokinase is the main glucose-phosphorylating enzyme in methanol-grown *H. polymorpha*, triggering of glucose repression not only by hexokinase but also by glucokinase should be advantageous for methylotrophic yeasts. We assume that due to the catalytic activity of hexose kinases a signaling metabolite is produced that acts as a mediator in the cascade of repression reactions. The signaling molecule can be glucose 6-phosphate or some nonglycolytic product derived from it. However, our data do not rule out the possibility that phosphorylation of the sugar is crucial for the conformational change of hexokinase and glucokinase proteins that might trigger the repression through interaction with other proteins. Generation of hexokinase and glucokinase disruption mutants and expression of specifically mutated hexokinase and glucokinase genes in these mutants is needed to specify the mechanism of sugar repression triggering in *H. polymorpha*.

This work was financed by *Estonian Science Foundation* (grants 2328 and 3923). The authors thank M. Kaljusmaa for the isolation of double kinase-negative mutants and A. Kahru for revising the manuscript. We also thank R. Roggenkamp and P. Sudbery for providing the *H. polymorpha* strains LR9 and A16.

REFERENCES

ALAMÄE T., SIMISKER J.: Isolation and preliminary characterization of *Pichia pinus* mutants insensitive to glucose repression. *Yeast* **10**, 1459–1466 (1994).

ALAMÄE T., LIIV L.: Glucose repression of maltase and methanol-oxidizing enzymes in the methylotrophic yeast *Hansenula polymorpha*: isolation and study of regulatory mutants. *Folia Microbiol.* **43**, 443–452 (1998).

ANGELL S., SCHWARZ E., BIBB M.J.: The glucose kinase gene of *Streptomyces coelicolor* A3(2): its nucleotide sequence, transcriptional analysis and role in glucose repression. *Mol.Microbiol.* **6**, 2833–2844 (1992).

BARNETT J.A.: The catabolism of acyclic polyols by yeasts. *J.Gen.Microbiol.* **52**, 131–159 (1968).

VAN DIJK R., FABER K.N., KIEL J.A.K.W., VEENHUIS M., VAN DER KLEI I.: The methylotrophic yeast *Hansenula polymorpha*: a versatile cell factory. *Enzyme Microb.Technol.* **26**, 793–800 (2000).

EGGELING L., SAHM H.: Derepression and partial insensitivity to carbon catabolite repression of the methanol dissimilating enzymes in *Hansenula polymorpha*. *Eur.J.Appl.Microbiol.Biotechnol.* **5**, 197–202 (1978).

ENTIAN K.-D.: Genetic and biochemical evidence for hexokinase PII as a key enzyme involved in catabolite repression in yeast. *Mol. Gen.Genet.* **178**, 633–637 (1980).

ENTIAN K.-D.: Sugar phosphorylation in yeast, pp. 67–79 in F.K. Zimmermann, K.-D. Entian (Eds): *Yeast Sugar Metabolism*. Lancaster, Basel 1997.

ENTIAN K.-D., FRÖHLICH K.-U.: *Saccharomyces cerevisiae* mutants provide evidence of hexokinase PII as a bifunctional enzyme with catalytic and regulatory domains for triggering carbon catabolite repression. *J.Bacteriol.* **158**, 29–35 (1984).

*Formerly 'dihydroxyacetone'.

ESPINEL A.E., GOMEZ-TORIBIO V., PEINADO J.: The inactivation of hexokinase activity does not prevent glucose repression in *Candida utilis*. *FEMS Microbiol.Lett.* **135**, 327–332 (1995).

GANCEDO J.M.: Yeast carbon catabolite repression. *Microbiol.Mol.Biol.Rev.* **62**, 334–361 (1998).

GANCEDO J.M, CLIFTON D., FRAENKEL D.G.: Yeast hexokinase mutants. *J.Biol.Chem.* **252**, 4443–4444 (1977).

GELLISSEN G., HOLLENBERG C.P.: Application of yeasts in gene expression studies: a comparison of *Saccharomyces cerevisiae*, *Hansenula polymorpha* and *Klyuveromyces lactis*. *Gene* **190**, 87–97 (1997).

GLEESON M.A., WAITES M.J., SUDBERY P.E.: Development of techniques for genetic analysis in the methylotrophic yeast *Hansenula polymorpha*, pp. 228–235 in R.L. Crawford, R.S. Hanson (Eds): *Microbial Growth on C1 Compounds*. American Society for Microbiology, Washington (DC) 1984.

GOFFRINI P., FICARELLI A., FERRERO I.: Hexokinase activity is affected in mutants of *Kluyveromyces lactis* resistant to glucose repression. *Microbiology* **141**, 441–447 (1995).

HEREDIA M.F., HEREDIA C.F.: *Saccharomyces cerevisiae* acquires resistance to 2-deoxyglucose at a very high frequency. *J.Bacteriol.* **170**, 2870–2872 (1988).

HERRERO P., GALINDEZ J., RUIZ N., MARTINEZ-CAMPA C., MORENO F.: Transcriptional regulation of the *Saccharomyces cerevisiae HXK1*, *HXK2* and *GLK1* genes. *Yeast* **11**, 137–144 (1995).

HERRERO P., MARTINEZ-CAMPA C., MORENO F.: The hexokinase 2 protein participates in regulatory DNA-protein complexes necessary for glucose repression of the *SUC2* gene in *Saccharomyces cerevisiae*. *FEBS Lett.* **434**, 71–76 (1998).

HIRAI M., OHTANI E., TANAKA A., FUKUI S.: Glucose-phosphorylating enzymes of *Candida* yeasts and their regulation *in vivo*. *Biochim.Biophys.Acta* **480**, 357–366 (1977).

KLEIN C. J.L., OLSSON L., NIELSEN J.: Glucose control in *Saccharomyces cerevisiae*: the role of *MIG1* in metabolic functions. *Microbiology* **144**, 13–24 (1998).

LOBO Z., MAITRA P.K.: Resistance to 2-deoxyglucose in yeast: a direct selection of mutants lacking glucose-phosphorylating enzymes. *Mol.Gen.Genet.* **157**, 297–300 (1977).

MA H., BOTSTEIN D.: Effects of null mutations in the hexokinase genes of *Saccharomyces cerevisiae* on catabolite repression. *Mol. Cell.Biol.* **6**, 4046–4052 (1986).

MA H., BLOOM L.M, WALSH C.T, BOTSTEIN D.: The residual enzymatic phosphorylation activity of hexokinase II mutants is correlated with glucose repression in *Saccharomyces cerevisiae*. *Mol.Cell.Biol.* **9**, 5643–5649 (1989).

MAITRA P.K.: A glucokinase from *Saccharomyces cerevisiae*. *J.Biol.Chem.* **245**, 2423–2431 (1970)

MAZON M., GANCEDO J.M., GANCEDO C.: Hexose kinases from *Rhodotorula glutinis*. *Arch.Biochem.Biophys.* **167**, 452–457 (1975).

PARPINELLO G., BERARDI E., STRABBIOLI R.: A regulatory mutant of *Hansenula polymorpha* exhibiting methanol utilization metabolism and peroxisome proliferation in glucose. *J.Bacteriol.* **180**, 2958–2967 (1998).

PETIT T., GANCEDO C.: Molecular cloning and characterization of the gene *HXK1* encoding the hexokinase from *Yarrowia lipolytica*. *Yeast* **15**, 1573–1584 (1999).

PRIOR C., MAMESSIER P., FUKUHARA H., CHEN X.J.: The hexokinase gene is required for transcriptional regulation of the glucose transporter gene *RAG1* in *Kluyveromyces lactis*. *Mol.Cell.Biol.* **13**, 3882–3889 (1993).

RANDEZ-GIL F, HERRERO P, SANZ P., PRIETO J.A., MORENO F.: Hexokinase PII has a double cytosolic-nuclear localization in *Saccharomyces cerevisiae*. *FEBS Lett.* **425**, 475–478 (1998).

ROGGENKAMP R.: Constitutive appearance of peroxisomes in a regulatory mutant of the methylotrophic yeast *Hansenula polymorpha*. *Mol.Gen.Genet.* **213**, 535–540 (1988).

ROGGENKAMP R., HANSEN H., ECKART M., JANOWICZ Z., HOLLENBERG C.P.: Transformation of the methylotrophic yeast *Hansenula polymorpha* by autonomous replication and integration vectors. *Mol.Gen.Genet.* **202**, 302-308 (1986).

RONNE H.: Glucose repression in fungi. *Trends Genet.* **11**, 12–17 (1995).

ROSE M.: Molecular and biochemical characterization of the hexokinase from the starch-utilizing yeast *Schwanniomyces occidentalis*. *Curr.Genet.* **27**, 330–338 (1995).

ROSE M., ALBIG W., ENTIAN K.-D.: Glucose repression in *Saccharomyces cerevisiae* is directly associated with hexose phosphorylation by hexokinases PI and PII. *Eur.J.Biochem.* **199**, 511–518 (1991).

RUIJTER G.J., PANNEMAN H., VAN DEN BROECK H.C., BENNETT J.M., VISSER J.: Characterization of the *Aspergillus nidulans* frA1 mutant: hexose phosphorylation and apparent lack of involvement of hexokinase in glucose repression. *FEMS Microbiol. Lett.* **139**, 223–228 (1996).

SAKAI Y., SAWAI T., TANI Y.: Isolation and characterization of a catabolite repression-insensitive mutant of a methanol yeast *Candida boidinii* A5 producing alcohol oxidase in glucose-containing medium. *Appl.Environ.Microbiol.* **53**, 1812–1818 (1987).

SIBIRNY A.A., TITORENKO V.I., GONCHAR M. V., UBIYVOVK V.M., KSHEMINSKAYA G.P., VITVITSKAYA O.P.: Genetic control of methanol utilization in yeasts. *J.Basic Microbiol.* **28**, 293–319 (1988).

VEALE R.A., GIUSEPPIN M.L.F., VAN EIJK H.M.J., SUDBERY P.E., VERRIPS C.T.: Development of a strain of *Hansenula polymorpha* for the efficient expression of guar α-galactosidase. *Yeast* **8**, 361–372 (1992).

WALSH R.B., CLIFTON D., HORAK J., FRAENKEL D.G.: *Saccharomyces cervisiae* null mutants in glucose phosphorylation: metabolism and invertase expression. *Genetics* **128**, 521–527 (1991).

WEDLOCK D.N., THORNTON R.J.: A hexokinase associated with catabolite repression in *Pachysolen tannophilus*. *J.Gen.Microbiol.* **135**, 2013–2018 (1989).

WEDLOCK D.N., JAMES A.P., THORNTON R.J.: Glucose-negative mutants of *Pachysolen tannophilus*. *J.Gen.Microbiol.* **135**, 2019–2026 (1989).

ZIMMERMANN F.K., SHEEL I.: Mutants of *Saccharomyces cerevisiae* resistant to catabolite repression. *Mol.Gen.Genet.* **154**, 75–82 (1977).