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Transport of Glucose in the Wine Spoilage Yeast *Dekkera anomala*

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Summary

When grown in media with 0.1 and 2 % of glucose Dekkera anomala IGC 5153 presented specific, relatively low, growth rates and the yield coefficient decreased from 0.40 to 0.28 g/g as the initial sugar concentration in the medium increased. From transport assays carried out in vivo and in vitro, it was observed that the yeast produced two transport systems for glucose which could be distinguished by their kinetic parameters and substrate specificity: (i) a high-affinity system able to accept 2-deoxy-D-glucose and D(+)galactose, and (ii) a low-affinity system able to accept 2-deoxy-D-glucose and D(-)fructose, but not D(+)galactose. When 2 % of glucose or fructose was used in the culture medium only the low-affinity carrier was found, while in acetic acid- or glycerol-grown cells only the high-affinity one was present. If a lower sugar concentration (0.1 %, w/v) or ethanol was used in the medium, both systems were operating. These results suggest that the most probable mechanism involved in glucose transport by both carriers was a facilitated diffusion. The values of the specific glucose transfer rates in cultures with 0.1 and 2 % of glucose were very similar to those of the corresponding V_{max} of glucose transport.

Key words: Dekkera anomala, sugar transport system, facilitated diffusion

Introduction

Species of the genus *Dekkera/Brettanomyces* are among the most troublesome yeasts that can grow in juice and wine, their involvement in wine spoilage being reported from all wine-producing areas of the world. They are responsible for important economic losses not only from overtly spoiled unmarketable wines, but also for wines of diminished quality. The presence of such species during the fermentative growth phase not only results in formation of obnoxious flavours and odours that develop and intensify during ageing, but it also disturbs the activity of the wine yeast *Saccharomyces cerevisiae*. Currently, concerns regarding the early detection and control of such species are important issues among winemakers, but it is clear from the wine literature that species of *Brettanomyces/Dekkera* appear to be peculiar and difficult to control. They are slow growers, clearly fastidious, requiring complex sources of exogenous nutrients and attempts to isolate them from grapes in the vineyard have been largely unsuccessful. Some believe that the availability of minute amounts of unfermented sugar predisposes wines to growth of these species once they are bottled, while other research groups support the view that growth rate is enhanced with increasing concentrations of glucose although substantial populations of *Dekkera/Brettanomyces* may develop at levels of sugar of less than 0.2 %. For a comprehensive review on the subject see Fugelsang *et al.* (*1*) and Chatonet *et al.* (*2*). Studies on the elucidation of this peculiar behaviour, besides being important in acquiring basic knowledge on the biology of a genus poorly understood, may have an important practical dimension.

In the present work, after evaluating the growth of the yeast *Dekkera anomala* IGC 5153 in batch cultures with different glucose concentrations, emphasis was given to the elucidation of the glucose transport and its regulation. The studies were carried out either in intact

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cells or in hybrid plasma membrane vesicles as a cellular model devoid of metabolic activity.

Materials and Methods

Microorganism and growth conditions

Dekkera anomala IGC 5153 was maintained on a medium containing glucose (2 %)*, peptone (1 %), yeast extract (0.5 %), and agar (2 %). The cells were grown in 500 mL flasks with 200 mL of liquid mineral medium containing vitamins (*3*) supplemented with glucose (0.1 or 2 %), fructose (2 %), ethanol (0.5 % volume fraction), glycerol (0.5 % volume fraction) or acetic acid (0.5 % volume fraction). All cultivations were carried out at initial pH = 5.5 and 26 °C, with mechanical shaking (150 rpm). Growth was monitored by measuring the A_{640} (Spectronic 21, Bausch & Lomb) for determination of maximum specific growth rates (μ_{max}). Yield coefficient (Y) was based on dry weight determinations and consumption of glucose monitored by the glucose oxidase method (Test-Combination, Boehringer Mannheim). Specific transfer rate of glucose was calculated as the ratio μ_{max}/Y.

Transport assays in intact cells

Growing cells were harvested in mid-exponential phase, centrifuged, washed twice with ice-cold distilled water, and suspended in distilled water at a final concentration (dry weight) of about 40 mg/mL.

To estimate uptake rates of labelled glucose, 10 μL of yeast suspension was mixed with 30 μL of 0.1 M potassium phosphate buffer at pH = 5.0 in 10 mL conical tubes. After 2 min of incubation at 26 °C in a water bath, the reaction was started by the addition of 10 μL of an aqueous solution of the radiolabelled sugar with about 4000 dpm/nmol at the desired concentration. Sampling times were 0, 5 and 10 s, time periods over which the uptake of labelled sugar was linear. The reaction was stopped by dilution with 4 mL of ice-cold water and the mixtures were filtered immediately through GF/C filters (Whatman, Clifton, NJ). The filters were washed with 8 mL of ice-cold water, and introduced in vials containing scintillation fluid OptiPhase HiSafe II. The radioactivity was measured in a Packard Tri-Carb 2200 CA liquid scintillation counter (Packard Instruments, Rockville, MD). For assessing non-specific ^{14}C adsorption, labelled sugar was added after cold water. Inhibition by non-labelled sugars was assayed by adding simultaneously the labelled and non-labelled substrate. The concentration range of labelled substrate varied from 0.01 to 2 mM and the final concentration of the cold substrate was at least 10-fold higher than the K_m value estimated for the carrier.

A standard pH meter (PHM 85, Radiometer, Copenhagen, Denmark) connected to a recorder (Kipp and Zonen), as described earlier (*4*), was used to investigate the presence of proton movements upon addition of the sugars.

Preparation of plasma membranes

Following the procedure described by van Leeuwen *et al.* (*5*), exponentially growing yeast cells obtained as indicated above were used to prepare plasma membrane vesicles. The membrane fractions were resuspended in 10 mM Tris, 1 mM EDTA, pH = 7.4 (about 3 mg protein per ml) and stored in liquid nitrogen. The purity of the plasma membrane preparations was assayed by the activity of the marker enzymes azide-sensitive mitochondria ATPase and vanadate-sensitive plasma membrane ATPase (*6*). The results obtained were very similar to those reported for plasma membrane preparations from the yeast *Candida utilis* (*7*), indicating that the experimental procedure allowed to obtain a plasma membrane fraction of the yeast *D. anomala* not significantly contaminated with mitochondrial membranes. Protein was determined according to Lowry *et al.* (*8*), using bovine serum albumin as a standard.

Preparation of hybrid plasma membrane vesicles

Commercial *Escherichia coli* phospholipids were purified by the method of Viitanen *et al.* (*9*) and stored at -20 °C in a chloroform solution. To prepare the liposomes, the chloroform was removed under a nitrogen stream and the lipids were resuspended by sonication in 50 mM potassium phosphate pH = 6.2, to a final concentration of about 30 mg/mL. Unless stated otherwise, the hybrid plasma membrane vesicles were prepared by mixing 100 μL of liposomes with 0.15 mg of yeast plasma membrane protein in 50 mM potassium phosphate pH = 6.2, 1 mM $MgCl_2$ to a final volume of 200 μL. Then, the suspension was frozen in liquid nitrogen, thawed at room temperature and sonified 15 s (Elma, Transsonic TP 690 sonifier) using intervals of 5 and 20 s recovery at 4 °C. The fusion of yeast plasma membranes with liposomes by freeze thaw sonication was confirmed by the fluorescence dequenching of membranes labelled with octadecyl rhodamine-β-chloride (*10*) and an efficiency of more than 98 % was obtained.

To obtain hybrid vesicles able to generate a transvesicular proton-motive force (PMF) the yeast plasma membranes were fused with liposomes with cytochrome *c* oxidase incorporated. To reconstitute the cytochrome *c* oxidase in liposomes, the enzyme (22.5 nmol) isolated from bovine heart (*11*) was mixed with 100 mg of sonicated lipids in 2.5 mL 50 mM potassium phosphate pH = 6.2, in the presence of 22.5 mg n-octyl-β-D-glucopyranoside and dialysed against 50 mM potassium phosphate pH = 6.2 (*5*). The fusion procedure between the plasma membranes and liposomes and the evaluation of its efficiency were performed as indicated above. The proton-motive force generated by these hybrid vesicles was evaluated as follows. The transvesicular pH gradient (ΔpH) was measured according to Clement and Gould (*12*). Hybrid vesicles with incorporated pyranine were suspended in 2 mL of 50 mM potassium phosphate pH = 6.2, 1 mM $MgCl_2$, and the energization of the

* Compositions expressed in percantage refer to mass per volume fraction *w/v* unless otherwise stated.

system was performed by the addition of the following electron donors: 150 μM *NNN'N'*-tetramethyl-*p*-phenylenediamine (TMPD), 15 μM cytochrome *c* and 15 mM ascorbate (adjusted to pH = 6.2 with KOH). The transvesicular membrane potential (ΔY) was inferred from the distribution of the lipophilic cation tetraphenylphosphonium (TPP^+) by using a TPP^+-selective electrode (*13*). The system was energized using the same electron donors as for the ΔpH determination. $\Delta\Psi$ was calculated according to Lolkema *et al.* (*14*) and de Vrij *et al.* (*15*), assuming symmetric binding.

Internal volume

To estimate the internal vesicle volume, 1 μCi of labelled D-glucose was included in the vesicles during the fusion procedure. The vesicles were separated from the medium by filtration, washed on the filters and the radioactivity was counted, as indicated in transport assays. An internal volume of 2.6 μL per mg of phospholipid was estimated.

Transport assays in hybrid vesicles

For estimating the initial uptake rates of D- or L-glucose, 3 μL of the radiolabelled sugar (60000 and 6000 dpm/nmol for the high- and low- affinity systems, respectively), at desired concentration, were added to 22 μL of vesicle suspension. The reaction was stopped after 30 s of incubation by dilution with 2.0 mL ice-cold 100 mM LiCl. The mixtures were filtered through cellulose nitrate filters (pore size 0.45 μm, Schleider & Schuell) and washed with 2.0 mL 100 mM LiCl. The filters were introduced in vials containing scintillation liquid and their radioactivity was measured as indicated above for transport assays in intact cells.

To measure the glucose transport along time, the uptake was initiated by the addition of D- or L-[U-^{14}C] glucose at desired concentration, to 200 μL of hybrid vesicles. Aliquots of 15 μL were withdrawn at appropriate intervals, the reaction was stopped and the samples were treated as indicated elsewhere.

To measure the influence of the proton motive force (PMF) on glucose uptake, hybrid vesicles with incorporated cytochrome *c* oxidase were used. The glucose transport along time was performed as indicated above and the mixture was energized with 15 mM ascorbate, 150 μM TMPD and 15 μM cytochrome *c*. All the experiments were carried out at 26 °C.

Calculation of kinetic parameters

The data of the initial uptake rates of labelled glucose were analysed by a computer-assisted non-linear regression analysis (GraphPad software; San Diego, CA). By this method, the transport kinetics best fitting to the experimental initial uptake rates was determined, and then estimates for the kinetic parameters were obtained. Substrate uptake is presented as mean values ± S.E. and *N* denotes the number of independent experiments.

Chemicals

D-[U-^{14}C] glucose (305 mCi/mmol) and L-[1-^{14}C] glucose (55 mCi/mmol) were obtained from Amersham International. Miscellaneous chemicals were obtained as follows: L-α-phosphatidylethanolamine (type IX from *E. coli*), *n*-octyl-β-D-glucopyranoside, carbonyl cyanide *m*-chlorophenylhydrazone (CCCP), *N,N,N'N'*-tetramethyl-*p*-phenylenediamine (TMPD), ascorbic acid, sodium orthovanadate (Sigma); sodium azide, cytochrome *c*, tetraphenylphosphoniumchlorid (Merck); pyranine (Eastman); octadecyl rhodamine-β-chloride (Molecular Probes Inc.); Scintillation fluid OptiPhase HiSafe II (LKB FSA Laboratory Supplies, Loughborough, UK). All other chemicals were reagent grade and obtained from commercial sources.

Results

Growth in batch cultures on glucose. Aerobic growth of the yeast *Dekkera anomala* IGC 5153 in batch cultures in media with 0.1 and 2% glucose was evaluated. The results are presented in Table 1.

Table 1. Growth parameters (specific growth rate, μ_{max}; yield coefficient, *Y*; specific glucose transfer rate, *q*) of *D. anomala* IGC 5153 in batch cultures in mineral medium with different glucose concentrations. Values of μ_{max} are mean ± S.E. (*N*=7) and values of *Y* are mean of two independent experiments.

Growth conditions	μ_{max}	Y	q
	h^{-1}	$g\ g^{-1}$	$mmol\ g^{-1}\ h^{-1}$
Glucose (2 %)-grown cells	0.18 ± 0.01	0.28	3.53
Glucose (0.1 %)-grown cells	0.12 ± 0.01	0.40	1.67

The maximum specific growth rates (μ_{max}) were relatively low and of the same order of magnitude with both sugar concentrations. Even so, growth was faster in 2 % of glucose. Additionally, the increase of the initial sugar concentration in the medium led to a decrease of the yield coefficient (*Y*), and an increase of the specific glucose transfer rate (*q*). The utilization of glucose by yeasts requires its transport across the plasma membrane which, in turn, may constitute an important step for the control of cell growth. In the following approach, emphasis on glucose transport and its regulation was given. The studies were carried out either in intact cells or in hybrid plasma membrane vesicles as a cellular model devoid of metabolic activity.

Transport of glucose in intact cells. The methodology used to measure sugar transport in other yeasts showed to be suitable for *D. anomala* IGC 5153, and the initial uptake of labelled D-glucose was linear up to 10 s. When transport was measured in cells grown in a medium with 2 % glucose or fructose with labelled D-glucose at concentrations from 0.01 to 10 mM, pH = 5.0, the Eadie-Hofstee plots of the initial velocities were linear. Fig. 1A shows the results obtained with 2 % glucose-grown cells. The application of a computer-assisted simulation to these data was consistent with the presence of only one, carrier-mediated, transport system with the kinetic parameters presented in Table 2.

The estimates of initial uptake rates of 0.01 to 2 mM-labelled D-glucose obtained after 30 s incubation

Table 2. Kinetic parameters for glucose transport by the high- and low-affinity components characterized in cells of the yeast *D. anomala* IGC 5153 grown on the carbon sources indicated
The kinetic parameter values were estimated by the application of a computer-assisted non-linear regression analysis to the data, as indicated in Materials and Methods. Data represent means ± S.E. (*N* varied between 4 and 9)

Carbon source for growth	Low-affinity component		High-affinity component	
	K_m / mM	V_{max} / mmol g^{-1} h^{-1}	K_m / mM	V_{max} / mmol g^{-1} h^{-1}
2 % glucose	0.694 ± 0.036	3.150 ± 0.040	n.m.	n.m.
2 % fructose	0.776 ± 0.086	1.487 ± 0.065	n.m.	n.m.
0.1 % glucose	0.774 ± 0.276	1.706 ± 0.122	0.014 ± 0.008	0.220 ± 0.115
0.5 % ethanol	0.375 ± 0.096	0.724 ± 0.036	0.010 ± 0.006	0.191 ± 0.054
0.5 % acetic acid	n.m.*	n.m.	0.030 ± 0.005	0.508 ± 0.014
0.5 % glycerol	n.m.	n.m.	0.013 ± 0.002	0.666 ± 0.022

* n.m., not measurable

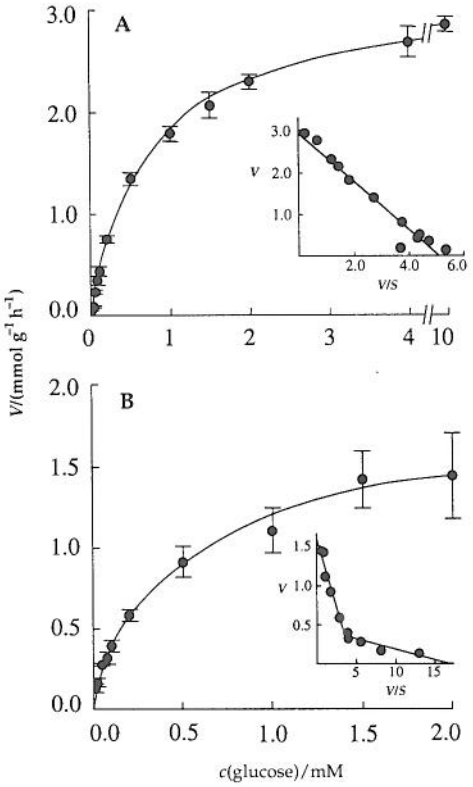

Fig. 1. Initial uptake rates of labelled D-glucose at pH = 5.0 by cells of *D. anomala* IGC 5153 grown with glucose at the following fractions: 2 % (A) and 0.1 % (B). Values are mean ± S.E., *N*=9 (A) and *N*=4 (B). *Inserts*: Eadie-Hofstee plots of the initial uptake rates. Data were fitted by linear regression

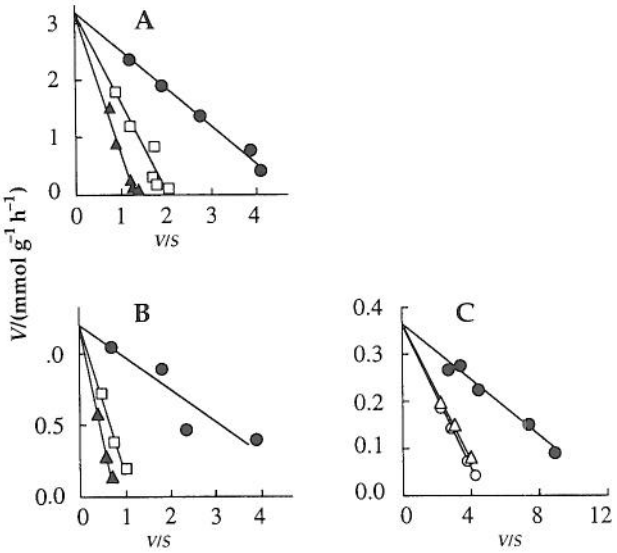

Fig. 2. Eadie-Hofstee plots of the initial uptake rates at pH = 5.0 of labelled D-glucose in cells of *D. anomala* IGC 5153 grown with glucose at the following fractions: 2 % (A) and 0.1 % (B and C). B and C, represent the uptake of labelled D-glucose by the low- and the high-affinity transport systems, respectively. Symbols: ● – absence of other sugars; □ – presence of 10 mM fructose; presence of 2 (Δ) or 10 mM (▲) 2-deoxy-D-glucose; ○ – presence of 2 mM galactose

with 100 μM carbonyl cyanide *m*-chlorophenylhydrazone (CCCP), at pH = 5.0, showed that the protonophore did not induce any inhibitory effect (not shown). These results indicate that the transport system was not dependent on the transmembrane proton-motive force, consistent with the hypothesis of glucose transport by facilitated diffusion. Accordingly, no proton movements associated to the addition of glucose to cell suspensions, were detected. Estimates of the initial uptake rates of labelled D-glucose in the presence of several sugars showed that D(-)fructose and 2-deoxy-D-glucose were competitive inhibitors for the glucose transport system indicating that they shared a common carrier; L-glucose, D(+)galactose, D(+)xylose, L(-)sorbose, D(-)ribose, D(+)trehalose, D(-)arabinose, L(+)rhamnose had no significant effect on the transport of glucose, thus appearing not to be recognized by the glucose transporter. Results of representative experiments are shown in Fig. 2A.

When the yeast was grown in a mineral medium with a lower glucose concentration (0.1 %), capacity to transport D-glucose was also observed but the Eadie-

-Hofstee plot of the initial uptake rates of labelled glucose was biphasic (Fig. 1B). The computer-assisted non-linear regression analysis of the data agreed with the presence of two distinct transport modes for glucose with the kinetic parameters presented in Table 2. Similarly to that observed in 2 % glucose-grown cells, no inhibition was observed by the addition of CCCP when the uptake was measured over labelled D-glucose concentration range 0.01-0.1 mM (high-affinity component) or 0.1-2 mM (low-affinity component). Furthermore, no proton movements associated to the sugar transport were detected, over the concentration range where both systems were operating. The results indicated that the systems were not dependent on the transmembrane proton-motive force. To determine whether the specificity of these transporters was similar to that observed for the glucose carrier in 2 % glucose-grown cells, inhibition studies were performed. Estimates of the initial uptake rates of labelled glucose in the presence of the various sugars tested in 2 % glucose-grown cells, showed that the lower-affinity component of glucose transport was competitively inhibited by 2-deoxy-D-glucose and D(-)fructose (Fig. 2B); the remaining sugars had no significant effect on the transport of glucose. Inhibition uptake studies in the higher-affinity range indicated that 2-deoxy-D-glucose and D(+)galactose used this transport system (Fig. 2C). It was found that 0.1 % glucose-grown cells produced a transport system which exhibited the same substrate specificity pattern of the carrier present in 2 % glucose-grown cells and, in addition, a higher-affinity transport system able to accept D(+)galactose, besides D-glucose and 2-deoxy-D-glucose.

Measurements of the initial velocities of labelled D-glucose in cells grown with ethanol, glycerol or acetic acid also showed the presence of saturable mechanisms for glucose transport. In the first type of cells, the Eadie-Hofstee plot of the initial velocities of glucose uptake was biphasic (Fig. 3A), while in acetic acid- (Fig. 3B) and glycerol-grown cells (not shown) monophasic kinetics were observed.

Table 2 shows kinetic parameters for these glucose transport systems, estimated by the application of the computer assisted simulation to the data. To investigate the substrate specificity of these carriers, typical inhibition experiments as those present in Fig. 2 were carried out (not shown). Taking into account the substrate specificity exhibited by the carriers and the values of their kinetic parameters, it was concluded that ethanol-grown cells displayed activity for both low- and high-affinity transport systems, while acetic acid- and glycerol-grown cells produced only the high-affinity one.

The uptake of labelled D-glucose was also measured in cells grown in media containing mixtures of glucose (0.1 %) and 0.5 % (volume fractions) ethanol, glycerol or acetic acid. In all these conditions, the yeast exhibited a diauxic growth. Glucose was the first substrate to be utilized, the second one being consumed only after the sugar exhaustion from the medium (not shown). In cells from the first exponential growth phase, the transport of labelled D-glucose occurred via the low- and high-affinity components. Cells from the second growth phase exhibited activity for glucose transporters, as follows: ethanol consuming phase, activity for the low- and the high- affinity systems; glycerol or acetic acid consuming phase, activity only for the high-affinity system.

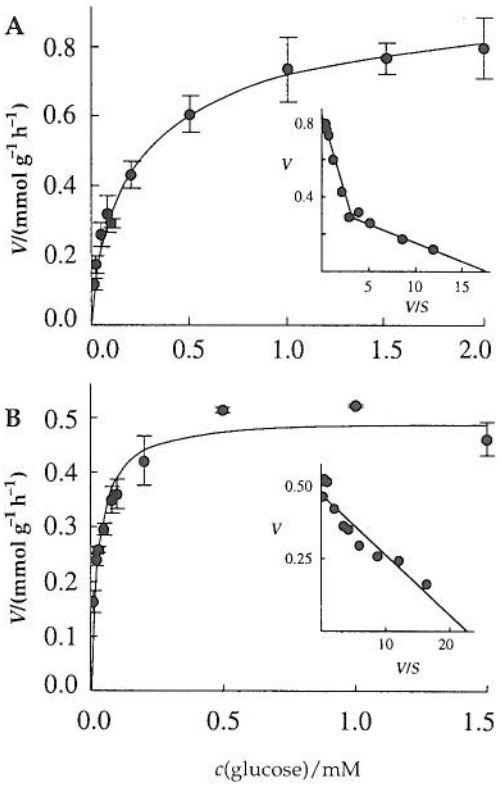

Fig. 3. Initial uptake rates of labelled D-glucose at pH = 5.0 by cells of *D. anomala* IGC 5153 grown with 0.5 % (volume fraction) of ethanol (A) or acetic acid (B). Values are mean ± S.E. (*N*=6). *Inserts*: Eadie-Hofstee plots of the initial uptake rates. Data were fitted by linear regression

Transport of glucose in hybrid vesicles. According to the studies in whole cells, both high- and low-affinity glucose transport systems did not depend on the transmembrane PMF. In order to test this hypothesis and further study the transporter protein(s), the transport of glucose was evaluated in plasma membrane vesicles. However, when using this technique some aspects have to be taken into account such as: (*i*) the isolated plasma membranes by themselves do not form well sealed vesicles, and (*ii*) when secondary active transporters are involved the vesicles must be prepared in order to be able to generate and maintain ion gradients. A usual procedure to circumvent these obstacles is the fusion of yeast plasma membrane fractions with liposomes where cytochrome *c* oxidase as a proton-motive force generating system was reconstituted (*5,7,16,17*). Thus, in our studies, membranes obtained either from acetic acid-grown cells (presence of activity only for the high-affinity system) or from 2 % glucose-grown cells (presence of activity only for the low-affinity system), were fused with *E. coli* liposomes containing cytochrome *c* oxidase (plasma membrane protein to lipid ratio = 1:20). These vesicles, at pH = 6.2, upon energization by TMPD, cytochrome *c* and ascorbate, as described in Materials and Methods, generated and maintained, over at least 15 min., a PMF of about -150 mV, alkaline and negative inside (not

shown). In a first set of experiments, energized and non-energized hybrid vesicles were used to evaluate the transport of glucose. In both situations, the uptake of labelled D-glucose followed over time, in the concentration range where the low- and the high-affinity systems were operating, was identical and the presence of a transvesicular electrochemical gradient of protons did not promote the glucose accumulation beyond diffusional equilibrium (not shown). The results supported the hypothesis that the activity for high- and low-affinity glucose transport systems did not require the involvement of PMF. Therefore, all the subsequent assays on the transport of glucose were carried out in vesicles prepared from plasma membranes fused with liposomes without cytochrome *c* oxidase incorporated.

When the initial uptake rates of labelled D-glucose were measured in hybrid vesicles obtained from acetic acid- or glucose-grown cells, a Michaelis-Menten kinetics was observed for the glucose transport, over the sugar concentration range used in intact cells (Fig. 4).

To estimate the kinetic parameters of the carriers, the percentage of vesicles containing glucose transporters and the weight of the simple diffusion of the sugar across the vesicle membrane were evaluated. For such studies, L-glucose was used, since (*i*) in intact cells, uptake of labelled L-glucose was negligible and it was not recognized as substrate for the glucose carriers, and (*ii*) initial uptake rates of labelled L-glucose and D-glucose in vesicles prepared without plasma membrane (absence of transporters) were equal and linearly related to the external sugar concentrations (Fig. 4). To estimate the percentage of vesicles containing glucose carriers, the uptake of labelled D-glucose and L-glucose was followed with time in hybrid vesicles obtained from acetic acid- and 2 % glucose-grown cells prepared with different membrane protein to lipid ratios. In both type of vesicles, the increase of the amount of membrane protein led to a substantial difference between the uptake of the sugars, D-glucose reaching the diffusional equilibrium more rapidly than the L-glucose (Fig. 5).

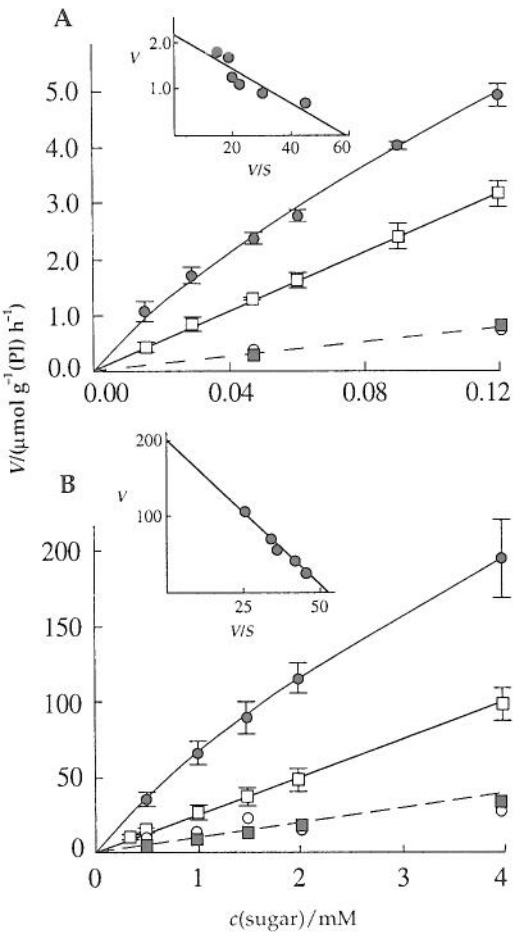

Fig. 4. Initial uptake rates of labelled D- and L-glucose at pH = 6.2 in membrane vesicles prepared from 0.5 % acetic acid-grown cells (A) and 2 % glucose-grown cells (B). Total uptake of D-glucose (●) and L-glucose (❑) in hybrid membrane vesicles prepared at 1:20 protein to *E. coli* phospholipid ratio. Dotted lines represent the average of two independent uptakes of D-glucose (○) and L-glucose (■) in vesicles without plasma membrane protein. Vertical bars denote S.E. of the mean (N=4). *Inserts*: Eadie-Hofstee plots of the initial D-glucose-specific uptake (D-glucose minus L-glucose). Data were fitted by linear regression. Pl= phospholipid.

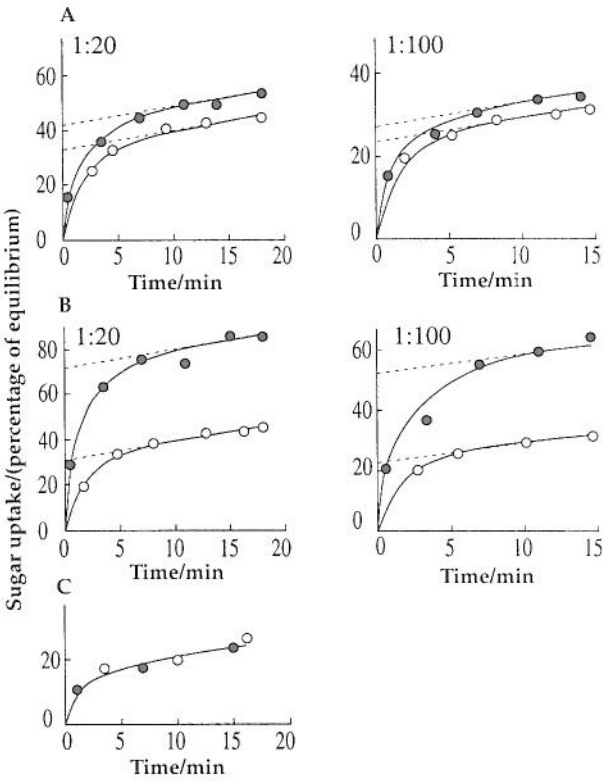

Fig. 5. Time course of labelled D-glucose (●) and L-glucose (○) uptake at pH = 6.2 in vesicles prepared as follows: A, liposomes fused with plasma membrane from 0.5 % acetic acid-grown cells; B, liposomes fused with plasma membrane from 2 % glucose-grown cells and C, liposomes without plasma membrane protein. The initial extravesicular concentration of sugar was 4 mM. The fraction of equilibrium was calculated dividing the internal sugar concentration per sample by the internal sugar concentration at the diffusion equilibrium; an internal vesicle volume of 2.6 μL per mg of phospholipid was used. Numbers indicate plasma membrane protein to *E. coli* phospholipid ratio. Representative experiments performed in duplicate are shown.

Based on the difference between the two distinct sugar uptake curves, and according to the methodology described in the literature (*5,7,18*), the following values for the percentage of hybrid vesicles with D-glucose carriers were obtained, at the protein-to-lipid ratio indicated in brackets: for the high-affinity system 9 % (1:20) and 4 % (1:100); and for the low-affinity one 40 % (1:20) and 29 % (1:100). Vesicles with higher protein-to-lipid ratios did not show a significant increase on the D-glucose-specific uptake (not shown). These results suggested that maximal capacity to incorporate plasma membrane fractions in liposomes to form hybrid vesicles had been achieved, being the 1:20 protein-to-lipid ratio used for subsequent transport studies. In these conditions, a value of about 0.4 μL/min per mg of phospholipid was obtained for the L-glucose diffusion coefficient. The kinetic parameters for the glucose transporters corrected for the simple diffusion and for the percentage of vesicles containing glucose carriers were the following: for the low-affinity system K_m, 3.90 ± 0.27 mM and V_{max}, 504 ± 18 μmol of glucose per g of phospholipid per h; for the high-affinity K_m, 0.036 ± 0.016 mM and V_{max}, 36 ± 3.6 μmol of glucose per g of phospholipid per h.

In order to test whether the substrate specificity of low- and high-affinity systems reconstituted in the hybrid vesicles conformed to the pattern observed in intact cells, inhibition uptake studies were performed. In hybrid vesicles from acetic acid-grown cells, the uptake of D-glucose was competitively inhibited by D(+)galactose and 2-desoxy-D-glucose while D(-)fructose and D(+)xylose did not show any inhibitory effect. On the other hand, in vesicles from 2 % glucose-grown cells, 2-deoxy-D-glucose and D(-)fructose, but neither D(-)galactose nor D(+)xylose, shared this glucose transporter (results not shown). This behaviour was identical to that found in intact cells and constituted an additional proof that the activity(ies) of the glucose carrier(s) by the low- and high-affinity systems was (were) reconstituted in plasma membrane vesicles.

Discussion

In yeasts, sugar transport occurs by two basic mechanisms, namely facilitated diffusion and active transport, essentially proton symports. Transport systems depend on the yeast strain, growth and experimental conditions and the nature of the carbohydrate (*19–21*). According to our results, obtained *in vivo* and *in vitro* assays, *D. anomala* IGC 5153, produced two transport systems for glucose which could be distinguished by their kinetic parameters and substrate specificity: (*i*) a high-affinity system able to accept 2-deoxy-D-glucose and D(+)galactose, and (*ii*) a low-affinity system able to accept 2-deoxy-D-glucose and D(-)fructose but not D(+)galactose. When 2 % of glucose or fructose was used in the culture medium only the low-affinity carrier was found, while in acetic acid- or glycerol-grown cells only the high-affinity one was present. If a lower sugar concentration (0.1 %) or ethanol was used in the medium, both systems were operating. When the cells were cultivated in higher concentrations of sugars such as 2 % glucose or fructose, activity for the high-affinity transporter was not detectable, suggesting that it is constitutive and subject to sugar repression. The low-affinity glucose transporter apparently did not behave as constitutive since it was not found in acetic acid- and glycerol-grown cells. Further studies will be necessary to clarify its regulation.

Regarding the mechanisms involved in these glucose transport systems, the presence of facilitated diffusion for either the low- or the high-affinity components was supported altogether by the following pieces of evidence: (*i*) absence of inhibitory effect of the ionophore CCCP, and (*ii*) no alkalinization signals associated with the glucose transport. Facilitated diffusion systems for glucose transport with values for K_m of the same magnitude of those measured for the low-affinity system of *D. anomala*, are widespread among yeasts. In *Sacch. cerevisiae*, it has been shown that glucose transport is also possible by a facilitated diffusion mechanism, the affinity depending on the physiological state of the cells (*22,23*). On the other hand, lower values of K_m, as those obtained for the high-affinity system of *D. anomala*, are uncommon. Instead, they are more frequently associated to sugar-proton symporters like those described in a number of non-*Saccharomyces* yeasts, including *Candida* sp. (*24-26*), *Rhodotorula* sp. (*27,28*), *Kluyveromyces marxianus* (*29*) and *Brettanomyces intermedius* (*30*). In the case of *Dekkera anomala* IGC 5153, the results obtained from the studies in intact cells, point to facilitated diffusion as the most probable mechanism involved in glucose carriers even for the highest-affinity one.

In the second part of this work, an attempt was made at reconstitution of the glucose carrier activities in plasma membrane vesicles from 2 % glucose- or acetic acid-grown cells, exhibiting activity for either the low- or high-affinity component, respectively. In these vesicular systems, the transport of labelled D-glucose occurred by Michaelis-Menten kinetics. The K_m values for the glucose transport by the low- or high-affinity systems as well as the substrate specificity patterns were identical to those obtained in intact cells. Additionally, the establishment of a PMF through the membrane of the hybrid vesicles did not lead to glucose accumulation. Therefore, the driving force for glucose translocation appeared to be provided exclusively by the concentration gradient. These results pointed to the involvement of facilitated diffusion mechanisms in the glucose transport either by the high- or the low-affinity components. We must point out that the magnitude of the PMF generated through the vesicular membranes (about -150 mV, negative and alkaline inside), was enough to support the accumulation of glucose, galactose and maltose by proton symports in similar hybrid vesicles prepared with plasma membranes from *C. utilis* (*16*), *K. marxianus* (*5*) and *Sacch. cerevisiae* (*17*), respectively. In the case of the high-affinity system, one issue that can be raised is that with low amounts of carrier protein, as in the vesicular system from acetic acid-grown cells (only about 10 % of vesicles contained glucose carriers), accumulation of solutes through PMF driven transports could be low and not easily distinguished from the passive diffusion background. However, it doesn't appear to be the present case, since the specific uptake of labelled D-glucose through this high-affinity system (*i*) conformed to Michaelis-Menten kinetics rather than to a first order one,

with a K_m value similar to that measured in intact cells, and (*ii*) was competitively inhibited by D(+)galactose and 2-desoxy-D-glucose and not by D(-)fructose. These results strongly suggested that the activity for the high-affinity system was indeed reconstituted in such vesiclar system.

Another aspect explored in our work was the analysis of the possible relationship between sugar transport and growth of the yeast in media with 0.1 and 2 % glucose. It was observed that the values of the yield coefficient ranged from 0.28 to 0.40 g/g with the decrease of sugar concentration. In the first case, the yeast appeared to be preferentially fermenting the sugar while in the second one a higher contribution of respiration for the sugar catabolism seemed to be occurring. Relating transport with growth parameters, we found that the values of the specific glucose transfer rates, obtained as the ratio μ_{max}/Y, were very similar to those of the corresponding V_{max} of glucose transport. On the other hand, as it has been reported in several yeasts, the strains used in this study appeared to be able to adapt their glucose transport systems to the sugar concentration in the culture, a carrier with a high affinity ($K_m \approx 0.02$ mM) being synthesized when growth was carried out with low glucose supply. This could be relevant from an ecological point of view, allowing that species to be better adapted to survival in environments with low sugar levels. In the fermentative yeast *Sacch. cerevisiae*, glucose transport occurs by lower affinity carriers (*19*) which, during grape must fermentation, could account for the relatively high residual sugar concentrations often present in wine. The presence of a glucose carrier with a higher affinity constant for the sugar in *D. anomala* could allow this species to have a competitive advantage over *Sacch. cerevisiae* in wine. This property of *D. anomala* may be associated with its ability to use minute amounts of unfermented sugar thus spoiling the wine.

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Transport glukoze u *Dekkera anomala*, vinskom kvascu kvarenja

Sažetak

Kada je rastao u podlozi s 0,1 i 2 % glukoze, Dekkera anomala IGC5153 imao je relativno malu specifičnu brzinu rasta, a koeficijent iskorištenja smanjivao se od 0,40 na 0,28 g/g kako se povećavala početna koncentracija šećera u podlozi. Ispitivanjem transporta, in vitro i in vivo, nađeno je da kvasac proizvodi dva transportna sustava za glukozu koji su se mogli razlikovati prema kinetičkim parametrima i specifičnosti supstrata:

(1) visokoafinitetni sustav sposoban da prihvati 2-deoksi-D-glukozu i D(+)-galaktozu i

(2) niskoafinitetni sustav sposoban da prihvati 2-deoksi-D-glukozu i D-(-) fruktozu, ali ne D(+) galaktozu. Kada se u podlozi nalazilo 2 % glukoze ili fruktoze nađen, je samo niskoafinitetni sustav, dok je u stanica koje su rasle na octenoj kiselini ili glicerolu bio prisutan samo visokoafinitetni sustav. Ako je u podlozi bila mala koncentracija šećera (0,1 % maseni udjel po volumenu podloge) ili etanol, djelovala su oba sustava. Rezultati pokazuju da je olakšana difuzija najvjerojatniji mehanizam u transportu glukoze s pomoću oba prijenosnika. Vrijednosti specifičnih brzina transporta glukoze u kulturama s 0,1 ili 2 % glukoze vrlo su slične vrijednostima V_{max} dobivenih pri transportu glukoze.