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Stimulation of Zero-*trans* Rates of Lactose and Maltose Uptake into Yeasts by Preincubation with Hexose To Increase the Adenylate Energy Charge▽

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Initial rates of sugar uptake (zero-*trans* rates) are often measured by incubating yeast cells with radiolabeled sugars for 5 to 30 s and determining the radioactivity entering the cells. The yeast cells used are usually harvested from growth medium, washed, suspended in nutrient-free buffer, and stored on ice before they are assayed. With this method, the specific rates of zero-*trans* lactose uptake by *Kluyveromyces lactis* or recombinant *Saccharomyces cerevisiae* strains harvested from lactose fermentations were three- to eightfold lower than the specific rates of lactose consumption during fermentation. No significant extracellular β-galactosidase activity was detected. The ATP content and adenylate energy charge (EC) of the yeasts were relatively low before the [^{14}C]lactose uptake reactions were started. A short (1- to 7-min) preincubation of the yeasts with 10 to 30 mM glucose caused 1.5- to 5-fold increases in the specific rates of lactose uptake. These increases correlated with increases in EC (from 0.6 to 0.9) and ATP (from 4 to 8 μmol · g dry yeast^{-1}). Stimulation by glucose affected the transport V_{max} values, with smaller increases in K_m values. Similar observations were made for maltose transport, using a brewer's yeast. These findings suggest that the electrochemical proton potential that drives transport through sugar/H$^+$ symports is significantly lower in the starved yeast suspensions used for zero-*trans* assays than in actively metabolizing cells. Zero-*trans* assays with such starved yeast preparations can produce results that seriously underestimate the capacity of sugar/H$^+$ symports. A short exposure to glucose allows a closer approach to the sugar/H$^+$ symport capacity of actively metabolizing cells.

The ability of the yeast *Kluyveromyces lactis* to metabolize lactose results from the presence of a lactose permease and a lactase (β-galactosidase). The β-galactosidase is thought to be intracellular (8, 36, 41) and hydrolyzes lactose into glucose and galactose. Lactose uptake by *K. lactis* is mediated by a transport system inducible by lactose and galactose (9) and is an active process that permits the intracellular accumulation of lactose against a concentration gradient (6, 9). A lactose/H$^+$ symport is believed to be responsible in this and related *Kluyveromyces* species (4, 7, 9, 44).

The maltose uptake system of *Saccharomyces cerevisiae* has been studied extensively because of the importance of maltose in industrial processes such as beer production. Maltose uptake occurs via a proton symport mechanism, in which one proton is cotransported with each maltose molecule (39). In *Saccharomyces* yeasts, maltose transporters are encoded by several genes, including at least *MALx1* (where x = 1 to 4 and 6 and indicates one of five *MAL* loci, each on a different chromosome), *AGT1* (17, 19, 23, 43), and the relatively recently discovered *MTT1* gene (11, 37). All the proteins encoded by these genes are thought to be maltose/H$^+$ symporters. Some can carry other α-glucosides as well as maltose.

Solute/H$^+$ symports couple the transport of solute into the cell with the thermodynamically favorable transport of protons into the cell. The proton motive force that drives protons into the cell results from the transmembrane electrochemical gradient of protons (Δp). Δp has two components: the difference in pH, ΔpH, between the (usually acidic) medium and the near-neutral cytosol, and the membrane potential, ΔΨ (50 to 200 mV, the cytosol negative compared to the cell exterior) (18, 40, 42). In *Saccharomyces* yeasts, Δp is generated largely by the plasma membrane ATPase (Pma1p), which is the major membrane protein and pumps protons out of the cell with a stoichiometry of 1 proton/ATP hydrolyzed to ADP (reviewed in references 2 and 46). This ATPase accounts for a large proportion of ATP consumption during yeast growth, at least 10 to 15% and over 25% during fermentative growth on actively transported disaccharides such as maltose (50) or lactose, where one proton must be pumped out for every sugar molecule entering the cell.

Zero-*trans* rates of sugar uptake into yeasts are most often measured using cells that are harvested, washed to remove growth medium, and stored at 0 to 5°C in nutrient-free buffer for minutes or hours before they are assayed. Rapid handling and storage at low temperatures are attempts to preserve the often nonconstitutive transporters in the state existing at the moment of harvest. These starved cell suspensions are then assayed after they are equilibrated to the assay temperature, either by short (typically 5-s to 30-s) incubations with radiolabeled sugar, followed by the determination of the amount of radioactivity incorporated into the cells or by rather longer

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incubations in weakly buffered solutions of the sugar, during which the pH changes caused by the operation of sugar/H^+ symports are recorded (see, e.g., references 1, 5, 27, 32, 39, and 48). Here we report a study of lactose transport by *K. lactis* and two recombinant *S. cerevisiae* strains expressing the *K. lactis LAC* genes. The zero-*trans* lactose uptake rates measured by using yeast harvested while growing on lactose and suspended in nutrient-free buffer were severalfold lower than the lactose consumption rates at the time of harvest. A short preincubation period of the starved cell suspensions with glucose or fructose immediately before the uptake assays increased the lactose uptake rates. This stimulation of lactose transport correlated with increases in the yeast ATP level and adenylate energy charge (EC) {where EC = ([ATP] + 0.5 × [ADP])/([ATP] + [ADP] + [AMP])} during the preincubation with glucose. Similar observations were made for maltose transport in brewer's yeast.

MATERIALS AND METHODS

Yeasts. The lactose-consuming yeasts used were *K. lactis* strain CBS2359 and *S. cerevisiae* recombinant strains T1 and T1-E. Strain T1 carries the *LAC12* (lactose permease) and *LAC4* (β-galactosidase) genes of *K. lactis* on a multicopy plasmid (12). Strain T1-E is a derivative of strain T1, obtained by a long-term evolutionary adaptation experiment (15). Strain A63015 (A15) is an industrial brewer's lager yeast from VTT's collection. The maltose-negative, Ura3-Δ, laboratory strain S150-2B was transformed with a multicopy plasmid carrying *URA3* and a *MALx1* gene isolated from strain A15, yielding the strain S150-2B/MALx1. The *MALx1* gene is expressed from a PGK1 promoter. Construction of this strain will be described elsewhere (V. Vidgren, A. Huuskonen, M.-L. Vehkomäki, L. Ruohonen, and J. Londesborough, unpublished data). It was grown on minimal medium lacking uracil and containing 2% glucose.

Yeasts for stocks were grown to early stationary phase (strains A15 and S150-2B/MALx1) or to late growth phase (lactose-consuming strains) and then harvested and stored at −80°C as suspensions in 30% glycerol containing 200 mg of fresh yeast mass · ml^{-1}.

Radioactive sugars. [D-glucose-1-^{14}C]lactose and [U-^{14}C]maltose were CFA278 and CBF182, respectively, purchased from Amersham Biosciences (Espoo, Finland). According to high-performance liquid chromatography analysis, [^{14}C]glucose accounted for about 2% of the total label in the [D-glucose-1-^{14}C]lactose used.

Lactose fermentations and lactose transport assays. The lactose-consuming yeasts were grown in the defined mineral medium described by Verduyn et al. (47) but with doubled concentrations of trace elements and vitamins. The lactose was autoclaved separately and added after the medium was heat sterilized, to a concentration of 20 to 25 g · $liter^{-1}$, as indicated. To avoid a major drop in pH during cultivation, the medium was supplemented with 100 mM potassium hydrogen phthalate. The initial pH was adjusted to 4.5 with NaOH. The cultivations were carried out in Erlenmeyer flasks filled with medium to 40% of the total volume (0.5, 1.0, or 2.0 liters). Media were inoculated with glycerol stocks to an initial optical density at 600 nm (OD_{600}) of 0.03 to 0.10 and shaken (150 rpm) at 30°C (or at 18°C when so stated).

Some fermentations were sampled at 1- to 3-h intervals to determine biomass and residual lactose. Lactose consumption rates were calculated from changes in the residual lactose concentration and the average dry yeast concentration over the interval when dry yeast concentration increased from about 0.6 to 1.5 g · $liter^{-1}$, which corresponded to an increase in lactose consumption from about 15 to 60% (10 to 20% for the *K. lactis* strain at 18°C) of the initial lactose. Over shorter time intervals within this time frame, specific consumption rates fluctuated markedly (up to 50%) but with no reproducible pattern among the experiments. These fluctuations probably resulted from random errors in the small differences between large lactose concentrations, so we used only the more reliable data from longer time intervals.

Yeast samples for zero-*trans* lactose uptake assays were harvested when the dry yeast concentration was 0.6 to 1.5 g · $liter^{-1}$ (the same interval that was used to calculate the lactose consumption rates, as described above) by centrifugation (5 min at 9,000 × *g*), washed twice with ice-cold water, and suspended to 200 mg fresh yeast mass · ml^{-1} in ice-cold 0.1 M tartrate-Tris buffer (pH 4.2). The strongly flocculating strains T1 and T1-E were washed with a solution of ice-cold NaCl at 15 g · $liter^{-1}$ (pH 3.0) instead of water. Zero-*trans* rates of lactose uptake were determined at 2 mM or 20 mM lactose and at 18, 20, or 30°C, essentially as described previously for maltose uptake (14, 27, 39). Portions of the yeast suspension (0.3 to 0.6 ml) were equilibrated to 18 or 20°C for 5 min or to 30°C for 10 min immediately before they were assayed. Reactions were started by adding 40 μl of equilibrated yeast suspension to 20 μl of labeled lactose solution (6 mM [^{14}C]lactose at about 1,000 cpm · $nmol^{-1}$ for assays at 2 mM lactose or 60 mM [^{14}C]lactose at about 100 cpm · $nmol^{-1}$ for assays at 20 mM lactose). Reactions were stopped after 10 s by adding 10 ml of ice-cold water. Assays were done in duplicate, and linearity with respect to time was confirmed by 15-s and/or 20-s assays. The rates determined with the longer times were >90% of those determined with the 10-s assays, with the exception of the *K. lactis* samples assayed at 30°C with 20 mM lactose. In this case, the rates calculated from the 15-s and 20-s assays were, respectively, about 85% and 65% of those calculated from the 10-s assays, suggesting that the results from the 10-s assays may also have been significantly lower than the true initial rates. Zero time assays (which give the amount of radioactivity on the membranes after zero seconds of incubation of yeast in the reaction mixture) were performed by first adding 10 ml of ice-cold water to the 20 μl of [^{14}C]lactose solution and then adding the 40 μl of yeast suspension. Rates were normalized to the yeast dry mass, determined by washing the yeast with water, and drying it overnight at 105°C.

For the preincubation step with glucose (or fructose), 500 μl of yeast suspension was equilibrated to 18 or 20°C for 5 min or to 30°C for 10 min and then mixed with 20 μl of 280 or 700 mM hexose to give 11 or 27 mM hexose in the yeast suspension. After samples were incubated further for the times indicated, zero-*trans* sugar uptake was assayed as described above, so that the final reaction mixtures contained ≤7 mM or 18 mM hexose. In control experiments, water instead of hexose solution was added to the yeast suspension. The possible instantaneous effects (e.g., competitive inhibition) of hexose on lactose transport were checked by assays in which hexose was mixed with the labeled lactose before the yeast suspension was added, to give final hexose concentrations of up to 110 mM.

To determine the effect of glucose stimulation on the kinetic parameters of lactose uptake, the *K. lactis* strain grown at 18°C was assayed at 18°C with lactose concentrations of between 0.5 and 20 mM. The suspension of harvested yeast was used directly or preincubated with 27 mM glucose for 5 or 10 min before it was assayed with [^{14}C]lactose. Differences between the rates of the samples preincubated for 5 min and the samples preincubated for 10 min were <5%. Replicate experiments were done with independently grown yeast suspensions. The V_{max} and K_m values were calculated by using the direct linear plot of Eisenthal and Cornish-Bowden (13).

Maltose transport assays. Strain A15 was grown in Erlenmeyer flasks filled to 40% of their total volume with YP (1% yeast extract, 2% peptone) medium containing 40 g · $liter^{-1}$ maltose and shaken (150 rpm) at 24°C. For strain S150-2B/MALx1, minimal medium lacking uracil and containing 20 g · $liter^{-1}$ glucose was used. Yeasts were harvested when the dry yeast concentration was 1.0 to 2.3 g · $liter^{-1}$ (OD_{600} of 3 to 6.5) and then washed and suspended in 0.1 M tartrate-Tris (pH 4.2), as described above. Zero-*trans* rates of maltose uptake were determined with or without glucose stimulation at 5 mM maltose (about 1,000 cpm · $nmol^{-1}$), unless stated otherwise, at 20°C as described above for lactose uptake.

Trehalose and glucose analyses. Trehalose was extracted from washed cells by boiling them in water for 10 min and converted to glucose by treatment with trehalase (Sigma-Aldrich, Helsinki, Finland). Glucose was assayed enzymatically with hexokinase and glucose-6-phosphate dehydrogenase (both from Sigma-Aldrich).

Preparation of spheroplasts. Spheroplasts were prepared by modifications of standard methods (38). The *S. cerevisiae* recombinants T1 and T1-E were grown to an OD_{600} of 2 to 3 and harvested by centrifugation (5 min at 5,000 × *g*). The yeast pellet was suspended to 100 mg of fresh yeast · ml^{-1} in 0.1 M EDTA-2% mercaptoethanol (pH 7.0) and incubated at 30°C for 30 min. Yeast cells were collected and washed with 10 to 15 ml of 1 M sorbitol in buffer A (25 mM potassium phosphate buffer [pH 6.5] containing 2 mM $MgCl_2$, 1 mM EDTA, and 0.1 mM dithiothreitol) and resuspended to 40 mg fresh yeast · ml^{-1} in buffer A. Pepstatin A and phenylmethylsulfonyl fluoride (PMSF) were added to final concentrations of 10 μg · ml^{-1} and 170 μg · ml^{-1}, respectively. Zymolyase 100T (Seikagaku, Japan) was added to a final concentration of 30 μg · ml^{-1}, and the cell suspension was incubated at 30°C until spheroplast formation (estimated by the decreasing OD_{600} of periodic dilutions of 100-μl samples into 3 ml of water) was nearly complete. The spheroplast suspension was centrifuged (10 min at 1,000 × *g*), and the supernatant was collected. The pellet was washed with 1 M sorbitol in buffer A containing pepstatin A and PMSF, and the resulting supernatant was combined with the previous supernatant to give the cell wall/periplas-

mic fraction. The pellet was then suspended in 10 to 15 ml of buffer A containing pepstatin A and PMSF, and the spheroplasts were allowed to lyse to give the cytoplasmic fraction. For *K. lactis* CBS2359, the sorbitol concentration was increased to 1.2 M, and the zymolyase concentration was decreased to 6 $\mu g \cdot ml^{-1}$ to avoid premature lysis of the spheroplasts, and the incubation time was extended to 60 min.

Enzyme assays. The β-galactosidase activity was assayed by using *p*-nitrophenyl-β-D-galactopyranoside (pNPG) as the substrate (31). Briefly, 800-μl samples of appropriate dilutions (at least 1:10) of the cell extract in buffer Z (100 mM sodium phosphate buffer [pH 7.0], 10 mM KCl, 1 mM $MgSO_4$, 0.28% [vol/vol] 2-mercaptoethanol) were transferred to a spectrophotometer cuvette, and the reaction was started by adding 200 μl of 4 $mg \cdot ml^{-1}$ pNPG. The reaction at room temperature (ca. 23°C) was followed by reading the A_{405} over time. The assay was calibrated by reading the A_{405} of standard solutions (0.01 to 0.2 mM) of *p*-nitrophenol in the same buffer.

Phosphoglucoisomerase (PGI) activity was assayed by coupling its catalyzed conversion of fructose-6-phosphate to glucose-6-phosphate with the activity of glucose-6-phosphate dehydrogenase (G6PDH). The conversion of NADP to NADPH was followed by measuring the change in A_{340} at room temperature (ca. 23°C). The reaction mixture consisted of 50 mM HEPES-KOH (pH 7.5) containing 10 mM $MgCl_2$, 0.1 mM EDTA, 0.4 mM NADP, 10 mM fructose-6-phosphate, and 1.5 $U \cdot ml^{-1}$ G6PDH (Sigma).

Invertase activity was assayed by following the glucose released from sucrose. The invertase reaction mixture consisted of 50 mM sodium acetate buffer (pH 4.5) containing 20 mM sucrose. The reaction was started by adding the extract and then stopped after 10 or 20 min of incubation at room temperature (ca. 23°C) by transferring the mixture to a boiling water bath for 5 min. The glucose formed during this reaction was then measured using hexokinase and glucose-6-phosphate dehydrogenase.

One unit of enzyme activity (U) catalyzes the conversion of 1 μmol of substrate per min under the stated conditions. Specific enzyme activities are expressed as U per g of fresh yeast mass.

Total protein was determined by the Lowry method (25), using ovalbumin as the standard.

Adenine nucleotide analyses. Intracellular adenine nucleotides were determined as described previously (16). For analyses of total adenine nucleotides (intracellular plus extracellular) in fermentation, 9-ml samples were collected and immediately injected into 1.0 ml of ice-cold 5.0 M perchloric acid (PCA). Parallel samples (20 to 40 ml) were withdrawn and centrifuged (5 min at 10,000 × *g*), and 9 ml of the clear supernatant was injected into 5.0 M PCA for the determination of extracellular adenine nucleotides. The yeast pellet was washed with ice-cold water and dried overnight at 105°C for the determination of yeast dry mass. Intracellular adenine nucleotide levels were calculated by the difference between the total adenine nucleotides (intra- plus extracellular) and the extracellular adenine nucleotides.

Changes in adenine nucleotides during glucose stimulation were assayed by using yeast harvested and suspended to a fresh yeast mass of 200 $mg \cdot ml^{-1}$ in ice-cold 0.1 M tartrate-Tris buffer (pH 4.2), as described above for the transport assays. For the analyses of total adenine nucleotides, 1.5-ml portions of the yeast suspension were first equilibrated to 20°C for 5 min or to 30°C for 10 min, and 60 μl of 700 mM glucose was then added (27 mM final glucose concentration). After further incubation for up to 20 min at 20°C or 30°C, the yeast suspension was quenched with 8.5 ml of ice-cold 0.59 M PCA. For the estimation of extracellular adenine nucleotides, 100 μl of 700 mM glucose was added to 2.5 ml of the yeast suspension that had been previously equilibrated to 20°C for 5 min or to 30°C for 10 min. The suspension was incubated at the respective temperature for another 10 min and filtered through a 0.45-μm membrane, and 1.5 ml of the filtrate was added to 8.5 ml of ice-cold 0.59 M PCA. Extracellular ATP levels were very low (<0.6%) compared to the intracellular levels. Extracellular ADP and AMP levels were also low but sometimes accounted for up to 20% of the total (intra- plus extracellular) levels. Nevertheless, these extracellular levels were low enough so they did not disturb calculations of the EC by more than 0.01. Control experiments were done in which water was added to the yeast suspension instead of glucose solution.

PCA extracts were handled and adenine nucleotides were assayed by using firefly luciferase, essentially as described by Lundin (29) and modified by Guimarães and Londesborough (16).

RESULTS

Lactose consumption rates during fermentation. Figure 1 shows specific rates of lactose consumption during shake-flask fermentations of lactose by *K. lactis* or the recombinant *S. cerevisiae* strains T1 and T1-E. Specific consumption rates were calculated from the changes in lactose concentration and the average dry mass concentration over the interval when the dry yeast concentration increased from about 0.6 to 1.5 $g \cdot liter^{-1}$ (see Materials and Methods). The *K. lactis* strain and the evolved *S. cerevisiae* recombinant strain T1-E exhibited about 2.5-fold higher lactose consumption rates than the nonevolved recombinant strain T1. For the *K. lactis* strain, specific rates of consumption were about 2.2-fold higher at 30°C than at 18°C.

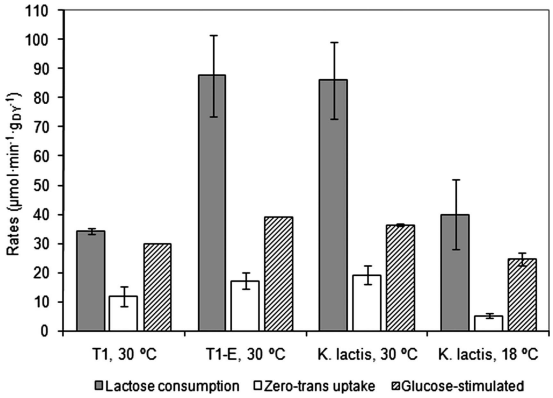

FIG. 1. Lactose consumption rates (gray columns) during fermentation for recombinant *S. cerevisiae* strains T1 and T1-E at 30°C and for *K. lactis* strain CBS2359 at 30 and 18°C. The zero-*trans* uptake rates at 20 mM lactose (white columns) and the glucose-stimulated rates (striped columns) are also shown for direct comparison. The assay temperature (the same as that in fermentation) is indicated. Lactose consumption rates are the average values with standard deviations (SDs) of 2 to 5 independent fermentations. Zero-*trans* rates are the averages with SDs of 3 to 8 determinations with independently grown yeast suspensions. Glucose-stimulated rates are the zero-*trans* lactose (20 mM) uptake rates obtained after optimal preincubation of the yeast suspension with 27 mM glucose (single experiments with T1 and T1-E and averages with SDs of 2 to 3 independent experiments with *K. lactis*).

Zero-*trans* lactose uptake rates. For uptake assays, the yeasts were harvested from lactose fermentations when the dry yeast concentration was between 0.6 and 1.5 $g \cdot liter^{-1}$ (i.e., in the same interval that was used to calculate the specific rates of lactose consumption shown in Fig. 1). After yeasts were washed and suspended in ice-cold tartrate-Tris (pH 4.2), they were equilibrated to the assay temperature, and zero-*trans* rates of lactose uptake were determined (Fig. 2). At 2 mM lactose, zero-*trans* uptake rates were two- to threefold higher at 30°C than at 20°C. At 30 and 18°C, rates were 1.5- to 2-fold higher at 20 mM than at 2 mM lactose, which is consistent with K_m values of 1 to 3 mM (6, 9). The zero-*trans* rates at a saturating lactose concentration (20 mM) were clearly lower than those of lactose consumption in fermentations at the same temperature (Fig. 1). The highest difference (7.5-fold) was for the *K. lactis* strain at 18°C, while the lowest (2.9-fold) was for T1 at 30°C.

Eliminating the known differences in experimental conditions between the fermentations and the zero-*trans* assays did not decrease the large differences in their specific rates. For example, when harvested yeast (strain T1-E) was suspended in and the zero-*trans* assays were performed in defined mineral

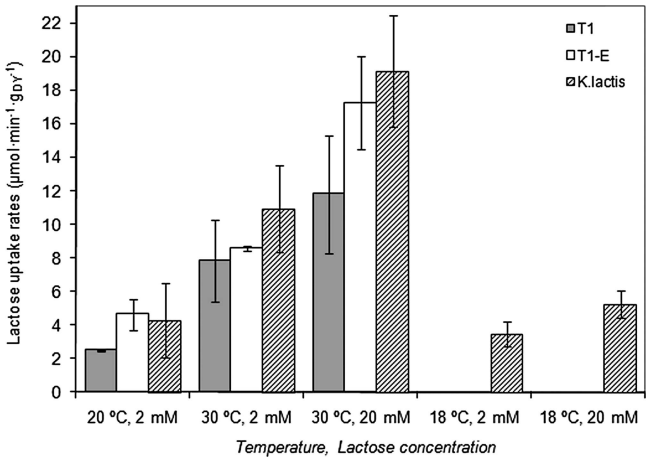

FIG. 2. Zero-*trans* lactose uptake rates of the recombinant *S. cerevisiae* strains T1 (gray columns) and T1-E (white columns) and the *K. lactis* strain CBS2359 (striped columns). The three yeasts were grown at 30°C, and the rates were measured by transport assays using ^{14}C-labeled lactose at 20°C with 2 mM lactose and at 30°C with 2 and 20 mM lactose, as indicated. *K. lactis* was also grown at 18°C and assayed at 18°C with 2 and 20 mM lactose. Error bars are the standard deviations of 2 to 8 determinations with independently grown yeast suspensions.

medium (pH 4.5) without lactose or in potassium hydrogen phthalate (pH 4.2), the zero-*trans* uptake rates were the same or 20 to 40% lower than those measured in tartrate-Tris (pH 4.2). Evidently, zero-*trans* uptake rates determined under conditions (temperature, pH, buffer composition, and saturating lactose concentration) very close to those in the fermentation could not account for the observed lactose consumption rates.

β-Galactosidase activity is intracellular. Carvalho-Silva and Spencer-Martins (7) reported a cell-bound extracellular β-galactosidase activity for some but not all *Kluyveromyces marxianus* strains. We reinvestigated whether *K. lactis* strain CBS2359 and the lactose-utilizing recombinant *S. cerevisiae* strains showed extracellular β-galactosidase activity, which might account for the discrepancy between zero-*trans* lactose uptake rates and lactose consumption rates. No activity (<0.05 U · g fresh yeast^{-1}) was detected in supernatants from the cultivations, showing that β-galactosidase was not excreted in a stable form. To investigate the possibility of cell wall or periplasmic β-galactosidase activity, spheroplasts were prepared, and the activities of β-galactosidase, PGI, and invertase in cell wall/periplasmic, and cytoplasmic fractions were compared (Table 1). Cytoplasmic β-galactosidase activity was 21-fold higher in T1-E than in T1 (in agreement with our previous results; 15) and 1.5-fold higher in T1-E than in the *K. lactis* strain. The activity of the cytosolic marker enzyme PGI in the periplasmic fraction was 7 to 12% of its total (cytoplasmic plus periplasmic) activity, indicating that some spheroplasts broke during the preparation. Invertase activity was two- to fivefold higher in the cell wall/periplasmic fraction than in the cytoplasm, as expected for this mainly cell wall-bound enzyme (34). For T1 and T1-E, the proportions of total activity found in the periplasmic fractions were lower (1.5 to 2%) for β-galactosidase than for PGI (7 to 10%), indicating that β-galactosidase was located intracellularly in these recombinant strains. For the *K. lactis* strain, the proportion of β-galactosidase activity in the periplasmic fraction was slightly higher (15%) than that (12%) of PGI, but the difference was close to the experimental error. These results show that the proportions of total β-galactosidase activity outside the cell membrane were very small compared to the intracellular activities for all three yeasts. The β-galactosidase activities shown in Table 1 were determined at pH 7.0, and β-galactosidase from *K. lactis* has at least 10-fold lower activity at pH 4.5 than at pH 7.0 (8, 20, 33). The absolute amounts of possible extracellular enzyme activity at the pH of fermentation (pH <4.5) were therefore also small compared to that of the lactose transport activities and could not account for the discrepancy between lactose consumption rates and zero-*trans* uptake rates.

Stimulation of zero-*trans* lactose uptake by incubation of the yeast suspension with glucose or fructose. Because lactose uptake by *Kluyveromyces* yeasts occurs by a proton symport (4, 7, 9), it depends on the transmembrane proton motive force that is generated by the plasma membrane H^+/ATPase. The low ATP levels and adenylate EC expected (3) in yeasts harvested before diauxie and then starved, even briefly, might restrict zero-*trans* lactose uptake. Yeast suspensions were therefore preincubated with glucose for various times immediately before lactose uptake was assayed. At 30°C, the incubation of *K. lactis* suspensions with 11 or 27 mM glucose caused a rapid (30-s) increase of about 60% in the uptake of 20 mM lactose (Fig. 3). The peak stimulation (about 90%) was

TABLE 1. Distributions of enzyme activities and protein in cytoplasm and periplasm[a]

Strain	Cell fraction measured	U enzymatic activity · g fresh yeast^{-1} ± SD (% of total)			mg protein · g fresh yeast^{-1} ± SD (% of total)
		β-Galactosidase	PGI	Invertase	
T1	Cytoplasm	13.3 ± 0.7	261 ± 15	3.7	65.4 ± 2.0
	Periplasm	0.2 ± 0.1 (1.5)	28.1 ± 2.5 (9.7)	7.2 (66)	63.8 ± 3.0 (49)
T1-E	Cytoplasm	272 ± 19	155 ± 7	2.5	61.6 ± 6.9
	Periplasm	5.5 ± 1.3 (2.0)	11.7 ± 1.1 (7.0)	11.5 (82)	61.6 ± 5.4 (50)
K. lactis CBS2359	Cytoplasm	179 ± 29	128 ± 3	0.5	52.3 ± 3.2
	Periplasm	32.6 ± 6.0 (15)	17.8 ± 0.4 (12)	1.9 (79)	70.4 ± 4.6 (57)

[a] Spheroplasts were prepared from the recombinant *S. cerevisiae* strains T1 and T1-E and *K. lactis* strain CBS2359. Enzyme activities and protein amounts in the cytoplasmic and cell wall/periplasmic fractions were assayed. Except for invertase data, results are averages ± standard deviations (SDs) of two to four assays. Values in parentheses are the enzyme activities or protein amounts found in the periplasm as percentages of the total enzyme activity or protein amount.

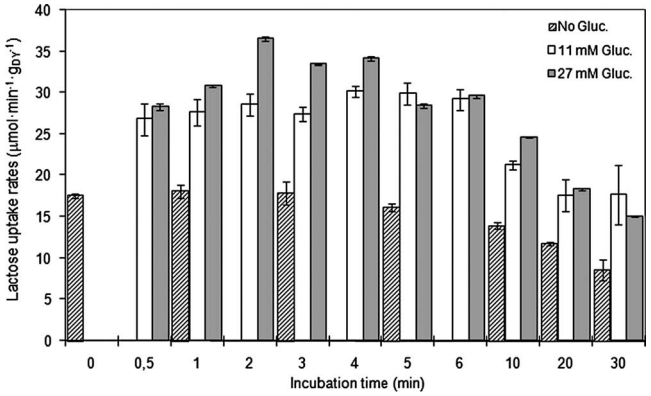

FIG. 3. Stimulation of *K. lactis* CBS2359 zero-*trans* lactose uptake rates by preincubation of the yeast suspension with glucose. The yeast suspension was first equilibrated for 10 min at 30°C, after which glucose was added to a final concentration of 11 mM (white columns) or 27 mM (gray columns). Lactose uptake assays (30°C, 20 mM lactose) were then performed after further incubation of the suspension at 30°C for the times indicated. Results of control experiments, without glucose addition, are also shown (striped columns). Error bars show the range of duplicate assays.

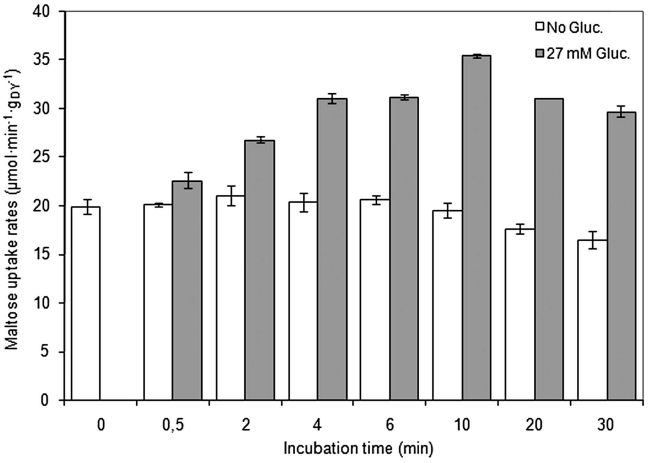

FIG. 4. Stimulation of brewer's yeast (strain A15) zero-*trans* maltose uptake rates by preincubation of the yeast suspension with glucose. The yeast suspension was first equilibrated for 5 min at 20°C, after which glucose was added to a final concentration of 27 mM (gray columns). Maltose uptake assays (20°C, 5 mM maltose) were then performed after further incubation of the suspension at 20°C for the times indicated. Results of control experiments, without glucose addition, are also shown (white columns). Error bars show the range of duplicate assays.

seen between 2 and 4 min, followed by a decline over the next 26 min. Without the glucose addition, the uptake rate was stable for the first 5 min after the 10-min preincubation and then declined by about 50% over the next 25 min. For the initial concentration of 27 mM glucose, 90% of the glucose in the yeast suspension was consumed within 10 min (data not shown). Fructose (27 mM) had essentially the same effect as glucose, causing a twofold stimulation of lactose uptake (30°C, 20 mM lactose) between 1 and 10 min after its addition to the suspensions (data not shown). Similar results were obtained with the recombinant *S. cerevisiae* strains T1 and T1-E (stimulations of 2- to 2.3-fold by 2- to 5-min preincubation with 11 or 27 mM glucose). The largest stimulation observed was 4.9-fold for the *K. lactis* strain harvested from lactose fermentation at 18°C and assayed at 20 mM lactose and 18°C, after preincubation with 27 mM glucose.

These 1.6- to 4.9-fold stimulation values were not observed when glucose was added to the [^{14}C]lactose solution, before the addition of the yeast suspension. In this case, instead of being preincubated with glucose before the uptake assay, the yeasts were exposed to the same final concentrations of glucose (7 or 18 mM) or to higher concentrations (up to 110 mM) only during the 10-s uptake assay. Within the experimental error (about ±15%) no activations were observed with the *K. lactis* strain, the T1 strain, or the T1-E strain under these conditions. At 20 mM lactose, the inhibition by glucose under these conditions was also quite small (<20% at 110 mM glucose for the *K. lactis* strain).

For the *K. lactis* strain grown and assayed at 18°C, glucose stimulation (5 to 10 min at 27 mM glucose) increased the V_{max} value by 4.9-fold (from 5.5 ± 0.6 to 27 ± 2 μmol · min^{-1} · g dry yeast^{-1}; mean value ± range of duplicate determinations) and caused a smaller increase in K_m (from 1.0 ± 0.1 to 1.8 ± 0.0 mM; mean value ± range of duplicate determinations).

Stimulation of zero-*trans* maltose uptake by the incubation of starved yeasts with glucose. Maltose uptake by *S. cerevisiae* also occurs by a proton symport (39). The preincubation of brewer's yeast suspensions with glucose increased their maltose transport capacity (Fig. 4). The stimulation (at 20°C) was slower than that observed for lactose transport by the lactose-utilizing yeasts at 30°C and reached a maximum of 1.8-fold after 10 min. After 10 min, the yeast had consumed 65% of the glucose and nearly all after 20 min. During incubation without glucose, the maltose uptake rate was stable for the first 6 min and showed a small decrease over the next 24 min. Glucose (at a final concentration in the assay of 18 mM) added to the [^{14}C]maltose solution before the addition of the yeast suspension caused only a slight inhibition (about 7%) of the maltose uptake. For a laboratory yeast containing a single maltose transporter encoded by the *MALx1* gene (S150-2B/MALx1; see Materials and Methods), stimulation by glucose increased the V_{max} value by about 50% but had no significant effect on the K_m value for maltose (unstimulated and glucose-stimulated K_m values of 4.7 ± 0.3 mM and 4.2 ± 0.8 mM, respectively; averages ± ranges; $n = 2$). For strain S150-2B/MALx1, glucose (at a final concentration of 11 or 28 mM) added to the [^{14}C]maltose solution before the yeast suspension had no effect on the maltose uptake rates.

In our hands, the maltose transport activity of yeasts harvested during growth on sugars was unstable during storage at 0°C, whereas yeasts harvested after diauxie seemed to retain their activities for longer periods of storage (data not shown). The S150-2B/MALx1 cells harvested at an OD_{600} of 3.6 contained <1 mg trehalose · g dry yeast^{-1}, whereas those harvested after diauxie at an OD_{600} of 9.5, contained 8 to 9 mg of trehalose · g dry yeast^{-1}. For S150-2B/MALx1 cells harvested at an OD_{600} of 3.5 to 5.0 during growth on glucose (20 g · liter^{-1}), maltose transport activity decreased about threefold during two days' storage on ice. However, when maltose transport assays were made after preincubation with glucose

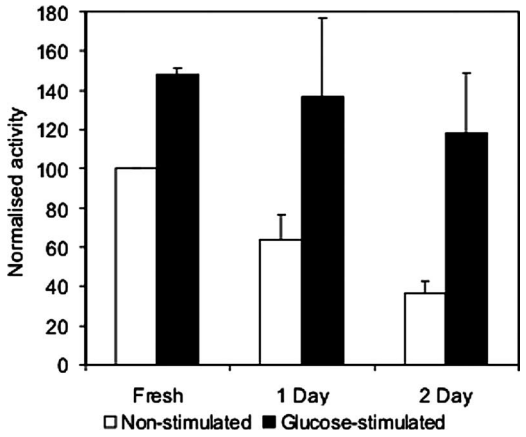

FIG. 5. Decay of nonstimulated and glucose-stimulated maltose transport during storage at 0°C of S150-2B/MALx1 yeast harvested from YP-2% glucose medium during growth on glucose (at an OD_{600} of 3.5 to 5). Yeasts were harvested and assayed after about 2.5 h (Fresh) and after storage for 24 h (1 Day) or 48 to 52 h (2 Day). Transport activities were normalized by setting the nonstimulated activity of each sample of fresh yeast to 100. Results are averages ± standard deviations for three independent suspensions of fresh yeast and 2-day old yeast and two independent suspensions of 1-day old yeast.

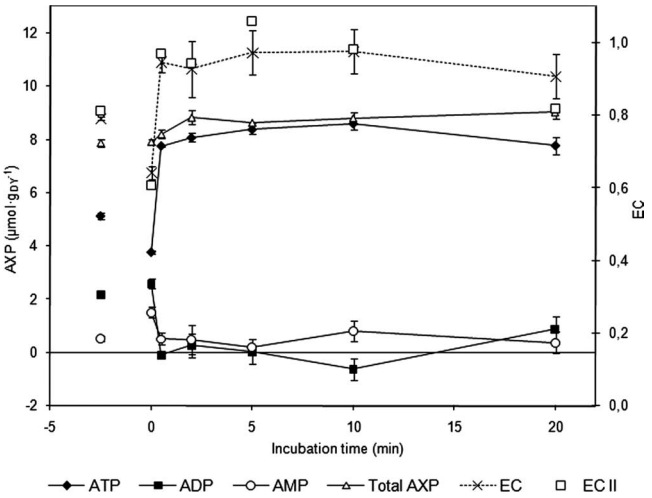

FIG. 6. Intracellular adenine nucleotide levels during the incubation of a buffered and starved suspension of *K. lactis* CBS2359 with glucose at 30°C. Portions of the suspension were preequilibrated for 10 min at 30°C, after which glucose was added (at 0 min) to 27 mM. Intracellular adenine nucleotides (AXP; ATP, ADP, AMP) and total adenine nucleotides (total AXP = ATP + ADP + AMP) were measured and the EC was calculated after incubation with glucose for the times indicated and (plotted at −2.5 min) in samples taken from the yeast culture immediately before harvesting. For the incubation with glucose, the error bars indicate the ranges between duplicate assays. For the culture, the error bars correspond to the ranges between two samples. EC data (EC II) from a replicate experiment using independently grown yeast are also shown.

(27 mM for 5 to 8 min at 20°C, as described above), stored cells were stimulated more (2- to 3.5-fold) than the freshly harvested cells (ca. 50%) (Fig. 5). This suggests that most of the activity loss during storage at 0°C is not caused by instability of the transporter molecules but by changes in the metabolic status of the yeast.

Intracellular adenine nucleotide levels during incubation of starved yeasts with glucose. Intracellular ATP levels (3.8 μmol · g dry yeast^{-1}) and EC (0.61 to 0.64) were low in the starved *K. lactis* strain suspensions prepared for zero-*trans* assays. During the incubation with 27 mM glucose at 30°C, the ATP concentration increased from 3.8 to 7.8 μmol · g dry yeast^{-1} in the first 30 s, reached 8.6 μmol · g dry yeast^{-1} after 10 min, and then fell to 7.8 μmol · g dry yeast^{-1} after 20 min, by which time all the glucose was consumed (Fig. 6). Similarly, the EC increased to 0.96 within 30 s and remained above 0.9 for 20 min (but fell to 0.82 at 20 min in a replicate experiment). The ADP level (initially 2.6 μmol · g dry yeast^{-1}) became very low in as soon as 30 s and did not start recovering until 20 min. The negative ADP values obtained at 0.5 and 10 min resulted from experimental error in the measurement of the small difference between ATP plus ADP and of ATP alone (16). Apparently negative ADP values were set to zero for the calculation of EC but have no significant effect on that calculation. AMP also decreased during the first 30 s (from 1.5 to about 0.5 μmol · g dry yeast^{-1}) and remained low thereafter. The total adenine nucleotide pool (ATP plus ADP plus AMP) increased a little (from 7.9 to 9.0 μmol · g dry yeast^{-1}) during the preincubation with glucose.

We also determined the adenine nucleotides levels in the culture just before harvesting the yeast (plotted at −2.5 min in Fig. 6). The EC in the culture was 0.79 to 0.81, while in the buffered yeast suspension equilibrated to 30°C for 10 min (0 min in Fig. 8), it was 0.61 to 0.64. In control experiments without glucose addition, incubation of the yeast suspension at 30°C for a further 5 or 10 min (after the initial 10-min equilibration) led to a further decrease in EC to 0.39 to 0.47, with a decrease in ATP to between 1.9 and 2.4 μmol · g dry yeast^{-1} (data not shown).

For brewer's yeast, incubation with 27 mM glucose at 20°C caused similar changes in adenine nucleotide levels (Fig. 7), as observed for the *K. lactis* strain, except that the changes were slower at the lower incubation temperature (as was the stimulation of maltose transport [compare Fig. 3 and 4]). The main increase in ATP (from 2.6 to 6.0 μmol · g dry yeast^{-1}) and EC (from 0.52 to 0.9) took place over 2 min, rather than within the 30 s seen with *K. lactis* at 30°C. For brewer's yeast (at 20°C), the ADP level never fell below that of AMP, whereas for the *K. lactis* strain (at 30°C), the ADP level was below that of AMP (and even undetectable) during the first 10 min of incubation with glucose. We do not know if this difference is species dependent or temperature dependent. During the incubation of the brewer's yeast suspension at 20°C without glucose for a further 10 min (after the initial 5-min equilibration to 20°C), ATP decreased by 25%, and the EC fell to 0.4 (data not shown).

DISCUSSION

When yeasts were harvested from sugar fermentation before diauxie and suspended in nutrient-free buffer, a short incubation of the starved yeasts with glucose increased their intracellular ATP and adenylate EC and simultaneously increased the V_{max} value for the active transport of lactose or maltose. For

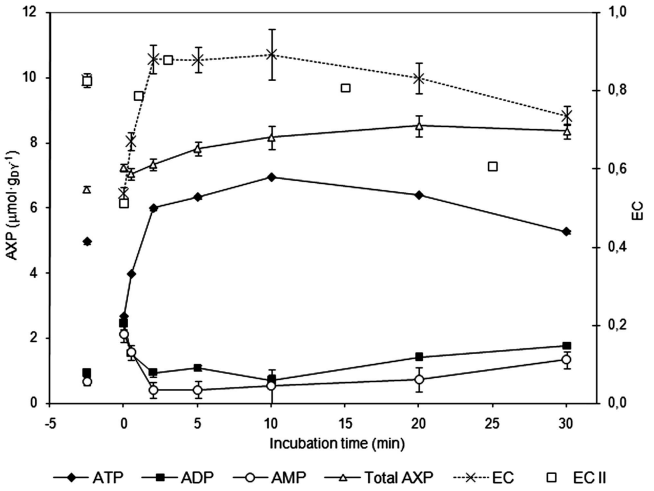

FIG. 7. Intracellular adenine nucleotides levels during incubation of a buffered and starved suspension of brewer's yeast (strain A15) with glucose at 20°C. Portions of the suspension were preequilibrated for 5 min at 20°C, after which glucose was added (at 0 min) to 27 mM. Intracellular adenine nucleotides (AXP; ATP, ADP, AMP) and total adenine nucleotides (total AXP = ATP + ADP + AMP) were measured, and the EC was calculated after incubation with glucose for the times indicated and (plotted at −2.5 min) in samples taken from the yeast culture immediately before harvesting. For the incubation with glucose, the error bars indicate the ranges between duplicate assays. For the culture, error bars correspond to the ranges between two samples. EC data (EC II) from a replicate experiment using independently grown yeast are also shown.

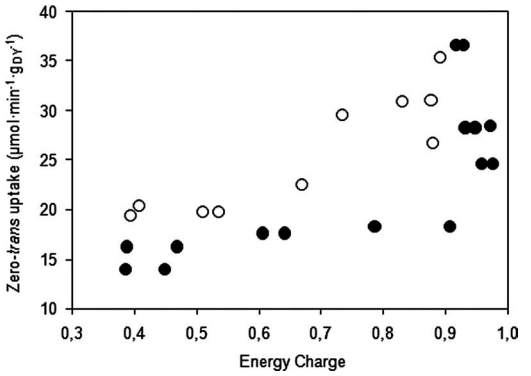

FIG. 8. Correlation between lactose uptake rates in the *K. lactis* strain at 30°C (●) or maltose uptake rates in A15 at 20°C (○) and EC.

lactose, a relatively smaller increase in K_m value also occurred, but for maltose, the K_m value was unchanged. The lactose transport results (with a *K. lactis* strain and two recombinant strains of *S. cerevisiae*) are consistent with those of earlier work by Van den Broek et al. (45), who showed that the rate of lactose transport by *K. marxianus* closely correlated with intracellular ATP levels when these were manipulated in various ways, including preincubation with glucose (to increase ATP) or with antimycin A (to decrease ATP). Also, for the glycerol/H^+ symport of *S. cerevisiae*, a short (20-s to 5-min) preincubation of starved cells with glucose increased the V_{max} value by about 30%, with no change in K_m (as reported by Lages and Lucas [22]; possible changes in adenylate nucleotides were not examined by these authors). However, our results with maltose transport (by a brewer's yeast lager strain) appear to contradict work by Serrano (39), who found that the rate of maltose transport by *S. cerevisiae* S-13 did not change when intracellular ATP was almost completely (≥98%) depleted by preincubation with antimycin A and 2-deoxyglucose. Serrano's (39) growth and assay conditions were very similar to ours (cells harvested from YP-2% maltose during growth on maltose and stored at low temperature in tartrate-Tris [pH 4.2] before being assayed at 20°C in the same buffer). A possible explanation for the apparent discrepancy between those results and ours is that Serrano (39) examined the effect of further decreasing the ATP and the EC below the levels in starved yeast suspensions, whereas we examined the effect of increasing ATP and the EC in such cells to the levels found in actively growing cells. In our experiments, increasing the EC from 0.4 to 0.67 had little effect on maltose transport, but increased transport rates occurred when the EC was raised to between 0.74 and 0.9 (Fig. 8). For lactose transport, the rate did not increase significantly until the EC was above 0.8. Serrano (39) showed that maltose transport was dependent upon an electrochemical gradient of protons across the cell membrane even when the extracellular concentration of maltose exceeded its intracellular concentration. We therefore suggest that starved yeast cells can maintain an electrochemical gradient of protons sufficient to support a basal level of maltose transport activity, even when ATP is depleted, but that increasing the EC to the levels (ca. 0.9) found in actively fermenting cells enhances both the Δp and the transport rate.

In our experiments, yeast cells growing on lactose or maltose were harvested and suspended in buffer lacking nutrients. Ball and Atkinson (3) showed that yeast cells growing on glucose were unable to maintain high levels of ATP and EC when transferred to a medium without glucose (the EC fell to below 0.6). In contrast, cells harvested after diauxie, when they are growing aerobically on ethanol produced earlier, can maintain EC values between 0.8 and 0.9. The different behaviors may be explained by the accumulation of reserve carbohydrates, glycogen and trehalose, beginning, respectively, just before or at diauxie (24) and the appearance of oxidative phosphorylation. Probably a drop in ATP and EC cannot be avoided when attempting to measure the activity of sugar/H^+ symports in yeast growing on the same sugar, because residual sugar must be removed from the cell suspensions before zero-*trans* uptake assays are carried out. A short (2-min) aeration of yeast cells before zero-*trans* assays are carried out has been used (10) but is not likely to restore cytosolic ATP levels in repressed cells harvested during growth on sugars and lacking reserve carbohydrates and a functional electron transport chain. Treatments to restore ATP and EC levels must be designed to avoid both the biosynthesis of new transporter molecules and the inactivation of existing transporters. It is known that maltose transporters are subject to glucose-triggered catabolite inactivation (26, 28, 30). The present method (5- to 10-min incubation with ≤27 mM glucose) will not induce the synthesis of new disaccharide transporters and is fast enough to avoid extensive catabolite inactivation. Typically, maltose transporters disappear with a half-life of 1.3 h when yeasts are exposed to 100 mM glucose at 30°C (26), corresponding to <10% loss of activity in 10 min.

The impetus for the present work was our finding that zero-*trans* uptake rates of lactose in *K. lactis* and in lactose-consuming *S. cerevisiae* recombinant strains were too small (by factors of 3 to 8) to account for the lactose consumption rates observed during shake-flask fermentations. Others have reported similar discrepancies. For example, Alves-Araújo et al. (1) found a V_{max} value for zero-*trans* maltose uptake of 0.66 nmol · s^{-1} · mg dry $yeast^{-1}$ compared to an estimated maltose consumption rate of 1.8 ± 0.3 nmol · s^{-1} · mg dry $yeast^{-1}$. Even for the facilitated transport of glucose, 5-s zero-*trans* assays were inhibited when the level of cytosolic ATP was decreased by respiratory inhibitors (49). In this case, the mechanism cannot be a decrease in Δp and is thought to be inhibition by intracellular glucose, which accumulates at low ATP levels. No inhibition was seen when reaction times were short enough (200 ms) to prevent significant accumulation of glucose.

We found no evidence of extracellular β-galactosidase activity that might resolve the discrepancy between the rates of lactose consumption and zero-*trans* uptake. Making the zero-*trans* reaction mixtures closer to the composition of the fermentation medium did not increase the zero-*trans* rates. Stimulation of zero-*trans* uptake by preincubation with glucose nearly resolved the discrepancy for the slower lactose-fermenting recombinant *S. cerevisiae* strain T1, in which the glucose-stimulated zero-*trans* rate was 29.8 ± 0.2 μmol · min^{-1} · g dry $yeast^{-1}$ and the lactose consumption rate was 34 ± 1.0 μmol · min^{-1} · g dry $yeast^{-1}$ (Fig. 1). Similarly, for the *K. lactis* strain at 18°C, the glucose-stimulated zero-*trans* rate (24.6 ± 2.3 μmol · min^{-1} · g dry $yeast^{-1}$) was close to the error limits of the lactose consumption rate during fermentation (40 ± 12 μmol · min^{-1} · g dry $yeast^{-1}$). However, for the faster fermenting recombinant strain T1-E and for the *K. lactis* strain at 30°C, the glucose-stimulated zero-*trans* rates were still markedly lower (2.2- to 2.4-fold) than the rates observed for lactose consumption (Fig. 1). At the high transport rates involved (80 to 100 μmol · min^{-1} · g dry $yeast^{-1}$), the 10-s zero-*trans* assays were possibly too long to measure true initial rates (at least for the *K. lactis* strain; see Materials and Methods). Assays by rapid reaction methods (e.g., 200 ms [49]) are needed to determine whether the true initial rates can account for the observed rates of lactose consumption under these conditions or whether there is still some unknown factor involved in lactose uptake.

The increases in K_m values for lactose caused by glucose stimulation were relatively small and have little practical consequence for industrial fermentations, where the concentration of lactose (or maltose) is much greater than the K_m values. The lactose K_m value of about 2 mM corresponds to 0.7 g · $liter^{-1}$ and the maltose K_m of about 5 mM to 1.8 g · $liter^{-1}$, which are low concentrations from an industrial viewpoint, met only at the end of fermentations. On the other hand, the increases in V_{max} values translate directly into increased rates at all lactose or maltose concentrations.

Rautio and Londesborough (35) reported close agreement between the specific rates of maltose consumption and zero-*trans* uptake assays (measured with each day's yeast in each day's wort) during the early and middle stages of fermentations of brewer's wort by brewer's yeast and concluded that maltose uptake was the dominant factor controlling the rate of maltose consumption under these conditions. In the final stages of these fermentations, the specific rates of maltose consumption were up to 50% lower than those determined by zero-*trans* assays, indicating that other factors also exerted significant control over the fermentation rate. Our present results (Fig. 4) suggest that the maximum maltose uptake rates were probably about 60% higher than those estimated by the zero-*trans* assays of Rautio and Londesborough (35), so that, also in early and mid fermentation, the rate of maltose consumption was limited by other factors, as well as transport. The importance of maltose transport to the speed of wort fermentation is shown by the acceleration obtained when maltose transport capacity is increased (21; Vidgren et al., unpublished results).

In conclusion, our results show that the zero-*trans* uptake assays done with yeast samples harvested from sugar fermentations and then washed and starved before being assayed can seriously underestimate the capacity of sugar/H^+ symports. A short preincubation with a moderate concentration of glucose provides a quick way, allowing little possibility for new synthesis or degradation of transporters, to approach more closely the sugar/H^+ symport capacity of the actively metabolizing cells.

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