Hxt-carrier-mediated glucose efflux upon exposure of Saccharomyces cerevisiae to excess maltose
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The disaccharide maltose is an important carbon source for *Saccharomyces cerevisiae* during beer fermentation and leavening of dough (3, 10, 20, 42). In addition to having applied significance, the maltose regulon in *S. cerevisiae* serves as a paradigm for metabolic regulation in eukaryotes (18, 23, 36, 37).

In *S. cerevisiae*, an intracellular maltase (α-glucosidase; EC 3.2.1.20) hydrolyzes maltose to glucose. The transport of maltose over the cell membrane differs from the transport of glucose (Fig. 1). In *S. cerevisiae*, glucose uptake occurs exclusively via facilitated diffusion (29, 43). Facilitated diffusion of glucose involves the 17 members of the *HXT* gene family (61), which encode hexose transporters that differ with respect to kinetic properties (12), transcriptional regulation, and intracellular localization (39). As Hxt-mediated glucose transport does not require input of metabolic energy, alcholic fermentation of glucose by *S. cerevisiae* results in a net yield of two ATP molecules per glucose molecule (30). Conversely, maltose is taken up via a maltose—one-proton symport mechanism (49). Extrusion of the symported proton via the plasma membrane ATPase costs one ATP molecule per proton (53, 59). Consequently, the net ATP yield from alcholic fermentation of one maltose molecule is only three ATP molecules (21).

The genes encoding the maltose permease are located in five highly homologous loci (*MAL1, MAL2, MAL3, MAL4*, and *MAL6*) (2, 8). The number and identity of *MAL* loci is strain dependent (35). Each *MAL* locus consists of three genes. The first gene (*MALx1*) encodes the maltose-proton symporter (9). Maltase is encoded by the *MALx3* gene (13, 22). The third *MAL* gene (*MALx2*) encodes a DNA-binding, maltose-dependent transcriptional activator that specifically controls expression of the *MALx1* and *MALx2* genes (7, 21).

Maltose metabolism in *S. cerevisiae* is strongly downregulated by glucose. At the transcriptional level, glucose represses transcription of the *MALx1* and *MALx2* genes via binding of the transcriptional repressor Mig1p in the *MAL* intergenic region (26, 27, 62). Moreover, glucose causes rapid catabolite inactivation of maltose permease activity (5). This glucose-induced inactivation can involve different signaling pathways (32). The first pathway uses Rgt2p as a sensor of extracellular glucose and induces degradation of the maltose permease protein. This degradation requires ubiquitination and endocytic internalization of the maltose transporter protein to the vacuole, where proteolysis takes place (32–34, 45). The second pathway depends on glucose transport and causes very rapid inactivation of maltose transport activity, followed by degradation of the maltose permease (24, 25). Which signal triggers this catabolite inactivation is still a matter of debate. Some authors have proposed that hexose transport via Hxt transporters is required for this pathway (24, 25), whereas other authors have stated that galactose and even maltose can also elicit catabolite inactivation (40, 46). In addition, trehalose and/or trehalose 6-phosphate have recently been mentioned as possible signals for catabolite inactivation (4). The glucose-induced loss of maltose transport activity is generally much faster than the loss expected from mere proteolytic degradation of the maltose transporter. This observation has been explained by glucose-induced phosphorylation of the maltose transporter that precedes proteolytic breakdown and immediately reduces transport activity (5).

Despite this multilayer regulation of maltose metabolism, several reports have indicated that *S. cerevisiae* has difficulty coping with sudden changes in the extracellular maltose concentration. Exposure of aerobic, maltose-limited chemostat cultures to excess maltose has even been reported to result in...
maltose-accelerated death (41). The loss of viability, accompanied by the release of glucose into the medium, was interpreted to be caused by nonrestricted maltose uptake and hydrolysis, with concomitant rapid intracellular accumulation of glucose and protons leading to cell death and lysis (41). Release of glucose upon exposure to excess maltose has also been observed in \textit{S. cerevisiae} mutants defective in glucose catabolite repression (5, 11, 26).

The aim of the present study was to investigate the mechanism responsible for glucose release during maltose fermentation by \textit{S. cerevisiae}. Special attention was paid to a possible role of the HXT-encoded glucose transporters in mediating glucose efflux.

**MATERIALS AND METHODS**

**Strains and maintenance.** The strains used in this study (Table 1) were grown to the stationary phase in shake flasks cultures on synthetic medium (55) adjusted to pH 6.0 and containing 2% (wt/vol) glucose. After addition of sterile glycerol to the stationary phase in shake cultures on synthetic medium (55), the strains were used to inoculate precultures for chemostat cultivation. Auxotrophic requirements were calculated as described by Oura (38), and the required concentrations were multiplied by 2 to prevent limitation by nutrients other than the sugar substrate. For chemostat cultivation, the glucose or maltose concentration in reservoir media was 7.5 g liter$^{-1}$ (0.25 mol of C liter$^{-1}$).

Media. Synthetic medium containing mineral salts and vitamins was prepared as described previously (55). To meet the auxotrophic requirements of strains CEN.PK.2-1C and EBY.VW.4000, media for cultivation of these strains were supplemented with uracil (113 mg liter$^{-1}$), L-leucine (180 mg liter$^{-1}$), and L-histidine (45 mg liter$^{-1}$). \textit{S. cerevisiae} auxotrophic strains were supplemented with uracil (113 mg liter$^{-1}$), L-leucine (180 mg liter$^{-1}$), and L-tryptophan (27 mg liter$^{-1}$).

**Table 1.** \textit{S. cerevisiae} strains used in this study

<table>
<thead>
<tr>
<th>\textit{S. cerevisiae} strain</th>
<th>Relevant genotype and/or phenotype</th>
<th>Source</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>CEN.PK113-7D</td>
<td>MAT$^a$, prototrophic</td>
<td>P. Kotter</td>
<td>50</td>
</tr>
<tr>
<td>DS28911</td>
<td>Aneuploid, prototrophic</td>
<td>DSM Bakery Ingredients, Delft, The Netherlands</td>
<td>51</td>
</tr>
<tr>
<td>CBS 8066</td>
<td>HO/HO, prototrophic</td>
<td>CBS$^*$</td>
<td>50</td>
</tr>
<tr>
<td>CEN.PK2-1C</td>
<td>MAT$^a$ leu2-3,112 ura3-52 trp1-289 his3-D1 htx17A</td>
<td>E. Boles</td>
<td>61</td>
</tr>
<tr>
<td>EBY.VW.4000</td>
<td>htx1 through -17A::loxP gal2Delta::loxP stl1Delta::loxP agr1Delta::loxP ydl247wDelta::loxP ypr100cDelta::loxP</td>
<td>E. Boles</td>
<td>61</td>
</tr>
</tbody>
</table>

$^a$ CBS, Centraalbureau voor Schimmelcultures, Utrecht, The Netherlands.

**FIG. 1.** Schematic representation of glucose and maltose transport in \textit{S. cerevisiae}. (A) Facilitated diffusion of glucose, driven by the concentration gradient of the sugar. (B) Maltose-proton symport driven by the proton motive force and the sugar concentration gradient. ATP hydrolysis by the plasma membrane ATPase is required to expel the protons that enter the cell together with the maltose. For each maltose molecule transported into the cell, one ATP molecule is hydrolyzed (53, 59). 1, Hxt transporter; 2, maltose permease; 3, H$^+$-ATPase complex.

Determination of culture dry weights. Culture dry weights were determined by filtration of samples with nitrocellulose filters and drying in a microwave oven as described previously (51).

Extracellular metabolite analysis. Glucose, maltose, ethanol, glycerol, acetate, and pyruvate concentrations in the supernatants of chemostat cultures were determined by high-performance liquid chromatography (HPLC) analysis by using an HPX-87H Aminex ion-exchange column (300 by 7.8 mm; Bio-Rad) at 60°C. The column was eluted with 5 mM sulfuric acid at a flow rate of 0.6 ml·min$^{-1}$. Pyruvate and acetate were detected at 214 nm with a Waters 441 UV meter coupled to a Waters 741 data module. Glucose, maltose, ethanol, and glycerol were detected with an ERMA type ERC-7515A refractive index detector coupled to a Hewlett-Packard type 3390A integrator. Glucose and maltose in reservoir media were also analyzed by HPLC.

Anaerobic fermentation assays. Samples containing exactly 200 mg (dry weight) of biomass were harvested from a steady-state chemostat culture by centrifugation (5,000 × g, 3 min) and were resuspended in 10 ml of fivefold-
concentrated synthetic medium (pH 5.6). Subsequently, the cell suspensions were introduced into a thermostat-controlled (30°C) vessel. The volume of each suspension was adjusted to 40 ml with demineralized water. After 10 min of incubation, 10 ml of a maltose solution (100 g liter\(^{-1}\)) was added, and samples (two 1-ml samples) were taken at appropriate time intervals for 2 h. The 10-ml headspace was continuously flushed with water-saturated carbon dioxide at a flow rate of approximately 30 ml min\(^{-1}\). Sugar concentrations and metabolite levels in the supernatants were determined by HPLC analysis. The ethanol concentration in the supernatant was determined by a colorimetric assay (56) by using phenol-purified alcohol dehydrogenase from H. polymorpha (a kind gift from Bird Engineering, Rotterdam, The Netherlands). At the end of the experiments (after 2 h) some growth had taken place (data not shown). Consequently, the levels of carbon recovery were only ca. 90\% if growth of biomass was not taken into account.

**Intracellular metabolite measurements.** Biomass samples (4 ml of a 4-g [dry weight]/liter suspension) were taken from an anaerobic fermentation assay mixture. The cells were washed twice with cold 60\% methanol, intracellular metabolites were extracted by resuspending the cell pellets in 5 ml of boiling 75\% ethanol and incubating them for 3 min at 80°C (19). Cell debris and intracellular metabolites were dried at room temperature with a vacuum evaporator (type AES1010 Savant Automatic Environmental SpeedVac system). Finally, 0.5 ml of demineralized water was added to each preparation. The resulting suspension was stored at -20°C. Intracellular metabolite analysis was performed by UV and fluorescence spectroscopy. Intracellular glucose contents were determined by HPLC analysis by using an Aminex HPX-87K column (300 by 7.8 mm; Bio-Rad) at 85°C. The column was eluted with demineralized water at a flow rate of 0.5 ml min\(^{-1}\).

**Fluorescent staining for yeast viability.** A commercial LIVE/DEAD yeast viability kit (Molecular Probes, Leiden, The Netherlands) was used to estimate the fractions of dead cells in samples obtained from anaerobic fermentation assays. FUN-1 and Calcofluor White M2R cell stain were added to yeast cell suspensions (10\(^{10}\) to 10\(^{10}\) cells/ml) at final concentrations of 5 to 20 and 25 \(\mu\)M, respectively. After staining, the suspensions were mixed thoroughly and incubated in the dark at 30°C for 3 min. Five microliters of a stained yeast suspension was trapped between a coverslip and an object slide and analyzed with a fluorescence microscope (Zeiss Axioplan 2 Imaging, Woesp, The Netherlands) by using appropriate filter sets (fluorescein isothiocyanate, Zeiss 450-490 FT510 LP515; and 4.6-diamidino-2-phenylindole [DAPI], Zeiss 02 G365 FT395 LP420).

**Maltase activity assay.** As a check for extracellular maltase activity, a standard anaerobic fermentation assay was performed. At 0, 30, and 60 min, a sample (2 ml) was centrifuged. Each supernatant (1 ml) was incubated at 30°C. Samples were taken at different times and analyzed for glucose by using the UV method (Boehringer kit no. 716251). A 10\% (wt/vol) maltose solution in water was used as a negative control.

**Protein concentration determination.** Protein concentrations in the supernatants of anaerobic fermentation assay mixtures and in cell extracts were estimated by the method of Lowry et al. (31). Dried bovine serum albumin (fatty acid free; obtained from Sigma, Zwijndrecht, The Netherlands) was used as a standard.

**Determination of viable counts.** Viable counts of \textit{S. cerevisiae} CEN.PK113-7D were determined on 2\% (wt/vol) YPD agar plates. This complex medium contained (per liter) 10 g of yeast extract (Difco, Detroit, Mich.), 20 g of peptone from casein (Merek, Darmstadt, Germany), 20 g of \(\alpha\)-glucose, and 20 g of agar (Difco). After appropriate dilution of the culture and plating (which yielded 30 to 400 colonies per plate), colonies were counted following 48 h of incubation at 30°C. At least 1,000 colonies were counted to calculate viable counts.

**RESULTS**

**Release of glucose during maltose fermentation.** When anaerobic maltose fermentation was studied with \textit{S. cerevisiae} CEN.PK113-7D pregrown in aerobic, maltose-limited chemostat cultures, substantial amounts of glucose were produced in addition to fermentation products like ethanol and glycerol (Fig. 2A). The highest rate of glucose production (dglucose/dt) took place during the first 45 min after maltose addition. During this period, ca. 0.6 mol of glucose was released for each 1 mol of maltose consumed (Fig. 2B), corresponding to 30\% of the maltose carbon. After 45 min, the amount of glucose released gradually decreased, and after ca. 2 h no further net production of glucose occurred (Fig. 2). When similar experiments were performed with cells pregrown in aerobic, glucose-limited chemostat cultures, the initial maltose consumption rates were low. No glucose release was observed during the slow induction of maltose-fermenting capacity (data not shown).

To investigate whether glucose release also occurs in other wild-type \textit{S. cerevisiae} strain backgrounds, anaerobic maltose fermentation experiments were performed with maltose-grown cultures of the industrial baker's yeast strain \textit{S. cerevisiae} DS28911 (51, 52) and the laboratory strain \textit{S. cerevisiae} CBS8066. Qualitatively, these two strains exhibited product formation profiles that were very similar to that of the CEN.PK113-7D strain (data not shown). The maximum glucose concentrations observed under standardized conditions were 9, 4, and 12 mM for strains CEN.PK.113-7D, DS28911, and CBS8066, respectively.

These results indicate that glucose release during maltose fermentation is a general phenomenon in \textit{S. cerevisiae} cultures but that the amount of glucose released is strain dependent.

**Glucose release is not caused by cell lysis.** Postma et al. (41) observed cell death and release of proteins after exposure of aerobic, maltose-limited chemostat cultures of \textit{S. cerevisiae} CBS8066 to excess maltose. To investigate whether the glucose release observed during anaerobic incubation of maltose-grown \textit{S. cerevisiae} CEN.PK113-7D with excess maltose (Fig. 2) was due to cell lysis, possibly accompanied by the release of maltose into the extracellular medium, several control experiments were performed.

Plating on complex medium did not reveal a marked decrease in viable counts during anaerobic maltose fermentation (Fig. 3A). During the first 1 h of the experiments, during which glucose release was most pronounced (Fig. 2), the viable counts were reduced by only ca. 4\% (Fig. 3A). These results were corroborated by a fluorescent live-dead staining technique, which indicated that throughout the pulse experiments virtually all cells remained metabolically active (data not shown).

As a further indicator of possible cell lysis, protein concentrations were analyzed in culture supernatants (29). No significant increase in the extracellular protein concentration was observed during the pulse (Fig. 3A). More specifically, the possibility that the extracellular glucose encountered during anaerobic maltose fermentation was due to an extracellular maltase was investigated. Incubation of supernatant samples
taken during the maltose fermentation experiments did not reveal any extracellular maltase activity (Fig. 3B).

**Absence of glucose release during maltose fermentation in an hxt null strain.** To investigate whether glucose release during anaerobic maltose fermentation was mediated by plasma membrane glucose transporters (Fig. 1), anaerobic maltose fermentation experiments were performed with the hxt null strain *S. cerevisiae* EBY.VW.400. In this strain, all members of the HXT gene family have been deleted, which eliminates glucose uptake via facilitated diffusion (58). *S. cerevisiae*
EBY.VW.4000 is a member of the CEN.PK strain family, but in contrast to the prototrophic CEN.PK113-7D strain, it carries four auxotrophic markers. Duplicate control experiments performed with cells from independent maltose-grown chemostat cultures of the isogenic, auxotrophic reference strain CEN.PK2-1C yielded the same glucose and ethanol profiles as the experiments performed with the prototrophic CEN.PK113-7D strain (data not shown). In contrast, hardly any extracellular glucose (maximum concentration, 1.6 \pm 0.1 \text{mM}) was found during anaerobic maltose fermentation experiments performed with the hxt null strain (Fig. 4). The concentrations of other extracellular metabolites (ethanol, glycerol, and acetate) were comparable to those in the reference strains (Fig. 2A and 4).

The strongly reduced glucose release by the hxt null strain suggests that one or more HXT-encoded hexose transporters are involved in glucose efflux. To investigate whether the absence of functional hexose transporters led to intracellular accumulation of glucose, intracellular metabolite assays were performed during anaerobic maltose fermentation by S. cerevisiae EBY.VW.4000. In this experiment, the intracellular glucose levels increased to 41.4 \pm 1.3 \text{mM} during anaerobic maltose fermentation by the hxt null strain (Fig. 5A). The intracellular concentrations of glucose 6-phosphate and ATP were 1.8 \pm 0.1 and 1.0 \pm 0.1 \text{mM}, respectively (Fig. 5B). High concentrations of intracellular maltose (ca. 130 \text{mM}) accumulated during anaerobic maltose fermentation, suggesting that maltase activity was saturated under the experimental conditions.

**DISCUSSION**

The reversibility of glucose transport has been demonstrated in previous studies with kinase-less mutants (14, 15). However, to our knowledge the present study provides the first experimental proof that Hxt transporters are involved in glucose export. There is no reason to assume that the ability to export glucose is confined to one or a few members of the Hxt family. However, as has been demonstrated for glucose uptake via the Hxt-encoded transporters, it is likely that the kinetic properties of the Hxt-encoded transporters for glucose efflux are different (44, 57).

The simultaneous uptake of maltose and efflux of glucose result in reduced ATP yields from maltose dissimilation. When the protons symported with maltose are expelled via the plasma membrane ATPase complex, the combination of maltose uptake via proton symport and glucose efflux via facilitated diffusion results in a net hydrolysis of ATP (Fig. 1). The physiological response of S. cerevisiae to excess maltose reported here is less dramatic than that reported in a previous study (41), in which exposure to excess maltose resulted in a loss of viability and cell lysis. The reason for this difference is not known but may be related to the use of a different strain background. Furthermore, the experimental conditions were different; in contrast to the data obtained in the present study, the data reported by Postma et al. (41) were obtained with aerobic, respiring cultures.

The detrimental effects of an imperfect balance between maltose uptake and glucose dissimilation are likely to be relevant for the development of maltose-constitutive S. cerevisiae strains for baker’s yeast production and brewing. For example, constitutive overproduction of maltose permease and maltase, which has been proposed as a means to increase fermentative capacity with maltose as the substrate, is likely to result in an imbalance between maltose uptake and glycolysis. This complication may also occur in other cases where disaccharides are
transported via proton symport and hydrolyzed intracellularly. A relevant example is the metabolic engineering of \textit{S. cerevisiae} for lactose fermentation by constitutive expression of a lactose-proton symporter and intracellular beta-galactosidase (1, 47, 48).

To prevent the dissipation of metabolic energy that results from noncoordinated uptake and hydrolysis of disaccharides, two regulatory mechanisms can be envisaged. One possible mechanism would involve downregulation of disaccharide hydrolysis (for instance, by glucose inhibition of the disaccharide hydrolyase). Although maltase in \textit{S. cerevisiae} is not known to be regulated via glucose inhibition or glucose catabolite inactivation, transcription of the \textit{MALx2} genes is subject to glucose repression (16, 17).

The second possibility is downregulation of maltose uptake to match the uptake rate to the glycolytic activity of the cells (for example, by glucose repression of the synthesis of maltose permease or glucose-induced inactivation of the maltose carriers). Albeit with different time scales, both mechanisms should lead to a situation where the level of maltose permease is adapted to the capacity of glucose metabolism. The intricate mechanisms described for glucose repression and glucose inactivation of the \textit{S. cerevisiae} maltose permease are generally explained by considering glucose the preferred carbon source (32, 62). Such a preferred status of glucose can be explained by the lower ATP yield from maltose due to an energy requirement for maltose uptake (49, 59). The present study offers an alternative, additional explanation: these control systems may have evolved to prevent ATP dissipation via simultaneous energy-dependent maltose uptake and glucose efflux.

**ACKNOWLEDGMENTS**

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