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Inhibition of *Saccharomyces cerevisiae* growth by simultaneous uptake of glucose and maltose

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Saccharomyces cerevisiae **expresses α-glucoside transporters, such as MalX1p (X = 1(Agt1p), 2, 3, 4, and 6), which are proton symporters. These transporters are regulated at transcriptional and posttranslational levels in the presence of glucose. Malt wort contains glucose, maltose, and maltotriose, and the assimilation of maltose is delayed as a function of glucose concentration. With the objective of increasing beer fermentation rates, we characterized α-glucoside transporters and bred laboratory yeasts that expressed various α-glucoside transporters for the simultaneous uptake of different sugars. Mal21p was found to be the most resistant transporter to glucose-induced degradation, and strain (HD17) expressing *MAL21* grew on a medium containing glucose or maltose, but not on a medium containing both sugars (YPDM). This unexpected growth defect was observed on a medium containing glucose and >0.1% maltose but was not exhibited by a strain that constitutively expressed maltase. The defect depended on intracellular maltose concentration. Although maltose accumulation caused a surge in turgor pressure, addition of sorbitol to YPDM did not increase growth. When strain HD17 was cultivated in a medium containing only maltose, protein synthesis was inhibited at early times but subsequently resumed with reduction in accumulated maltose, but not if the medium was exchanged for YPDM. We conclude that protein synthesis was terminated under the accumulation of maltose, regardless of extracellular osmolarity, and HD17 could not resume growth, because the intracellular concentration of maltose did not decrease due to insufficient synthesis of maltase. Yeast should incorporate maltose after expressing adequate maltase in beer brewing.**

[**Key words:** *Saccharomyces cerevisiae*; Growth inhibition; α-Glucoside transporters; Maltose; Glucose]

The first step of sugar assimilation by *Saccharomyces cerevisiae* is the transport of sugar into cells. *S. cerevisiae* produces sugar transporters that incorporate sugars from the medium, and *HXT1–HXT17* encode transporters for hexose such as glucose, fructose, and mannose. Each hexose transporter possesses a different kinetic property (1). *S. cerevisiae* regulates the expression of hexose transporters to respond to the environment with priority for glucose uptake (2,3). Hexose transporters transfer glucose through facilitated diffusion and therefore do not require energy (1).

To assimilate α-glucosides such as maltose and maltotriose, *S. cerevisiae* produces the α-glucoside transporters MalX1p (X = 1(Agt1p), 2, 3, 4, and 6) that are encoded on chromosomes VII, III, II, XI, and VIII, respectively (4). The allele type and copy number of α-glucoside transporter genes vary depending on the strain. Generally, the genomes of yeasts used to brew beer encode numerous numbers of α-glucoside transporter genes (5). Mal21p, Mal31p, Mal41p, and Mal61p, which are also called maltose transporters, can incorporate maltose and turanose. In contrast, the substrate specificity of Agt1p is broad. For example, Agt1p can transport maltose, sucrose, turanose, trehalose, and maltotriose as well as hexoses such as fructose and glucose (6,7).

Besides MalX1p and Agt1p, the genome of a lager-brewing yeast, which is a hybrid between *S. cerevisiae* and *Saccharomyces eubaynus* (8), encodes the Agt1p ortholog SeAgt1p as well as Mtt1p that is 90% identical to Mal61p (9–11). These α-glucoside transporters are encoded by the *MAL* locus, which comprises transporter (*MALX1*), maltase (*MALX2*), and activator (*MALX3*) genes. The transcription of genes encoding transporters and maltase are driven by the bidirectional promoter between them, and their transcription is repressed by glucose and induced by maltose through the binding of the transcriptional activator MalX3p at the upstream-activating promoter sequence (12,13).

The results of a binding experiment using strains carrying wild-type Mal63p, the constitutive mutant Mal63p, and the non-inducible mutant Mal63p revealed that native, inducible Mal63p binds strongly to Ssa1p, Hsp82p, and Sti1p in the presence of glucose but is released in response to the inducer, maltose, after the depletion of glucose (14).

When glucose is added to the medium, Mal61p and Mal31p are immediately phosphorylated and are subsequently ubiquitinated

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by Npi1p/Rsp5p. The ubiquitinated transporters are endocytosed, transferred to vacuoles, and rapidly degraded (15–17). These regulatory systems enable *S. cerevisiae* to primarily utilize glucose and then other sugars.

Malt wort contains maltose, maltotriose, and glucose comparable to approximately 20–25% of maltose, and beer-brewing yeast start to assimilate maltose and maltotriose after depletion of glucose. Fermentation with malt wort of a high specific gravity improves efficiency of beer production, although occasional problems are caused by the delay in assimilating maltose and maltotriose that is associated with the prolonged time required to consume glucose (18). Therefore, we characterized several α-glucoside transporters and attempted to breed yeast that can assimilate glucose and maltose simultaneously to achieve rapid fermentation. We report here that a strain that simultaneously incorporated glucose and maltose from the medium did not grow unexpectedly, and we therefore investigated the reasons for this growth defect. This would give a clue as to whether to select or breed an adequate brewing strain for rapid fermentation with malt wort of high specific gravity.

MATERIALS AND METHODS

Yeast strains, media, and culture conditions The *S. cerevisiae* strains used in this study are listed in Table S1. Strain JH1032 is the *ura3*-derivative of strain X2180-1A, created by integration of a constitutive expression unit of *MAL32* (19). The strains indicated by "HH" are derivatives of JH1032, in which an expression unit of a transporter gene is integrated into the *ura3* site. D152 was generated by disruption of *MAL61* with *TRP1* in strain ATCC96955 (19). D152U and D152MS are strains which were inserted *URA3* or *URA3::TDH3p::MAL62* (encoding maltase) to *ura3* in D152, respectively. HD93 and HD94 are strains with inserted β-galactosidase (*lacZ*) expressed under the control of the *TPI1* promoter in D152U or D152MS, respectively, that harbors *MAL21* on the plasmid pYCGPY (20). The strains with designations starting with "HD" are transformants of D152U or D152MS harboring a transporter gene (*MAL61, MAL21, AGT1-2HA, SeAGT1,* or *MTT1*) on the plasmid pYCGPY. YP5D, YP0.5M, YP5M, YP5S, YP5F, YP4D0.01M, YP4D0.05M, YP4D0.1M, YP4D0.25M, YP4D0.5M, YP4D0.75M, YP4D1M, and YP4D1S are designations of media based on 1% yeast extract and 2% yeast peptone. D, M, S, and F indicate glucose, maltose, sucrose, and fructose, respectively, and the numbers preceding D, M, S, and F indicate the percentage concentration of each sugar. YPD and YPM contain 2% glucose or 2% maltose, respectively. Glucose or maltose-based synthetic complete medium (SCD or SCM) was prepared as previously described (19). SCM medium with or without antimycin A (3 mg/L) was used to measure maltose uptake. To test the resistance of transporters to glucose-induced degradation, we used YNBMH medium (19) with 0–2 mM 2-deoxyglucose (2-DOG). YNBDS medium (19) was used for transporter degradation studies (21). When the strains designated HD were cultivated, geneticin (300 μg/mL) was added to all media to maintain the plasmids constructed using pYCGPY.

DNA manipulations and mutagenesis All PCR primers are listed in Table S2. The construction of plasmids pJHIMAL61 and pJHIMAL21 was previously described (19). *AGT1* was obtained from S288C, *SeAGT1* (*AGT1* ortholog of *Saccharomyces eubayanus-type* gene of *Saccharomyces pastrianus*) and *MTT1* from Weihenstephan German Lager Dry Yeast, SafLager W-34/70 (22) by PCR, and they were inserted downstream of *TPI1* promoter of pJHIXSB (19). Two tandem hemagglutinin tags (2HA) were inserted at *Xho*I site created immediately upstream of the stop codon of *AGT1* to generate pJHIAGT1-2HA. Mutant alleles of *MAL61[E161A]*, *MAL21[E161A]*, *AGT1-2HA[E51P]*, *AGT1-2HA[L55P]*, and *AGT1-2HA[E51G, L55H]* were created from pJHIMAL61, pJHIMAL21, and pJHIAGT1-2HA using a GeneArt mutagenesis kit (Thermo Fisher Scientific, Waltham, MA, USA). The pJHIXSB-based plasmids were integrated into the *ura3* site of JH1032. *MAL61*, *MAL21*, *MAL61[E161A]*, *MAL21[E161A]*, *AGT1-2HA*, *AGT1-2HA[L55P]*, *Se-AGT1*, and *MTT1* were transferred from each pJHIXSB plasmid into the centromeric expression vector pYCGPY under the control of the *PYK1* promoter. The pYCGPY-based plasmids were used to transform D152U and D152MS. The GenBank accession numbers of nucleotide sequences used in this study are as follows: *MAL21*, GenBank: AB453253.1; *MAL61*, GenBank: X17391.1; *MTT1*, GenBank: EF650853.1; and *AGT1*, GenBank: Z73074.1. *SeAGT1* is LBYG13187 (22) obtained from the WS34/70 genome sequence, GenBank: ABPO00000000.1.

Measurement of maltose and glucose uptake activities Using cells (10 OD_{660} unit = OD × mL) grown in each condition, maltose or glucose uptake rate was measured with [^{14}C]-labeled substrates as described in reference (19). The final concentration of sugar was 0.1 mM for HD17 and HD49, and 0.1, 1.0, or 2.0 mM for strains with designations starting with "HH."

Transporter degradation studies Degradation rates of transporters tagged with HA in the presence of glucose were measured by Western blot analysis as described in reference (19) with mouse anti-HA antibody (MMS-101P, Berkeley Antibody Company, Richmond, CA, USA). The assays were repeated three times.

Measurement of maltase activity Maltase activity of cell extract was measured using *p*-nitrophenyl-α-D-glucopyranoside as described in reference (23), and the relative maltase activity per mg protein was calculated.

Measurement of β-galactosidase activity The Yeast β-Galactosidase Assay Kit (Thermo Fisher Scientific) was used to determine β-galactosidase activity according to the manufacturer's manual. Briefly, cells (1.0 OD unit) were collected and extracted in a mildly alkaline solution. Hydrolysis of *o*-nitrophenyl-β-D-galactopyranoside to *o*-nitrophenol and galactose was measured at 420 nm.

Quantitative PCR RNA samples were prepared using the RNeasy Mini Kit (Qiagen, Hilden, Germany). Complementary DNA was synthesized using PrimeScript RT Master Mix (Takara Bio, Shiga, Japan). Quantitative PCR was performed using the SYBR premix Ex Taq kit (Takara Bio) with primer sets for *MAL32* (25 + 26), *lacZ* (27 + 28), and *PDA1* (29 + 30) with a StepOnePlus System (Thermo Fisher Scientific). Expression levels of *MAL32* and *lacZ* were normalized to those of *PDA1*.

Intracellular sugar analysis Cells (5 OD units = OD × mL) were collected from the culture, washed with ice-cold water and lyophilized, and then 1 mL of chloroform:methanol:water (2:5:2), with 8.9 μg/mL ribitol as the internal standard for GC/MS analysis, was added to the cells, and the mixture was vortexed. The sample was centrifuged at 16,000 ×*g* and 4°C for 3 min, and 900 μL of the supernatant was collected and mixed with 400 μL of water. The sample was centrifuged, and 500 μL of the supernatant was centrifugally dried and then freeze-dried. Samples were derivatized as follows: (i) Oximation, 50 μL of methoxyamine hydrochloride in pyridine (20 mg/mL) was added before incubation at 30°C for 90 min. (ii) Trimethyl silylation, 50 μL of *N*-methyl-*N*-trimethylsilyltrifluoroacetamide was added before incubation at 37°C for 30 min.

GC/MS analysis was performed using a GC-2010 Plus gas chromatograph (Shimadzu, Kyoto, Japan) with an AOC-20 in-series injector/autosampler (Shimadzu) and a GCMS-QP2010 Ultra mass spectrometer (Shimadzu). We used a fused silica capillary column (30 m long × 0.25 mm inner diameter) coated with 0.25 μm CP-SIL 8-CB low bleed/MS (Agilent Technologies, Tokyo, Japan). The front inlet temperature was 230°C. The flow of helium through the column was 1.12 mL/min. The column temperature was held at 80°C for 2 min and then ramped from 80°C to 330°C at 15°C/min and held isothermally for 6 min. The transfer line and ion-source temperatures were 250°C and 200°C, respectively. Scans (20/s) were recorded from 85 to 500 *m/z*. Sugar concentration was quantified using an external standard, and the intracellular sugar concentration was calculated using a cell size that was measured using a CDA-500 particle counter (Sysmex Corporation), according to the electrical sensing zone method (24), and the cell number per OD unit was counted using a hemocytometer.

Analysis of nucleotide species and metabolites produced by glycolysis Cultured cells (10 OD units of cells per sample) were collected by filtration using 0.2 μm isopore membrane filters (Millipore Corporation, Billerica, MA, USA) and washed twice with ice-cold water. The cells were sonicated in 1.6 mL of methanol for 30 s to inactivate enzymes. Next, the cell extract was treated with 1.1 mL of Milli-Q water containing internal standards (H3304-1002, Human Metabolome Technologies, Inc., Tsuruoka, Japan) and incubated for another 30 s. To remove proteins, the extract was centrifuged at 2300 ×*g* at 4°C for 5 min, and then 1.6 mL of the upper aqueous layer was centrifugally filtered through a Millipore 5 kDa cutoff filter at 9100 ×*g* at 4°C for 120 min. For CE/MS analysis, the filtrate was centrifugally concentrated and resuspended in 50 μL of Milli-Q water. Human Metabolome Technologies, Inc. performed the metabolome analyses. Analysis was conducted using independent triplicate samples.

Determination of intracellular pH Intracellular pH was determined using flow cytometry with the fluorescent pH-indicator carboxy SNARF-4F AM acetate from Thermo Fisher Scientific, according to the reference method (25).

RESULTS AND DISCUSSION

Characterization of α-glucoside transporters Each α-glucoside transporter expression unit driven from *TPI1* promoter was integrated into JH1032, which overexpresses Mal32p maltase. Maltose uptake activities of α-glucoside transporters were measured using these strains. The activities of Agt1p, SeAgt1p, and Mtt1p, which have broad substrate specificities, were much lower than those of Mal61p and Mal21p (Table 1). To compare the degradation of transporters in the presence of glucose, each strain was spotted on maltose-based SCM containing 2-DOG