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Alleviation of Glucose Repression of Maltose Metabolism by *MIG1* Disruption in *Saccharomyces cerevisiae*

CHRISTOPHER J. L. KLEIN,[1] LISBETH OLSSON,[1]* BIRGITTE RØNNOW,[2] JØRN DALGAARD MIKKELSEN,[2] AND JENS NIELSEN[1]

Department of Biotechnology, Center for Process Biotechnology, Technical University of Denmark, DK-2800 Lyngby,[1] and Danisco Biotechnology, Danisco A/S, DK-1001 Copenhagen K,[2] Denmark

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The *MIG1* gene was disrupted in a haploid laboratory strain (B224) and in an industrial polyploid strain (DGI 342) of *Saccharomyces cerevisiae*. The alleviation of glucose repression of the expression of *MAL* genes and alleviation of glucose control of maltose metabolism were investigated in batch cultivations on glucose-maltose mixtures. In the *MIG1*-disrupted haploid strain, glucose repression was partly alleviated; i.e., maltose metabolism was initiated at higher glucose concentrations than in the corresponding wild-type strain. In contrast, the polyploid Δ*mig1* strain exhibited an even more stringent glucose control of maltose metabolism than the corresponding wild-type strain, which could be explained by a more rigid catabolite inactivation of maltose permease, affecting the uptake of maltose. Growth on the glucose-sucrose mixture showed that the polyploid Δ*mig1* strain was relieved of glucose repression of the *SUC* genes. The disruption of *MIG1* was shown to bring about pleiotropic effects, manifested in changes in the pattern of secreted metabolites and in the specific growth rate.

Industrial carbon sources, like molasses and wort, as well as dough contain mixtures of sugars, which the yeast *Saccharomyces cerevisiae* metabolizes in a certain order. The most important underlying regulation is known as glucose control; i.e., the presence of glucose inhibits the uptake and metabolism of other sugars, like sucrose, galactose, and maltose.

Maltose utilization in *S. cerevisiae* requires the presence of at least one of the five *MAL* gene loci: *MAL1* through *MAL4* and/or *MAL6*. Each of these unlinked, highly homologous loci contains at least one copy of *MALR*, *MALS*, and *MALT*, coding for a regulatory protein, a maltase (α-D-glucopyranoside glucohydrolase [EC 3.2.1.20]), and maltose permease, respectively. In one of the alternative systems of nomenclature, a number is added to the name of the gene locus: e.g., *MAL61*, *MAL62*, and *MAL63* correspond to *MALT*, *MALS*, and *MALR*, respectively, of the *MAL6* locus (for a review, see Vanoni et al. [47]). Maltose permease transports maltose across the cell membrane, and subsequently, the cytoplasmic maltase hydrolyzes maltose into two units of glucose, which then are channelled through the glycolytic pathway. Recently, the gene *AGT1*, which codes for an α-glucoside transporter with characteristics similar to those of MalTp but with a wider substrate specificity, has been characterized and could turn out to be as important as the maltose permease (21).

The control over *MAL* gene expression is exerted at three levels (Fig. 1). The presence of maltose induces, whereas glucose represses the transcription of the *MALS* and *MALT* genes (12, 13, 27). The constitutively expressed regulatory protein (MalR) binds near the *MALS* and *MALT* promoters (4, 30) and mediates the induction of *MALS* and *MALT* transcription (8) (Fig. 1, arrows 1). Furthermore, experiments with *MALR*-disrupted strains led to the conclusion that MalRp is involved in glucose repression (15, 48). Relatively little attention has been paid to posttranscriptional control, i.e., the control of translational efficiency (42), or mRNA turnover, as mechanisms complementing glucose repression. The addition of glucose to induced cells has been reported to cause a 70% increase in the lability of a mRNA population containing a fragment of *MALS* (12) (Fig. 1, arrow 2). The third level of control is posttranslational modification. In the presence of glucose, maltose permease is either reversibly converted to a conformational variant with a decreased affinity (32, 41) or irreversibly proteolytically degraded (17, 26, 33), depending on the physiological conditions. The latter phenomenon is called catabolite inactivation (22) (Fig. 1, arrow 3). The transmembrane transport of maltose is regarded to be the limiting step for maltose metabolism (10, 16).

Glucose repression has been extensively reviewed (14, 24, 34, 45). In 1990 Nehlin and Ronne demonstrated that *MIG1* encodes a repressor protein (28). This gene is identical to *SSN1* (3, 46) and *CAT4* (11, 39). The Mig1p repressor protein has been proposed to confer specificity upon a repression complex consisting of the Tup1p and Ssn6p proteins (44). Hu et al. have shown that Mig1p represses the transcription of all three *MAL* genes (Fig. 1) by binding upstream of them (23). Previously, *SUC2* and most genes of the *MEL-GAL* regulon were also found to be under Mig1p control (28, 29).

In this study, the *MIG1* gene was disrupted in a laboratory strain and in an industrial polyploid strain. The effects of these disruptions on glucose control of both maltose and sucrose metabolism were investigated in batch cultures.

MATERIALS AND METHODS

Yeast and bacterial strains. *S. cerevisiae* G26 and SMH8 were generated from the industrial strain DGI 342 (Danisco Distillers, Copenhagen, Denmark). Two copies of *MIG1* in strain G26 and four copies of *MIG1* in SMH8 were disrupted at its chromosomal locus with *MEL1* by the method of Rothstein (36). *S. cerevisiae* T408 was generated from strain B224 in which *MIG1* was disrupted with *MEL1* (see Table 1). *Escherichia coli* DH5α (Bethesda Research Laboratories) was used for transformation and plasmid amplification and preparation.

Preparation and analysis of DNA and RNA. Plasmid DNA from *E. coli* was

* Corresponding author. Mailing address: Center for Process Biotechnology, Department of Biotechnology, Building 223, Technical University of Denmark, DK-2800 Lyngby, Denmark. Phone: 45 45 25 26 84. Fax: 45 45 88 41 48. Electronic mail address: LO@ibt.dtu.dk.

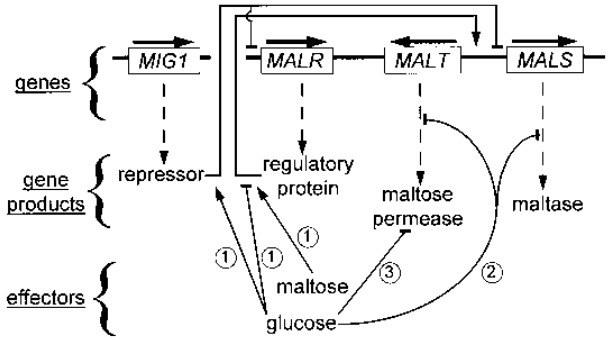

FIG. 1. Control of *MAL* gene expression and related gene products on the transcriptional (arrows 1), posttranscriptional (arrow 2), and posttranslational (arrow 3) levels. The thick arrows indicate the direction of transcription, the broken arrows indicate gene expression, and the thin arrows indicate either inductive effects (arrowheaded) or repressive effects (hammerheaded).

prepared with Qiagen columns (Diagen GmbH, Düsseldorf, Germany) following the manufacturer's instructions. Total yeast DNA was prepared by the method of Struhl et al. (43) with minor modifications and by using Zymolyase 100T for spheroplast formation.

For restriction analysis, gel electrophoresis, and Southern blot experiments, standard protocols were followed (37). For the purification of DNA fragments used for cloning experiments, the desired fragments were separated on 0.7% agarose gels, excised, and recovered from agarose by using BIOTRAP BT1000 (Schleicher und Schüll, Düren, Germany). After synthesis of oligonucleotides on an Applied Biosystems 392 DNA/RNA Synthesizer, they were desalted over NAP10 columns (Pharmacia, Uppsala, Sweden). The *MALS* (accession no. SCYBR299W) and *MALT* (accession no. SCYBR298C) sequences used as hybridization probes in Northern (RNA) blot analyses were amplified by PCR. *MALS* was amplified with the primers 5′-CAA GCC AAT TCC TCC AAA CA-3′ and 5′-AAT TCA AAG CAG CAA ACA GC-3′, and *MALT* was amplified with the primers 5′-AAT CGG TCT ATG TCT ATG CT-3′ and 5′-AGT TTC CAA ATC TTC CTT CG-3′. The *MIG1* sequence used as a hybridization probe in Southern blot analysis was a 2.0-kb *Cla*I restriction endonuclease fragment from pMIG1, and the *MEL1* sequence used as a hybridization probe in Southern blot analysis was a 2.8-kb *Bam*HI-*Sal*I restriction endonuclease fragment from pMP550. Klenow Fill-in Kit (Stratagene, La Jolla, Calif.) was used to fill in restriction endonuclease sites according to the manufacturer's instructions.

For preparation of mRNA, 20 mg of the cell mass harvested during cultivation was transferred to a Falcon tube and spun down for 1 min at 4°C. After the supernatant was discarded, the tube was immediately put into liquid nitrogen and subsequently stored at −80°C. Isolation of cytoplasmic RNA was performed by the method of Schmitt et al. (38). RNA was separated on a 1% agarose–2.2 M formaldehyde gel. DNA and RNA bound to positively charged nylon membranes (Boehringer GmbH, Mannheim, Germany) were hybridized, and DNA probes were randomly primed and labelled with digoxigenin by using the DIG DNA Labelling and Detection Kit from Boehringer GmbH according to the manufacturer's instructions.

Genetic transformation and analysis. Both *E. coli* and *S. cerevisiae* were transformed by electroporation, using a Gene Pulser/Pulse Controller (Bio-Rad, Richmond, Calif.) according to the manufacturer's instructions. Mating, sporulation, and tetrad analysis were performed as previously described by Rose et al. (35).

Pulsed-field electrophoresis of intact yeast chromosomes. Preparation of chromosome-size DNA molecules was performed by the method of Schwartz and Cantor (40) with minor modifications (31). Electrophoretic separation of the yeast chromosomal DNA was carried out with contour-clamped homogeneous electric fields (7) by using the CHEF-mapper (Bio-Rad, Richmond, Calif.).

Plasmid constructions. The plasmids used for disruption of the *MIG1* gene were made by the following procedure. The 2.0-kb *Cla*I restriction endonuclease fragment from pMIG1 (28) containing the 5′ untranslated region and most of the coding region was subcloned into the *Cla*I site of pBluescript II SK (Stratagene) in which the *Eco*RI sites had previously been destroyed. The 90-bp *Eco*RI fragment within the coding region of *MIG1* was removed from the resulting plasmid and replaced with the selection marker *MEL1*. This marker was excised from pMP550 as a 2.8-kb *Bam*HI-*Sal*I fragment, to which *Eco*RI linkers were added, and the resulting plasmid was termed pHJ26.

Media for strain development. Bacterial strains were grown in Luria-Bertani medium (37). When needed for plasmid maintenance, ampicillin was present at 100 mg · liter^{-1}.

Yeast cells used in the molecular biology work were grown on solid or liquid YP medium (35) containing carbon sources at the following concentrations: glucose (0.67 C-mol · liter^{-1}), galactose (0.07 C-mol · liter^{-1}), ethanol (1.30 C-mol · liter^{-1}), and glycerol (0.65 C-mol · liter^{-1}) (35). (One C-mole of a compound is the quantity of the compound $C_aH_bO_cN_d$ containing 1 g-atom (12.011 g) of carbon and corresponds to the compound formula weight, with the carbon subscript a taken as unity, i.e., $CH_{b/a}O_{c/a}N_{d/a}$ [1].) For appropriate screening and selection, 5-bromo-4-chloro-3-indolyl-α-D-galactopyranoside (X-α-Gal) and G418 were used in concentrations of 25 and 200 mg · liter^{-1}, respectively.

The sporulation regimen utilized presporulation medium containing 8 g of yeast extract liter^{-1}, 3 g of peptone liter^{-1} and 3.3 C-mol of glucose liter^{-1}, and sporulation medium containing 10 g of potassium acetate liter^{-1}, 1 g of yeast extract liter^{-1}, and 0.017 C-mol of glucose liter^{-1}.

Media for physiological investigations. Adjusting the medium composition of Bruinenberg et al. (2) to a final cell concentration of 4 and 12 g · liter^{-1}, the following mineral media were used. The precultures grew in flasks with 100 ml of medium of the following composition: $(NH_4)_2SO_4$, 7.5 g · liter^{-1}; KH_2PO_4, 14 g · liter^{-1}; $MgSO_4 \cdot 7H_2O$, 0.74 g liter^{-1}; trace element solution, 10 ml · liter^{-1}; vitamin solution, 1 ml · liter^{-1}; Synperonic antifoam, 50 μl · liter^{-1}; and glucose, 0.33 C-mol · liter^{-1}. The pH was adjusted to 6.5 with NaOH. The medium used in the batch cultures contained: $(NH_4)_2SO_4$, 7.5 g · liter^{-1}; KH_2PO_4, 3.5 g · liter^{-1}; $MgSO_4 \cdot 7H_2O$, 2.7 g · liter^{-1}; trace element solution, 20 ml · liter^{-1}; vitamin solution, 2 ml · liter^{-1}; Synperonic antifoam, 50 μl · liter^{-1}; glucose, 0.7 C-mol · liter^{-1}; and either maltose (0.7 C-mol · liter^{-1}) or sucrose (0.6 C-mol · liter^{-1}). Glucose, maltose, and sucrose were autoclaved separately from the minerals. The medium for auxotrophic strains B224 and T408 was supplemented with 50 mg of histidine liter^{-1} and 100 mg of uracil liter^{-1}.

The trace element solution had the following components: EDTA, 3.00 g · liter^{-1}; $CaCl_2 \cdot 2H_2O$, 0.90 g · liter^{-1}; $ZnSO_4 \cdot 7H_2O$, 0.90 g · liter^{-1}; $FeSO_4 \cdot 7H_2O$, 0.60 g · liter^{-1}; H_3BO_3, 200 mg · liter^{-1}; $MnCl_2 \cdot 2H_2O$, 156 mg · liter^{-1}; $Na_2MoO_4 \cdot 2H_2O$, 80 mg · liter^{-1}; $CoCl_2 \cdot 2H_2O$, 60 mg · liter^{-1}; $CuSO_4 \cdot 5H_2O$, 60 mg · liter^{-1}; and KI, 20 mg · liter^{-1}. The vitamin solution contained the following ingredients (all in 1 liter); d-biotin, 50 mg; *para*-aminobenzoic acid, 200 mg; nicotinic acid, 1.00 g; Ca-pantothenate, 1.00 g; pyridoxine · HCl, 1.00 g; thiamine · HCl, 1.00 g; and *m*-inositol, 25 g. The vitamin solution was sterilized by filtration, and the trace elements were autoclaved together with the minerals.

Preparation of inoculum. The strains were kept at −80°C in YPD medium (35) containing 200 ml of glycerol liter^{-1}. They were spread onto YPD agar plates, and one colony was taken from a plate to inoculate a shake flask.

The shake flasks were baffled, cotton-stopped, 500-ml Erlenmeyer flasks, each containing 100 ml of medium. Cells were grown at 30°C at 120 rpm until the cell mass had reached 1.5 g · liter^{-1} and were harvested in the exponential growth phase. The bioreactors were inoculated to give 1.50 mg of cell mass (dry weight) per liter.

Bioreactor culture conditions. All strains were grown in well-controlled carbon-limited batch cultures at 30°C in in-house-manufactured, four-baffle 5-liter bioreactors with a working volume of 4 liters and fitted with two disk turbine impellers rotating at 800 rpm. The pH was controlled at pH 5.0 ± 0.1 by the automatic addition of 4 M NaOH or 2 M H_2SO_4. The air flow was 1 vvm (4 liter · min^{-1}), and the off gas was led through a condenser.

Determination of cell mass concentration. The dry weight was determined by using nitrocellulose filters with a pore size of 0.45 μm (Gelman Sciences, Ann Arbor, Mich.). The filters were predried in a microwave oven at 150 W for 10 min. A known volume of cell culture was filtered; the residue was washed with distilled water and dried on the filter for 15 min at 150 W.

The optical density was determined at 525 nm using a Shimadzu Micro Flow Spectrophotometer CL-720. Samples were diluted either 1:10 or 1:50 with distilled water, if the optical density exceeded 0.3.

Analysis of sugars and metabolites. For measurements of extracellular metabolites, cultivation medium was sampled, immediately filtered through a 0.45-μm-pore-size cellulose acetate filter (Sartorius AG, Göttingen, Germany), subsequently frozen, and kept at −20°C until analysis.

Glucose, maltose, ethanol, glycerol, acetate, and pyruvate were separated on an Aminex HPX-87H column (Bio-Rad, Hercules, Calif.) at 65°C, using 5 mM H_2SO_4 as a mobile phase at a flow rate of 0.6 ml · min^{-1}. Glucose, maltose, ethanol, and glycerol were detected refractometrically (Waters 410 Differential Refractometer Detector [Millipore Corp., Milford, Mass.), whereas acetate and pyruvate were monitored spectrophotometrically with a Waters 486 Tunable Absorbance Detector set at 210 nm.

Sucrose was separated on a Waters Sugar-Pak column (Millipore Corp.), kept at 90°C, using 0.1 mM Ca-EDTA as the mobile phase, at a flow rate of 0.5 liter · min^{-1}, and then subjected to refractometrical analysis with the detector mentioned above.

Off gas analysis. The concentration of carbon dioxide in the off gas was measured either with a Brüel & Kjær 1308 acoustic gas analyzer (6) (Brüel & Kjær, Nærum, Denmark) or with a Servomex 1410 infrared analyzer (Servomex Ltd., Crowborough, Sussex, United Kingdom).

Enzyme assays. For maltase determination, 12 mg of cell mass was taken out of the bioreactor, sedimented by centrifugation (4°C), and washed twice with cold PK-DTT buffer (0.1 M potassium phosphate, 1.0 mM dithiothreitol; pH 6.8). The pellet was resuspended in 3 ml of PK-DTT buffer and divided into three 2-ml Eppendorf tubes. A 0.5-ml sample of acid-treated glass beads (diameter, 0.75 to 1.0 mm) was added to each tube. The tubes were frozen in liquid nitrogen and subsequently stored at −80°C. After the cells were thawed, they were disintegrated in a Retsch MM2 vibrator (Retsch, Haan, Germany) by using

TABLE 1. Yeast strains used in the genetic work

Strain(s)	Relevant genotype	Origin
B224 (wild type)	*MAT***a** *cdc6 his4 ura1 MAL2 gal1 SUC*	Glenn Kawasaki, 1979
T408	*MAT***a** *cdc6 his4 ura1 MAL2 gal1 SUC mig1*::*MEL1*	This study
DGI 342 (wild type)	*MIG1*/*MIG1*/*MIG1*/*MIG1*	Danisco Distillers
T302, T304, T306	*mig1*::*MEL1*/*MIG1*/*MIG1*/*MIG1*	This study
D1776, D1800	*mig1*::*MEL1*/*MIG1*	This study
D1784, D1809	*mig1*::*MEL1*/*MIG1*	This study
G1 to G28	*mig1*::*MEL1*/*mig1*::*MEL1*/*MIG1*/*MIG1*	This study
SM25, SM26, SM37, SM39, SM41 to SM44, SM46	*mig1*::*MEL1*/*mig1*::*MEL1*	This study
SMH1 to SMH8	*mig1*::*MEL1*/*mig1*::*MEL1*/*mig1*::*MEL1*/*mig1*::*MEL1*	This study
D286-2A	*MAT***a** *ade1 his1*	Cold Spring Harbor Laboratory
D273-11A	*MATα ade1 his1*	Cold Spring Harbor Laboratory

maximum vibration for 30 min at 4°C. Cell debris was removed by centrifugation (20,000 × *g*, 20 min, 0°C). Part of the supernatant was diluted 1:30 and 1:100 in PK-DTT buffer, and the remainder was frozen again, for a later protein determination. The maltase assay described by Halvorson and Ellias (20) was modified for use in microtiter plates: each well of a microtiter plate was filled with 100 μl of PK-DTT buffer, 50 μl of supernatant (in triplicate), and 50 μl of a solution of 5 mM PNPG (*p*-nitrophenyl-α-D-glucopyranoside) in PK-DTT buffer. A dilution row of 1.2 mM *p*-nitrophenol served as a standard. Within half an hour, the change of A_{405} was measured at 5-min intervals with the help of an Anthos Reader 2001 with a thermostat set at 30°C (Anthos Labtec Instruments, Salzburg, Austria). The obtained slope was taken as a measure of PNPG-hydrolyzing activity, one unit being defined as 1 μmol of released *p*-nitrophenol per min. Six runs of the same sample revealed a standard deviation of about 6%.

In order to exclude any major α-glucosidase activity other than maltase, some samples were also measured as follows: maltose was used instead of PNPG as a substrate, and the liberated glucose was monitored in a spectrophotometric assay. The PNPG assay was confirmed to give a reliable measure of maltase activity.

The protein measurement was based on the Bradford assay (1a) and was also modified for microtiter plates. Twenty microliters of sample or bovine serum albumin standard (0 to 150 mg of protein · liter^{-1}) was pipetted in quadruplicate into the wells of a microtiter plate, before 230 μl of protein assay dye reagent concentrate (Bio-Rad GmbH, Munich, Germany) that had been diluted 5 times was added. After 20 min, the absorption was measured at 620 nm. Six runs of the same sample revealed a standard deviation of about 5%.

The maltose permease activity was assayed as described previously by Loureiro-Dias and Peinado (25), with the following minor modifications; a sample of 40 mg of cells was used to obtain two 4-ml portions of a cell suspension with a concentration of 5 g · liter^{-1}. The in-house-manufactured jacketed cell for the kinetic measurements was maintained at 30°C. Calibrations were performed with 40 μl of 10 mM NaOH. The assay was started by adding 0.5 ml of 1 M maltose solution to the cell suspension, giving a concentration higher than 20 K_m (5).

RESULTS

Yeast strains. In order to investigate the effects of disruption of the *MIG1* gene, both a haploid laboratory strain (B224) and a polyploid industrial strain (DGI 342) were genetically engineered as follows. Upon cotransformation of B224 with 3 μg of pBEJ16 (18), containing the heterologous *PGK* promoter-driven G418 resistance determinant for *S. cerevisiae*, and 10 μg of the excised 4,780-bp *Cla*I restriction fragment from pHJ26, approximately 6,000 clones resistant to G418 were isolated. After replica plating the bacteria onto the chromogenic substrate X-α-Gal under inducing conditions with galactose and glycerol, 16 clones were obtained. One clone was randomly chosen, subjected to plasmid loss under nonselective conditions, and restreaked on medium containing G418 and X-α-Gal. Of these 16 clones, six clones were chosen, and Southern blot analysis of extracted genomic DNA, cut with *Cla*I, employing digoxigenin-labelled *MIG1* and *MEL1* as hybridization probes, confirmed correct integration in all six clones. For the physiological experiments, one of these six clones, T408, was chosen (Table 1).

As a first step in obtaining Δ*mig1* strains, 8 μg of the excised 4,780-bp *Cla*I restriction endonuclease fragment from pHJ26 and 1 μg of pBEJ16 were used to cotransform strain DGI 342, resulting in 560 clones resistant to G418. Of these 560 clones, 3 clones, T302, T304, and T306 (Table 1), turned green on medium containing X-α-Gal. These three clones were subsequently subjected to plasmid loss and restreaked onto medium containing G418 and X-α-Gal. Correct integration was confirmed as described above. T302, T304, and T306 were sporulated, and a total of 120 tetrads were dissected, of which 55 spore clones were viable. Spores from this initial sporulation are expected to be diploid; mating spores would be *MAT***a**/*MAT***a** or *MATα*/*MATα*, and sporulating spores would be *MAT***a**/*MATα*. Screening for sporulation resulted in 43 nonsporulating spore clones which were mating type tested with strains D286-2A and D273-11A. Two spore clones with mating type **a**, D1776 and D1800 (Table 1), and two spore clones with mating type α, D1784 and D1809 (Table 1), were isolated and restreaked on medium containing X-α-Gal. The results of Southern analysis done as described above confirmed that in these strains one *MIG1* copy was intact, whereas the other was disrupted with *MEL1*.

Strains D1776, D1800, D1784, and D1809 were subsequently crossed with each other. A total of 28 hybrids (G1 to G28) were isolated, and for the physiological experiments, G26, which is a hybrid between D1800 and D1809, was chosen.

mig1$^-$ strains are able to grow on raffinose in the presence of 2-deoxyglucose (28), so D1776, D1800, D1784, and D1809 were screened on this medium in order to select homozygous Δ*mig1*/Δ*mig1* spontaneous recombinants. A total of 63 recombinants were isolated, and Southern blot analysis, performed as previously described, revealed that nine true recombinants (SM25, SM26, SM37, SM39, SM41 to SM44, and SM46) were derived from D1800 and one true recombinant, SM52, was derived from D1809.

Strain SM52 was crossed with SM25, SM26, SM37, SM39, SM41 to SM44, and SM46, and from each cross, one hybrid was isolated, resulting in strains SMH1 to SMH8. For the physiological experiments, SMH1, SMH2, SMH7, SMH8, and SM44 were chosen, and SMH8 was investigated in detail.

Molecular hybridization of the *MALR* probe to a DNA blot of size-separated yeast chromosomes released from strains B224, T408, DGI 342, G26, and SMH8 showed that DGI 342 (and G26 and SMH8) possess the following loci: *MAL1*,

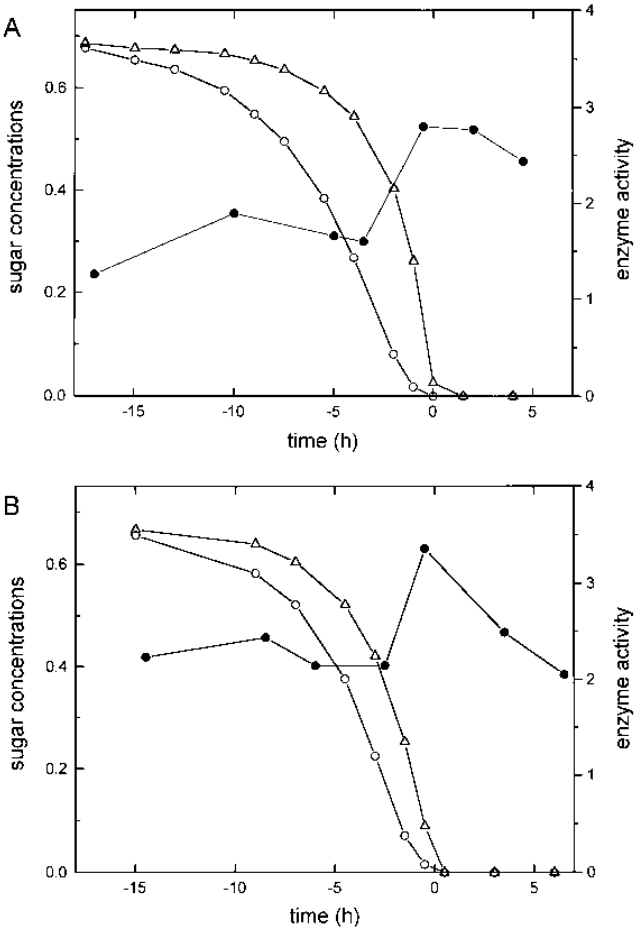

FIG. 2. Maltase activity and concentrations of glucose and maltose in aerobic batch cultivations of strains B224 (A) and T408 (B) on a glucose-maltose mixture, plotted as a function of time. The time point of sugar depletion is set to zero. Symbols: ○, glucose concentration (in C-moles · liter^{-1}); △, maltose concentration (in C-moles · liter^{-1}); ●, maltase activity (in units milligram of protein^{-1}).

MAL3, and *MAL4* on chromosomes VII, II, and XI, respectively, whereas B224 (and T408) possesses *MAL3*.

Batch cultivations on glucose-maltose mixtures. The impact of *MIG1* disruption was first investigated in the laboratory strain and compared with disruption in an industrial strain.

In batch cultivations of the haploid strains B224 (wild type) and T408 (Δ*mig1*), neither a fully simultaneous uptake nor a distinctly biphasic uptake could be observed, but in both cultures, glucose uptake preceded maltose uptake (Fig. 2). The time span between the point when half of the glucose had been metabolized and the point when half of the maltose had been metabolized was 1.9 h in the genetically modified strain compared with 3.4 h in the wild-type strain.

T408 exhibited a higher maximum specific growth rate (μ = 0.25 h^{-1}) than B224 (μ = 0.21 to 0.22 h^{-1}) (Table 2). Beside cell mass, ethanol was the main product, caused by respiro-fermentative growth (Crabtree effect). As sugar was consumed, the concentrations of ethanol, glycerol, and pyruvate increased to reach maximum levels at the time of sugar depletion, whereas the concentration of acetate continued to increase after sugar depletion. Final metabolite concentrations of ethanol and pyruvate were found to lie within the same range for both strains (Table 2). At the end of the first growth phase, the

TABLE 2. Growth characteristics from aerobic batch cultivations of five selected strains on a glucose-maltose mixture[a]

Strain	Max. sp. growth rate (h^{-1})[b]	Cell mass yield (g · C-mol^{-1})	Max. sp. sugar uptake (C-mol · g^{-1} · h^{-1})	Max. ethanol concn (C-mol · liter^{-1})	Max. glycerol concn (C-mmol · liter^{-1})	Max. acetate concn (C-mmol · liter^{-1})[c]	Max. pyruvate concn (C-mmol · liter^{-1})	No. of cultivations	Illustrated in Fig.
Haploids									
B224 (wt)[d]	0.21–0.22	2.5	NC[e]	0.69	38	27	4.1	1	2A
T408	0.25 ± 0.00	2.3 ± 0.1	NC	0.69 ± 0.04	78 ± 15	6 ± 1	3.2 ± 0.2	2	2B
Tetraploids									
DGI 342 (wt)	0.45 ± 0.03	3.7 ± 0.1	0.12 ± 0.00	0.67 ± 0.06	21 ± 2	9 (14) ± 4	6.1 ± 0.0	2	4A, 5
G26	0.35 ± 0.01	3.2 ± 0.0	0.10 ± 0.01	0.63 ± 0.06	36 ± 2	10 (13) ± 2	2.6 ± 0.2	2	4B, 5
SMH8	0.34 ± 0.01	3.4 ± 0.1	0.10 ± 0.01	0.65 ± 0.03	45 ± 4	7 (12) ± 2	2.3 ± 0.7	3	4C, 5

[a] Values are means ± standard deviations. Abbreviations: Max. sp., Maximum specific; Max., maximum.
[b] Maximum specific growth rates are calculated on the basis of optical density at 525 nm.
[c] Values reflect a local minimum or shoulder of acetate concentration before the point of complete sugar depletion. Maximum concentrations after depletion are given in parentheses.
[d] wt, wild type.
[e] NC, not calculated.

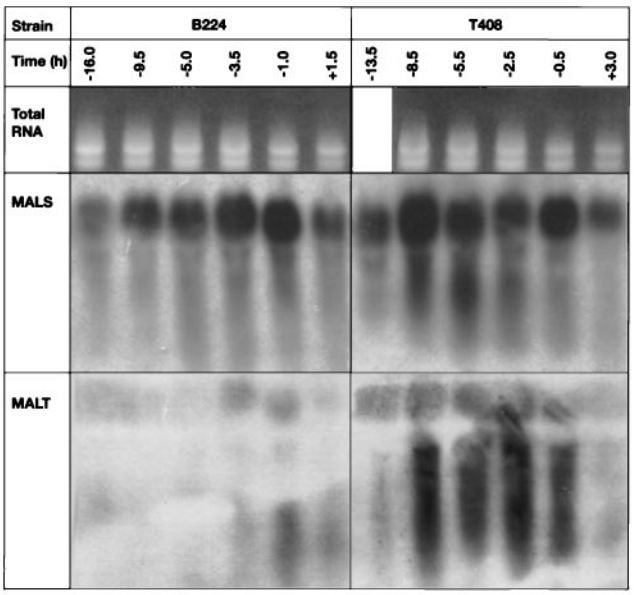

FIG. 3. Northern blot analysis of *MALS* and *MALT* expression from strains B224 (*MIG1*) and T408 (Δ*mig1*). Samples containing 20 mg of cell mass were taken at different times during the glucose-maltose cultivations of B224 and T408 (Fig. 2), and the total RNA was extracted, separated by electrophoresis, blotted, and hybridized with digoxigenin-labelled *MALS* and *MALT* probes. Equal loading was confirmed by ethidium bromide staining (total RNA, top panels) of the gel before blotting.

B224 culture had accumulated 38 C-mmol of glycerol liter^{-1} and 27 C-mmol of acetate liter^{-1}, whereas T408 had accumulated 78 C-mmol of glycerol liter^{-1} and 6 C-mmol of acetate liter^{-1}. The ethanol concentration reached 0.69 C-mol · liter^{-1} accounting for half the amount of metabolized carbon added to the cultures in the form of glucose and maltose. Cell mass yields were between 2.3 and 2.5 g (dry weight) · C-mol^{-1} in both strains (Table 2).

In strain B224, the maltase activity started at 1.5 U · mg of protein^{-1} and increased to 2.5 to 3 U · mg of protein^{-1}, whereas in strain T408, it started at 2 U · mg of protein^{-1} and increased to 3 U · mg of protein^{-1} (Fig. 2). The maltose permease activity was initially about 1 mmol · g (dry weight)$^{-1}$ · h^{-1} for both strains and increased an order of magnitude upon glucose depletion during cultivation. The highest activity in T408 was 1.5 times the highest activity of B224 (data not shown). Northern blots indicated concomitant induction profiles for mRNA of *MALT* and *MALS* in both cultivations and higher initial levels of these mRNAs in a T408 cultivation than in a B224 cultivation (Fig. 3).

In batch cultivations of the polyploid strains DGI 342 (*MIG1*/*MIG1*/*MIG1*/*MIG1*), G26 (*MIG1*/*MIG1*/Δ*mig1*/Δ*mig1*), and SMH8 (Δ*mig1*/Δ*mig1*/Δ*mig1*/Δ*mig1*), glucose uptake preceded maltose uptake. Maltose uptake was not apparent from sugar measurements until at least half of the initial glucose had been consumed (Fig. 4). This delay was more pronounced in G26 than in DGI 342, and in SMH8 there was not any simultaneous uptake of glucose and maltose (Fig. 5). In this plot, a curve parallel with the abscissa at low glucose concentrations would reflect total glucose control; i.e., all glucose would be consumed before maltose would commence to be consumed. On the other hand, simultaneous uptake would be reflected as a linear curve with a slope of 1 for equal starting concentrations of glucose and maltose. The polyploid genetically modified strains, G26 and SMH8, obviously exhibited tighter glucose control of maltose metabolism than the wild-type strain DGI 342.

The polyploid genetically modified strains, G26 and SMH8, had lower maximum specific growth rates (0.35 and 0.34 h^{-1}, respectively) than the wild-type strain, DGI 342, with $\mu = 0.45$

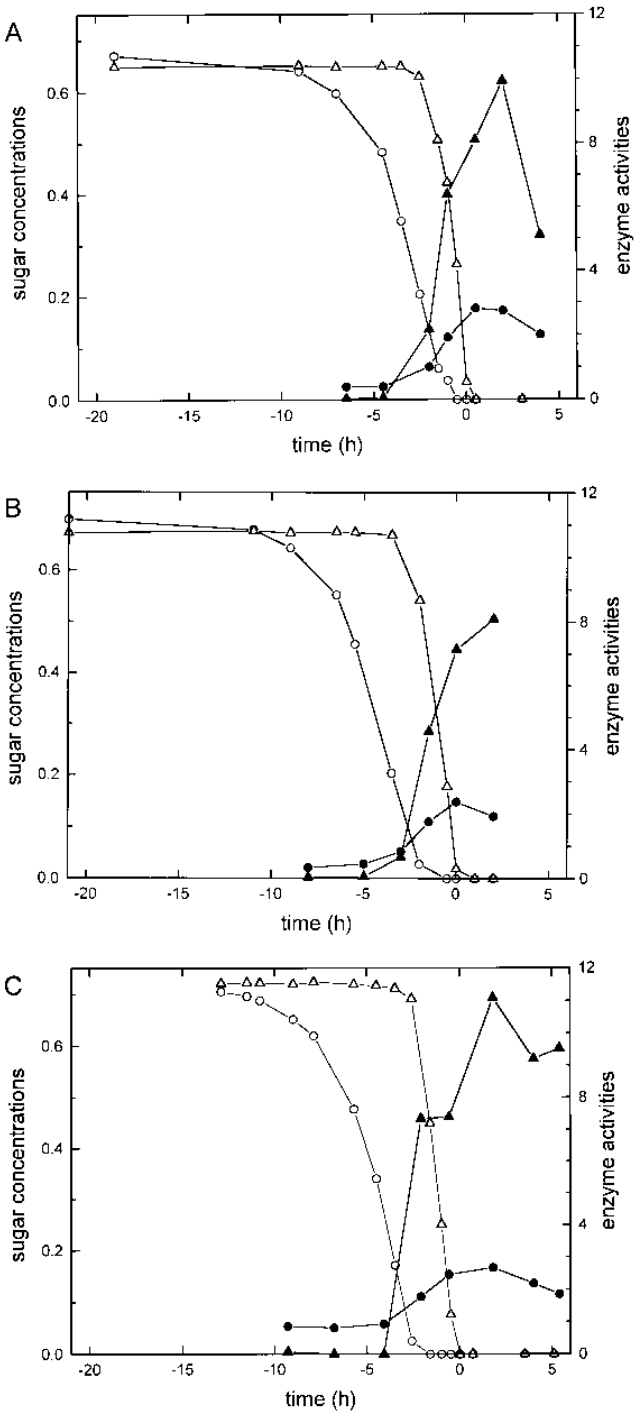

FIG. 4. Sugar concentrations and maltase and maltose permease activities in aerobic batch cultivations of strains DGI 342 (A), G26 (B), and SMH8 (C) on a glucose-maltose mixture, plotted as a function of time. Symbols: ○, glucose concentration (in C-moles · liter^{-1}); △, maltose concentration (in C-moles · liter^{-1}); ●, maltase activity (in units milligram of protein^{-1}); ▲, maltose permease activity (in millimoles of proton gram of cell mass^{-1} hour^{-1}).

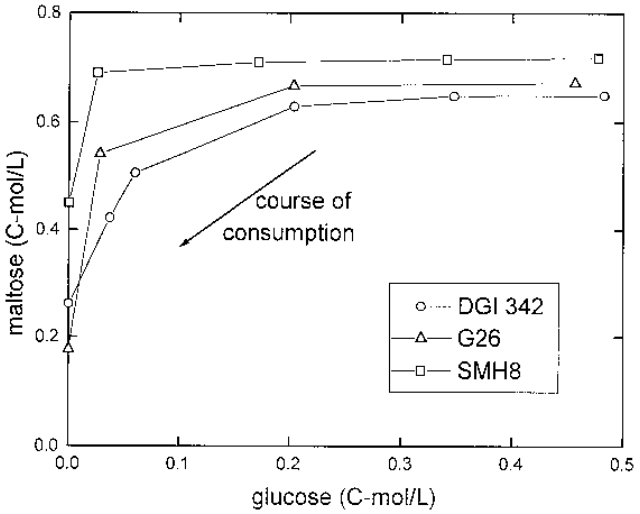

FIG. 5. Plot of the maltose concentration as a function of the glucose concentration for the batch cultivations presented in Fig. 4.

h^{-1} (Table 2). The maximum specific rate of sugar uptake was 0.12 C-mol · g (dry weight)$^{-1}$ · h^{-1} in DGI 342 and 0.10 C-mol · g (dry weight)$^{-1}$ · h^{-1} in G26 and SMH8. The cell mass yield in G26 was 3.2 g (dry weight) · C-mol^{-1} compared with 3.4 to 3.7 g (dry weight) · C-mol^{-1} in the other two strains (Table 2).

The glycerol concentration in the medium of G26 and SMH8 reached 36 and 45 C-mmol · liter^{-1}, respectively, whereas 21 C-mmol · liter^{-1} was found in the DGI 342 cultures. The concentration of pyruvate in the G26 and SMH8 cultures was 2 to 3 C-mmol · liter^{-1} compared with 6 C-mmol · liter^{-1} in the DGI 342 culture. Both ethanol and acetate concentrations were in the same range for the three strains and together accounted for half the amount of carbon metabolized from the sugars (Table 2).

The increase in maltase and maltose permease activities on glucose depletion followed the similar profile in all three strains. The initial maltase activity in DGI 342 and G26 was below 0.5 U · mg of protein^{-1}, whereas SMH8 was characterized by a higher initial maltase level (0.8 U · mg of protein^{-1}) (Fig. 4). After the glucose concentration had decreased below 0.3 mol · liter^{-1}, the maltase activity started to increase to its maximum level of 2.4 to 2.8 U · mg of protein^{-1}, which in our experiments was reached after depletion of both sugars (Fig. 4). Maltose permease activity was not detectable at a glucose concentration higher than 0.4 C-mol · liter^{-1} but increased simultaneously with increasing maltase activity to a value of about 10 mmol · g (dry weight)$^{-1}$ · h^{-1} after glucose was depleted (Fig. 4), with maximum activity obtained 2 h after both sugars were depleted.

Northern blots indicated a simultaneous induction of *MALT* and *MALS* in all three strains, with maximum mRNA levels at the time of glucose depletion (Fig. 6). In G26, there are also indications of higher initial mRNA levels than in DGI 342, when glucose was still present in the medium, and even higher levels in SMH8.

Cultivations of SMH1, SMH2, SMH7, and SM44 confirmed that SMH8 is a representative member of the strains that do not have any *MIG1* allele intact (data not shown).

Batch cultivations on glucose-sucrose mixtures with polyploid strains. The investigation of the influence of *MIG1* disruption upon the maltose metabolism in polyploid strains was extended to the regulation of the *SUC* genes (24). Expression of *SUC* genes is repressed only by glucose and not induced by sucrose, and consequently, no inducer exclusion can occur. *SUC* genes encode invertase, which hydrolyzes sucrose into glucose and fructose.

Strains DGI 342 and SMH8 were cultivated on mixtures of 0.7 to 0.8 C-mol of glucose liter^{-1} and 0.6 C-mol of sucrose liter^{-1} (Fig. 7). While sucrose hydrolysis was not detectable in DGI 342 before the glucose concentration had decreased below 0.2 C-mol · liter^{-1} (Fig. 7A), immediate sucrose hydrolysis was apparent in SMH8 from the start of the cultivation and resulted in an increase in the fructose concentration to 0.3 C-mol as well as in the glucose concentration (Fig. 7B). The intermittent accumulation of fructose and glucose reflects that the rate of sucrose hydrolysis was higher than the subsequent uptake and metabolism of glucose and fructose. Sucrose had been completely hydrolyzed in SMH8 4 to 5 h before total sugar depletion, whereas this time span was 1 h for DGI 342.

Glucose pulse to maltose-grown polyploid strains. Pulse experiments were performed in order to gain insight into the kinetics of the glucose control system.

Batch cultivations of strains DGI 342 and SMH8 were started on 0.7 C-mol of maltose liter^{-1}, and after they had consumed 0.2 to 0.3 C-mol · liter^{-1}, a glucose pulse to give a concentration of 0.7 C-mol · liter^{-1} was given (Fig. 8). Subsequent to the pulse addition, the volumetric maltose uptake rate (slope of maltose concentration in Fig. 8) decreased and became constant after 45 min, whereafter it remained constant for 3 to 4 h. Maltose uptake accelerated again when the glucose concentration had decreased to 0.3 to 0.4 C-mol · liter^{-1} for DGI 342 and to 0.1 C-mol · liter^{-1} for SMH8. After the pulse, the maltase activity decreased to about 50% of the activity at the time of pulse addition and the activity recovered almost completely when the volumetric maltose uptake accelerated (Fig. 8).

DISCUSSION

An alleviation of glucose control of *MAL* genes by *MIG1* disruption was shown in a haploid *S. cerevisiae* strain. The

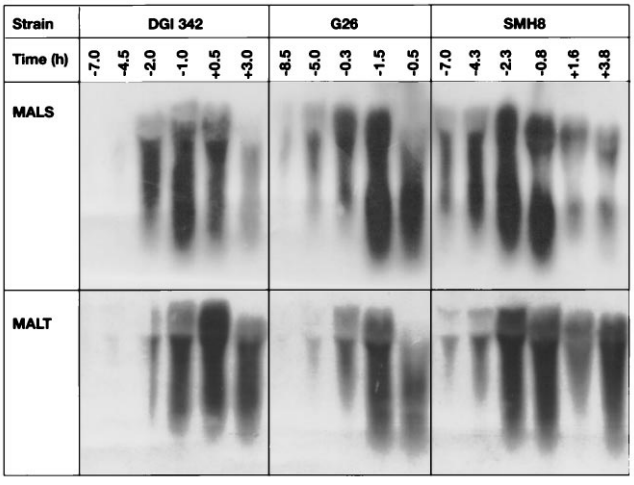

FIG. 6. Northern blot analysis of *MALS* and *MALT* expression originating from strains DGI 342 (*MIG1*/*MIG1*/*MIG1*/*MIG1*), G26 (*MIG1*/*MIG1*/Δ*mig1*/Δ*mig1*), and SMH8 (Δ*mig1*/Δ*mig1*/Δ*mig1*/Δ*mig1*). Samples containing 20 mg of cell mass were taken at different times during the glucose-maltose cultivations of DGI 342, G26, and SMH8 (Fig. 4), and the total RNA was extracted, separated by electrophoresis, blotted, and hybridized with digoxigenin-labelled *MALS* and *MALT* probes.

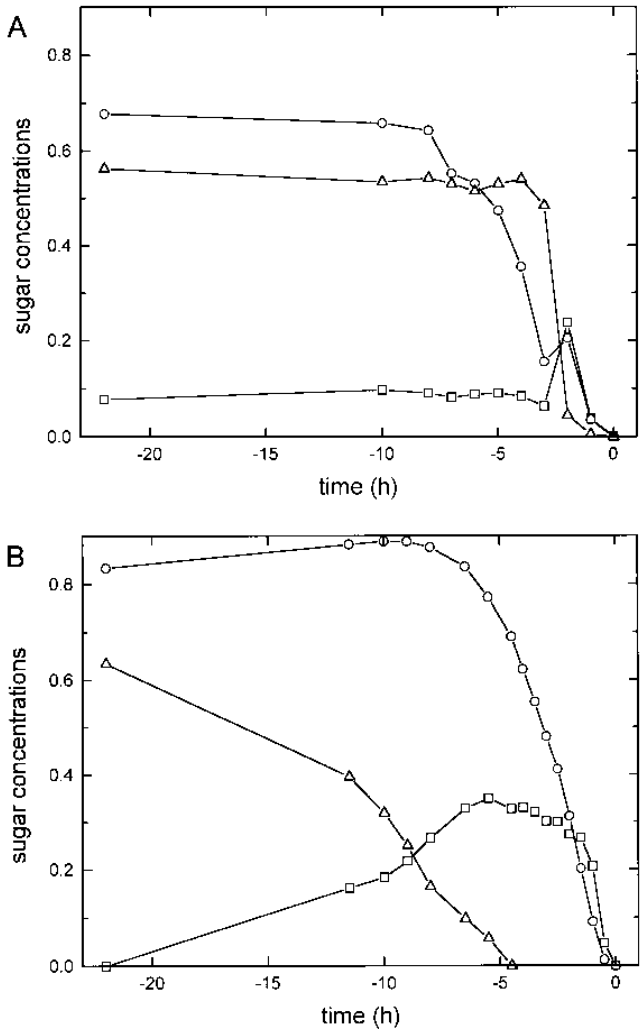

FIG. 7. Concentrations of sucrose, glucose, and fructose during aerobic batch cultivations of strains DGI 342 (A) and SMH8 (B) on a sucrose-glucose mixture. Fructose was present from the start in the DGI 342 cultivation as a result of sucrose hydrolysis during sterilization. Symbols: ○, glucose; △, sucrose; □, fructose. All concentrations are given in C-moles · liter^{-1}.

temporal separation of glucose and maltose uptake was less pronounced in the *MIG1*-disrupted strain T408 than in the corresponding wild-type strain B224 (Table 1 and Fig. 2). The alleviation of glucose repression could be corroborated by indications of higher levels of *MALS* and *MALT* mRNA under inducing-repressing conditions (in the presence of glucose and maltose) in T408 than in B224 (Fig. 3).

The impact of *MIG1* disruption was also investigated with the polyploid strains DGI 342, G26, and SMH8 (Table 1). It was shown that glucose control could be alleviated for the sucrose-metabolizing system (Fig. 7) but not for the maltose-metabolizing system (Fig. 4 and 5). A main difference between the two systems is that the *SUC* genes do not need to be induced, whereas all *MAL* genes require the presence of maltose for induction (see the introduction). The more-rigid glucose control of maltose metabolism is not due to stricter glucose repression of *MAL* genes, as indicated by higher *MALS* and *MALT* mRNA levels in G26 and SMH8 (Fig. 6), than in DGI 342.

The sequential utilization of glucose and maltose illustrated in Fig. 2 and 4 is consistent with reported results from shake flask cultivations (19). The critical glucose threshold, under which maltose utilization ceased to be glucose controlled, is estimated to be between 0.10 and 0.15 C-mol · liter^{-1} for DGI 342 (Fig. 5) and is concordant with a reported threshold of 0.12 C-mol · liter^{-1} in a commercial baker's yeast strain (41). Maltase and maltose permease activities increased concomitantly (Fig. 2 and 4) (9), and Northern blots indicated that *MALS* and *MALT* gene expression is induced simultaneously (Fig. 3 and 6).

When DGI 342 and SMH8 were compared both under inducing-repressing conditions (<0.5 U · mg of protein in DGI 342 compared with 0.8 U · mg of protein in SMH8) and under inducing conditions (2.5 to 3 U · mg of protein for both strains), maltase activity turned out to be less sensitive to glucose repression in the *MIG1*-disrupted strain than in the wild type (Fig. 4). This observation was corroborated by Northern blots for *MALS* and *MALT* mRNA (Fig. 6) and is consistent with results reported by Hu et al. (23).

The low or absent activity of maltose permease under inducing-repressing conditions indicates catabolite inactivation (Fig. 2 and 4). Pulse experiments pointed to maltose uptake as the critical factor of glucose control, as the specific maltase activity could not account for the restricted volumetric maltose uptake (slope of maltose curve in Fig. 8). The specific uptake rate of

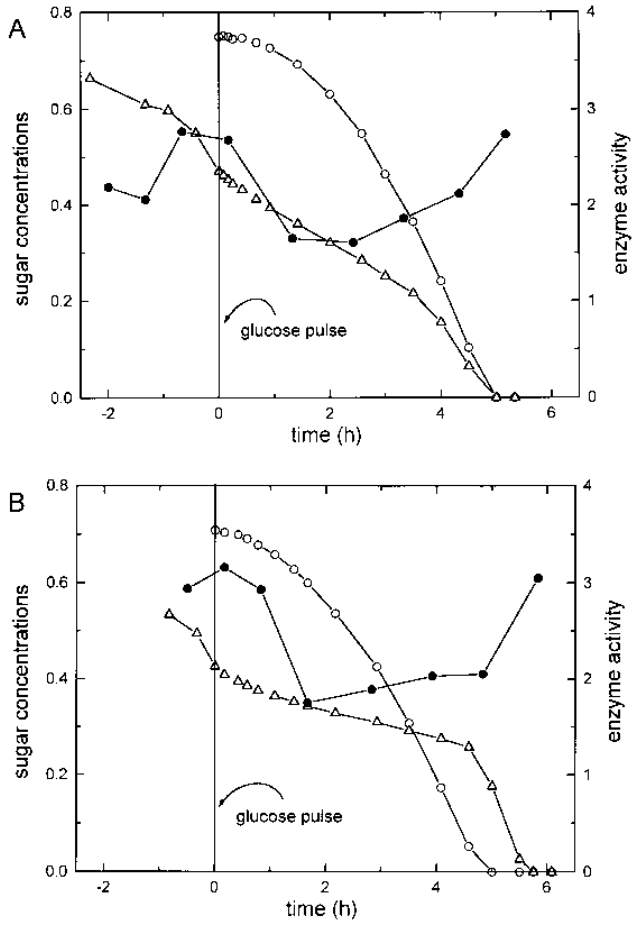

FIG. 8. Pulse experiments with strains DGI 342 (A) and SMH8 (B). At time zero, a glucose pulse was added to cultures that had been growing on maltose. Symbols: ○, glucose concentration (in C-moles · liter^{-1}); △, maltose concentration (in C-moles · liter^{-1}); ●, maltase activity (in units milligram of protein^{-1}).

maltose as a function of cell mass is more than double the value in DGI 342 compared with SMH8 throughout the period of constant volumetric maltose uptake (plot not shown), underlining the more-rigid glucose control in SMH8 found in the batch cultivations. The decreased specific maltose uptake after the glucose pulse can be explained with a stop in *MAL* gene expression and with the reversible conversion of maltose permease to a lower-affinity form under inducing-repressing conditions. The latter phenomenon, reported to occur immediately (41), is characteristic for growing cells that are not nitrogen depleted (32). The transition period, lasting 45 min after the pulse, is suggested to comprise the time needed to inhibit *MAL* gene expression; a transition period of 30 min has been reported for complete maltase inhibition (12).

Hu et al. have identified a *MIG1*-independent glucose control of *MAL* genes, which is stronger than *MIG1*-dependent control, and have attributed it to inducer exclusion (23). This supports our assumption that catabolite inactivation of maltose permease remains the limiting factor in maltose metabolism, which could be counteracted by overexpression of *MALT* (Fig. 1, arrow 3). In addition to the altered glucose control, the disruption of *MIG1* was shown to bring about pleiotropic effects like significant changes in the maximum specific growth rate, maximum specific sugar uptake rate, and pattern of maximum metabolite concentrations in the medium (Table 2). The disruption of all four *MIG1* alleles resulted in a decrease of the maximum specific growth rate from 0.45 (DGI 342) to 0.34 (SMH8) h^{-1}, a decrease of the maximum specific sugar uptake rate from 0.12 to 0.10 C-mol $\cdot$ g^{-1} h^{-1}, and an increase of the maximum glycerol concentration in the medium from 21 to 45 C-mmol $\cdot$ $liter^{-1}$.

In conclusion, *MIG1* disruption resulted in an alleviation of glucose repression of the *MAL* and *SUC* genes. However, the glucose control in strains G26 and SMH8 was stricter than in strain DGI 342, indicating that Mig1p may have an impact on the translational efficiency, and/or posttranslational modification, in the polyploid strains (Fig. 1). As part of their more complex genetic background, the polyploid strains possess three *MAL* loci (*MAL1*, *MAL3*, and *MAL4*), while the haploid strains carry only *MAL3*. The pleiotropic effects of *MIG1* disruption in the haploid and polyploid strains make it clear that the role of Mig1p still remains to be fully elucidated.

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