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Glucose and Maltose Metabolism in *MIG1*-disrupted and *MAL*-constitutive Strains of *Saccharomyces cerevisiae*

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Summary

The alleviation of glucose control of maltose metabolism brought about by MIG1 disruption was compared to that by MAL overexpression in a haploid Saccharomyces cerevisiae strain. The sugar consumption profiles during cultivation of the wild type, single transformants and a double transformant in a mixed glucose-maltose medium revealed that the MAL- constitutive strains were more alleviated than the single MIG1-disrupted transformant. While all transformants exhibited higher maximum specific growth rates (0.24–0.25 h^{-1}) in glucose--maltose mixtures than the wild type strain (0.20 h^{-1}), the MAL-constitutive transformants grew even faster (0.27–0.30 h^{-1}) in pure glucose medium than the wild type strain (0.24 h^{-1}).

Keywords: *Saccharomyces cerevisiae*, baker's yeast, glucose repression, catabolite inactivation, *MIG1*, *MAL*, genetic engineering

Introduction

Maltose metabolism in *Sacch. cerevisiae* plays an important role in the utilisation of industrial carbon sources like brewer's wort and flour. In malt-derived brewer's wort maltose accounts for 50–60% of the total sugar *(1)*. The main component in flour (dough) is starch, which is hydrolysed to maltose by the action of amylases from the wheat flour or by that of industrial enzymes added as a processing aid. In *Sacch. cerevisiae* maltose is taken up into the cell by maltose permease (encoded by *MALT*), before it is hydrolysed by maltase (encoded by *MALS*) into glucose (Fig. 1). The transcription of *MALT* and *MALS* is subject to maltose induction and glucose repression *(2)*. Glucose control is not limited to the mechanism of glucose repression of transcription, but also comprises higher rates of *MALS* mRNA (and probably *MALT* mRNA) degradation in the presence of glucose, and higher rates of degradation of maltose permease in the presence of glucose, referred to as catabolite inactivation *(3–6)*. Since glucose is unavoidably present in brewer's wort, dough and most other industrial carbon sources, processes employing these are delayed, which lowers the economic efficiency.

Glucose repression acts through a signalling cascade, important elements of which are encoded by *HXK2*, *SNF1* and *MIG1* (Fig. 1; for reviews see *7–11*). Hxk2 is a hexokinase assumed to function as a glucose sensor. Snf1 is a serine/threonine protein kinase, which possibly controls the activity of the Mig1 protein *(12)*. Mig1 binds to the promoter of glucose-repressible genes, recruiting the Tup1- Ssn6 repressor complex *(13)*.

The lower part of the glucose signalling cascade is well understood, which makes it suitable for genetic engineering. Mutation of *MIG1* is one option; in fact, maltose metabolism in *MIG1*-disrupted strains has already been reported to be partly glucose-derepressed *(14,15)*. The alternative, relief of glucose repression by silencing *TUP1* and/or *SSN6*, is less feasible since the Tup1-Ssn6 complex is also recruited by other effectors for the transcription of haploid-specific and **a**-specific genes *(16)*, glucose induced genes *(17)* and hypoxic genes *(18)*. The undesired pleiotropic effects caused by mutation of *TUP1* or *SSN6* have been reviewed by Johnston and Carlson *(19)*.

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Overexpression of glucose-repressed genes is another strategy to alleviate the effects of glucose control/repression. *MALS* and *MALT* overexpression resulted in higher maltose utilisation rates in a baker's yeast strain *(20, 21)*. The same effect has been observed for a *MALT*^{c} brewer's yeast strain *(22)*.

In this paper, we quantify and compare the degree of alleviation of glucose control of maltose metabolism brought about by *MIG1* disruption and/or by overexpression of the two essential genes for maltose utilisation, *MALT* and *MALS*.

Material and Methods

Yeast and bacterial strains

Sacch. cerevisiae T408 was generated from B224 (*MAT***a** *cdc6 his4 ura1 MAL2 MAL3 gal1 SUC*) in which *MIG1* was disrupted by *MEL1* as described by Klein *et al. (15)*. Strains T442 and T433 were generated from B224 and T408, respectively, by transforming these strains with the integrative plasmid pTZ18RhygBMAL *(21)*. The plasmid pTZ18RhygBMAL contains *MALT* controlled by the *ADH* promoter, and *MALS* controlled by the *TEF* promoter. *Escherichia coli* DH5α (Bethesda Research Laboratories) was used for transformation, plasmid amplification and preparation.

Preparation and analysis of DNA, genetic transformation and analysis

Plasmid DNA from *E. coli* was prepared with Qiagen columns (Qiagen GmbH, Düsseldorf, Germany) following the manufacturer's instructions. Total DNA was prepared by the method of Struhl *et al. (23)* with minor modifications and by using Zymolyase 100T for spheroblast formation.

For restriction analysis, gel electrophoresis, and Southern blot experiments, standard protocols were followed *(24)*. 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 BP1000 (Schleicher und Schüll, Düren, Germany). Southern analysis was performed using a 4660 bp HindIII fragment from pTZ18RhygBMAL, containing the *hygB* resistance gene, as a probe. DNA bound to positively charged nylon membranes (Boehringer GmbH, Mannheim, Germany) was hybridised. The 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.

Both *E. coli* and *Sacch. cerevisiae* were transformed by electroporation, using a Gene Pulser/Pulse Controller (Bio-Rad, Richmond, California, U.S.A.).

Media for strain development

Bacterial strains were grown in LB medium *(24)*. When needed for plasmid maintenance, ampicillin was present at 100 mgL^{-1}. Yeast cells were grown on solid or liquid YP media *(25)* containing carbon sources at the following concentrations: glucose (0.67 C-mol L^{-1}), galactose (0.07 C-mol L^{-1}), ethanol (1.30 C-mol L^{-1}), and glycerol (0.65 C-mol L^{-1}). (The unit C-mol signifies moles of carbon atoms; its use facilitates the comparison of consumed sugar and formed metabolite concentrations *(26)*). For appropriate screening and selection, 5-bromo-4-chloro-3-indolyl-α-D-galactopyranoside (X-α-Gal), and hygromycin were used in concentrations of 25 and 300 mg L^{-1}, respectively.

Construction of yeast strains

In yeast strain T408 *MIG1* was disrupted with *MEL1* as described earlier *(15)*. Yeast strains B224 and T408 were genetically engineered as follows. Plasmid pTZ18-RhygBMAL was cut with Bg1II, a unique restriction site located within *MALS*. Ten μg of the isolated, linearised plasmid was used to transform B224 and T408, and 20 transformants were obtained after transformation of B224 whereas 82 transformants were obtained after transformation of T408. Six transformants were chosen from each transformation, and correct integration at the *MALS* locus was confirmed by Southern blot analysis of extracted genomic DNA, cut with HindIII, employing digoxigenin-labelled *hygB* as hybridisation probe. Subsequently, T442, a transformant of B224, and T433, a transformant of T408, were chosen for the physiological studies.

Culture media and culture conditions

The composition of defined media employed for precultures and for batch cultivations was similar to that used by Bruinenberg *et al. (27)*, with appropriate uracil and histidine supplementation *(15)*. From cryocultures a loop full of stock culture was streaked onto a YPD plate, from which precultures were inoculated. These were grown at 30 °C in baffled flasks containing 100 mL of 0.33 C-mol L^{-1} glucose medium, until the cell mass had reached 1.5 g L^{-1}. The bioreactors were then inoculated to give 1.50 mg of cell mass (dry weight) per litre.

The in-house four-baffled bioreactors had a working volume of four litres and were fitted with two disk turbine impellers. The temperature was controlled at (30.0±0.2) °C, the pH value at 5.0±0.1, the agitation at 800 RPM, and the air flow at 1 vvm (four litres per minute). The total initial sugar concentration was 1.3 C-mol L^{-1}. The strains were grown either on glucose or maltose alone, or on a mixture containing 0.7 C-mol L^{-1} glucose and 0.6 mol L^{-1} maltose (21 and 18 gL^{-1}, respectively).

Analytical methods

The analytical methods used in this study have been described previously *(15)*, and are only briefly mentioned here. The cell mass concentration was determined by dry weight measurements and absorbance measurements at 525 nm, both performed in duplicate. About eight measurements were taken in the range from 0.5 to 3 gramme cell mass per litre for the determination of the specific growth rate, which was calculated by linear regression through the logarithmic values. For the analysis of sugars (glucose and maltose) and metabolites (ethanol, glycerol, acetate and pyruvate) cultivation medium was sampled, immediately filtered, separated by HPLC and detected refractometrically or spectrophotometrically *(15)*.

Maltase activity was assayed spectrophotometrically measuring the absorbance of the *para*-nitrophenol released on hydrolysis of the substrate *para*-nitrophenyl-α--D-glucopyranoside (PNPG). The specific activity is given in units per mg of protein, one unit being defined as 1 μmol of released *para*-nitrophenol per minute at 30 °C. The maltose uptake system is a maltose – proton symport. Maltose permease activity on whole washed cells was determined by monitoring the increase in pH after a maltose pulse in the suspension medium. The specific activity is given in mmol of protons taken up by one gramme of dry cell weight per hour at a temperature of 30 °C.

Results

Batch cultivations on glucose – maltose mixtures

The first set of experiments was intended to compare the impact of *MIG1* disruption with that of *MAL* overexpression in mixed sugar batch cultivations. After preculturing in glucose medium, the four strains B224 *(MIG1, MAL)*, T408 *(Δmig1, MAL)*, *T442* (*MIG1, MAL^c*) *and T433* (*Δmig1, MAL^c*) were cultivated on a glucose – maltose mixture. All strains tested exhibited rather a simultaneous uptake pattern, *i.e.* no distinctly biphasic sugar uptake (Fig. 2). In wildtype B224, however, maltose uptake was delayed considerably with respect to glucose uptake. In *MIG1* disruption transformant T408 the time span between the point when half of the glucose had been consumed and when half of the maltose had been consumed was decreased by 50% (Fig. 2A and B). In *MAL^c* transformants T442 and T443 maltose con-

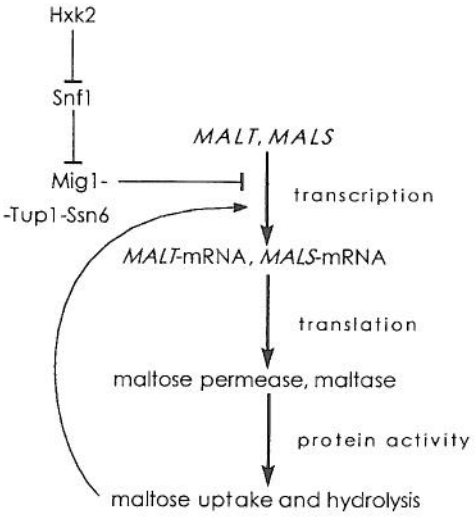

Fig. 1. Gene expression of *MALS* and *MALT*. Thick arrows indicate *MAL* gene expression; thin arrows indicate either inductive effects (arrow-headed) or repressive effects (hammer-headed). Transcription is induced by maltose taken up into the cell and is repressed by glucose, which is mediated by Mig1.

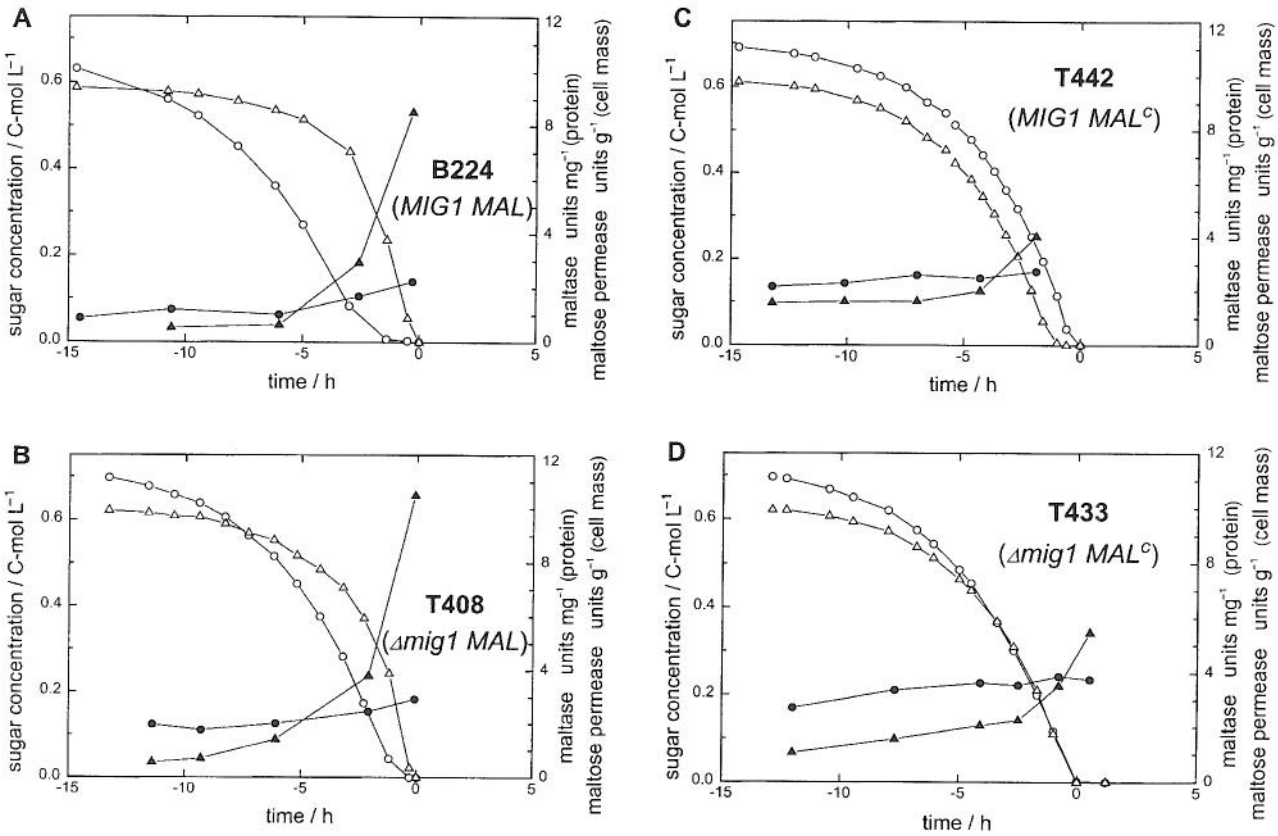

Fig. 2. Sugar concentrations and maltase and maltose permease activities in aerobic batch cultivations of (A) strain B224 *(MIG1, MAL)*, (B) strain T408 *(Δmig1, MAL)*, (C) strain T442 *(MIG1, MAL^c)*, (D) strain T433 *(Δmig1, MAL^c)* in a glucose – maltose mixture, plotted as a function of time.
(○ glucose, Δ maltose, ▲ maltose permease, ● maltase)

sumption caught up with that of glucose, and in T442 maltose consumption even preceded that of glucose (Fig. 2C and D). The results of sugar consumption in the four batch cultivations are collected in the same phase plot (Fig. 3). In this plot total glucose control would be reflected by a horizontal line for glucose concentrations greater than zero (pure glucose consumption), followed by a vertical line on the ordinate after full depletion of glucose (consumption of remainder maltose). Alleviated glucose control would be reflected by curves closer to the straight line that links the origin with the point of starting sugar concentrations (constant sugar uptake ratios). From Fig. 3 the order of decreasing glucose control is B224 → T408 → T433 → T442.

While the B224 and T408 cultivations passed from inducing-repressing conditions (presence of maltose and glucose until 1–2 hours before sugar depletion) to inducing conditions (presence of maltose the remaining 1–2 hours), T442 passed from inducing-repressing to repressing conditions (presence of glucose), and T433 remained under inducing-repressing conditions throughout the whole cultivation.

In all four strains half the amount of carbon supplied by the sugars was metabolised to ethanol (Table 1). At the end of the first growth phase significantly higher glycerol and lower acetate concentrations had been accumulated in *Δmig1* strains (71–79 C-mmol L^{-1} and 6–9 C-mmol L^{-1}, respectively) compared with their parental strains (46–57 C-mmol L^{-1} and 16–21 C-mmol L^{-1}, respectively). The final pyruvate concentrations were in the same order of magnitude for all strains (4–7 C-mmol L^{-1}) (Table 1). The maximum specific growth rates calculated from dry weight measurements for the three transformants T408, T442 and T433 (μ = 0.24–0.25 h^{-1}) were significantly higher than that for the wild type strain B224 (μ = 0.20 h^{-1}). Cell mass yields were between 2.3 and 2.5 g C-mol^{-1} (Table 2).

The maltase activity increased slightly during the course of each cultivation. It was lowest in the wild type strain (0.9 to 2.2 U/mg protein, greater in T408 and T442 (1.8 to 2.9 U/mg protein and 2.2 to 2.7 U/mg protein, respectively), and highest in T433 (2.7 to 3.9 U/mg pro-

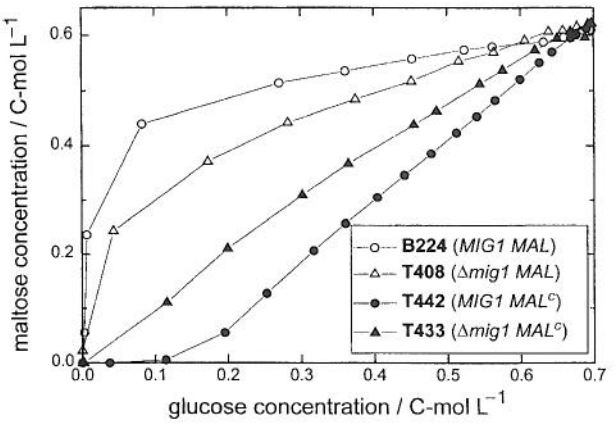

Fig. 3. Plot of the maltose concentration as a function of the glucose concentration for the batch cultivations presented in Fig. 2.

Table 1. Metabolite formation in aerobic batch cultivations of *Sacch. cerevisiae* laboratory strains in a glucose – maltose mixture[a]

Strain	Genotype	Max. ethanol concentration	Max. glycerol concentration	Max. acetate concentration	Max. pyruvate concentration	Number of experiments
		C-mol L^{-1}	C-mol L^{-1}	C-mol L^{-1}	C-mol L^{-1}	
B224	*(MIG1, MAL)*	0.65 ± 0.02	46 ± 8	21 ± 8	5 ± 1	6
T408	*(Δmig1, MAL)*	0.65 ± 0.04	71 ± 10	6 ± 3	4 ± 1	6
T442	*(MIG1, MALᶜ)*	0.69	57	16	7	1
T433	*(Δmig1, MALᶜ)*	0.66 ± 0.04	79 ± 16	9 ± 2	7 ± 2	4

[a] The initial sugar concentration is 0.7 C-mol L^{-1} glucose plus 0.6 C-mol L^{-1} maltose

Table 2. Specific growth rates and cell mass yields of *Sacch. cerevisiae* laboratory strains

Strain	Genotype	Sugars in medium[a]	μ *(DCW)* h^{-1}	$\mu(A^b)$ h^{-1}	Yield/g C-mol^{-1}	No. of exper.
B224 (wt)[c]	*(MIG1, MAL)*	glucose	0.24 ± 0.01	0.24 ± 0.02	2.6 ± 0.1	3
		maltose	0.22	0.23	2.6	1
		glucose-maltose	0.20 ± 0.01	0.21 ± 0.02	2.4 ± 0.1	6
T408	*(Δmig1, MAL)*	glucose	0.23	0.26	2.6	1
		maltose	0.21	0.21	2.6	1
		glucose-maltose	0.25 ± 0.03	0.25 ± 0.02	2.5 ± 0.2	6
T442	*(MIG1, MALᶜ)*	glucose	0.27 ± 0.02	0.29 ± 0.02	2.6 ± 0.1	5
		maltose	0.25 ± 0.01	0.26 ± 0.00	2.6 ± 0.1	2
		glucose-maltose	0.25	0.25	2.4	1
T433	*(Δmig1, MALᶜ)*	glucose	0.30 ± 0.02	0.31 ± 0.03	2.6 ± 0.1	3
		maltose	0.21 ± 0.02	0.22 ± 0.02	2.7 ± 0.2	2
		glucose-maltose	0.24 ± 0.01	0.27 ± 0.02	2.3 ± 0.1	4

[a] The initial concentrations are 1.3 C-mol L^{-1} for glucose, 1.3 C-mol L^{-1} for maltose, and 0.7 C-mol L^{-1} plus 0.6 C-mol L^{-1} for glucose-maltose mixtures.;
[b] Absorbance was measured at 525 nm;
[c] wild type

tein). The maltose permease activity of B224 and T408 increased from <1 to >8 units/g (dry weight) h^{-1}, whereas that of T442 and T433 was between 1 and 6 units/g (dry weight) h^{-1} (Fig. 2).

Batch cultivations on pure glucose and pure maltose media

In the second set of experiments, the four strains B224, T408, T442 and T433 were cultivated in both pure glucose and pure maltose media, in order to determine their maximal specific growth rates as well as the cell mass yields on each single sugar. The cultivations yielded 2.3 to 2.7 g of cell mass per C-mol of consumed sugar (Table 2). The maximum specific growth rates were in the range $\mu = 0.20–0.30\ h^{-1}$ (Table 2). All four strains grew faster on glucose than on maltose. While strain T433, grew at 0.21 h^{-1} on maltose and at 0.30 h^{-1} on glucose, the difference in the maximum specific growth rates for the two sugars was only $\Delta\mu = 0.02\ h^{-1}$ for B224, T408 and T442 (Table 2). Moreover, *MAL*^{c} transformants T442 and T433 exhibited higher specific growth rates on glucose (0.27 h^{-1} and 0.30 h^{-1}, respectively) than wild type strain B224 (0.24 h^{-1}).

Discussion

Glucose control of maltose metabolism was shown to be (partly) alleviated both by *MIG1* disruption and by *MAL* gene overexpression in a haploid strain of *Sacch. cerevisiae* (Figs. 2 and 3). Interestingly, the sugar consumption profiles suggest that the single Δ*mig1* transformant T408 *(Δmig1, MAL)* still was more glucose-controlled than the *MAL*^{c} transformants T442 *(MIG1, MAL*^{c}*)* and T433 *(Δmig1, MAL*^{c}*)*. Thus, *MAL* overexpression is concluded to be more effective than silencing of *MIG1*. This can be explained by the different involvement of Mig1 and *MAL* gene products in the mechanisms of glucose control. Glucose control comprises the mechanisms of transcription repression by glucose *(2)*, of glucose-stimulated decay of relative mRNAs *(3)*, and, especially, of catabolite inactivation (maltose permease) *(5,6)*. While a *MIG1* disruption/deletion is in its effects limited to the level of transcriptional control, *MAL* overexpression results in unnaturally high concentrations of mRNA counteracting all mechanisms of glucose control, and thereby may be more effective (Fig. 1).

In strain T433 maltase activity was about 40% higher throughout the cultivation than in T442, which is consistent with the fact that it is not only *MAL*-constitutive, like T442, but also *MIG1*-disrupted, and therefore should be more alleviated of glucose control than T442. However, maltose consumption was more delayed in the cultivation of T433, pointing to an apparently weaker alleviation of glucose control (Figs. 2C and D, 3).

The sugar consumption profiles can be explained by an increased control of maltose uptake in *MIG1* disruption mutant T433, which is also consistent with the maltose permease activity levels in Fig. 2C and D. An increase in catabolite inactivation by *MIG1* disruption/deletion has been concluded from mixed glucose – maltose cultures, where Northern blot analysis proved increased levels of *MALT* mRNA, but maltose permease activity was absent under inducing-repressing conditions until full depletion of glucose *(15)*.

Apart from glucose control, the sugar consumption profiles depend both on the maltose and glucose consumption; *i.e.*, the uptake and hydrolysis of maltose into glucose and its subsequent cytosolic phosphorylation compete with the uptake of glucose and its phosphorylation. Thus, a relatively higher consumption of glucose in T433 could explain the unexpected profile for T433. Indeed, the maximum specific growth rates of T442 and T433 are on glucose $\mu = 0.27\ h^{-1}$ and $\mu = 0.30\ h^{-1}$, on maltose $\mu = 0.25\ h^{-1}$ and $\mu = 0.21\ h^{-1}$, respectively (Table 2). When the maximum specific growth rate on each single sugar was assumed proportional to the specific sugar consumption in a glucose – maltose mixture, the sugar consumption profile could be mathematically simulated (data not shown) and explain the apparently higher degree of glucose control in *MIG1*- disrupted strain T433. Mig1 has been reported to mediate transcriptional repression of glucose transporter and/or sensor genes, like *HXT2, HXT3, HXT4* and *SNF3*, as well as of the genes that encode hexokinase I *(HXK1)* and glucokinase I *(GLK1) (28–30)*. Thus, in the *MIG1*-disrupted strains there could be a higher glucose influx resulting in higher specific growth rates; and if the further processing of intracellular glucose becomes rate-controlling one could expect that the relative maltose uptake rate decreases.

The higher specific growth rates of *MAL*^{c} transformants on glucose compared with B224 point to a further function of *MALS* and *MALT* in addition to encoding proteins that are assigned to maltose uptake and cleavage. An increased activity in trehalose-6-phosphate synthase has been reported for *MAL*^{c} transformants grown on glucose *(31)*. Since *GGS1/TPS1* encodes a subunit of trehalose-6-phosphate synthase, there might be a functional link of the *MAL* genes to this gene. The *MAL* gene function might in some way affect the maximum specific growth rate, as *GGS1/TPS1* is essential for trehalose synthesis, affects the flux of glucose to glycolysis, and is a positive regulator of stimulated mRNA degradation and catabolite inactivation *(32–34)*.

The higher allevation of glucose control by *MAL* overexpression compared to *MIG1* disruption is consistent with the finding of a *MIG1*-independent mechanism of glucose control of *MAL* gene expression *(14)*. One line of research currently investigates the glucose-stimulated degradation of mRNA, like that of *MALT* and *MALS* mRNAs *(34–36)*. A second line of study has investigated the mechanism of catabolite inactivation, like that of maltose permease *(6,37–39)*. These efforts contribute to the knowleddge that is essential to produce genetically engineered strains that are more alleviated from glucose control than *MIG1* disruption strains and that are alleviated for more than one metabolic function.

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Metabolizam glukoze i maltoze u *MIG1*-razgrađenim i *MAL*-konstitutivnim sojevima *Saccharomyces cerevisiae*

Sažetak

Razgradnjom MIG1 smanjena je glukozom uvjetovana represija metabolizma maltoze, te je uspoređena s MAL prekomjernom ekspresijom u haploidnom soju Sacch. cerevisiae. Način utroška šećera u miješanom mediju glukoza-maltoza, tijekom uzgoja divljeg tipa te jednostruko i dvostruko izmijenjenih sojeva, pokazao je da je represija u MAL-konstitutivnim sojevima mnogo manja nego u jednostruko izmijenjenom soju s razgrađenim MIG1. Dok svi izmijenjeni sojevi, u podlogama s glukozom i maltozom, pokazuju veću maksimalnu brzinu specifičnog rasta (0,24–0,25 h^{-1}) od soja divljeg tipa (0,20 h^{-1}), MAL-konstitutivni izmijenjeni soj rastao je brže (0,27–0,30 h^{-1}) u mediju sa čistom glukozom od soja divljeg tipa (0,24 h^{-1}).