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Microarray karyotyping of maltose-fermenting *Saccharomyces* yeasts with differing maltotriose utilization profiles reveals copy number variation in genes involved in maltose and maltotriose utilization

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Abstract

Aims: We performed an analysis of maltotriose utilization by 52 *Saccharomyces* yeast strains able to ferment maltose efficiently and correlated the observed phenotypes with differences in the copy number of genes possibly involved in maltotriose utilization by yeast cells.

Methods and Results: The analysis of maltose and maltotriose utilization by laboratory and industrial strains of the species *Saccharomyces cerevisiae* and *Saccharomyces pastorianus* (a natural *S. cerevisiae*/*Saccharomyces bayanus* hybrid) was carried out using microscale liquid cultivation, as well as in aerobic batch cultures. All strains utilize maltose efficiently as a carbon source, but three different phenotypes were observed for maltotriose utilization: efficient growth, slow/delayed growth and no growth. Through microarray karyotyping and pulsed-field gel electrophoresis blots, we analysed the copy number and localization of several maltose-related genes in selected *S. cerevisiae* strains. While most strains lacked the *MPH2* and *MPH3* transporter genes, almost all strains analysed had the *AGT1* gene and increased copy number of *MALx1* permeases.

Conclusions: Our results showed that *S. pastorianus* yeast strains utilized maltotriose more efficiently than *S. cerevisiae* strains and highlighted the importance of the *AGT1* gene for efficient maltotriose utilization by *S. cerevisiae* yeasts.

Significance and Impact of the Study: Our results revealed new maltotriose utilization phenotypes, contributing to a better understanding of the metabolism of this carbon source for improved fermentation by *Saccharomyces* yeasts.

Keywords

AGT1; gene copy number variation; *MAL* genes; maltotriose; *Saccharomyces*

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Introduction

Saccharomyces yeast strains have been used by humans for millennia for brewing, baking and the production of wine and diverse distilled beverages. The yeast species *Saccharomyces* cerevisiae is considered the predominant agent present in these fermentations, but other species of the *Saccharomyces sensu stricto* complex (such as *Saccharomyces bayanus* and *Saccharomyces paradoxus*) have also been isolated from these industrial processes. Hybrid strains between species in the *Saccharomyces* complex have also been described in wine and beer fermentations, and strains of *Saccharomyces pastorianus*, a natural hybrid yeast between *S. cerevisiae* and *S. bayanus*, are traditionally used to brew lager beers (Querol and Bond 2009).

Many of the industrial applications of *Saccharomyces* yeasts rely on the efficient fermentation of starch hydrolysates rich in the α-glucosides maltose and maltotriose. In the brewing industry, for example, these two sugars are of special importance as they are the predominant sugars in wort (typically 50–60% is maltose, 15–20% is maltotriose), followed by glucose (10–15%) and other minor carbohydrates. Of these sugars, glucose is preferentially and rapidly utilized by yeast cells, but both process efficiency and product quality require the complete fermentation of all sugars, including maltose and maltotriose. Although maltose is easily fermented by the majority of yeast strains after glucose exhaustion, maltotriose is not only the least preferred sugar for uptake by these *Saccharomyces* cells, but many yeasts may not use this α-gluco-side at all (Zheng *et al*. 1994b; Yoon *et al*. 2003). Slow and incomplete yeast sugar fermentation represents a significant economic loss for these industries, and consequently most strain development programmes aim to select yeasts with improved fermentation performance.

Utilization of these α-glucosides requires the active transport of the sugar across the plasma membrane by maltose permeases and its subsequent hydrolysis by cytoplasmic α-glucosidases (maltases). The genetic and biochemical analysis of maltose fermentation by yeast cells revealed a series of five unlinked telomere-associated multi-gene *MAL* loci: *MAL1* (chromosome VII), *MAL2* (chromosome III), *MAL3* (chromosome II), *MAL4* (chromosome XI) and *MAL6* (chromosome VIII). Each locus contains at least one copy of three different genes encoding a maltose permease (*MALx1*, where *x* stands for one of the five loci, e.g. *MAL11* is the permease at the *MAL1* locus on chromosome VII), a maltase (*MALx2*) and a positive regulatory protein (*MALx3*) that induces the transcription of the two previous genes in the presence of maltose (Novak *et al*. 2004). The genes in the *MAL* loci show a high degree of sequence and functional similarity, but there can be extensive variability, and several different alleles that determine distinct phenotypes (i.e. *MAL*-inducible and *MAL*-constitutive strains) have been described. The *MAL1* locus is considered the progenitor locus from where all other *MAL* loci were derived, as all *S. cerevisiae* strains, and even its closest related yeast species *S. paradoxus*, contain *MAL1* sequences near the right telomere of chromosome VII. This holds true even for many maltose nonfermenting strains, which may harbour partially functional *mal1p* (*mal11 mal12 MAL13*), *mal1g* (*MAL11 MAL12 mal13*) or *mal1*^{0} (*mal11 MAL12 mal13*) loci containing only a functional regulator, only a functional permease and maltase or only a functional maltase, respectively (Charron and Michels 1988; Naumov *et al*. 1994). Indeed, the genome

sequence of strain S288C, a maltose-negative laboratory strain, contains a *mal1g* and a *mal3g* loci (each containing only a functional permease and maltase, but nonfunctional regulatory genes) and two other maltose permease genes, *MPH2* and *MPH3*, located at the telomeres of chromosome IV and X, respectively (Feuermann *et al*. 1995; Volckaert *et al*. 1997; Day *et al*. 2002a).

All α-glucoside transport systems so far characterized in yeast are H^+-symporters that use the electrochemical proton gradient to actively transport these sugars into the cell, even for downhill transport of the sugar (Crumplen *et al*. 1996; Stambuk and de Araujo 2001). Maltose transport into the cell is required for full induction of MAL genes, and several reports have shown that maltose uptake is also the rate-limiting step for fermentation (Kodama *et al*. 1995; Wang *et al*. 2002; Rautio and Londesborough 2003). Maltose transport has thus been extensively studied in both laboratory and industrial yeast strains, revealing complex kinetics that indicate the presence of high- and low-affinity transporters (Crumplen *et al*. 1996; Zastrow *et al*. 2001; Rautio and Londesborough 2003). At least three different maltose transporters have been identified in *S. cerevisiae* cells, and while the *MALx1* transporters (and probably the two *MPH2* and *MPH3* alleles) encode high-affinity (K_m 2–4 mmol l^{-1}) maltose permeases, the *AGT1* permease (a gene present in partially functional *mal1g* loci) transports maltose with lower (K_m *c*. 20 mmol l^{-1}) affinity (Han *et al*. 1995; Stambuk and de Araujo 2001; Day *et al*. 2002a; Alves *et al*. 2007, 2008).

Significantly less well-characterized than maltose transport, maltotriose uptake by yeast cells also shows complex kinetics indicating the presence of high- and low-affinity transport activities, and studies on sugar utilization by yeast cells also revealed that maltose and maltotriose are apparently transported by different permeases (Zheng *et al*. 1994a; Zastrow *et al*. 2001). Two known permease genes have been described as transporting maltotriose in yeasts, the *S. cerevisiae* AGT1 transporter and the *S. pastorianus MTY1* (also known as *MTT1*) permease, both having relatively low affinity (K_m *c*. 20 mmol l^{-1}) for maltotriose (Stambuk and de Araujo 2001; Salema-Oom *et al*. 2005; Dietvorst *et al*. 2005; Alves *et al*. 2007, 2008).

However, there have been other reports regarding the observed patterns of maltose and maltotriose utilization by yeast cells that contradict the results described earlier. Some years ago, Day and co-workers presented data indicating that all known α-glucoside transporters present in *S. cerevisiae*, including the maltose permeases *MAL31*, *MAL61*, *MPH2* and *MHP3*, allowed growth of the yeast cells on both maltose and maltotriose (Day *et al*. 2002a,b). Furthermore, their kinetic analysis of maltose and maltotriose uptake by the cells indicated that all these transporters, including the *AGT1* permease, could transport both sugars with practically the same affinities and capacity. Thus, aiming to better understand maltotriose utilization by yeast strains, we performed an analysis of maltose and maltotriose utilization by 52 laboratory and industrial *Saccharomyces* yeast strains; we then used microarray comparative genome hybridization (aCGH), to correlate the observed phenotypes with copy number variations (CNVs) in genes known to be involved in maltose and maltotriose utilization by yeasts.

Materials and methods

Strains, media and growth conditions

The *Saccharomyces* strains analysed in the present study are described in Tables 1 and 2. The *AGT1* gene was deleted from the genome of yeast strains according to a previously described PCR-based gene replacement procedure (Batista *et al.* 2004; Alves *et al.* 2008). Rich YP medium (1% yeast extract and 2% Bacto peptone) was supplemented with 2% of the indicated carbon source (maltose or maltotriose), and the pH of the medium was adjusted to pH 5·0 with HCl. Yeast cells were pregrown overnight in 3 ml of YP-2% maltose, and 1 : 100 dilutions of these precultures were used to inoculate 100 μl of rich YP medium containing the indicated sugars in 96-well plates in a Tecan GENios microplate reader (Tecan, Männedorf, Switzerland), to determine the growth at 30°C. All wells in the plate were tightly sealed with Accu-Clear Sealing Film for qPCR (E&K Scientific, Santa Clara, CA), and growth of each culture was monitored by measuring the OD_{600} every 15 min, with high intensity orbital shaking between measurements. All growth curves were performed in duplicate, and controls in rich YP medium without a carbon source were included; the no-carbon curves were subtracted from the growth curves obtained in the presence of the sugar to yield normalized growth curves. All growth experiments were repeated at least twice, and we observed that differences between strains were highly reproducible. Alternatively, cells were batch grown (160 rev min^{-1}, 30°C) in cotton-plugged Erlenmeyer flasks filled to 1/5 of the volume with medium, and culture samples were harvested regularly, centrifuged (5000 ***g***, 1 min), and their supernatants used for the determination of sugars and ethanol as described in the following.

Microarray karyotyping

The microarray karyotyping analysis of the industrial yeast strains was performed essentially as described previously (Dunn *et al.* 2005). We used microarrays onto which had been spotted PCR products corresponding to full-length ORFs from the S288C strain of *S. cerevisiae* (DeRisi *et al.* 1997), and thus the reference DNA used in all hybridizations was isolated from this strain. Genomic DNA was isolated with YeaStar columns (Zymo Research, Orange, CA) and then cut with *Hae*III (New England Biolabs, Ipswich, MA). Approximately, 1 mg of this DNA was labelled with fluorescently tagged nucleotides (Perkin-Elmer, Waltham, MA), usually Cy3-dUTP for the reference strain (thus giving a green signal for every spot) and Cy-5 dUTP for the industrial strains, using the BioPrime random-prime labelling system (Invitrogen, Frederick, MD). After labelling, the reactions were heat-inactivated, the experimental (Cy5-labelled) and reference (Cy3-labelled) DNAs were mixed, purified away from unincorporated label using Zymo Clean&Concentrate columns (Zymo Research) and then hybridized to the microarrays at 65°C as described (Dunn *et al.* 2005). Arrays were scanned with an Axon 4000A scanner, and the data were extracted using GenePix (Molecular Devices Corp., Union City, CA, USA) software. The array data were treated and analysed as described previously (Dunn *et al.* 2005; Stambuk *et al.* 2009); note that all arrays contained duplicated spots for each gene, and all data presented are the average of the values from the duplicate spots. As all array data were normalized by setting the average log fluorescence hybridization ratio of all array elements to a value of zero, differences in hybridization intensity because of ploidy differences are eliminated.

Therefore, even if the industrial strains are of diploid or higher ploidy, the normalization process allows direct comparison to the haploid reference strain so that relative CNVs of a given gene within a strain, as determined by the red:green (R/G) hybridization ratio, will be relative to its haploid genome.

PFGE, chromosome blotting and hybridization

Yeast chromosomes were prepared as previously described (Guerring *et al.* 1991), and the pulsed-field gel electrophoresis (PFGE) performed in 1% agarose gels in 50 mmol l^{-1} Tris, 50 mmol l^{-1} boric acid, 1 mmol l^{-1} EDTA, pH 8·3, at 10°C using a Gene Navigator pulsed-field system (Pharmacia Biotech, Little Chalfont, UK) for a total of 27 h at 200 V. The pulse time was stepped from 70 s after 15 h to 120 s for 12 h. The chromosomes separated by PFGE were transferred to a nylon membrane (Ausubel *et al.* 1995), and prehybridization, hybridization, stringency washes and chemiluminescent signal generation and detection were performed using an AlkPhos kit (GE Healthcare/Amersham Biosciences, Little Chalfont, UK). Probes corresponding to nucleotides +1 through +1848 of the *AGT1* ORF, or −73 through +1845 of the *MAL31* gene, were generated by PCR using primers AGT1-F (AG GAGCTCATGAAAAATATCATTTCATTGG) and AGT1-R (TTGGATCCACATTTATCAGCT GC), and MAL31-F (CCATACTTGTTGTGAGTGG) and MAL31-R (TCATT TGTTCACAACAGATG), respectively, and genomic DNA from strain CEN.PK2–1C as the template.

Sugar and ethanol quantification

Maltose and maltotriose were determined spectrophotometrically at 540 nm with methylamine in 0·25 mol l^{-1} NaOH, while the ethanol produced by the yeast cells was assessed with alcohol oxidase and peroxidase, as previously described (Alves *et al.* 2007, 2008).

Results

Analysis of maltose and maltotriose utilization by *Saccharomyces* strains

To analyse the maltose and maltotriose utilization patterns of a fairly large number of laboratory and industrial yeast strains, while also taking into account the high price of maltotriose, we decided to use 96-well microplates and a Tecan GENios reader to obtain growth curves for the 53 different yeast strains described in Tables 1 and 2. These yeast strains included nine laboratory and 20 industrial *S. cerevisiae* strains, as well as one *S. bayanus* and three *S. cerevisiae* × *S. bayanus* hybrids, and 20 lager brewing strains of the species *S. pastorianus*. All the strains analysed utilized maltose efficiently, reaching the stationary phase of growth with this carbon source after 15–30 h of incubation, although the rate of growth and the maximal OD reached varied to different extents depending on the strain analysed (Fig. 1).

However, three different patterns of growth during maltotriose utilization by these same yeast cells were observed. The 27 strains belonging to Group 1 – cells that efficiently used both maltose and maltotriose as carbon sources for growth (Fig. 1a) – included only nine of the 29 *S. cerevisiae* strains (two laboratory, six baker's and one ale brewing yeast strain), but

included the majority (17 out of 20) of the *S. pastorianus* lager brewing yeasts and also the *S. pastorianus* (*monacensis*) distiller's yeast (Table 3). All these strains reached the stationary phase of growth in 15–30 h of incubation in the presence of maltotriose, and the analysis of ethanol production by several strains belonging to this Group 1 revealed efficient maltose and maltotriose fermentation, with ethanol concentrations reaching 5–7 g l^{-1} at the moment of sugar exhaustion from the medium (data not shown).

The yeast strains belonging to Group 2 (12 strains, see Table 3) had a very different pattern of maltotriose utilization when compared to the strains in Group 1 (Fig. 1b). These yeasts utilized and fermented maltose efficiently, but when cultivated in the presence of maltotriose a very slow growth rate was observed; in some cases, the growth rate improved only after several days of incubation in this carbon source. The majority of strains belonging to this group were industrial *S. cerevisiae* strains (only one, LCM001, was a laboratory strain), as well as three *S. pastorianus* brewing strains. Finally, the remaining yeast strains (a total of 13 strains, including the eight remaining *S. cerevisiae* laboratory strains) belonged to Group 3 (see Table 3) and were characterized by their complete inability to grow on maltotriose (see Fig. 1b), while maltose fermentation and utilization by these cells was normal. Within this group was also the *S. bayanus* var. *uvarum* CBS7001 yeast strain as well as two *S. cerevisiae* × *S. bayanus* hybrid strains used for wine and cider production.

Microarray karyotyping of *Saccharomyces cerevisiae strains*

We hypothesized that gene CNVs between the yeast strains may account for some of the differences in observed maltotriose utilization and fermentation efficiency, and thus we performed aCGH on each of the strains to determine whether there were observable CNVs. As microarray karyotyping detects changes in DNA copy number relative to a reference genome (in this case the genome of laboratory strain S288C), gene duplications or deletions that result in CNV can be easily detected. As shown in Fig. 2a, the aCGH results can easily detect the presence of a deleted *AGT1* gene in the genome of strain LCM001, but not in the otherwise isogenic wild-type strain 1403–7A from which it derives, while the same data indicate that these two strains have amplifications relative to S288C of both the *MALx1* and *MALx2* genes (known from previous work to be represented by the *MAL31* maltose permease, and the *MAL12* and *MAL32* maltases present in the genome of strain S228C, see Feuermann *et al.* 1995 and Volckaert *et al.* 1997).

Chromosome blotting and hybridization with *MAL31* and *AGT1* probes revealed indeed the presence of several (*MAL21, MAL31* and *MAL41*) maltose permease genes in the chromosomes of these two (1403–7A and LCM001) strains, while, as expected, only strain LCM001 lacked the *AGT1* permease (Fig. 2b). Most of the *S. cerevisiae* strains analysed also contained several maltose permease genes. For example, strains VR-1 and SA-1 had maltose perm-eases on both chromosomes VII (*MAL11*) and chromo-some II (*MAL31*), while strains CAT-1 and UFMG-A1007 also had these two maltose transporters genes but were heterozygous for chromosome VII, having both the *AGT1* and *MAL11* permease genes in each of the two chromosomes of these diploid yeast strains (see data for strain VR-1 and CAT-1 in Fig. 2b). Strain PE-2 was similar to strains CAT-1 and UFMG-A1007 (*AGT1/MAL11* and *MAL31*), but also contained the *MAL41* permease (chromosome XI) in its

genome (Fig. 2b). Indeed, a good correlation was observed between gene CNV of these α-glucoside transporters present in the genome of the yeast strains, as revealed by chromosomal blotting, and the aCGH data for the corresponding *AGT1* and *MAL31* genes (Fig. 2c).

The aCGH data of *MAL* genes present among selected *S. cerevisiae* yeast strains shown in Fig. 3 indicates that the copy number of *MPH2-MPH3* genes probably has little influence on maltotriose (and maltose) utilization by the *S. cerevisiae* yeasts analysed, as strains lacking these two genes were found among all three groups of strains with differing maltotriose utilization profiles described previously. Regarding the *MALx1* transporters, practically all strains analysed in Fig. 3 showed increased copy number of this gene relative to S288C, indicating that more than one functional *MAL* locus is present in these strains, allowing efficient maltose utilization.

The CGH results revealed a very different pattern regarding the *AGT1* permease (Fig. 3). All the efficient maltotriose utilization strains (Group 1) analysed by microarray karyotyping have this gene in their genome, while most of the strains belonging to either Group 2 (slow/delayed maltotriose utilization) or Group 3 (no maltotriose utilization) had a lower copy number (just one gene per diploid genome), or even lacked the *AGT1* gene (Fig. 3, see also Fig. 2c). Finally, although our microarrays were designed to determine gene CNVs in *S. cerevisiae* genomes, Fig. 3 also shows the *S. cerevisiae*-related aCGH data for an interspecific hybrid between this species and *S. bayanus* (strain CLIB180). As can be seen for this strain, which belongs to Group 1, the aCGH results indicate the presence of the *AGT1* permease and amplification of the *MALx1* genes, similar to what is seen in the *S. cerevisiae* Group 1 strains.

New maltotriose utilization phenotypes in *Saccharomyces* yeasts

The results shown in Fig. 1 regarding the slow/delayed maltotriose utilization (Group 2) pattern observed for some yeast strains were quite unexpected and prompted us to perform a more detailed analysis of these new phenotypes. Figure 4 shows the patterns of maltotriose utilization by two laboratory *S. cerevisiae* strains (CEN.PK2–1C and 1403–7A) and their corresponding *agt1*Δ isogenic yeast strains (LCM003 and LCM001, respectively). As we have already reported for strain LCM003 (Alves *et al.* 2008), these *agt1*Δ cells are completely unable to utilize maltotriose, even after an extensive (>8 days) incubation in the presence of this carbon source. However, and in accordance with the growth assays shown in Fig. 1, strain LCM001 (also *agt1*Δ) had an unexpected different phenotype: it did not grow on maltotriose during the first 3–4 days of incubation, but after this extensive lag phase the cells started to consume the sugar, allowing efficient aerobic growth on maltotriose (note, though, that no ethanol was produced during sugar consumption, see Fig. 4c). This extensive lag phase phenotype was always reproducible and takes place even if cells growing exponentially in maltotriose (for example after 100 h of incubation in this carbon source) are diluted back into new YP-2% maltotriose medium; this shows that the lag phase phenotype is not merely because of selection of new maltotriose fermentative yeast mutants (data not shown).

Similar maltotriose utilization phenotypes were observed for some other *S. cerevisiae* industrial yeast strains belonging to Group 2. For example, cells of the industrial strain VR-1 also displayed long lag times (3–4 days) before starting to consume maltotriose for growth, and no ethanol was produced from this carbon source, although this yeast consumes and ferments maltose efficiently (Fig. 5c). Thus, this VR-1 yeast strain resembles the phenotype observed for the *agt1*Δ LCM001 strain shown above; indeed our aCGH and chromosomal blotting results indicate that this strain lacks the *AGT1* permease in its genome (see Fig. 2b–c). However, for the remaining members of the Group 2 yeasts (the industrial fuel ethanol strains CAT-1, SA-1 and PE-2), yet another novel phenotype was observed: like LCM001 and VR-1, these strains consumed maltotriose only after an extensive lag phase, but unlike LCM001 and VR-1, they produced ethanol from maltotriose. Note that maltose consumption and fermentation was normal in the CAT-1, SA-1 and PE-2 strains. Nevertheless, in most cases, the ethanol yields on maltotriose were significantly lower than those obtained during maltose fermentation, as shown in Fig. 5c for the industrial *S. cerevisiae* yeast strain PE-2.

Discussion

The fermentative performance of *Saccharomyces* yeast strains has long been recognized to differ between strains, and the extent of utilization of maltose, for example, has been shown to vary especially between industrial and laboratory yeast strains (Naumov *et al*. 1994; Han *et al*. 1995; Bell *et al*. 2001; Meneses and Jiranek 2002; Meneses *et al*. 2002). Furthermore, the difficulty that some industrial yeast strains have in consuming maltotriose leads to one of the problems experienced by many breweries, namely sluggish fermentations with a high content of fermentable sugars in the finished beer, lower ethanol yields and atypical beer flavor profiles. Previous studies with industrial ale (*S. cerevisiae*) and lager (*S. pastorianus*) brewing yeast strains showed that in general lager yeasts consume maltotriose from wort faster, and thus residual maltotriose is more common at the end of ale fermentations (Zheng *et al*. 1994b).

In this study, our analysis of maltotriose utilization by 52 efficient maltose-fermenting *Saccharomyces* yeast strains (Fig. 1) confirmed the superior performance of *S. pastorianus* lager brewing yeast strains for maltotriose fermentation, as the majority of the *S. pastorianus* strains analysed (18 in 23) utilized this carbon source efficiently; only three of the lager yeasts, and two other *S. cerevisiae* × *S. bayanus* hybrids, showed patterns of no or slow/delayed maltotriose utilization. The genome of the lager brewing yeast is composed of two subgenomes originated from *S. cerevisiae* and *S. bayanus* that underwent extensive chromosomal translocations and re-arrangements, including higher copy number of selected chromosomes (Dunn and Sherlock 2008; Nakao *et al*. 2009). The *MTY1* (*MTT1*) α-glucoside transporter of *S. pastorianus*, identified as a unique yeast transporter with higher affinity for maltotriose than for maltose (Dietvorst *et al*. 2005; Salema-Oom *et al*. 2005), is present at the MAL1 (chromosome VII) locus of the *S. bayanus* subgenome present in lager yeasts (Nakao *et al*. 2009). Unfortunately, the two-species arrays developed and used previously to study the origin and evolution of this complex genome did not contain probes for this gene, as the sequences present in the microarray were based in the genome of the *S. bayanus* (var. *uvarum*) strain CBS7001 (Dunn and Sherlock 2008), a maltose fermenting but maltotriose nonfermenting yeast strain (see Table 3). Nevertheless, while several of the

efficient maltotriose utilization lager yeast strains had a full-length *S. cerevisiae* chromosome VII in their genomes (especially the right arm where the *AGT1*-containing *MAL1* locus is located), three lager strains that showed no or slow/delayed maltotriose utilization (strains CBS2440, DBVPG6258 and DBVPG6261) had lost most of their *S. cerevisiae* chromosome VII during the genome re-arrangements typical of this hybrid yeast (Dunn and Sherlock 2008); this shows a correlation between the lack of the AGT1 gene and the slow/delayed maltotriose utilization phenotype, similar to what is seen in the *S. cerevisiae* strains as described in the following.

In the case of the *S. cerevisiae* yeast strains analysed, more diverse maltotriose utilization phenotypes were observed. Besides efficient maltotriose-fermenting *S. cerevisiae* yeast strains (Zastrow *et al*. 2001; Londesborough 2001), previous reports have shown that several industrial *S. cerevisiae* yeast strains experience nonfermentative (respiratory) growth on this sugar, i.e. growth without concomitant ethanol production; as expected, growth was impaired with the addition of the mitochondrial inhibitor antimycin A (Zastrow *et al*. 2000, 2001; Dietvorst *et al*. 2005; Salema-Oom *et al*. 2005). Other industrial strains have been previously shown to have an extended lag phase (*c*. 1 day) during growth in maltotriose, especially when the cells were pregrown on glucose (Londesborough 2001), but the new maltotriose utilization phenotypes that we found among some laboratory and industrial *S. cerevisiae* strains (see Figs 1, 4 and 5) add new complexity into the utilization patterns of this important carbon source by yeast cells.

Of all the maltose-fermenting *S. cerevisiae* yeast strains we analysed, only approx. one-third of them could utilize maltotriose efficiently (Table 3). As expected, strains selected for efficient fermentation of starch hydrolysates (one ale brewing, and seven baker's yeast strains) were able to consume this carbon source efficiently, a phenotype shared with two of the laboratory yeast strains. The results obtained with the laboratory *S. cerevisiae* yeast strains are in accordance with the microarray data (shown in Fig. 3) that indicate that the *AGT1* permease is required for efficient maltotriose consumption and fermentation by this yeast (see Alves *et al*. 2008). For example, strain YJM789 is a pathogenic isolate that is *MAL1* and *MAL3* (Wei *et al*. 2007), and thus contains a normal *MAL11* gene at the *MAL* locus in chromosome VII and consequently lacks the *AGT1* gene; it is unable to utilize maltotriose. This phenotype is shared with the homothalic *MAL1* diploid Y55 strain (lacking the *AGT1* gene), with strain CMY001 (having a *MAL61*::*HA* allele at the *MAL1* locus, and thus lacking the *AGT1* gene), and with the wine strain RM11–1a (Brem *et al*. 2002) that contains a nonfunctional version of the *AGT1* gene (lacking a cytosine at position +996 of the ORF, and truncating the permease). All of these strains are unable to utilize maltotriose, while maltose fermentation is normal. We found that some industrial strains (e.g. strains CAT-1, PE-2, G34, UFMG-905 and UFMG-1007) had the *AGT1* gene in their genomes (see Figs 2 and 3), but nevertheless these strains belonged to Group 2 (slow/delayed maltotriose utilization), or even Group 3 (no maltotriose utilization). We have sequenced the *AGT1* present in some of these strains (e.g. CAT-1 and PE-2) and found that they encode bona-fide full-length permeases (S.L. Alves Jr, J.M. Thevelein and B.U. Stambuk, unpublished data). However, we have failed to find an UAS_{MAL} upstream of the *AGT1* gene in strains CAT-1 and PE-2, which might explain why these strains fail to efficiently ferment maltotriose. In accordance with the importance of this gene for

maltotriose fermentation, transcriptome studies have also shown a high expression of this gene during wort fermentation (James *et al*. 2003), and we have recently shown that *AGT1* overexpression improves maltotriose fermentation by industrial yeast strains (Stambuk *et al*. 2006).

Our results clearly show that the presence of *MALx1* (*MAL11, MAL21, MAL31, MAL41* or *MAL61*) genes in the yeast genome does not allow efficient maltotriose consumption or fermentation by *S. cerevisiae* cells, contrary to the early claims of Day *et al*. (2002a,b). However, some alleles of these *MALx1* transporter genes might be implicated in the novel slow/delayed growth phenotypes we have observed for some *S. cerevisiae* yeast strains (Figs 4 and 5). In this regard, the *MAL3* locus is an interesting candidate, as it is present in almost all strains analysed (including several members of Group 2), and a detailed mapping and molecular analysis of this locus revealed a complex structure with several *MALx1*-related alleles repeated in tandem (Michels *et al*. 1992); unfortunately, these *MAL31* alleles have not been functionally analysed regarding substrate specificity. Although *MALx1* genes show a high (98–99%) degree of sequence identity, minor sequence variations among these transporters can impart quite distinct transport properties to the permeases, as recently shown for a *MAL21* allele (Hatanaka *et al*. 2009), or even for *AGT1* alleles found in some distillers yeasts (Smit *et al*. 2008). The novel delayed maltotriose utilization profile we observed, i.e., where maltotriose utilization occurs only after an extensive lag phase, could be because of the expression of an extracellular glucoamylase, as is typically found in *S. cerevisiae* var *diastaticus* yeasts (Pretorius *et al*. 1991). Indeed, maltotriose is classically used as substrate to measure the activity of this enzyme at the surface of yeast cells. These *diastaticus* yeasts strains also grow on starch after an extensive lag phase (up to 4 days), when expression of the polymorphic *STA1-STA3* genes encoding the extracellular glucoamylase takes place (Pretorius *et al*. 1991; Vivier *et al*. 1999). However, we have not been able to detect the presence of *STA* genes in the genome of several of the strains belonging to Group 2, using PCR with specific primers (S.L. Alves Jr, J.M. Thevelein and B.U. Stambuk, unpublished data), and assays performed to measure extracellular maltotriose hydrolysis by the late maltotriose consuming yeast strains also failed. Further studies will be required to reveal the molecular basis of the slow/delayed maltotriose utilization phenotypes uncovered in the present high throughput screening study of *Saccharomyces* yeasts.

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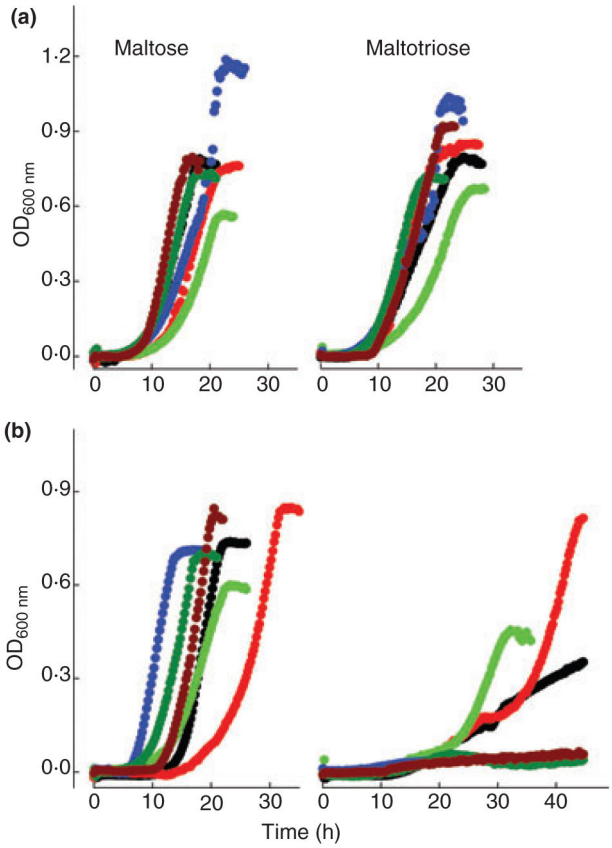

Figure 1.

Growth on rich YP medium containing 2% maltose (left panel) or maltotriose (right panel) by representative yeast strains (a) belonging to Group 1 (G1), or (b) belonging to Group 2 (G2) and Group 3 (G3). G1: (●) 1403–7A; (●) CBS1513; (●) CBS1260; (●) DBVPG6283; (●) WY2124 and (●) CLIB180; G2: (●) LCM001; (●) SA-1 and (●) CBS1503; G3: (●) LCM003; (●) CBS1486 and (●) GDB-379.

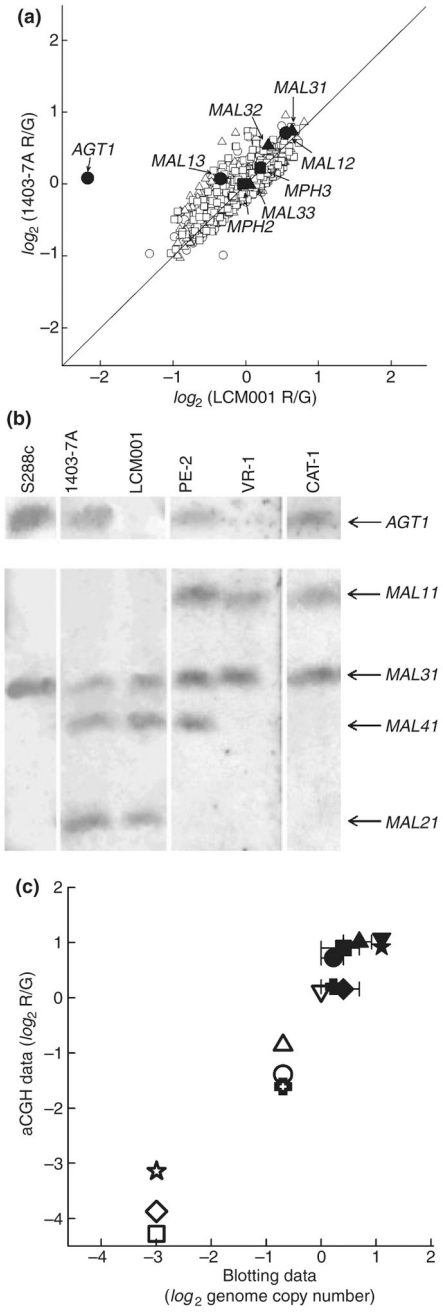

Figure 2.

Correlation between the microarray karyotyping of the laboratory strain 1403–7A and its isogenic Δ*agt1* strain LCM001 (a). The microarray comparative genome hybridization (aCGH) data (in log_2 of the R/G ratio) for the *MAL* genes analysed are highlighted. Chromosomal blotting (b) of the indicated yeast strains with the *AGT1* gene (upper panel) or *MAL31* gene (lower panel) as probe, and (c) correlation of copy number variation of the *AGT1* (open symbols) and *MALx1* (black symbols) genes, as determined by aCGH (expressed as log_2 of the R/G ratio) and chromosomal blotting (log_2 of gene copy number per diploid genome), present in strains CAT-1 (circles), PE-2 (triangles), VR-1 (diamonds),

SA-1 (squares), 1403–7A (inverted triangles), LCM001 (stars) and UFMG-1007 (crosses). For the chromosomal blotting, an arbitrary value of <0·05 was assumed for strains lacking the *AGT1* gene.

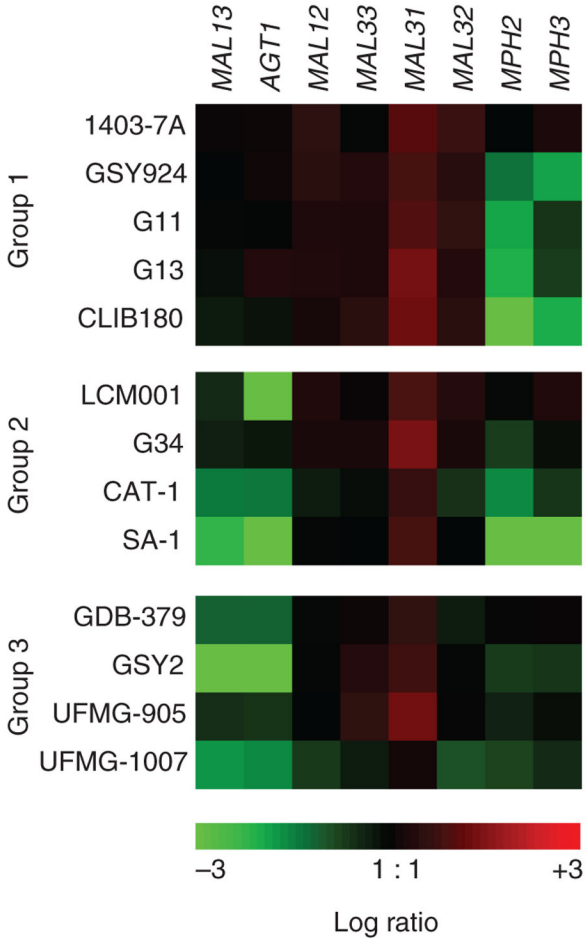

Figure 3.

Microarray karyotyping data of relative gene copy number variation (compared to strain S288C) present in the genomes of selected yeast strains from Group 1 (G1), Group 2 (G2) or Group 3 (G3).

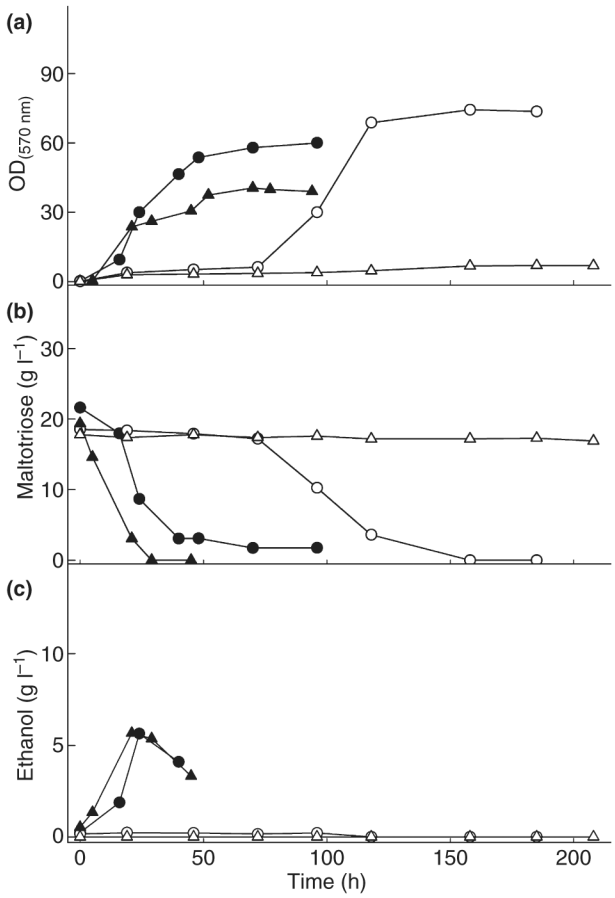

Figure 4.

Typical cell growth (a), sugar consumption (b) and ethanol production (c) during growth on maltotriose by cells of the laboratory strains CEN.PK2–1C (black triangles) and its isogenic Δ*agt1* strain LCM003 (open triangles), or by strain 1403–7A (black circles) and its isogenic Δ*agt1* strain LCM001 (open circles).

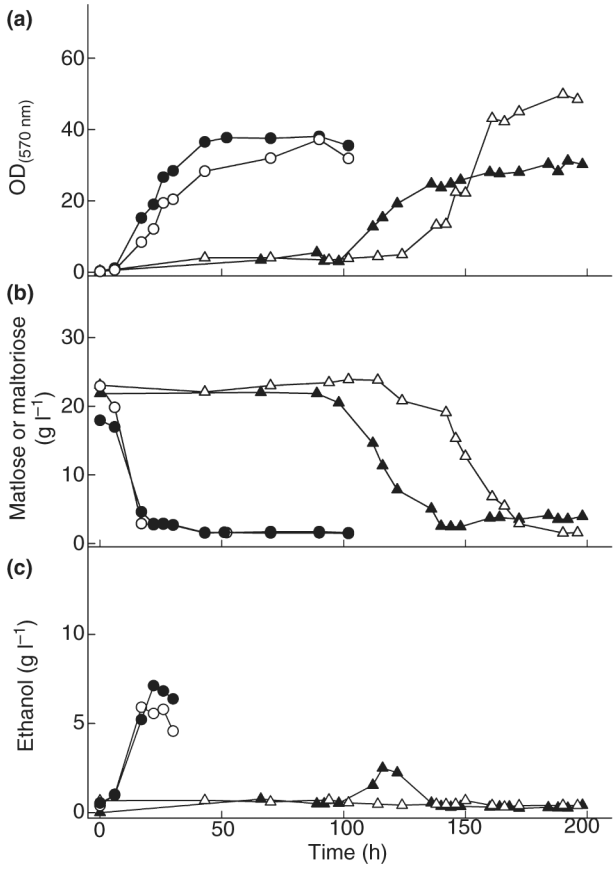

Figure 5.

Typical cell growth (a), sugar consumption (b) and ethanol production (c) during growth on maltose (circles) or maltotriose (triangles) by cells of the industrial strains PE-2 (black symbols) or VR-1 (open symbols).

Table 1

Laboratory *Saccharomyces* cerevisiae yeasts analysed

Strain	Genotype	Reference
S288C	*MATα mal13 AGT1 MAL12 mal33 MAL31 MAL32 mel gal2 flo1 flo8–1 hap1 ho bio1 bio6 SUC2*	Mortimer and Johnston (1986)
RM11–1a	*MAT***a** *mal13 agt1 MAL12 MAL3 leu2Δ ura3Δ ho::Kan*	Brem *et al.* (2002)
VJM789	*MAT***a** *MAL1 MAL3 ho::hisG lys2 gal2*	Wei *et al.* (2007)
CMY001	*MAT***a** *mal11::MAL61/HA MAL12 MAL13 GAL ura3–52 leu2his3–200 trp1-Δ63 lys2–801 ade2–101*	Wang *et al.* (2002)
CEN.PK2–1C	*MAT***a** *MAL2–8^{C} MAL3 mal13 AGT1 MAL12 SUC2 ura3–52 his3Δ1 leu2–3, 112 trp 1–289*	Alves *et al.*(2008)
LCM003	*agt1Δ::kanMX6* derivative of CEN.PK2–1C	Alves *et al.* (2008)
1403–7A	*MAT***a** *MAL4^{C} mal33 MAL31 MAL32 mal13 AGT1 MAL12 MAL21 MGL3 gal3 gal4 trp1 ura3 suc^{-}*	Alves *et al.* (2008)
LCM001	*agt1A::kanMX6* derivative of 1403–7A	Alves *et al.* (2008)
Y55	*MATα/MAT***a** *HO/HO gal3/gal3 MAL1/MAL1 SUC1/SUC1*	Houghton-Larsen and Brandt (2006)

Table 2

Other *Saccharomyces* yeasts analysed

Strain	Description	Reference
Saccharomyces bayanus var. *uvarum*		
CBS7001		Dunn and Sherlock (2008)
Saccharomyces cerevisiae industrial strains		
GSY2; UCD522	Montrachet wine yeasts	Dunn *et al.* (2005)
UCD819	Prise de Mousse wine yeast	Dunn *et al.* (2005)
GSY924	Leinenkugel Ale Brewing yeast	Dunn and Sherlock (2008)
LBCC-A3	Ale Brewing yeast	Batistote *et al.* (2006)
G11; G13; G14; G15; G30; G34; G35	Baker's yeasts	-*
CAT-1; SA-1; PE-2; VR-1	Fuel ethanol yeasts	Basso *et al.* (2008)
GDB-178; GDB-379	Fuel ethanol yeasts	da Silva-Filho *et al.* (2005)
UFMG-A905; UFMG-A1007	Cachaça production yeasts	Gomes *et al.* (2007)
Saccharomyces pastorianus industrial strains DBVPG6033; DBVPG6047; DBVPG6257; DBVPG6258; DBVPG6261; DBVPG6282; DBVPG6283; DBVPG6284; DBVPG6285; DBVPG6560	Lager Brewing yeasts	Dunn and Sherlock (2008)
CBS1174; CBS1483; CBS1484; CBS2156; CBS2440; CBS6903	Lager Brewing yeasts	Dunn and Sherlock (2008)
LBCC-L52	Lager Brewing yeasts	Batistote *et al.* (2006)
WY2124	Bohemian Lager Brewing yeast	-†
WY2206	Bavarian Lager Brewing yeast	-†
W-34/70	Weihenstephan Lager Brewing yeast	Nakao *et al.* (2009)
Other *S. cerevisiae* × *S. bayanus* hybrid strains		
Y251	Wine yeast	-‡
Y165	Cider yeast	-‡
CLIB180	*S. pastorianus (monacensis)* distiller's yeast	dos Santos *et al.* (2007)

*Strains provided by M. Ettayebi, University Sidi Mohamed Ben Abdallah, Fez, Morocco.

†Strains kindly provided by B. Maca, Miller Brewing Co., Milwaukee, WI, USA.

‡Strains provided by J. Piskur, Department of Microbiology, Technical University of Denmark.

Table 3

Patterns of maltose and maltotriose utilization by the *Saccharomyces* yeast strains *

Group 1	Efficient maltose and maltotriose utilization
Strains	*1403–7A; CEN.PK2–1C; G11; G13; G14; G15; G30; G35; GSY924*;CBS1174; CBS1483; CBS1484; CBS2156; CBS6903; CLIB180; DBVPG6033; DBVPG6047; DBVPG6257; DBVPG6282; DBVPG6283; DBVPG6284; DBVPG6285; DBVPG6560; LBCC-L52; W-34/70; WY2124, WY2206
Group 2	Efficient maltose utilization, and slow/delayed maltotriose utilization
Strains	*CAT-1; G34; LBCC-A3; LCM001; PE-2; SA-1; UCD522; UCD819; VR-1*; CBS2440; DBVPG6258; DBVPG6261
Group 3	Efficient maltose utilization, no maltotriose utilization
Strains	*CMY001; G5Y2; GDB-178; GDB-379; LCM003; RM11–1a; Y55; YJM789; UFMG-A905; UFMG-A1007*; CBS7001; Y251; Y165

* *Saccharomyces cerevisiae* strains are in italics.