Dissertation

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Maltose and maltotriose transport into ale and lager brewer´s yeast strains

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Abstract

Maltose and maltotriose are the two most abundant sugars in brewer's wort, and thus brewer's yeast's ability to utilize them efficiently is of major importance in the brewing process. The increasing tendency to utilize high and very-high-gravity worts containing increased concentrations of maltose and maltotriose renders the need for efficient transport of these sugars even more pronounced. Residual maltose and maltotriose are quite often present especially after high and very-high-gravity fermentations. Sugar uptake capacity has been shown to be the rate-limiting factor for maltose and maltotriose utilization. The aim of the present study was to find novel ways to improve maltose and maltotriose utilization during the main fermentation.

Maltose and maltotriose uptake characteristics of several ale and lager strains were studied. Genotype determination of the genes needed for maltose and maltotriose utilization was performed. Gene expression and maltose uptake inhibition studies were carried out to reveal the dominant transporter types actually functioning in each of the strains. Temperature-dependence of maltose transport was studied for ale and for lager strains as well as for each of the single sugar transporter proteins Agt1p, Malx1p and Mtt1p. The *AGT1* promoter regions of one ale and two lager strains were sequenced by chromosome walking and the promoter elements were searched for using computational methods.

The results showed that ale and lager strains predominantly use different maltose and maltotriose transporter types for maltose and maltotriose uptake. Agt1 transporter was found to be the dominant maltose/maltotriose transporter in the ale strains whereas Malx1 and Mtt1-type transporters dominated in the lager strains. All lager strains studied were found to possess an *AGT1* gene encoding a truncated polypeptide unable to function as maltose transporter. The ale strains

were observed to be more sensitive to temperature decrease in their maltose uptake compared to the lager strains. Single transporters were observed to differ in their sensitivity to temperature decrease and their temperature-dependence was shown to decrease in the order Agt1≥Malx1>Mtt1. The different temperature-dependence between the ale and lager strains was observed to be due to the different dominant maltose/maltotriose transporters ale and lager strains possessed. The *AGT1* promoter regions of ale and lager strains were found to differ markedly from the corresponding regions of laboratory strains and instead were similar to corresponding regions of *S. paradoxus*, *S. mikatae* and natural isolates of *S. cerevisiae*. The ale strain was found to possess an extra MAL-activator binding site compared to the lager strains. This could, at least partly, explain the observed differential expression levels of *AGT1* in the ale and lager strains studied. Moreover, the *AGT1*-containing *MAL* loci in three *Saccharomyces sensu stricto* species, *i.e. S. mikatae*, *S. paradoxus* and the natural isolate of *S. cerevisiae* RM11-1a were observed to be far more complex and extensive than the classical *MAL* locus usually described in laboratory strains.

Improved maltose and maltotriose uptake capacity was obtained with a modified lager strain where the *AGT1* gene was repaired and placed under the control of a strong promoter. Integrant strains constructed fermented wort faster and more completely, producing beers containing more ethanol and less residual maltose and maltotriose. Significant savings in the main fermentation time were obtained when modified strains were used. In high-gravity wort fermentations 8-20% and in very-high-gravity wort fermentations even 11–37% time savings were obtained. These are economically significant changes and would cause a marked increase in annual output from the same-size of brewhouse and fermentor facilities.

Preface

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Espoo, December 2010

Virve

Contents

List of publications

This thesis is based on the following original publications, referred to in the text by their Roman numerals I–IV.

I Vidgren, V., Ruohonen, L., and Londesborough, J. 2005. Characterization and functional analysis of the *MAL* and *MPH* loci for maltose utilization in some ale and lager yeast strains. *Appl. Environ. Microbiol.* 71: 7846–7857.

II Vidgren, V., Huuskonen, A., Virtanen, H., Ruohonen, L., and Londesborough, J. 2009. Improved fermentation performance of a lager yeast after repair of its *AGT1* maltose and maltotriose transporter genes. *Appl. Environ. Microbiol.* 75: 2333–2345.

III Vidgren, V., Multanen, J.-P., Ruohonen, L., and Londesborough, J. 2010. The temperature dependence of maltose transport in ale and lager strains of brewer's yeast. *FEMS Yeast Res.* 10: 402–411.

IV Vidgren, V., Kankainen, M., Londesborough, J. and Ruohonen, L. Identification of regulatory elements in the *AGT1* promoter of ale and lager strains of brewer's yeast. Submitted to *Yeast* 2010.

List of abbreviations

AA	apparent attenuation
ADP	adenosine diphosphate
AGT1	transporter gene (alpha-glucoside transporter)
ATP	adenosine triphosphate
BGL2	beta-glucanase gene
BLASTN	basic local alignment search tool nucleotide
bp	base pair(s)
Can1	arginine transporter, confers canavanine resistance
CE	current apparent extract
Chr	chromosome
CoA	coenzyme A
COMPASS	complex proteins associated with Set1
DNA	deoxyribonucleic acid
FSY1	fructose transporter gene, fructose symport
Fur4	uracil permease, 5-fluorouracil sensitivity
GAL	galactose (utilization)
GMO	genetically modified organism
HG	high-gravity
Hxt	hexose transporter
IPR	intellectual property rights
K_m	Michaelis-Menten constant

MAL	maltose (utilization)
MEL	melibiose (utilization)
Mig1	multicopy inhibitor of *GAL1* promoter
MPH	transporter gene (maltose permease homologue)
mRNA	messenger ribonucleic acid
MTT1	transporter gene (mty1-like transporter)
MTY1	transporter gene (maltotriose transport in yeast)
NCBI	National Center for Biotechnology Information
OE	original extract
ORF	open reading frame
°P	degree Plato (measure of the sum of dissolved solids in wort)
PCR	polymerase chain reaction
PEST	peptide sequence rich in proline (P), glutamic acid (E), serine (S) and threonine (T)
PGK	phosphoglycerate kinase
PMA1	gene for plasma membrane ATPase
SbAGT1	*Saccharomyces bayanus*-derived *AGT1*
SER2	phosphoserine phosphatase gene
Set1	histone methyltransferase
SGD	*Saccharomyces* Genome Database
SUC	sucrose (utilization)
TAT2	gene for tryptophan amino acid transporter
TCA	tricarboxylic acid
V_{max}	maximum velocity
v/v	volume/volume
VHG	very-high-gravity
w/v	weight/volume

1. Introduction

Beer is one of the oldest biotechnological products. It has been manufactured for thousands of years and nowadays beer brewing is an important field of industry. In Finland alone, 400–425 million litres of beer is sold yearly (statistics of years 2003–2009). This is approximately 80–90 litres of beer consumed per person annually (www.panimoliitto.fi/panimoliitto/tilastot). After understanding the role of yeast in the fermentation process in the early nineteenth century (reviewed by Boulton and Quain, 2001), there has been continuously increasing interest in improving and accelerating the brewing process, for example by means of developing better performing yeast strains.

In the fermentation process, sugars of the wort are converted to ethanol and carbon dioxide by the metabolism of the yeast cell. A major factor determining the rate and extent of the fermentation is the utilization rate of sugars. A lot of effort has been made to accelerate the fermentation of maltose and maltotriose sugars, which usually are not consumed immediately at the beginning of the fermentation but instead have a rather long lag phase before their utilization is initiated. Sometimes maltose and especially maltotriose are left unfermented at the end of the main fermentation. This lowers the efficiency of the process and also has an impact on the final quality of the beer by impairing the flavour. Delay in the utilization of maltose and maltotriose is mostly due to the fact that glucose is the preferred sugar for yeast as a carbon and energy source. When there is glucose present the utilization of alternative fermentable sugars is hindered. Mechanisms by which glucose causes this delay occur by catabolite repression and catabolite inactivation of enzymes and transporters that are needed for the utilization of alternative sugars.

Several studies have shown that the rate-limiting step in the utilization of maltose and maltotriose is the transport capacity of sugars into the yeast cell (Kodama *et al.*, 1995; Rautio and Londesborough, 2003; Meneses *et al.*, 2002; Alves

et al., 2007). Improving the ability of yeast cells to transport maltose and maltotriose has been the subject of many studies. Over the last years new maltose/maltotriose transporters have been identified and characterized (Day *et al*., 2002a; Salema-Oom *et al*., 2005; Dietvorst *et al*., 2005) or just identified and not yet characterized (Nakao *et al*., 2009). Ways to improve the transport efficiency have been obtained, for example by over-expressing the corresponding maltose or maltotriose transporter genes (Kodama *et al*., 1995; Stambuk *et al*., 2006). These strains have been shown to have improved sugar uptake capacity and are able to intensify the fermentation process. However, since these strains are genetically modified their commercial use in the breweries is not, at least yet, accepted because of the current negative attitude towards GMO of consumers. Nonetheless, these strains have given important knowledge about the bottlenecks of the fermentation process and information has been gained in how the process could be improved and what magnitude of intensification could be obtained.

Efficient utilization of sugars is even more important nowadays when there is a tendency to move to a greater extent to ferment high-gravity (HG) or even very-high-gravity (VHG) worts, which have increased concentrations of sugars compared to traditional worts. Incomplete utilization of sugars, especially maltotriose, is sometimes a problem even in standard fermentations and even more when HG and VHG worts are used (Piddocke *et al*., 2009).

1.1 Outline of malting and brewing processes

A schematic diagram of malting and brewing processes is presented in Figure 1. Malt is the main starting material in the brewing process together with water and hops. Malt is produced from barley grains by a three-phase malting process including steeping, germination and kilning. In steeping, barley grains are soaked in water to obtain the right moisture content. After that, germination is carried out in carefully controlled temperature, moisture and aeration conditions. Kilning is performed to stop the biochemical reactions in the kernel and to produce a dry product. The main purpose of malting is so that the natural enzymes in the barley grain are activated. These enzymes then assist the conversion of the storage carbohydrate material, starch, composed mainly of amylose and amylopectin, to fermentable sugars. Degradation of starch starts during malting and continues at wort production phase.

In wort production, malt is first milled to release the contents of the grains. In mashing, milled malt is suspended in water and heated to prepare an aqueous

extract. During mashing the malt components are solubilized and hydrolysed by the enzymes produced during germination. Heating disrupts the crystalline structure of starch granules and makes them susceptible to attack by amylases. Most of the degradation of starch to fermentable sugars takes place during mashing where α- and ß-amylases degrade it. The ß-amylases are more heat labile than the α-amylases and thus their activity is lost in high temperature mashes. In the production of lager beer the mash mixture is heated gradually to certain temperatures (50–72°C), which are suitable for enzymatic reaction, whereas in traditional ale brewing a single mashing temperature (65°C) is used. Conditions used in mashing, especially the temperature range, have a significant effect on the sugar spectrum formed. The combined action of α- and ß-amylases produces mostly maltose and to a lesser extent maltotriose and glucose. Also, a significant share of undegraded dextrins remain. The debranching enzyme, limit dextrinase, which is present in barley and is activated during germination, is able to convert branched dextrins into linear glucose polymers, which can after that be degraded by other amylolytic enzymes. However, limit dextrinase is heat labile and is rapidly denatured during mashing. After mashing, solids are removed, and clarified wort is obtained.

In the next step, wort is boiled together with hops. Liquid sugar syrup adjuncts, if used, are added at this point before boiling. Boiling serves many purposes. It sterilizes the wort and inactivates malt enzymes. It also assists with clarification and removes substances that would interfere with downstream processes. After boiling, solids in the form of trub and any hop material are separated from the hot wort. After that, wort is cooled and delivered to the fermentation vessel.

Before the main fermentation, wort is oxygenated. Oxygenation is important for yeast cells to be able to synthesize sterols and unsaturated fatty acids, which are necessary for the correct composition of yeast membranes. These lipids cannot be synthesized under anaerobic conditions and thus yeast must rely on lipids synthesized at the early phases of the fermentation during the rest of the fermentation. At the moment yeast is added (pitching), the main fermentation starts. During the main fermentation, fermentable sugars are converted by the yeast metabolism to ethanol, CO_2 and to a minor extent to higher alcohols, organic acids and esters. Quite soon yeast cells have used up all the oxygen and conditions change to anaerobic. It is characteristic of brewer’s yeast that even under aerobic conditions metabolism is both respiratory (oxidative phosphorylation) and fermentative (substrate level phosphorylation). Ethanol is therefore formed

also during the aerobic phase. The main fermentation has reached its end when the major part of the fermentable sugars has been used. In some cases fermentation stops earlier when there still is a significant amount of fermentable sugars present but for some reason yeast cells are not able to utilize them further.

Ale and lager fermentations differ in several respects. Lager fermentations are performed with lager strains, which perform better at low temperatures. Main fermentations performed with lager strains last approximately 7–10 days and are carried out at 6–14°C. Whereas main fermentations with the ale strains are carried out at higher temperatures, 15–25°C, and need less time to be completed. Ale and lager strains differ also in their flocculation and sedimentation performance. Ale strains tend to float and ferment on top of the beer. Whereas lager strains tend to form flocs, which sediment to the bottom of the fermentation vessel at the late stages of the fermentation. Yeast cells can be collected from the fermentation tank at the end of the main fermentation. The yeast cells collected can be stored and used to repitch a new main fermentation.

The product of the main fermentation is called green beer. It is not potable since it contains unwanted flavour components like diacetyl. For maturation of the beer flavour, secondary fermentation is needed. Removal of diacetyl is the rate-limiting step in the maturation of beer. Maturation requires the presence of viable yeast cells since diacetyl must be taken into and metabolised by the remaining yeast cells. For lager strains the secondary fermentation, which is performed traditionally near 0°C, is a slow process taking approximately 1 to 3 weeks. However, with use of an immobilized yeast technique it is possible to significantly reduce the secondary fermentation time (Pajunen *et al*., 1991). For ale beers instead only three to four days maturation at 4°C is needed.

After the secondary fermentation, downstream processing, *i.e*. filtration and pasteurization (or strerile filtration) and finally bottling takes place.

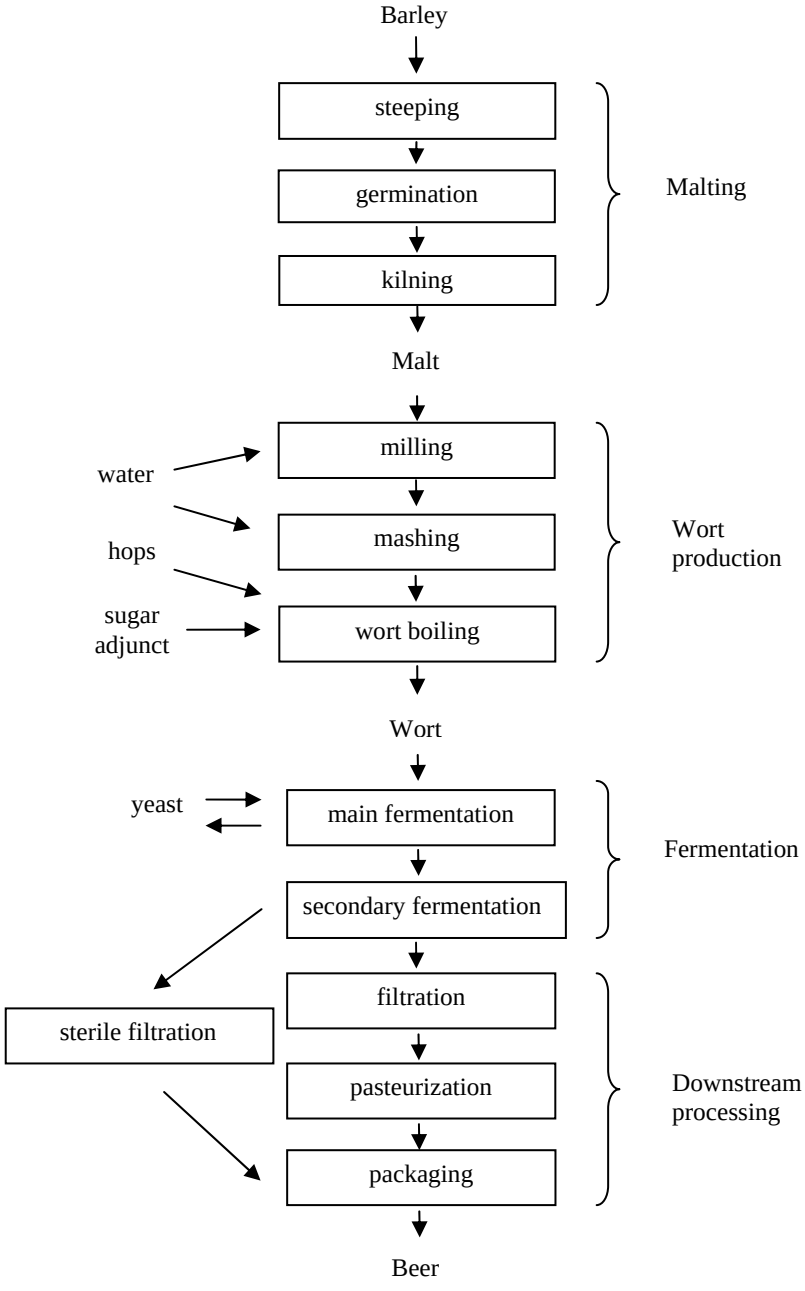

Figure 1. Shematic diagram of malting and brewing processes.

Some brewing terms are introduced below.

Extract, Degrees Plato is a measure of the sum of dissolved solids in wort, *i.e.* mostly fermentable sugars plus nonfermentable soluble carbohydrates of wort: a solution with an extract of x°P has the same density as a water solution containing x g of sucrose in 100 g of solution.

Apparent extracts measured during fermentation and not corrected for ethanol density. Apparent extracts can be corrected to real extracts if the ethanol concentration is separately determined.

Attenuation measures the proportion of carbohydrates that have been consumed from wort.

Apparent attenuation is the difference between the original extract (OE) of the wort and the current apparent extract (CE) divided by the original extract ([OE-CE]/OE).

Apparent attenuation limit is apparent attenuation measured after exhaustive fermentation with excess yeast, measure of total amount of fermentable sugars in wort.

1.2 Brewer's yeast strains

Brewer's yeast strains are divided into ale (*Saccharomyces cerevisiae*) and lager (*Saccharomyces pastorianus*, earlier referred to as *S. carlsbergensis*) strains. "Top-fermenting" ale strains are ancient strains, which have been used in beer brewing for thousands of years. "Bottom-fermenting" lager strains emerged presumably only a few hundred years ago when the low temperature fermentation technique was introduced in Bavaria (Hornsey, 2003). Since ale strains have been in use for a longer time than lager strains their diversification is much greater. Chromosomal fingerprinting showed that lager strains throughout the world essentially have only one or two basic fingerprints with small differences between the strains. Instead ale strains didn't have any common form of fingerprint (Casey, 1996).

Ale strains constitute a broad variety of strains, most of which seem to be closely related to *S. cerevisiae* (Kobi *et al*., 2004; Tornai-Lehoczki and Dlauchy, 2000). However, it has been shown recently that there are strains included, *e.g.* isolated from Trappist beers, which are actually hybrids between *S. cerevisiae* and *S. kudriavzevii* (González *et al*., 2008). Also, some other strains previously classified as *S. cerevisiae* may be hybrids (Querol and Bond, 2009). All lager strains are regarded as hybrids of two species. Parental species of the lager

hybrid were most probably diploids, which fused to generate an allotetraploid strain (Aigle *et al*., 1983). One component of the hybrid has uniformly been described as *S. cerevisiae* but there have been different suggestions for the other component during the last decades. However, in recent years it has been confirmed that lager strains are actually hybrids of *S. cerevisiae* and *S. bayanus* (Naumova *et al*., 2005; Caesar *et al*., 2007; Dunn and Sherlock, 2008). Moreover, *S. bayanus* strains consist of two subgroups, *i.e*. *S. bayanus* var. *uvarum* and *S. bayanus* var. *bayanus* and it has been shown that the *S. bayanus* component in lager strains is more related to *S. bayanus* var. *bayanus* (Nakao *et al*., 2009). Genomes of lager yeasts are reported to be dynamic and able to undergo rearrangements (Smart, 2007). Changes such as chromosome loss and/or duplications have resulted in unequal numbers of chromosomes in the present-day strains, a state referred to as aneuploidy (Querol and Bond, 2009). Also, copies of each sister chromosome are not necessarily identical, for example sister chromosomes derived from *S. cerevisiae* have diverged from each other with time. The hybrid lager strain formed between *S. bayanus* and *S. cerevisiae* species probably had selective advantage in cold brewing temperatures. Cryophilic performance of lager yeasts is suggested to derive from characteristics of *S. bayanus* (Sato *et al*., 2002). However, it has been observed that the parental species *S. cerevisiae* and *S. bayanus* are less capable of metabolizing the available sugars to ethanol at cold brewing temperatures than the hybrid (Querol and Bond, 2009). Thus, it seems that the combination of parental types is needed for efficient fermentation performance at low temperatures. The hybrid genome of lager yeast is suggested to confer a high degree of resistance to various stresses such as temperature, low pH, high alcohol concentrations, high osmotic pressure and anaerobiosis stress met during the fermentation (Querol and Bond, 2009).

Recent genome-wide sequencing of a lager strain WS34/70 further confirmed that lager brewing yeast is a hybrid between *S. cerevisiae* and *S. bayanus*. Part of the WS34/70 genome was observed to be related to the *S. cerevisiae* genome, whereas another part of WS34/70 was observed to be highly similar to *S. bayanus*. In the genome of WS34/70 there were both *S. cerevisae* and *S. bayanus*-type chromosomes found as well as 8 hybrid chromosomes consisting partly of *S. cerevisiae* and partly of *S. bayanus*. Presence of hybrid chromosomes shows that the hybrid genome has reorganized markedly after the hybridization event (Nakao *et al*., 2009). Dunn and Sherlock (2008) also report that significant reorganization of the hybrid genome took place after the hybridization event. They divide lager strains into two subgroups, which they show originate from two

separate hybridization events between *S. cerevisiae* and *S. bayanus*. They propose that in both events the *S. cerevisiae* partner was a different, but closely related, ale strain and hybridization was followed in group 1 by a loss of large portion of *S. cerevisiae* genome whereas in group 2 the loss of the *S. cerevisiae* portion of the genome was minor (Dunn and Sherlock, 2008). The loss of portions of the *S. cerevisiae* genome indicates that these parts, at least, of the *S. cerevisiae* genome were redundant in the hybrid strains under the conditions of fermentation at low temperature in which the hybrids have further evolved. It also appears that lager strains vary in the copy number of the parental chromosomes and the number and type of hybrid chromosomes they possess (Querol and Bond, 2009).

Physiological differences between ale and lager strains are most probably an outcome of their considerable genetic difference. Ale strains are called top-fermenting because they form a head yeast at the top of the wort during fermentation, whereas bottom-fermenting lager strains flocculate and sediment to the bottom of the fermentation tank in the late phase of fermentation. This difference has been explained by the different surface hydrophobicity between ale and lager strains. Ale strains are suggested to be more hydrophobic and because of this more able to adhere to CO_2 bubbles and to form yeast heads at the top of the fermentor (Dengis *et al*., 1995). However, recent process development has somewhat changed these features. Use of large cylindroconical fermenting vessels and selection have resulted in some ale yeast becoming bottom-fermenting (Boulton and Quain, 2001).

Optimum growth temperature for the ale strains is higher than for the lager strains (Giudici *et al*., 1998). The ale strains also ferment better at higher temperature (approximately 20°C) than the lager strains, which prefer 6–14°C for their optimum performance (Bamforth, 1998). This difference can, at least partly, be explained by their different capability for sugar utilization at low temperatures. Both maltose and maltotriose utilization were observed to be affected more in an ale strain compared to a lager when temperature was decreased from 14°C to 8°C (Takahashi *et al*., 1997).

Ale and lager strains differ in their sugar utilization abilities and this has been one method for their classification. The most pronounced difference is the ability of lager strains to utilize melibiose (disaccharide of galactose and glucose subunits). Lager strains possess *MEL* genes, which encode the melibiase enzyme, which is secreted into the periplasmic space of the yeast cell and is able to hydrolyse melibiose (Boulton and Quain, 2001; Turakainen *et al*., 1993). Lager

yeast strains also possess the *FSY1* gene encoding a fructose transporter not present in the ale strains (Gonçalves *et al*., 2000). It has been also shown that the lager strains use maltotriose more efficiently than the ale strains and less residual maltotriose is usually left after lager fermentation (Zheng *et al*., 1994a).

1.3 Carbohydrates of wort

A typical sugar spectrum for 11–12°Plato wort is shown in Table 1. Worts supplemented with sugar adjuncts have markedly changed sugar concentrations as described in section 1.10. Wort contains both fermentable (accounting for 70–80%) and non fermentable (20–30%) carbohydrates. Of fermentable sugars, the most abundant is maltose, which is a disaccharide of two glucose subunits joined together via α-1,4-linkage. Maltose accounts for 60–65% of the total fermentable sugars. Two other main sugars of wort are glucose and maltotriose, each accounting for approximately 20% of the total fermentable sugars. Maltotriose is a trisaccharide consisting of three glucose subunits joined together via α-1,4-linkages. Both maltose and maltotriose are hydrolysed by the yeast to glucose subunits by an intracellular α-glucosidase enzyme (maltase) capable of hydrolysing terminal 1,4-linked α-D-glucoside residues with a release of α-D-glucose. The α-glucosidase has the same affinity for both of these sugars (Zastrow *et al*., 2000) (K_m 17 mM for both, Needleman *et al*., 1978).

In addition to the three main sugars, there is a minor amount of sucrose (disaccharide of glucose and fructose subunits) and fructose present in the wort. The unfermentable fraction of wort consists mostly of dextrins which are carbohydrates with four or more glucose subunits linked by α-1,4 or α-1,6 glycosidic bonds. In addition to dextrins unfermentable fraction contains a fraction of β-glucans (polysaccharides consisting of glucose molecules linked together by β-1,3 and β-1,4 bonds) and a small fraction of pentose sugars such as arabinose and xylose.

Table 1. Typical sugar spectrum of 11–12°Plato wort. Share of each sugar is shown as a percentage (%) (modified from Stewart, 2009).

Wort concentration	11–12°Plato
Maltose	50–60
Maltotriose	15–20
Glucose	10–15
Sucrose	1–2
Fructose	1–2
Total fermentable sugars	70–80
Total dextrins	20–30

1.4 Sugar uptake and assimiliation during fermentation

The barrier between the outside and inside of the yeast cell consists of cell wall, plasma membrane and periplasmic space, which is located in between these two. The cell wall of the yeast cell is porous and sugars are able to pass through it. Thus, it is the plasma membrane that forms a barrier between the inside and outside of the yeast cell. Sugars do not freely permeate biological membranes and cellular uptake of sugars requires the action of transporter proteins. Sugar transporters specifically bind their substrate sugar and subsequently carry it into the yeast cell. Some of the sugar transporters are highly specific whereas some have a wide substrate range (Bisson *et al*., 1993; Lagunas, 1992). Sugar transporters mediate two types of transport processes in the yeast cells: energy-independent facilitated diffusion, in which solutes are transported down a concentration gradient, and energy-dependent transport via proton symport mechanism where solutes can be accumulated also against the concentration gradient (Bisson *et al*., 1993; Lagunas, 1992).

Brewer's yeasts can utilize a wide variety of sugars but when several sugars are present simultaneously yeast tend to use them in sequential manner. Most easily assimilated sugars, *i.e*. monosaccharides glucose and fructose, are used first (Fig. 2). Both glucose and fructose are carried into the yeast cell by members of the hexose transporter (HXT) family that consists of 18 transporters (Wieczorke *et al*., 1999). Hxt transporters mediate energy-independent facilitated diffusion of glucose and fructose. Uptake of both glucose and fructose is initiated at an early phase of the fermentation. Hxt transporters are more efficient

carriers of glucose compared to fructose and, for this, glucose is taken up faster than fructose (D'Amore *et al*., 1989a). Thus, glucose is usually used up before fructose (Meneses *et al*., 2002), even if the initial concentration of glucose was higher. Differently to other sugars, sucrose is usually not carried into the yeast cell but is hydrolysed in the periplasmic space by the secreted invertase enzyme encoded by the *SUC* genes (Hohmann and Zimmermann, 1986). Hydrolysis of sucrose to glucose and fructose by invertase and slower uptake of fructose compared to glucose may even cause a transient increase in the concentration of fructose at the beginning of the fermentation (Meneses *et al*., 2002).

Glucose is the substrate preferred over all the other carbohydrates by the yeast and in the presence of glucose uptake of other less preferred sugars, like the maltose and maltotriose, is delayed. The most important mechanisms by which glucose causes this delay are catabolite repression and catabolite inhibition (discussed in more detail in chapter 1.9). Usually, uptake of maltose starts only when approximately 60% of the glucose has been utilized (D'Amore *et al*., 1989a).

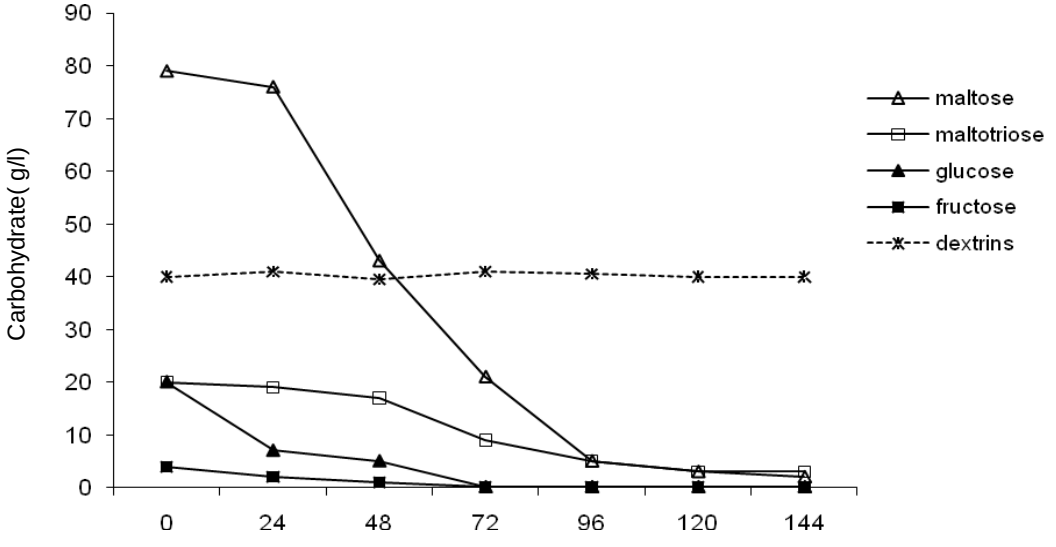

Figure 2. Order of uptake of sugars by yeast from wort (modified from Stewart, 2009).

A schematic representation of sugar uptake by brewer's yeast cell is shown in Figure 3. Maltose and maltotriose are carried into the yeast cell by energy-dependent transport through a symport mechanism, in which one proton is co-transported with each maltose or maltotriose molecule (Serrano, 1977; van Leeuwen *et al*., 1992). The driving force for this transport is an electrochemical transmembrane proton gradient generated largely by plasma membrane ATPase, which pumps protons out of the cell with a stoichiometry of 1 proton/ATP hydrolysed to ADP.

Maltotriose does not have its own specific transporters, but is transported with some, but not all, of the maltose transporters (Han *et al*., 1995; Day *et al*., 2002a; Salema-Oom *et al*., 2005). Most of the transporters capable of carrying both of these sugars carry maltose more efficiently than maltotriose (Han *et al*., 1995; Day *et al*., 2002a) and thus its uptake is faster. Competition for the same transporters and maltose being the preferred substrate leads to maltotriose being utilized only after most of the maltose has been assimilated.

Several studies have shown that the overall fermentation rate of maltose and maltotriose is correlated with their maltose and maltotriose transport activity and correlates poorly with maltase activity (Meneses *et al*., 2002; Rautio and Londesborough, 2003; Kodama *et al*., 1995; Alves *et al*., 2007). Transport rather than hydrolysis is therefore the rate limiting step in the utilization of these two sugars.

The higher polysaccharides dextrins are not utilized by brewer's yeasts and contribute to the beer flavour by imparting fullness. Attempts have been made to utilize dextrins, for example, by introducing appropriate enzymes into the brewing yeast by genetic engineering or by addition of dextrinase enzyme to the wort (Hammond, 1995). Both of these approaches have been successful in the production of diet beer.

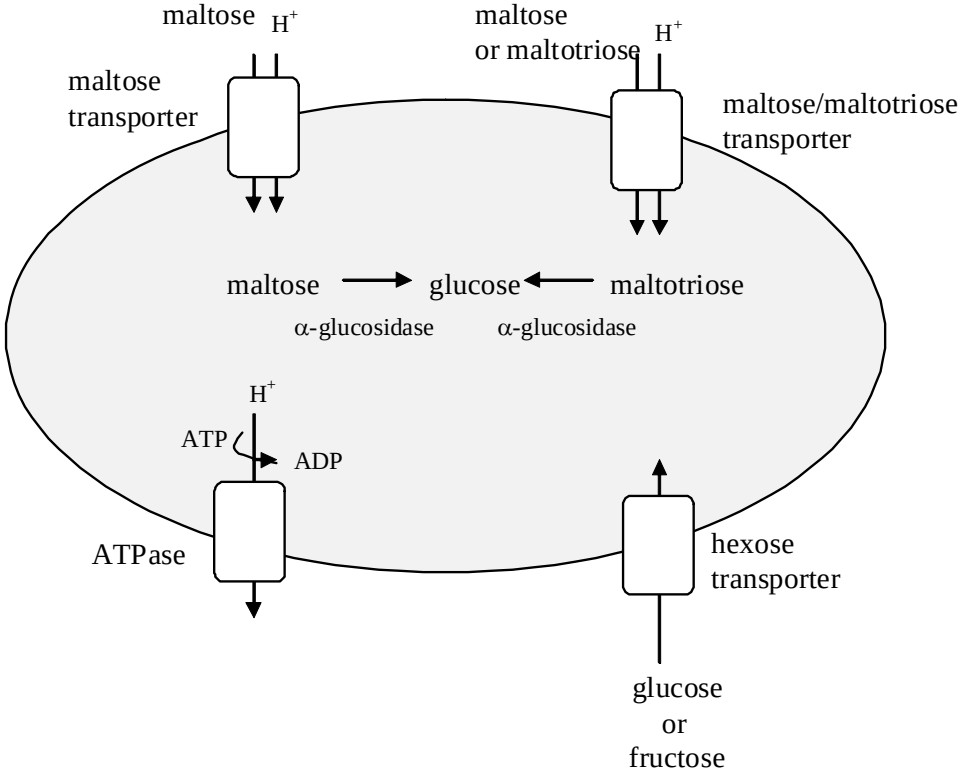

Figure 3. Uptake of wort sugars by brewer´s yeast.

The sugar uptake profile of brewer's yeast differs markedly from that of laboratory strains. Laboratory strains are not usually able to use maltose or maltotriose at all. In laboratory strains, sucrose hydrolysis by invertase is delayed by glucose (Meijer *et al.*, 1998), mostly because glucose represses the expression of the *SUC2* gene encoding invertase (Neigeborn and Carlson, 1984). In contrast, many brewer's yeast strains are characterized by rapid depletion of sucrose in the presence of glucose (D'amore *et al.*, 1989b; Meneses *et al.*, 2002) implying that invertase activity is constitutive. In brewer's yeast strains direct uptake of sucrose also occurs. Agt1 transporters are able to carry sucrose with high affinity (Salema-Oom *et al.*, 2005) and, once inside the cell, α-glucosidase is able to hydrolyse it to subunits (Needleman *et al.*, 1978). However, since *AGT1* genes are known to be glucose repressed, there is practically no importance in direct sucrose uptake in the brewery fermentations because, by the time glucose re-

pression is lifted, sucrose has already been hydrolysed by invertase. In addition the lager strains have been shown to possess specific fructose transporters. These are fructose/proton symporters encoded by the *FSY1* gene not present in the ale strains (Gonçalves *et al.*, 2000). Glucose is known to repress also the *FSY1* genes (Rodrigues de Sousa *et al.*, 2004) so that direct fructose transport does not have significance in brewery fermentations for the same reason as described for the sucrose direct transport.

Glucose, transported into the yeast cell by Hxt transporters or produced by intracellular hydrolysis of maltose and maltotriose, has the same fate, *i.e.* it is channelled to glycolysis. Also, fructose can enter directly to the glycolysis pathway after its phosphorylation to fructose 6-phosphate. In glycolysis, glucose is degraded to pyruvate and energy in the form of ATP is produced. Pyruvate intermediate is a branchpoint where respiration or fermentation is selected. Pyruvate can either be converted into acetyl-CoA, the fuel of the TCA-cycle (respiration), or be decarboxylated and reduced to ethanol (fermentation). In principle, oxygen availability will determine whether yeast respires or ferments pyruvate. However, despite fully aerobic conditions some yeast including brewer's yeast can exhibit alcoholic fermentation.

A further level of complexity in maltotriose utilization by *S. cerevisiae* yeast cells was revealed by Zastrow *at al*. (2000) who observed that several industrial strains could utilize maltotriose only aerobically, *i.e*. grow on this carbon source in the absence of ethanol production. However, Londesborough (2001) showed that two brewer's yeast strains could grow anaerobically on pure maltotriose as sole carbon source, but the lag phase was very long. Salema-Oom *et al*. (2005) concluded that the relative fraction of maltotriose fermented versus respired is strain-dependent and varies with the efficiency of maltotriose transport into the cell. Salem-Oom *et al*. (2005) suggested that this is because the rate of glycolysis is diminished when maltotriose transport occurs slowly and reduced glycolytic flux leads to an increase in respirative metabolism.

1.5 Factors affecting maltose and maltotriose uptake efficiency

Ability to utilize maltose and maltotriose varies widely between different brewer's yeast strains. Widest variation is seen in the ability to utilize maltotriose (Dietvorst *et al.*, 2005; Meneses *et al.*, 2002), *i.e*. there are strains with

severe difficulties, whereas some of the strains utilize it fast and efficiently (Meneses *et al*., 2002).

Both maltose and maltotriose transport velocities have been observed to decrease significantly at late phase of cultivation in both ale and lager strains (Zheng *et al*., 1994b). In the late fermentation, sugar uptake is generally inhibited by deteriorated circumstances, *i.e*. increased ethanol concentration, nutrient deprivation, inhibited yeast metabolism, *etc*. Guimarães *et al*., (2006) have shown that maltose transport activity is affected by the lipid composition of the yeast. The proper function of maltose transporters was shown to require adequate amounts of ergosterol in the yeast. This effect may partly explain the low maltose (and maltotriose) uptake rates in the secondary half of brewery fermentations when the sterol content of the yeast has fallen. Inactivation of plasma membrane transporters has been connected also to nitrogen starvation in resting cells (laboratory strains) (Riballo *et al*., 1995; Peñalver *et al*., 1998). Nitrogen starvation was observed to lead to endocytosis and degradation of Mal61 transporters expressed in laboratory strains (Lucero *et al*., 2002). However, it is not known if this phenomenon takes place also in brewer's yeast cells. Since several different causes seem to deteriorate the maltose and maltotriose uptake at late phases of fermentation, an early onset and high rate of maltose and maltotriose utilization is important.

Fermentation temperature is also an important factor affecting the uptake capacity of maltose and maltotriose. Raising the fermentation temperature from 15 to 21°C increased markedly the rate of maltotriose utilization in both ale and lager strains (Zheng *et al*., 1994a). Takahashi *et al*. (1997) observed that when temperature was raised from 8 to 14°C there was no significant effect on the glucose utilization but the maltose and maltotriose utilization rates were both increased.

Maltose and maltotriose uptake velocities have been shown to be dependent on pH of the medium. An external pH rise from 5.5 to 7.0 decreased maltose uptake from 8.7 to 0.4 nmol/min/mg dry wt (Van Leeuwen *et al*., 1992). Similar results were obtained by Visuri and Kirsop (1970) for both maltose and maltotriose uptake. Visuri and Kirsop (1970) suggest that the pH optimum for the uptake of both maltose and maltotriose is pH 5.

Wort extract changes (in range between 7°P to 15°P) did not have a notable effect on glucose, maltose or maltotriose utilization ability in either ale or lager strains (Takahashi *et al*., 1997). However, when wort osmotic pressure was increased with sorbitol (15–30% w/v) significant decrease in the maltotriose up-

take was observed in the lager strains indicating that in very-high-gravity wort lager strains may have lowered maltotriose uptake ability (Zheng *et al*., 1994a).

1.6 Kinetics of maltose and maltotriose transport

The transporters work practically like enzymes. They show specific binding for the substrates after which they catalyse uptake of the substrate and, while doing so, undergo some conformational change. Affinities and maximal velocities can be determined for transporters similarly as for enzymes. Because of the finite number of binding sites, both enzymes and transporters are saturable. There can be several substrates for each transporter and in this case they inhibit each others' binding to the transporter proteins. Analogously to the velocity of enzymatic reactions sugar transport velocity also follows Michaelis-Menten kinetics. Reaction velocity approaches a maximum when substrate concentration is increased. If the initial rate of the reaction is measured over a range of substrate concentrations (denoted as [S]), the reaction rate (v) increases as [S] increases. However, as [S] gets higher, the enzyme becomes saturated with substrate and the rate reaches V_{max}, the enzyme's maximum rate. K_m is defined as the substrate concentration where reaction velocity is ½ V_{max}.

Some sugar transport systems, like glucose transport in *S. cerevisiae*, exhibit biphasic kinetics, where there appear to be two distinct K_m values (Busturia and Lagunas, 1986). Biphasic kinetics has also been observed for maltose transport in both ale and lager strains. A high affinity system with a K_m of 1.3–4 mM and V_{max} of 28 nmol/min/μg dry wt and a low affinity system with K_m 15–70 mM and V_{max} of 17–20 nmol/min/μg dry wt have been described for both ale and lager strains (Crumplen *et al*., 1996; Rautio and Londesborough, 2003). Some authors have suggested that the low-affinity component for maltose transport is due to the function of low affinity maltose transporters such as Agt1 and Mtt1 (Salema-Oom *et al*., 2005; Alves *et al*., 2008). Alves *et al*. (2008) studied natural isolates of *S. cerevisiae* strains and observed that they exhibited biphasic maltose transport kinetics with both high (K_m 5 mM) and low affinity (K_m 30 mM) systems. For maltotriose transport only the low affinity (K_m 36 mM) system was observed. When the *AGT1* gene was deleted from these strains, maltotriose transport ability was completely lost as well as the maltose low affinity transport. Thus, Agt1 transporters seem to be responsible for the low affinity maltose transport system as well as for maltotriose transport in these strains.

For maltotriose transport, only the low affinity component has most often been found in both *S. cerevisiae* and in brewer's yeast strains (Zastrow *et al*., 2001; Salema-Oom *et al*., 2005; Alves *et al*., 2008). An exception is a study by Zheng *et al*. (1994b) where it is reported that in both ale and lager strains there exists also a high affinity system for maltotriose transport, which was observed to be almost completely inhibited by maltose.

Two approaches have been used to measure the maltose or maltotriose uptake into yeast cells. In the first approach uptake studies are performed with [^{14}C] labelled maltose or maltotriose and velocity of the transport is calculated from the radioactivity remaining inside the yeast cells after Zero-*trans* transport assay (Lucero *et al*., 1997). Another approach is to calculate the rate of H^+ symport activity determined from the increased alkalinity of the medium due to concomitant uptake of protons with sugars (Serrano, 1977).

1.7 Maltose and maltotriose transporters

At present there are four different types of maltose and/or maltotriose transporters characterized from *S. cerevisiae* and/or *S. pastorianus*. These are Malx1, Agt1, Mphx and Mtt1 transporters. Substrate ranges determined in different studies for each of the transporters are shown in Table 2. Michaelis-Menten constants K_m and V_{max} for each transporter are shown in Table 3. K_m and V_{max} values have been obtained by cloning a single transporter gene and expressing the gene from a plasmid in a laboratory strain lacking endogenous α-glucoside transporter activity. Thus, affinities and V_{max} values can be compared between maltose and maltotriose when a study is performed with a single construct. Whereas results obtained in different studies with different constructs are only indicative, since there can be differences in the expression levels of genes, stability of the transporters in the plasma membrane, *etc*. so that the number of transporters per g of yeast in each case is not known.

Table 2. Substrate range of α-glucoside transporters.

	Maltose	**Turanose**	**Maltotriose**	**Trehalose**	**α-methyl glucoside**	**Sucrose**	**Reference**
Agt1	√	√	√	√	√	√	Salema-Oom *et al*., 2005; Han *et al*., 1995
Mtt1	√	√	√	√	-	-	Salema-Oom *et al*., 2005
Mphx	√	√	√	-	√	n.d.	Day *et al*., 2002a
Mal31	√	√	√	-	-	-	Day *et al*., 2002a
Mal31	√	√	-	-	-	-	Salema Oom *et al*., 2002
Mal61	√	√	-	-	-	-	Han *et al*., 1995

√ = substrate,
- = not a substrate
n.d. = not determined

Table 3. Affinities and maximal velocities of transporters.

	Maltose		**Maltotriose**		**Reference**
	K_m (mM)	V_{max}	K_m	V_{max}	
Mal21	4.7 ±0.2	115 ±3	n.d.	n.d.	Stambuk and de Araujo, 2001
Mal31	4.2 ± 1.1	42	5.7 ± 1.0	41	Day *et al*., 2002b
	4.3 ± 0.6	n.d.	335 ± 165	n.d.	Multanen, 2008
Mal61	2–4	n.d.	n.d.	n.d.	Han *et al*., 1995
	2.7 ± 0.6	36	7.2 ± 0.9	40	Day *et al*, 2002b
Agt1	5–10	n.d.	n.d.	n.d.	Han *et al*., 1995
	17.8 ±1.3	13.2 ± 0.5	18.1 ± 1.8	7.8 ± 0.3	Stambuk and de Araujo, 2001
	5.1 ± 0.6	41	4 ± 0.7	39	Day *et al*., 2002b
	30	n.d.	36 ±2	n.d.	Alves *et al*., 2008
Mphx	4.4 ± 0.5	39 ± 4.2	7.2 ± 1.0	49 ± 5.4	Day *et al*., 2002a
Mtt1	61–88	n.d.	16–27	n.d.	Salema-Oom *et al*., 2005
	41 ± 0	n.d.	23 ± 4	n.d.	Multanen, 2008

V_{max} = nmol/min/µg dry wt; n.d.= not determined

1.7.1 Malx1 transporters

Both ale and lager strains usually possess several copies of *MALx1* (maltose utilization) genes in their genomes (Jespersen *et al*., 1999). Several *MALx1* genes (*MAL11, MAL21, MAL31* and *MAL61*) have been cloned and sequenced. Most of them are derived from laboratory strains but *MAL61* gene was originally isolated from a lager strain (Needleman *et al*., 1984). All these genes have very similar sequences and they are observed to encode transporters bearing 98% identity at amino acid level, thus suggesting a conserved nature for Malx1 transporters. Lager strains also possess *S. bayanus*-derived Malx1 transporters approximately 80% identical to corresponding *S. cerevisiae* transporters (Nakao *et al*., 2009).

Malx1 transporters are reported to be high affinity maltose transporters (K_m 2–5 mM) (see Table 3; Day *et al*., 2002b; Han *et al*., 1995; Stambuk and de Araujo, 2001). It has been shown in many studies that the substrate range of Malx1 transporters is restricted to maltose and turanose (Han *et al*., 1995; Salema-Oom *et al*., 2005; Alves *et al*., 2008; Multanen, 2008; Duval *et al*., 2010) and that maltotriose is not carried by Malx1 transporters. This view has been challenged by Day *et al*. (2002b) who claimed that Mal61 and Mal31 transporters are actually able to carry maltotriose as efficiently as maltose. The reason for the significantly different results obtained by Day *et al*. could be due to the analysis method used. In results shown in Table 3 only Day *et al*. and Multanen measured the uptake of radioactive maltotriose whereas other authors have used the H^+ influx rate measurement method to assay maltotriose uptake. Dietvorst *et al*. (2005) have shown that commercial [^{14}C] maltotriose from the same supplier that Day *et al*. were using in their study actually is not pure but is heavily contaminated with [^{14}C] maltose and [^{14}C] glucose residues. It has been shown that use of commercially available [^{14}C] maltotriose without further purification can overestimate the rate of maltotriose transport by more than four-fold (Dietvost *et al*., 2005). It has been suggested that, due to the contaminations, maltotriose transport was strongly overestimated by Day *et al*. (Alves *et al*., 2008). In the study of Multanen, [^{14}C] maltotriose used has been further purified and results by Multanen actually show that Mal31 can't carry maltotriose. However, Day *et al*. (2002b) claim that they have verified and determined by chromatography that no degradation has occurred in [^{14}C] maltotriose used. Another possibility to explain conflicting results is that Mal31 and Mal61 transporter proteins used in the study of Day *et al*. have some changed amino acids that significantly affect

their sugar carrying ability. It is known that even one amino acid change in transporter protein can have a significant effect on sugar uptake characteristics (Smit *et al*., 2008).

1.7.2 Agt1 transporters

Charron and Michels (1988) isolated a *MAL1* locus with a maltose transporter gene clearly distinct from *MAL11*, as observed by restriction mapping and Southern analysis. This allele was referred to as *MAL1g* and the maltose transporter gene located in it was characterized later by Han *et al*. (1995). This new transporter gene was referred to as *AGT1* (alpha-glucoside transporter) since it was found to carry several different α-glucosides (Han *et al*., 1995). Its preferred substrates were observed to be trehalose and sucrose with K_m 8 mM for both. Significantly lower affinity (K_m 20 to 35 mM) was detected for maltose, maltotriose and even lower affinities for α-methylglucoside, turanose, isomaltose, palatinose and melezitose (Han *et al*., 1995.). The Agt1 transporter was observed to be an α-glucoside/proton symporter (Han *et al*., 1995; Stambuk *et al*., 1999) similar to Malx1 transporters.

Recently, results of the whole genome sequencing of lager strain WS34/70 have revealed that the WS34/70 strain possesses another putative maltose/maltotriose transporter not earlier described (Nakao *et al*., 2009). There was an ORF found, referred to as LBYG13187 and believed to be the *S. bayanus* homologue of *S. cerevisiae AGT1*. This is because its closest homology showed 79% identity to the *AGT1* sequence in the *Saccharomyces* Genome Database (Nakao *et al*., 2009). Here we call this gene *Sb-AGT1*, although nothing is yet known about its functionality and sugar carrying properties.

1.7.3 Mphx transporters

MPHx (maltose permease homologue) genes were originally identified by *S. cerevisiae* (laboratory strain) genomic sequence data clustering and grouping. Two ORFs, YDL247w and YJR160c, were grouped with a cluster of maltose transporter genes and referred to as *MPH2* (YDL247w) and *MPH3* (YJR160c) (Nelissen *et al*., 1995). These ORFs have identical sequences but are located on different chromosomes, *MPH2* is located on chromosome IV and *MPH3* on chromosome X. Sequence identity of *MPHx* to *MALx1* and *AGT1* genes is 75% and 55%, respectively (Day *et al*., 2002a). Day *et al*. (2002a) cloned *MPHx* gene

from a lager strain and characterized its ability to transport sugars. Day *et al.* showed that Mphx transporters are able to carry maltose, maltotriose, α-methylglucoside and turanose. Rather high affinities for both maltose (K_m 4.4 mM) and maltotriose (K_m 7.2) were observed. There are no reports on whether Mphx transporters function as α-glucoside/proton symporters as with other maltose/maltotriose transporters.

Several studies have questioned the role of Mphx transporters in maltose and maltotriose transport. Jespersen *et al.* (1999) have suggested that Mphx transporters most probably play a secondary role in the maltose uptake since they have not been found in functional analysis screenings performed but were identified via genomic sequencing of a laboratory strain. Moreover, Alves *et al.* (2008) have shown that *MPH2* and *MPH3* genes derived from a laboratory strain do not allow efficient transport of maltotriose. Duval *et al.* (2010) suggest that *MPHx* genes probably have little influence on maltotriose (and maltose) utilization since in 21 brewer's yeast strains included in their study, the utilization of maltotriose (maltose) didn't correlate with the presence of *MPHx* genes. Whereas there was significant correlation observed in the presence of other maltose/maltotriose transporter genes.

1.7.4 Mtt1 transporters

A new type of maltose and maltotriose transporter gene was identified in 2005 by two independent research groups (Salema-Oom *et al.*, 2005; Dietvorst *et al.*, 2005). Both groups found the transporter gene by screening genomic libraries of lager strains for the ability of cells to grow on maltotriose either aerobically (Salema-Oom *et al.*, 2005) or when the respiration of the cell was blocked by antimycin A (Dietvorst *et al.*, 2005). Salema-Oom *et al.* (2005) referred to the new α-glucoside gene they found as Mty1 (maltose transport in yeast) and Dietvorst *et al.* (2005) as Mtt1 (mty1-like transporter) because they noted the similarity to the *MTT1* sequence deposited by Salema-Oom in EMBL gene bank before publication. *MTY1* and *MTT1* genes are identical in their sequence and are hereafter referred to as *MTT1*. *MTT1* share 90% and 54% identity to *MALx1* and *AGT1* genes, respectively. Mtt1 transporters can carry maltose, maltotriose, trehalose and turanose but trehalose is the preferred substrate (Salema-Oom *et al.*, 2005).

Interestingly, Mtt1 displays higher affinity (K_m 16–27 mM) for maltotriose than for maltose (K_m 61–88 mM). This is a unique characteristic among all α-glucoside transporters and this feature makes Mtt1 particularly important in re-

gard to brewery fermentations. Mtt1 transporters were shown to be α-glucoside/proton symporters (Salema-Oom *et al*., 2005), similar to Malx1 and Agt1 transporters.

Dietvorst *et al*. (2005) also obtained an altered version of *MTT1* in the screening. This version lacks 66 base pairs from the 3'–end of *MTT1* gene but instead contains 54bp of the cloning vector. This altered version referred to as *MTT1alt* was found to encode maltose/maltotriose transporter with more efficient uptake of maltotriose than the original *MTT1* encoded version. The ratio of maltotriose uptake versus maltose uptake was also observed to be raised with this altered version in favour of maltotriose. Increase in transport ability could be due to the deletion of catabolite inactivation signal as discussed in the chapter 1.9.

1.8 *MAL* loci

Genes required for maltose and maltotriose utilization are located in *MAL* loci (except *MPHx* and *Sb-AGT1*). In the classical *MAL* locus there are three genes, all of which are needed for the efficient utilization of maltose. There is a gene encoding the maltose transporter, a gene encoding α-glucosidase (maltase) and a gene encoding the activator protein needed for the efficient expression of the two other genes of the locus. Structure of the classical *MAL* locus is shown in Figure 4. There are five known *MAL* loci in *S. cerevisiae*; *MAL1* (located on chromosome VII), *MAL2* (Chr III), *MAL3* (Chr II), *MAL4* (Chr XI) and *MAL6* (Chr VIII). Genes of the locus are referred to as *MALx1* for maltose transporter (where x refers to the *MAL* locus, *i.e* 1 to 4 and 6), *MALx2* for maltase and *MALx3* for MAL-activator encoding gene. The regulatory protein from one locus can act in *trans* to activate *MALx1* and *MALx2* genes from another locus. Laboratory strains are not usually able to use maltose or maltotriose at all because the *MAL* loci they possess are non-functional due to presence of non-functional MALx3 activators (Bell *et al*., 2001). Glucose is known to repress *MAL* genes in a Mig1-mediated manner. There are Mig1 binding elements present in promoters of all the three genes of the locus (Hu *et al*., 1995; Wang and Needleman, 1996). Maltose is an inducing agent of *MAL* genes. It has been suggested that MAL-activators are bound by maltose and this yields a conformation with functional activity (Wang *et al*., 1997). Active conformation would then be capable of entering the nucleus and/or activate the transcription (Danzi *et al*., 2000).

There are usually several *MAL* loci present in each yeast strain. *MAL* loci studied by restriction fragment analysis and Southern hybridization studies are

shown to be highly similar in their structure (Charron *et al*., 1989). Also, sequence data obtained has revealed highly conserved sequences for at least *MALx1* and *MALx2* genes between different loci and also between different strains.

All *MAL* loci are located near telomeres. Regions near telomeres are known to be more prone to chromosomal rearrangements since recombination events between different chromosomes are common near telomeres (Bhattacharyya and Lustig, 2006). In addition, genes that are located close to the telomeres can become transcriptionally repressed by an epigenetic process known as telomeric silencing, *i.e.* variation in chromatin structure near the telomeres leading to the silencing of genes located in this region (Pryde and Louis, 1999; Loney *et al*., 2009). The role of chromatin remodelling in the regulation of expression of *MAL* genes has been reported (Houghton-Larsen and Brandt, 2006; Dietvorst and Brandt, 2008). It has been observed that telomeric silencing does not occur uniformly but there is significant variation between different strains (Pryde and Louis, 1999). A specific complex consisting of several subunits, the COMPASS complex is known to be involved in the telomeric silencing in the yeast cells (Miller *et al*., 2001). In a study where the COMPASS complex was rendered non-functional, strain-dependent differences in the telomeric silencing of *MAL* genes were observed. The *MAL* genes were found to be strongly silenced in some strains, whereas in other strains the non-functional COMPASS complex did not cause any changes in the expression of *MAL* genes (Houghton-Larsen and Brandt, 2006). It has also been observed that in a single strain some chromosome ends are more prone to the telomeric silencing than others (Loney *et al*., 2009). Thus, it is possible that the different *MAL* loci, located at different chromosome ends, are not uniformly regulated by telomeric silencing.

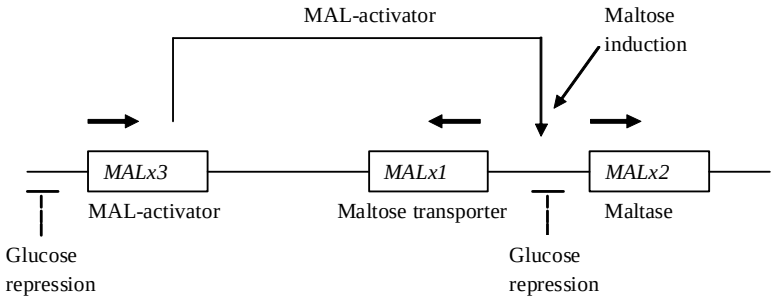

Figure 4. Structure of classical *MAL* locus.

In some *MAL1* loci there is a gene encoding a different type of transporter than *MAL11*. This gene, 57% identical to *MALx1* transporter, is referred to as *AGT1* and it has changed characteristics for sugar transport (Han *et al*., 1995). *MALx2* and *MALx3* genes were found upstream and downstream of the *AGT1* gene, respectively, referring otherwise conventional *MAL* locus. Also, the *MTT1* gene was observed to be located in a conventional *MAL* locus, at the place of *MALx1* gene, since *MALx2* and the *MALx3* genes were found upstream and downstream of the *MTT1* encoding sequence, respectively (Salema-Oom *et al*., 2005). The *MTT1* gene has been mapped to chromosome VII at right sub-telomeric region (Nakao *et al*., 2009) similarly to *MAL1* locus. Possibly *MAL13-MTT1-MAL12* is another version of *MAL1* locus but this remains to be verified in the future work. As an exception, *MPHx* and *SbAGT1* genes are not located in conventional *MAL* loci but exist as single genes without *MALx2* or *MALx3* genes in proximity (Day *et al*., 2002a; Nakao *et al*., 2009).

The *MALx1* and *MALx2* genes share a divergent promoter region (Bell *et al*., 1997). Similarly, there is a divergent promoter region also for *AGT1-MAL12* (SGD) and *MTT1-MAL12* (Salema-Oom *et al*., 2005; Dietvorst *et al*., 2005) gene complexes. Gene clustering and divergent promoters are found in the yeast genome like, for example, *GAL1-GAL10*. In many cases the two genes either function in the same metabolic pathway or the functions of their products are related. The regulation of neighboring genes by a common promoter element and regulatory proteins allows efficient and coordinate gene expression (Beck and Warren, 1988). *MALx1* and *MALx2* genes are coordinately induced several hundred-fold by maltose and repressed dramatically immediately following glucose addition (Vanoni *et al*., 1989). To mediate glucose repression there are two Mig1 binding sites found in the *MALx1-MALx2* divergent promoter regions (Hu *et al*., 1995) and one Mig1 binding site in *MALx3* promoters (Wang and Needleman, 1996). For maltose-based induction there are three binding sites in the *MALx1-MALx2* divergent promoter region for the MAL-activator (Levine *et al*., 1992, Sirenko *et al*., 1995). The *AGT1-MALx2* divergent promoter region has also three binding sites for the MAL-activator but only one binding site for the Mig1 element (SGD). The *MTT1-MALx2* intergenic region hasn't been studied in detail but it has been observed that sequence identity to the *MALx1-MALx2* divergent promoter region is very high ~99% (in ~540 bp upstream region from the start of the available *MTT1* gene sequence) (Dietvorst *et al*., 2005). The same promoter elements are most probably found in *MTT1-MALx2* intergenic region.

Although *MPHx* genes are not located in *MAL* loci and have significantly different promoter sequences compared to *MALx1* and *AGT1* promoters (43-45% identity, respectively), *MPHx* genes have been shown to be glucose repressed and maltose and maltotriose induced. There is a single MAL-activator binding site in the promoter region of *MPHx* as well as one putative Mig1 binding site (Day *et al*., 2002a). It has also been shown that *MPHx* genes need a MAL-activator for induction (Day *et al*. 2002a). *MPHx* genes actually showed very similar expression profile to *MALx1* and *AGT1* genes when expression was studied in repressing or inducing conditions (Day *et al*., 2002a).

Some *MAL* loci are known to possess extra copies of one or more *MAL* genes (Charron *et al*., 1989; Michels *et al*., 1992). Moreover, now that more sequence data has started to emerge from various whole genome sequencing projects of *S. cerevisiae* and other *Saccharomyces sensu stricto* strains, it has actually been observed that *MAL* loci are not so conserved as the classical model of the locus would suggest. There are, for example, *MAL* loci found where there are several copies of each *MAL* locus gene present, but not in equal numbers, for example there is a *MAL* locus found where there are three *MALx2*, two *MALx3*, one *AGT1* and one *MALx1* gene present in the same *MAL* locus (present in same continuous contig sequence spanning approximately 22 kbp region thus referred here as *MAL* locus) of *S. cerevisiae* RM11-1a strain. Interestingly, both *AGT1* and *MALx1* genes are located in the same *MAL* locus in this case (*S. cerevisiae* RM11-1a sequencing project, Broad Institute of Harvard and MIT (http://www.broad.mit.edu)).

1.9 Catabolite repression and inactivation

When glucose is present, the enzymes, transporters, *etc*. required for the utilization of alternative carbon sources are synthesized at low rates or not at all. This phenomenon is known as carbon catabolite repression or simply catabolite repression or glucose repression. Catabolite repression allows yeast to use the preferred (most rapidly metabolizable) carbon and energy source first (Gancedo, 1998).

Catabolite repression of maltose and maltotriose utilization is mostly mediated by the repression of gene expression although glucose has been shown to interfere also with the stability of at least α-glucosidase mRNA (Federoff *et al*., 1983). Repression of gene expression is mediated in a Mig1p mediated manner. Mig1p is a DNA-binding transcriptional repressor regulating the expression of several genes in response to glucose. As explained earlier, promoters of *MAL*

locus genes possess binding sites for Mig1 (Han *et al*., 1995; Dietvorst *et al*., 2005; Day *et al*., 2002a). These promoters also have MAL-activator binding sites and competition in the promoters between Mig1 and MAL-activator binding has been suggested to mediate the balance between repression versus induction (Wang *et al*., 1997). Probably MAL-activators are not able to bind when Mig1 repressors already cover the promoter. For example Kodama *et al*. (1995) have shown with a lager strain that when the MAL-activator was over-expressed from a multicopy plasmid, no increase in the *MALx1* and *MALx2* expression was seen under glucose repressive conditions (Kodama *et al*., 1995). Part of the glucose repression is due to a secondary effect, *i.e.* glucose represses *MALx3* genes, which in turn causes a lower level of induction of *MALx1* and *MALx2* genes (Hu *et al*., 1995).

In addition to the Mig1-dependent repression mechanism, a Mig1-independent mechanism has also been described. It was detected that in mig1 deletion strains, with constitutive MAL-activator expression, glucose repression was not completely alleviated (Hu *et al*., 2000). This could be because the MAL-activator needs intracellular maltose to obtain its active conformation (Wang *et al*., 1997). Another option is that when glucose is present, active conformation of the MAL-activator can't be obtained (Hu *et al*., 1995).

Maltose transport is affected also by catabolite inactivation. In particular, catabolite inactivation means glucose-triggered inactivation and/or proteolysis of proteins. By analogy to catabolite repression, this phenomenon has been called catabolite inactivation. Catabolite inactivation is a common mechanism for a number of plasma membrane proteins, which are observed to be removed from the plasma membrane and inactivated by glucose under different physiological conditions (Medintz *et al*., 1996). Catabolite inactivation has been mostly studied in laboratory strains but it has also been shown to occur at least in an ale strain (Rautio and Londesborough, 2003). Addition of glucose to maltose fermenting cells causes a rapid and irreversible loss of the ability to transport maltose (Görts *et al*., 1969). Maltose transporters but not maltase enzyme is subject to catabolite inactivation (Federoff *et al*., 1983; Rautio and Londesborough 2003), as expected since catabolite inactivation is particularly connected to the plasma membrane proteins.

There is an endocytosis and degradation targeting signal found in the N-terminal cytoplasmic domain of the Mal61 protein (Medintz *et al*., 2000). This signal sequence is referred to as the PEST sequence since it is rich in proline, glutamate, aspartate, serine and threonine. Glucose-triggered phosporylation of

serine and threonine residues in the PEST sequences is suggested to be associated with regulated degradation (Medintz *et al*., 2000). This occurs by rapid inactivation of the transporter from the membrane by endocytosis and subsequent vacuolar proteolysis (Riballo *et al*., 1995). Mal61 transporter mutants lacking the PEST sequence exhibit significantly reduced rates of glucose-induced proteolysis (Medintz *et al*., 2000). The PEST sequence has been reported to be present also in the Mal31 transporter (Day *et al*., 2002a) and is most probably found in other Malx1-type transporters due to the high conservation of sequences. Mal21p has been shown to be more resistant to glucose-induced degradation than Mal31p and Mal61p. This has been suggested to be due to D46G and L50H conversion in its amino acid sequence (Hatanaka *et al*., 2009).

Agt1 transporters lack the PEST signal sequence (Day *et al*., 2002a; Dietvorst *et al*., 2005). Nonetheless, Hollantz and Stambuk (2001) have shown that glucose induces a rapid catabolite inactivation of the Agt1 transporter. Possibly there is some other internalization signal present in the Agt1 protein. Dietvorst *et al*. (2005) observed that in Mtt1 transporter there is a RSTPS protein motif present, which they suggest to be an internalization signal. This signal sequence was missing in the altered version (Mtt1alt) of the transporter, which possessed increased maltose and maltotriose transport rates. This possibly suggests that Mtt1 type transporters are also under catabolite inactivation through this RSTPS signal motif.

It has been shown that yeast adaptation to maltose utilization diminishes the glucose regulation of maltose uptake. Cells pre-grown on maltose and harvested while the sugar is still present in the medium are more adapted to utilize maltose and maltotriose during the early stages of fermentation (Ernandes *et al*., 1993). Hazell and Attfield (1999) showed that pulsing yeast with maltose to induce expression of *MAL* genes prior to inoculation to mixed sugar medium enhanced sugar fermentation. The reason for this is probably that due to pre-growth on maltose there is already a significant amount of maltose transporters present in the yeast plasma membrane and thus yeast cells are able to start utilization of maltose earlier. This also suggests that glucose inactivation doesn't occur remarkably in these strains.

Glucose repression and glucose inactivation of the maltose transporters, result in rather strict exclusion of maltose from the cell (since there are no maltose transporters in the plasma membrane). This result is due to an inducer exclusion effect (Hu *et al*., 1995), *i.e.* while glucose is present, the inducing agent maltose is not transported into the cell in sufficient amounts to be able to create the in-

ducing effect. However, maltose transport at very low levels has been shown to suffice for the inducing effect (Hu *et al.*, 2000) so that practically complete exclusion of the inducer must be obtained for this effect.

Glucose regulation may be difficult to overcome completely since it is mediated at different levels and by different mechanisms. However, it has been shown by Meneses *et al*. (2002) that the actual rate of maltose or maltotriose transport into the cell once it begins is of major importance. Strains with efficient maltose and maltotriose transport can perform better than strains with early onset but not as efficient transport (Meneses *et al*., 2002).

1.10 High-gravity brewing

In traditional brewing, worts with original gravity of 11 to 12°Plato are fermented to beers with 4 to 5% (v/v) ethanol. In high-gravity brewing, concentrated wort with higher original gravity is fermented. Higher sugar concentration in the wort results in higher final alcohol concentrations and formation of stronger beer. Beer produced is diluted at some point to produce finished beer of desired alcohol content. Breweries often use carbohydrate adjuncts (*e.g*. sugar syrups) when making high-gravity worts. High-gravity wort contains more fermentable sugars for each degree plato of extract.

High-gravity brewing was originally introduced in the United States in the 1950s and is now a very common practice worldwide. Today, most major beer brands are made by fermenting high-gravity (14–18°P) worts and there is effort to increase wort strength into the very-high-gravity (VHG) range, *i.e.* a specific gravity of 18–25°P. There are several advantages in high-gravity brewing. Production capacity increases, *i.e*. more beer is produced with the same size of brewhouse and fermentation facilities at a given time. The closer to the final stage (packaging) that the concentrated beer is diluted, the more economical benefit is obtained. Use of high-gravity brewing decreases energy consumption of brewhouse and gives savings in labour and cleaning costs per unit volume of beer produced. With high-gravity brewing the capacity of existing plants can be increased 20–30% (Boulton and Quain, 2001) or even up to 50% if very-high-gravity worts are used (Blieck *et al*., 2007).

Attemps to use VHG worts have faced some problems. Beer fermentation using initial wort gravities above 18°P has a negative impact on yeast performance and often results in sluggish fermentations, *i.e*., lower specific growth rate and a longer lag phase before initiation of ethanol production (Pátková *et al*., 2000;

Piddocke *et al*., 2009). Also, unacceptably high concentrations of sugars, especially maltotriose, often remain in the final beer, with consequent decreases in yield and lower and variable product quality. There are several reasons linked to the deteriorated performance of yeast in very-high-gravity brewing. One of the reasons is increased osmotic pressure at the beginning of the fermentation due to high sugar concentration. Elevated osmotic pressure has been shown to have an effect on the uptake of maltose and maltotriose (whereas glucose uptake is only modestly affected) (Stewart *et al*., 1995). Decreased oxygen availability due to decreasing solubility of oxygen with increasing wort gravity could be one reason for decreased sugar uptake (D'Amore *et al*., 1991). An adequate amount of oxygen is needed for the synthesis of membrane lipids, which are necessary for efficient maltose/maltotriose transporter function.

Towards the end of the fermentation ethanol concentrations in HG and VHG wort fermentations are high. There is also a shortage of nutrients. It has been claimed that nutritional deficiency is a more severe problem than high alcohol concentrations as when nutritional deficiencies are remedied it has been demonstrated that brewer's yeast tolerates high ethanol concentrations well (Jakobsen and Piper, 1989). It has also been shown that when a nitrogen source, ergosterol, and oleic acid are added to worts it is possible to use even 31°P extract wort and beer up to 16.2% (v/v) ethanol can be produced (Casey *et al*., 1984).

Traditionally, brewers recycled yeast indefinitely from fermentation to fermentation so that present brewer's yeast strains result from hundreds of years of selection in traditional brewer's worts. However, this selection stopped some 40 years ago when brewer's started to store their strains as pure cultures. Nowadays, even if serial repitching is used, propagation of the yeast is nonetheless started from stored pure cultures at regular intervals. Thus natural evolution of yeast strains by selection for better performance in high-gravity conditions doesn't take place.

Sugar adjuncts are any added source of fermentable sugars used in the brewing process. Most often sugar syrups of different forms are used. Syrups are characterized by their concentration, purity, colour and sugar spectrum. Thus they can range from almost pure glucose syrup, which is entirely fermentable, to crude hydrolysates of cereal starch, which have been subjected to various amylases and thus contain a mixture of fermentable sugars and dextrins. Less pure cereal extract may also include significant concentrations of nitrogen. In Table 4, typical sugar spectra of VHG worts obtained by addition of two different types of sugar syrups are shown. Addition of barley syrup results in high-glucose wort

whereas addition of high-maltose syrup raises mostly the share of maltose. Addition of maltose-rich syrup as opposed to glucose-rich syrup has been shown to result in more balanced fermentation performance (Piddocke *et al*., 2009).

When sugar adjuncts are added it must be taken into consideration that this modifies the nutrient balance. For example nitrogen concentration is diluted. This leads to modified carbon to nitrogen ratio, which in turn if changed dramatically, can change the flavour profile (Piddocke *et al*., 2009).

Table 4. Typical composition of very-high-gravity worts (Huuskonen *et al*., 2010). In high-glucose wort barley syrup and in low-glucose wort high-maltose syrup was used as an adjunct.

Composition or characteristic of wort	**High-glucose 25°P wort**	**Low-glucose 25°P wort**	**All-malt 18°P wort**
Glucose (g /l)	62	25	13
Fructose (g/l)	5	4	3
Maltose (g /l)	105	120	80
Maltotriose (g /l)	24	40	24
Total conc of fermentable sugars (g/l)	198	193	121
Maltose/glucose ratio (g/g)	1.7	4.8	6.2
pH	4.9	5.04	4.98

1.11 Effect of temperature change on the plasma membrane and transporters embedded in it

Composition of the plasma membrane is essential for transporter function. This is because the transporter proteins are associated with specific lipids of the plasma membrane and are dependent on these lipids for their structural integrity and function (Bogdanov *et al*., 2002; Opekarová and Tanner 2003). Actually transporters can be regarded as protein-lipid complexes.

The existence of functional lipid rafts in the plasma membrane of yeast has been shown (Bagnat *et al*., 2000). Lipid rafts are less fluid membrane microdomains whose main components in yeast are sphingolipids and ergosterol. Many transporter proteins have been shown to be located in the lipid rafts, like, for example, uracil permease Fur4p (Hearn *et al*., 2003), tryptophan permease Tat2p (Umebayashi and Nakano, 2003), arginine/H^+ symporter Can1p and ATPase

Pma1 (Malínská *et al*., 2003). It has been shown that proteins located in the lipid rafts need certain amounts of ergosterol and/or sphingolipids for both efficient delivery to the plasma membrane and for their subsequent stabilization and function in the plasma membrane (Bagnat *et al*., 2000; Hearn *et al*., 2003; Mitsui *et al*., 2009). It has been suggested that proteins localized in lipid rafts associate with lipid rafts already in the endoplasmic reticulum and lipid rafts are essential for the delivery of these newly synthesized proteins to the plasma membrane (Bagnat *et al*., 2000; Hearn *et al*., 2003). Also, after the delivery to the plasma membrane these protein remains in the lipid rafts.

At the optimum growth temperature for a strain, membranes are colloidal solutions of lipids and proteins in a fluid (liquid crystalline) phase. Proteins embedded in the membrane, like transporters, function efficiently only when the membrane is in the fluid phase and loose their activity when the phase changes to rigid (McElhaney, 1982). It is possible to measure membrane fluidity with probes such as 1,6-diphenyl-1,3,5-hexatriene, which are fluorescent only in the liquid phase. A decrease in the temperature or an increase in pressure lead to a decrease in the mobility of these fluorescent molecular probes, and is interpreted as decrease in the membrane fluidity (MacDonald *et al*., 1988). Studies performed with such probes with membrane preparation have shown that there is no evidence of sharp phase transition with temperature but rather a continuous change of membrane fluidity when temperature decreases (Beney and Gervais, 2001).

The occurrence of phase-transition depends on the membrane lipid composition (*e.g*., the presence and type of sterols and fatty acids) and on external factors, such as the osmotic pressure (Guyot *et al.*, 2006). For example, ergosterols determine to a large extent the rigidity of the plasma membrane (van der Rest *et al*., 1995a). The lower the temperature the more unsaturated the membrane fatty-acid composition is (Watson, 1978). Increased portion of unsaturated lipids in the membranes is suggested to protect against the temperature decrease and reduce the temperature at which the membrane undergoes the phase change. Many organisms regulate their membrane lipid composition in response to environmental temperature so that membrane fluidity can remain optimal. The degree of lipid unsaturation has been observed to be affected by growth temperature in *S. cerevisiae* strains, *i.e*. the degree of unsaturation increased significantly (doupled) at 13°C compared to that at 25°C (Torija *et al*., 2003).

The rates of most enzyme-catalysed reactions approximately double for each 10°C rise in temperature because of more collisions between reactants per time

(until the denaturation temperature is reached). In chemical reactions a relationship between the temperature and velocity is usually expressed with the Arrhenius equation. In an Arrhenius plot the logarithm of transport velocity is plotted against the inverse temperature (1/T). For a single rate-limited thermally activated process, an Arrhenius plot gives a straight line. However, reactions catalysed by integral membrane proteins usually exhibit non-linear Arrhenius plots with increased temperature-dependence at lower temperatures. This is usually interpreted as reflecting change of state (“freezing”) of the lipids around the catalytic protein.

The substrate-translocation mechanism most commonly ascribed to secondary membrane transporters is the alternating access mechanism. In this mechanism, the transporter is suggested to have two major alternating conformations: inward-facing and outward-facing. In any moment, a single binding site in a polar cavity is accessible to any one side of the membrane (Huang *et al.*, 2003) and thus change of shape of the transporter requires that it moves against the surrounding lipid (Abramson *et al.*, 2003). When temperature decreases and plasma membrane becomes more rigid, conformational change occurring between inward and outward conformations is probably more tedious.

2. Materials and methods

Materials and methods for studies performed in publications I–IV are described in each publication. Transcriptional analysis results represented in Figure 5 were performed as described in Rautio *et al*. 2007. Industrial strains used in I–IV are listed in Table 5.

Table 5. Industrial strains used in the studies of I–IV.

Strain	**Origin**
Lager strains	
A-60012	Weihenstephan 1
A-62013	Weihenstephan 294
A-63015 (A15)	Nordic brewery
A-66024 (A24)	Nordic brewery
A-82064	Nordic brewery
A-85072	Nordic brewery
A-95143	Nordic brewery
WS34/70	Weihenstephan
CMB33	Belgium
Ale strains	
A-10179 (A179)	UK brewery
A-10180 (A180)	UK brewery
A-101181 (A181)	UK brewery
A-60055	NCYC 1200
A-60056	NCYC 240
A-75060 (A60)	Nordic brewery
A-93116	NCYC 1087
Baker's yeasts	
B-62001	Nordic baker's yeast
B-62003	Nordic baker's yeast
Distiller's yeasts	
C-72051	Nordic distillery
C-77076	Nordic distillery
C-91180A	Nordic distillery

3. Results and discussion

3.1 *MAL* locus distribution and integrity in brewer's yeast strains (Paper I, IV)

Distribution of *MAL* loci in the genomes of brewer's yeast strains was studied by hybridizing chromosome blots with *MAL* locus probes. In studies described in paper I, *AGT1*, *MALx1*, *MALx2* and *MALx3* probes were used to study the distribution of the *MAL* loci genes in several brewer's and in some laboratory yeast strains. Two specific *MALx3* probes for the detection of divergent *MALx3* sequences, ca. 75% identical to standard *MALx3* gene, found in the *MAL1* and the *MAL3* loci, respectively, were also used. These probes hybridize to *MAL33* and to the *MAL13* activator, here called *MAL13*(*AGT1*), found in the *MAL1* loci that contain *AGT1*. Earlier studies on *MAL* locus distribution in the brewer's yeast strains have been performed with α-glucoside transporter, *MALx1* and *AGT1* probes solely and thus do not give information on whether the two other genes of the locus are also present (Jespersen *et al*., 1999; Meneses and Jiranek, 2002). In the present study, the distribution of the *MAL* loci in the genomes of yeast strains, as well as the integrity of each *MAL* locus was revealed. It was observed that the *MAL* loci are highly conserved in possession of all three genes of the locus. Apart from two exceptions, only *MAL* loci with all three genes were found. These exceptions were weak hybridization of *MAL62* probe to chromosome IX in all brewer's yeast strains, while no hybridization of other probes (*MALx1*, *AGT1* or *MALx3*) to this chromosome was observed. Based on the S288C laboratory strain sequence (SGD), there is an ORF, 73% identical to the *MAL62* gene, present independently without other *MAL* loci genes, on chromosome IX. It is possible that the hybridization observed occurred with this ORF, which possibly has higher similarity to the *MAL62* probe in the brewer's yeast

strains. Another exception was very weak binding of the *MAL61* probe but not *MAL62* and *MAL63* probes to chromosome VIII, where the *MAL6* locus maps, in the brewer's yeast strains. However, in lager strain A72 all three probes bound strongly to the *MAL6* locus indicating that the locus is entire in this strain.

Some studies have shown that there can be extra copies of one or more of the three genes of the *MAL* locus present. For example, there are two MAL-activator encoding genes present in the *MAL6* loci of some yeast strains (Charron *et al*., 1989). In the S288C strain there is an extra *MALx2* gene, *i.e.* verified ORF YGR287c, 75% identical to *MAL12*, encoding isomaltase, present in the *MAL1* locus (*Saccharomyces* Genome Database data). Also partial tandem duplication of *MAL3* locus genes has been described where the copy number of *MAL31* and *MAL33* genes are increased compared to the *MAL32* genes (Michels *et al*., 1992). In the present study, hybridization intensities were observed to vary between the *MAL* loci with a single probe. Unequal hybridization might be due to extra copies of the *MAL* genes in certain loci as explained above. It also might be due to unequal distribution of the *MAL* loci in sister chromosomes, especially in lager strains, which are polyploid. Thirdly, unequal labelling could be due to the sequence divergence between the loci but since, at least *MALx1* and *MALx2* genes deriving from different *MAL* loci have been shown to be almost identical this option does not seem probable. Unequal labelling observed between the *MAL* loci was measured by taking the *MAL3* locus as a reference locus and the intensity of the hybridization signals obtained with *MAL61* and *MAL62* probes at each *MAL* locus were calculated relative to the intensities obtained at the *MAL3* locus (I, Table 4). In the lager strain A15, the signal intensities normalized this way were similar for both probes, *MAL61* and *MAL62*, suggesting that at each *MAL* locus of strain A15 the ratio of *MALx1* and *MALx2* genes is the same as in its *MAL3* locus. Thus, in the A15 strain the different signal intensities between the loci probably reflect different copy numbers of the *MAL* loci present (*MAL1>MAL3>MAL4>MAL2>MAL6*), *i.e*. there are more chromosomes VII where *MAL1* locus is present than chromosomes II where *MAL3* locus is present *etc*. For the other brewer's yeasts, the normalized signals at *MAL1* and *MAL2* loci were markedly stronger with the *MAL62* probe than with the *MAL61* probe. This would imply that there are more copies of *MALx2* compared to the *MALx1* in *MAL1* and *MAL2* loci than in the reference *MAL3* locus. A24 differed from other strains by having particularly strong signal strengths at *MAL2* (I, Table 4; I, Fig. 4), suggesting that *MAL2* is the most abundant *MAL* locus in this strain

and that in A24 either several copies of chromosome III carry the *MAL* locus or there is tandem duplication of *MAL* genes in some of the chromosomes III.

Both the *AGT1* and *MAL11* genes were present in all the strains studied (I, Table 3) on chromosome VII (I, Fig. 3), as has been described earlier for other brewer's yeast strains (Jespersen *et al*., 1999; Meneses and Jiranek, 2002). There possibly are two types of chromosomes VII in brewer's yeast strains, *i.e*. one possessing *AGT1-MALx2-MALx3* type *MAL1* locus and another possessing *MAL11-MALx2-MALx3* type *MAL1* locus. This is based on the observation that in the laboratory yeast strains either the *AGT1* or *MALx1* gene is found at each time in the *MAL1* locus together with *MALx2* and *MALx3* genes (Naumov *et al*., 1994; Charron and Michels, 1988). However, recently after obtaining sequence data from whole genome sequencing projects of several *S. cerevisiae* yeast strains, contig sequences have been presented where, for example, both *AGT1* and *MALx1* genes are located 10 kbp apart from each other on the same continuous stretch of DNA (IV, Fig. 3) (*S. cerevisiae* strain RM11-1a sequencing data http://www.broadinstitute.org/annotation/genome/saccharomyces_cerevisiae.3/Home.html). Thus, another option would be that *AGT1* and *MALx1* genes are actually both located on chromosome VII and are not allelic. Lager strain WS34/70 whole genome sequencing results do not answer the question of localization of *MAL11* and *AGT1* genes in respect to each other since there was no *MALx1* sequence mapped on chromosome VII of WS34/70. This is most probably because there are gaps in the sequence (sequence coverage was estimated to be 95.8%) and the *MAL11* gene might be in the non-sequenced 4.2%.

If *AGT1* and *MAL11* are alleles of each other, unequal labelling would be expected for the *MAL1* locus in the hybridization studies. There should be *MAL1* loci with *AGT1* sequences, which do not bind the *MAL61* probe but are accompanied by *MAL12* genes that do bind the *MAL62* probe. In all other brewer's yeast strains, except in A15, unequal labelling between *MALx1* and *MALx2* probes was observed in the *MAL1* locus (I, Table 4) that could imply the allelic nature of *MAL1* and *AGT1* genes. In the A15 strain, more or less equal binding of *MAL61* and *MAL62* probes to the *MAL1* locus was observed. This probably suggests a different organization of the *MAL1* locus in this strain like, for an example, an *AGT1* gene not accompanied by *MAL12* sequences.

A common characteristic of all brewer's yeast strains in the present study was that they all possessed *MAL1, MAL3* and *MAL4* loci. This was in accordance with the results obtained by Jespersen *et al*. (1999) and Meneses and Jiranek (2002), who found that *AGT1*, *MAL11, MAL31* and *MAL41* genes were present

in practically all ale and lager strains in their studies. Distribution of the *MAL2* locus was observed to be different between ale and lager strains. *MAL2* locus was found in all lager strains but only in one of the ale strains (A60 strain) (I, Table 3). In studies of Jespersen *et al*. (1999) and Meneses and Jiranek (2002) it was observed that the *MAL21* gene was found in 60% of the lager strains whereas all ale strains lacked this gene. Some *MAL21* gene-encoded transporters have been observed to possess amino acid changes, which make them resistant to glucose inactivation, *i.e*. endocytosis of maltose transporters from the plasma membrane caused by glucose (Hatanaka *et al*., 2009). Possession of such a *MAL2* locus would be beneficial and probably increase maltose uptake capacity. The *MAL6* locus was observed to be the least common. Only lager strain A72 possessed it in the present study and none of the ale or lager strains in the studies of Jespersen *et al*. (1999) and Meneses and Jiranek (2002).

Hybridization of *MAL* locus probes to other than known *MAL* loci was observed (I, Table 3). All three probes *MALx1*, *MALx2* and *MALx3* hybridized to ~1.3 Mbp chromosome in the A60 ale strain. In *S. cerevisiae* there is no chromosome of this size and therefore it is not known which chromosome this band represents. Binding with all three probes was slightly weaker than observed with other *MAL* loci suggesting that sequence homology is probably lower. Jespersen *et al*. (1999) found also hybridization of *MAL61* probe to this same size of chromosome (~1.3 Mbp) in one lager strain. They studied if the hybridization could be explained by chromosome size polymorphism of chromosome VII, which is in the same size range and contains *MAL* locus. However, the 1.3 Mbp chromosome was not detected with probes for the *BGL2* or *SER2* genes mapping to the chromosome VII (Jespersen *et al*., 1999). Taken together, these results suggest that there seems to be a new, not earlier identified *MAL* locus localized in this 1.3 Mbp chromosome found in both ale and lager strains. This putative *MAL* locus seems to be even more often present in the brewer's yeast genomes than the rarely met *MAL6* locus.

3.2 *AGT1* gene of lager strains encodes a non-functional transporter (Paper I, III)

The *AGT1* gene of all lager strains studied was found to possess an extra nucleotide at position 1183 leading to the frame shift mutation and formation of truncated polypeptide of 394 amino acids instead of the full length 616 amino acid protein (I). It was observed that this truncated polypeptide was unable to transport

maltose (III). It was also shown that *AGT1*, possessing the frame shift mutation, was characteristic for lager strains as it was found in all nine studied lager strains but in none of the ale, baker's or distiller's strains studied (III, Table 1).

The *AGT1* genes deriving from ale strains, A60 and A179, were observed to encode a full-length maltose transporter that was functional, *i.e.* Agt1 transporters of ale strains were observed to be high affinity maltose transporters strongly inhibited by maltotriose (85% and 79% inhibition in A60 and A179, respectively) and trehalose (94% and 85% inhibition in A60 and A179, respectively) (Paper III) as expected for a broad specificity Agt1 transporter.

3.3 Presence of *MPHx*, *MTT1* and *SbAGT1*genes (Paper I, III)

The presence of *MPHx* genes was studied similarly by hybridizing chromosome blots with the *MPHx* probe. It was found that the *MPH3* gene was missing in all the brewer's yeast strains but was present in the laboratory strains (I, Table 3; I, Fig. 5). In accordance, the *MPH3* gene was also found to be missing in the brewer's yeast strains studied by Jespersen *et al*. (1999) and Meneses and Jiranek (2002). The *MPH2* gene was observed to be differently distributed among the ale and lager strains. It was present in most of the lager strains but usually missing from the ale strains. In the A15 strain the *MPHx* probe hybridized to Chromosome VII/XV duplet instead of the known localizations for the *MPH2* and *MPH3* genes on chromosomes IV and X, respectively. Also Jespersen *et al*. found hybridization of the *MPHx* probe to the VII/XV duplet region in some of the lager strains. This suggests that, in addition to previously identified *MPH2* and *MPH3* loci, there is another *MPHx* locus present in the lager strain genome either on chromosome VII or XV.

At the time our chromosome blot hybridization studies were performed (I) *MTT1* gene had not yet been identified (first time reported 2005). The presence of the *MTT1* gene in relevant strains was performed as a separate analysis by PCR later on (III). It was found that *MTT1* was present in all lager strains studied but was missing in the ale strains (III, Table 1). *MTT1* gene was also present in some but not all baker's and distiller's yeasts. Nakao *et al*. (2009) have suggested that the *MTT1* gene is derived from the *S. bayanus* component of the lager yeast strain but since *MTT1* was found also in baker's and distiller's yeasts, which are related to *S. cerevisiae*, that option does not seem plausible.

Presence of the *Sb-AGT1* gene in ale and lager strains was screened for by PCR analysis (III). *Sb-AGT1* was found to be present in all lager strains studied

but missing from ale strains (III, Table 1). Sequencing of the *Sb-AGT1* gene of A15 and A24 lager strains revealed both of them to be 100% identical to *Sb-AGT1* of WS34/70 (Nakao *et al*. 2009) (III). Based on sequence analysis, *Sb-AGT1* seems to encode a full-length Sb-Agt1 protein with 85% identity at amino acid level to Agt1 protein. Substrate specificity or any sugar transport characteristics of this Sb-Agt1 protein haven't, however, yet been characterized. Possibly Sb-Agt1 protein has a role in maltose/maltotriose transport in lager strains but this remains to be elucidated in future work.

3.4 *MAL* and *MPHx* genotypes of laboratory strains (Paper I)

Laboratory strains differed from brewer's yeast strains in that they seem to possess only *MAL1* (*AGT1-MALx2-MALx3*) and *MAL3* loci, except the CEN.PK2-1D strain where the constitutive *MAL2-8c* locus has been integrated to its genome to obtain a mal-positive phenotype (Rodicio and Zimmermann, 1985). In the S288C strain both of the genes encoding MAL-activator, *MAL13* and *MAL33*, are known to be non-functional (Bell *et al*., 2001). This renders the S288C strain unable to utilize maltose, producing a Mal-negative phenotype. Also, other laboratory strains (except CEN.PK2-1D with constitutive *MAL* locus) of this study were observed to be Mal-negative. It is possible that possession of defective activators in *MAL1* and *MAL3* loci is a common characteristic for laboratory strains. The *MPH3* gene was found to be present in the genomes of all laboratory strains. There was also very weak hybridization to chromosome IV where the *MPH2* locus maps with the *MPHx* probe.

3.5 More prevalent α-glucoside transporter genotypes for ale and lager strains (Paper I, III)

Taking the genotype determination results together, the more prevalent genotype in regard to α-glucoside transporter genes can be represented as *agt1*, *Sb-AGT1*, *MAL11*, *MAL21*, *MAL31*, *MAL41*, *MPH2*, *MTT1* for a lager strain and *AGT1*, *MAL11*, *MAL31* and *MAL41* for an ale strain, respectively. The *AGT1* gene was shown to be non-functional in all studied lager strains and for this it is marked as defective in the genotype description above. It is also possible that some other of these α-glucoside transporter genes encode non-functional proteins. For example, functionality of Mphx transporters has been questioned (Duval *et al*., 2010).

In addition, nothing is known about the functionality of Sb-Agt1 transporter yet. There can also be strain-dependent differences in the functionality of each transporter. In general, lager strains seem to have wider distribution of α-glucoside transporter encoding genes in their genome. This was expected because of the hybrid nature of lager strains, *i.e.* they possess transporters originally derived from both species. Actually, there can even be more α-glucoside transporter genes present in the genomes of lager strains than now detected since, for example, the *S. bayanus* derived *MALx1* genes are not necessarily recognized with probes used, *i.e.* probes designed based on *S. cerevisiae* sequences.

3.6 Expression of α-glucoside transporter genes *AGT1*, *MALx1* and *MPHx* in brewer's yeast strains (Paper I, II)

The ale and lager strains were observed to differ markedly in their expression of *MALx1* and *AGT1* genes in all conditions tested [induced (maltose), repressed (glucose) or in the presence of both inducing and repressing sugars]. *MALx1* genes were strongly expressed in all three lager strains studied, whereas their expression in the ale strains was very weak (I, Fig. 6). In contrast to *MALx1*, the expression of *AGT1* gene was observed to be strong in the ale strains and very weak in the lager strains (I, Fig. 7). In these expression studies it is possible that *Sb-AGT1* expression would not have been detected because of low sequence identity (79%) to the probe. These results are consistent with the study of Meneses and Jiranek (2002), where it was shown that *AGT1* expression in a lager strain was hardly detectable whereas in the five ale strains of the study, strong *AGT1* gene expression was observed. The *MTT1* gene was not included in these studies since it was not yet identified at that time. However, it has been shown that in the brewery fermentations with a lager strain, *MTT1* expression has a very similar pattern to *MALx1* gene expression (Rautio *et al*., 2007). As a conclusion, *MALx1* and possibly also the *MTT1* genes seem to be the most dominantly expressed transporter genes in the lager strains, whereas the *AGT1* gene is dominantly expressed in the ale strains. It is seen that the α-glucoside transporter genes present most often in the genomes of ale and lager strains are not necessarily the most strongly expressed. For example, ale strains generally possess three different *MALx1* genes (*MAL11*, *MAL31* and *MAL41*) and the *AGT1* gene but nonetheless *AGT1* gene expression was observed to be much stronger compared to *MALx1*.

MPHx expression was not detected in any conditions (repressed, induced, or in the presence of both repressing and inducing sugar) in any of the ale or lager strains of the present study (I). This is in contrast to the studies of the Day *et al*., (2002b) who found strong expression of the *MPHx* gene in similar conditions. In the expression studies of Day *et al*., *MPHx* gene was cloned from a lager strain and expressed under its own promoter from a multicopy plasmid in a laboratory strain. Strong expression of *MPH2*, induced by both maltose and maltotriose and repressed by glucose, was observed (Day *et al*., 2002b). Also during wort fermentations with different lager strains strong expression of *MPHx* genes has been observed (James *et al*. 2003; Gibson *et al*., 2008). It is possible that the expression of *MPHx* genes is strain-dependent and in the three lager strains included in the present study it does not occur. Another explanation would be that the *MPHx* probe used in the present study was somehow defective although its sequence as well as radioactive labelling was verified.

Meneses and Jiranek (2002) have shown that there are strain-dependent differences in the degree of glucose repression of the *AGT1* and *MALx1* genes. They observed that in ale strains able to constitutively utilize maltose, the *AGT1* (or *MALx1*) genes were expressed constitutively and seemed not to be under glucose repression. In the present study, strong glucose repression of the *MALx1* and *AGT1* genes as well as strong induction by maltose was observed in all strains (I, Fig. 6; I, Fig. 7). In shake flask cultivations glucose repression was still present when glucose concentration was 5 g/l and only at a glucose concentration below 2 g/l was strong expression of *MALx1* and *AGT1* genes detected, even if the inducing sugar maltose was present at a concentration of ~10 g/l in the cultivation where both inducing and repressing sugars were present simultaneously (I, Fig. 6, sugar concentrations not shown). In shake flask cultivations the starting concentration for glucose was 20g/l and glucose was the sole fermentable sugar present, except in the mixed sugar cultivation where the inducing and repressing sugars, maltose and glucose, were both present at starting concentrations of 10 g/l. In brewery fermentations sugar concentrations are higher, for example, in 24°P wort used in studies described in paper II, glucose concentration was 24 g/l and concentration of total fermentable sugars as high as 180 g/l. Expression studies performed with samples collected during the main fermentation of VHG wort (Rautio and Londesborough, 2003) differed significantly from those obtained from shake flask cultivations. In a lager strain, strong coordinate increase in the expression of *MALx1*, *MALx2* and *MTT1* was observed when there was still a high concentration of glucose present (>20 g/l). Similar strong

expression of *MALx1* was observed also in the A15 strain when glucose concentration was still correspondingly high (Rautio and Londesborough, 2003). One explanation for this significant difference could be that maltose concentration is much higher in the VHG wort (24°P), 80–120 g/l, compared to the maltose concentration in shake flask cultivations (10 g/l). Thus, the inducing effect caused by maltose could be stronger in wort fermentations and possibly somehow overtake the glucose repression. In addition, there is also a significant amount of maltotriose present in VHG wort (20–40 g/l). Maltotriose is suggested to be a better inducer of maltose/maltotriose transporters than maltose (Salema-Oom *et al*., 2005; Dietvorst *et al*., 2005).

3.7 Effect of amino acid changes in the Agt1 sequence on maltose and maltotriose uptake (Paper I)

It has been shown that even a single change in the amino acid sequence of Agt1p can have significant effects on maltose and maltotriose transport ability. Smit *et al*. (2008) have reported two specific amino acid changes I505T and T557S that render the Agt1 transporter in *S. cerevisiae* unable to carry maltotriose. Whereas we observed in our studies that L117P change in the Agt1 sequence rendered the Agt1 transporter unable to carry maltose (unpublished results). *AGT1* genes were sequenced from A15, A24, A60 and A179 strains (I). There were several amino acid changes detected when translated sequences were compared to Agt1p of SGD data bank (I, Table 6). Altogether there were 33 amino acid changes between the SGD Agt1 sequence and the brewer's yeast sequences, 13 common to all of the brewer's yeast strains, 5 specific for the lager strains, and 15 specific to one or both ale strains. Agt1 tranporters of lager strains are known to be non-functional due frame-shift mutation but in ale strains these sequence changes most probably lead to at least slight differences in the characteristics of maltose and/or maltotriose uptake between these transporters. Interestingly, some of the amino acid changes described by Smit *et al*. (2008) were found in the ale strains of the present study. One of them was V548A change (referred to as V549A in Smit *et al*. (2008) because they use different alignment where Mal31p sequence is also included), which was found in the ale strain A60 of the present study (I, Table 6). According to Smit *et al*. (2008) V549A amino acid change decreased maltotriose transport capacity 3-fold. The second common change was T557S change (referred in our study as T556S, see above), which is reported to lead to the complete inability to carry maltotriose (Smit *et al*., 2008). The A179 ale

strain was observed to possess this change. The third change described by Smit *et al*. (2008), *i.e*. I505T change that as well renders Agt1 transporter unable to carry maltotriose was not found in the strains of the present study.

Although the changes described by Smit *et al*. (2008) change significantly maltotriose transport, ability of Agt1 transporter to uptake maltose, was observed to be not affected. It was actually shown that maltose transport was even more efficient by Agt1 transporter with T557S change than with the ordinary Agt1p (Smit *et al*., 2008). One of the amino acid changes that decrease or even completely abolish maltotriose transport ability of Agt1p were present in both ale strains of the present study. These strains are not known to be closer relatives to each other than ale strains are in general. If possession of such changes is characteristic of ale strains, it could, at least partly, explain the slower utilization rate of maltotriose observed in ale strain fermentations compared to those performed with lager strains (Zheng *et al*., 1994a). Ale strains are not known to possess other transporters able to carry maltotriose since they do not possess Mtt1 transporters and only rarely possess Mphx transporters.

3.8 Maltose and maltotriose uptake kinetics (Paper I)

Competitive inhibition studies of maltose uptake can be used to deduce the transporter types that are actually present in the plasma membranes of yeast cells. Trehalose is known to be a substrate for Agt1 and Mtt1 transporters but not for Mphx and Malx1 transporters (Han *et al*., 1995; Day *et al*., 2002a; Salema-Oom *et al*., 2005) and trehalose is therefore expected to inhibit competitively maltose transport by the Agt1 and Mtt1 transporters but not affect transport by the Malx1 and Mphx transporters. After growth on maltose, strong inhibition of maltose transport by trehalose for both ale strains, 82% for A60 and 88% for A179, was observed (I, Fig. 1). Lager strains showed more moderate inhibition in their maltose transport capability by trehalose, 30% for A24 strain and 55% for A64 strain, whereas maltose transport in A15 strain was not affected (I, Fig. 1).

Corresponding results were obtained when maltose uptake inhibition was performed with maltotriose and α-methylglucoside. Strong inhibition of maltose uptake was observed in the ale strains (average inhibition 59–66% with maltotriose and 41–74% with α-methylglucoside) whereas only moderate inhibition in the lager strains (12–24% with maltotriose and 10–23% with α-methylglucoside) was observed (I, Table 1).

Taken together, these kinetic results suggest that when these brewer's yeast strains are grown on maltose, high specificity maltose transporters, such as Malx1p, account for 0–15% of the maltose transport capacity of ale strains but for 40–80% of the maltose transport capacity of lager strains. The inhibition studies are in accordance with expression results of *AGT1* and *MALx1* genes in these strains. Both expression and inhibition studies suggest that maltose transport by the two studied ale strains was mainly mediated by broad specificity α-glucoside transporters that can carry also trehalose, maltotriose and α-methylglucosides, whereas transport by the three lager strains involved mainly Malx1 type transporters.

3.9 Improved fermentation performance of lager yeast strain after repair and 'constitutive' expression of its *AGT1* gene (Paper II, IV)

It has been earlier shown that fermentation performance of brewer's yeast can be enhanced by constitutively over-expressing *MAL61* maltose transporter gene in the brewer's yeast cells (Kodama *et al*., 1995). Significantly increased maltose uptake rates were observed with the *MAL61* over-expression strain and as significant as 30% reduction in the total fermentation time could be obtained in VHG wort (24°P) fermentation performed with the strain constructed. It was also observed that *MAL61* over-expression did not have effect on the glucose or maltotriose utilization pattern, which remained the same between the host strain and the modified strain. This result once more supports the prevailing view that maltotriose is not carried by *MALx1* transporters. Also, a baker's yeast strain over-expressing *AGT1* gene has been constructed (Stambuk *et al*., 2006). This over-expression strain carried maltotriose three-fold faster compared to the parent strain. Also, uptake of other α-glucosides, trehalose and sucrose, was increased by approximately three-fold.

In both of these over-expression strains described, the *MAL61* and *AGT1* genes are expressed from a multicopy plasmid where a gene conferring G418 antibiotic resistance for selection is present. Thus, constant presence of antibiotic in growth media is necessary to prevent the loss of the plasmids. For industrial-scale fermentations, maintenance of such selection is not desirable and not even economically profitable. Moreover, as the finished product, packaged beer, can possibly contain some residual yeast, use of antibiotic marker genes is not suitable. In addition, beer would contain antibiotics, which is not acceptable. For

this, another strategy, better suited for modification of food microbes, was used, *i.e*. stable integration of a cassette containing the desired gene and promoter to the genome of the host strain. Antibiotic resistance conferring plasmids was temporarily used to facilitate the screening phase of the transformants. During the co-transformation event, yeast tend to take inside both the integrating fragment and the selection plasmid. Selection plasmid is later removed from the cells when cultivated in the absence of selection (Blomqvist *et al*., 1991). As a result, stable integrant strains are obtained, which are not dependent on any additional agent for their stability. It has been earlier shown that brewer's yeast can be engineered this way and stable brewer's yeast strains have been obtained by using this method (Blomqvist *et al*., 1991; Liu *et al*., 2004).

3.9.1 Construction of integrant strain with repaired *AGT1* gene under the control of *PGK1* promoter

The approach of the present study to intensify the lager strain fermentation capacity was to repair the defective *AGT1* gene of the lager strain and at the same time put the *AGT1* gene under the control of the constitutive *PGK1* promoter. The latter was to avoid the glucose repression directed towards α-glucoside transporters at the early phases of the fermentation. Since brewer's yeast is a food microbe, it is important and desired that it does not contain any heterologous DNA. For this, an integration cassette was constructed in such a way that it contained only *S. cerevisiae* (ale) or *S. pastorianus* (lager) DNA. Structure of the integration cassette is shown in paper II, Figure 1. Lager strain A15 was co-transformed with integration cassette and antibiotic G418 resistance conferring plasmid pKX34. Homologous recombination, which occurs in *S. cerevisiae* spontaneously (Szostak *et al*., 1983) was expected to take place at the *AGT1* promoter and in the *AGT1* gene region, *i.e*. at both ends of the integration cassette leading to the integration of the cassette to the *AGT1* locus.

Selection of transformants pre-grown on glucose was performed on G418 plates by screening the ability of clones to grow on maltotriose when respiration was blocked with antimycin A (Dietvorst *et al*., 2005). It has been observed that there is a long lag phase before yeast pre-grown on glucose adapts to fermentative growth on maltotriose (Londesborough, 2001). Transformants containing a constitutively expressed maltotriose transporter gene were expected to start fermentative growth on maltotriose earlier. Colonies appearing first on the selection plates (II, Table 2) were collected and analysed by Southern blot analysis. This

analysis revealed that all the first appearing 36 colonies were transformants. Thus, the selection strategy was very efficient. Secondly, it was observed that there were two types of transformants. Most of the transformants (80%) contained both an endogenous *AGT1* gene and a transformed *AGT1* gene with *PGK1* promoter. The rest of the transformants contained only the transformed *AGT1* with *PGK1* promoter but not the endogenous *AGT1* (II, Fig. 2). This suggested that there must be at least two loci where *AGT1* resides in the genome of the lager strain.

PCR analyses confirmed that the integration had occurred as planned and as a result a chimeric *AGT1* gene was produced having sequence from nucleotide 1 up to 1478 from the A60 lager strain and the rest is the original A15 sequence previously present in the locus. Several transformants were sequenced at the corresponding region to verify that the second cross-over had occurred 3' to the frameshift in the A15 sequence. The actual cross-over place was observed to vary between different transformants but in most cases the crossover occurred 3' to the frame shift. In Integrant 1 there were two versions of the cross-over site. One cross-over site was found 5' to the frame shift and another 3' to the frame shift. This implies that there must actually be more than two *AGT1* loci present in the lager strain genome, *i.e*. at least three *AGT1* loci since there were two versions of the integrated *AGT1* gene and in addition also the native *AGT1* gene found in Integrant 1. Tandem integration was observed not to have occurred in any of the integrants studied as verified by Southern hybridization (II, Fig. 2). The resultant chimeric *AGT1* gene encodes Agt1 protein with up to 492 amino acids deriving from A60 lager strains and the rest from A15 strain. In this way the changed amino acid (V548A) of the ale strain A60, which has been reported to lead to lowered maltotriose transport velocity (Smit *et al*., 2008), is excluded from the resultant protein.

After the screening, the plasmid pKX34 was removed from the transformed strains. Removal of the plasmid was verified by Southern blot analysis with plasmid sequences. As a result, integrant strains, which contain only *S. pastorianus* and *S. cerevisiae* own DNA were obtained. Transformed strains were observed to be genetically stable, *i.e.* when they were grown in non-selective conditions over the several successive (more than 100) generations they still contained changed phenotypes (II, Table 4).

3.9.2 Characterization of the integrant strains

Integrant strains showed increased expression levels of the *AGT1* gene and the pattern of expression was rather similar when strains were grown in any of the three sugars maltose, glucose or maltotriose (II, Fig. 3), *i.e.* maltose and maltotriose were, in addition to glucose, good inducers of the *PGK1* promoter. Conversely, the endogenous *AGT1* gene was observed to be expressed at a very weak but constant level in the host strain when grown in any of the three sugars (II, Fig. 3). It was also observed that integration of the cassette DNA did not alter the expression of the endogenous maltose transporter genes (*MTT1* and *MALx1*) or the maltase gene (*MALx2*) (II, Fig. 3). The *AGT1* gene was expressed more strongly in the stationary phase cells than in the exponential growth phase in shake flask cultivations (II, Fig. 3). However, during wort fermentations expression of *AGT1* in the integrant strains was observed to be strongest during the first three days of fermentation and after that remained at a low level until the end of the fermentation (Fig. 5).

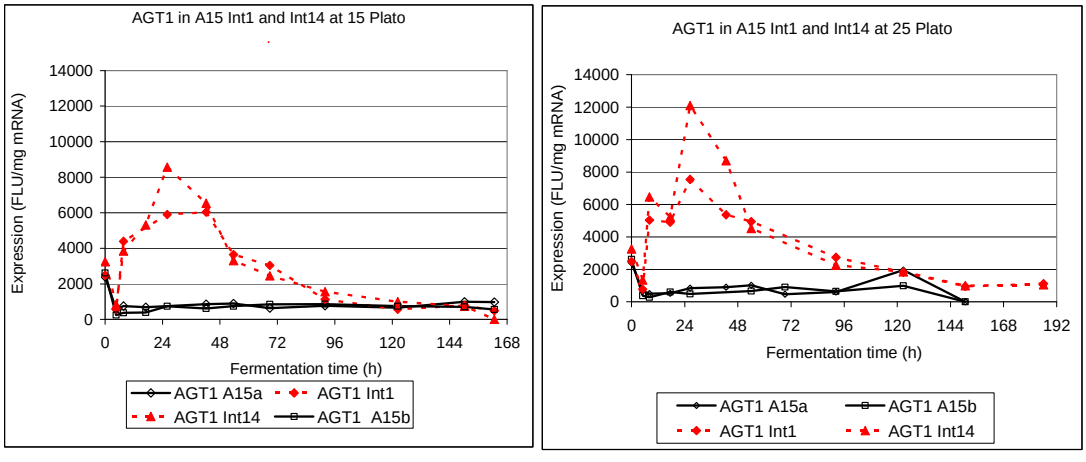

Figure 5. *AGT1* expression levels during the main fermentations with Integrant and host strains.

Maltose transport activity was observed to be higher in the integrant strains and this effect was clearly more pronounced when cells were grown on glucose, which represses the endogenous α-glucoside transporter genes and induces the *AGT1* under the control of *PGK1* promoter. After growth on glucose, an increase in maltose transport capacity (up to 6-fold) was observed in all the integrant

strains compared to the host strain (II). After growth on maltose, which induces the endogenous α-glucoside transporter genes, increase in the maltose transport capacity was smaller (10–20% compared to the A15 strain). Similarly, during the tall-tube fermentations (described in more detail in the next chapter) it was observed that when glucose concentration was high integrants had significantly better maltose transport capacity compared to the host, whereas after glucose had been used up and only maltose and maltotriose were present, a slight increase in the maltose transport capacity was observed. This could be due to limited capacity to functionally express membrane proteins in yeast cell membranes. When grown on maltose, endogenous maltose/maltotriose transporter genes are strongly expressed and this possibly limits the amount of Agt1 transporters that can be present on the plasma membrane.

Maltotriose uptake capability was observed to be increased 1.5–2.5-fold compared to the host strain when maltose was present and induction of the endogenous maltose/maltotriose transporters took place. This suggests that there is a certain amount of Agt1 transporters present in the plasma membrane when grown on maltose. Possibly Agt1- and Malx1-type transporters compete with each other for the yeast cell's capacity to functionally express them in the plasma membrane.

As a conclusion, maltotriose transport and utilization is observed to be intensified during the whole main fermentation process whereas maltose uptake is intensified mostly at the beginning of the fermentation when the glucose concentration is high.

3.9.3 Tall-tube fermentations with the integrant strains

Fermentation performance of some of the integrant strains and the host strain A15 were tested in static tall-tube fermentors that imitate industrial cylindroconical fermentation tanks. Fermentations were performed both in VHG (24°P) and HG wort (15°P). VHG wort was prepared by adding high-maltose syrup, which has been shown to result in more balanced fermentations than addition of, for example, glucose-rich syrup to the wort (Piddocke *et al.*, 2009). HG wort was prepared from the resultant VHG wort by diluting with water. A temperature of 14°C was used in HG fermentations but for VHG fermentations temperature was raised to 18°C to increase the efficiency of fermentation as a positive effect of temperature rise on high-gravity fermentation performance has been shown (Dragone *et al.*, 2003).

All the integrants fermented both 15°P wort and 24°P wort faster and more completely than did the parent, A15, lager strain (II, Fig. 4). The apparent attenuation limits of these worts were close to 86%. Brewers would like to reach an apparent attenuation as close as possible to this limit, but in practise some fermentable sugars always remain in the final beer. Time savings were estimated as the difference between the time required for host strain A15 to reach its highest apparent attenuation (AA) level (80–83% AA were reached in these experiments) compared to the time needed for the integrants to reach the same AA level.

For the 15°P wort, the differences in final apparent extract were small and increases in ethanol yield were close to experimental error (II, Table 5). Time savings were also small (2 to 8 h) at an apparent attenuation of 80% but more significant (13 to 32 h) at 83% apparent attenuation. The 13–32 hours time savings mean 8–20% decrease in the total fermentation time.

In the 24°P fermentations larger differences between integrants and host strain were observed. Differences in the final apparent extracts were 0.25 to 0.53°P whereas in HG fermentations corresponding values were 0.14 to 0.19°P (II, Table 5). Also, 2% more ethanol was produced with the integrant strains compared to the parent strain. Residual maltose and maltotriose in the final beers made by integrant strains were markedly decreased. All three integrants utilized maltose and maltotriose faster than did the parent strain. There were differences between the integrants in the utilization of maltotriose. Integrant 1 was observed to be fastest and it used all the maltotriose present in the VHG wort. In the 24°P fermentations, time savings on reaching the apparent attenuation of 80% were 13–57 hours (fermentation series A) meaning 10–30% decreases in the fermentation time. Greater proportional decreases in fermentation time (14–37%) were observed in the B fermentation series, performed 10 months later. These are significant time savings since the main fermentation, which usually lasts for seven days, could, based on these results, be reduced to only five days. This represents a marked increase in annual output from the same size brewhouse and fermentation facilities.

These results confirm the earlier findings (Kodama *et al*., 1995; Rautio and Londesborough 2003; Alves *et al*., 2007) that the rate of α-glucoside uptake is a major factor limiting the rate of wort fermentations. The extent of the time savings obtained in 24°P wort fermentations with these integrant strains were approximately similar to those observed by Kodama *et al*., (1995) in 24°P fermentations carried out with *MAL61* over-expression strain. Thus, it is seen that introduction of the integration cassette in the genome, where it is present probably

at a maximum of two or three copies, is enough to confer similar time savings as obtained in the strain where the α-glucoside transporter gene is over-expressed from a multicopy plasmid and thus present in several copies. This suggests that probably there are some constraints in the amount of transporter proteins, which can be localized to the yeast cell membrane. Possible reasons for reduced ability to functionally express a transporter are discussed in more detail in chapter 3.15.

When industrial production strains are engineered it is important that only the target properties are changed and other important characteristics of the yeast like, for example, growth characteristics and viability remain unaltered. In the case of brewing yeasts also flocculation and sedimentation properties are important and change in these properties can deteriorate the performance of yeast significantly. In the integrant strains, growth and flocculation/sedimentation properties were observed to be unchanged compared to the host strain (II, Fig. 5). Also, the viability after the propagation and the viability of the yeast collected after the main fermentations (cropped yeast viability) were unaltered in the host and the integrants (II, Table 5). Another important characteristic for each beer type is its flavour. Yeast metabolites, which contribute to beer flavour most include higher alcohols, esters and carbonyls. Higher alcohols, most important of which is 3-methylbutanol, intensify the beer taste and aroma. Esters impart floral and fruity flavours and aromas to beers and are regarded to be among the most important desirable flavour compounds. Low ester concentrations result in an empty and characterless taste. Aldehydes almost without exception have unpleasant flavour (Boulton and Quain, 2001) like, for example, acetaldehyde, which produces an unpleasant “grassy” flavour (Meilgaard, 1975). The most important yeast-derived volatile aroma compounds were analysed in beers collected at the end of the 24°P wort fermentations (II, Fig. 7). The greatest difference between fermentations performed with the integrant and A15 strains was increased acetaldehyde production in main fermentations by Integrant 14 strain. It was observed to produce 2.5-fold as much acetaldehyde as strain A15 and acetaldehyde at this level would be undesirable. All the three integrant strains produced also slightly more ester ethyl acetate (fruity flavour) and integrants 1 and 2 produced more ester 3-methylbutyl acetate (banana-like flavour). These flavours are generally regarded as beneficial and these beers would most probably meet the quality criteria.

3.9.4 Commercial applicability

Integrant strains constructed as described in paper II can be regarded as strains generated by self-cloning. The definition of self-cloning is that it refers to targeted genetic modifications generating an organism which contains DNA exclusively from species closely related to the host organism. That is, any foreign DNA temporarily used as a cloning tool, *e.g.* vector DNA and genetically selectable markers, has been removed (Nevoigt, 2008). For the exact definition of the European Legislature see Council Directive 98/81/EC (http://rod.eionet.europa.eu/instruments/569). After the selection plasmid has been removed only *S. cerevisiae* and *S. pastorianus* DNA remain in the integrant strains constructed. *S. cerevisiae* (ale) and *S. pastorinaus* (lager) strains are regarded as closely related species so that the criteria in the definition of self-cloning are met. There is a patent covering the use of constructs with intact genes in improving the sugar uptake of *Saccharomyces* genus (IPC A21D8/04;C12N1/19;A21D8/02). However, constructs made in the present study do not have interfering intellectual property rights (IPR) in regard to the patent because transforming DNA is not an intact gene and does not itself encode a functional protein.

The integration cassette constructed can be introduced (integrated) to the genomes of other brewer's yeast strains in addition to lager strain A15, as verified by the sequencing of promoter regions of different ale and lager strains. *AGT1* promoters of lager strains A15 and A24 as well as ale strains A60 and A179 were sequenced by chromosome walking (Paper IV). Sequences between ale and lager strains were observed to be very homologous in the whole region up to 1900 bp upstream from the *AGT1* gene (except one 22 bp deletion and two ~95 bp insertions between ale and lager promoters as described in paper IV) and practically identical in regions included in the cassette. Based on this, the same cassette can be used for integration in ale or lager strains. The benefits of constructing corresponding ale integrant strains would be that glucose repression of the *AGT1* gene would be avoided. It was observed that under the control of the *PGK1* promoter, strong expression of *AGT1* was observed already 6 h from the start of the fermentation (Fig. 5). Changed amino acids found in some ale strains decreasing the maltotriose transport ability should however be taken into consideration and the cassette possibly modified to repair these changes.

There are great differences between countries in relation to how GMOs are approved, classified and labelled (Nevoigt, 2008). There is very low acceptance by consumers of GMO in the food and beverage industry, especially within the

European Union (EU). European legislation on GMO is particularly strict. Although many advantageous brewer's, baker's and wine yeasts have been engineered during recent years (Donalies *et al.* 2008; Nevoigt, 2008) and some of them have obtained approval in respective countries for their commercial use (Akada 2002; Husnik *et al*., 2006) none of them has entered larger scale commercial use in Europe. The commercial application of genetically modified micro-organisms has been problematic due to public concerns (Sybesma *et al*., 2006). For example, two recombinant yeast strains received official approval from the British Government for commercial use already in 1990 and 1994. One was baker's yeast that displays a shorter rising time (Aldhous, 1990) and another was brewer's yeast designed for the production of diet beers (Hammond, 1995). However, due to negative public acceptance these strains have not been used on a larger scale. There is also a modified sake yeast strain FAS2-1250S that has received official approval for its industrial use in 2001 by the Japanese Government as a self-cloning yeast so that the yeast does not need to be treated as GM yeast (Akada, 2002). With this strain a higher amount of ethyl caproate, an apple-like flavour compound, was produced in the modified strains (Akada *et al*., 1999). The situation is approximately similar with the wine yeasts. At the moment there is one genetically modified wine yeast worldwide that has been approved for commercial use. This is a modified wine yeast strain where the formation of toxic bioamines, which are detrimental to health, is prevented. It has been approved for commercial use in USA and in Canada (and also Moldova) (Husnik *et al*., 2006).

3.10 Temperature-dependence of maltose uptake in ale and lager strains (Paper III)

Reactions catalysed by integral membrane proteins usually exhibit non-linear Arrhenius plots with increased temperature-dependence at low temperatures. This is usually interpreted as reflecting the change of state of the plasma membrane (freezing of the membrane). As temperature decreases, membranes become increasingly viscous with decreasing membrane fluidity and at some point will undergo a phase change to a gel (rigid) phase and biological function is impaired (Nedwell, 1999). However, it has been shown, at least for yeast cells, that there is no sharp phase transition but rather a continuous change of membrane fluidity when temperature decreases (Beney and Gervais 2001). Sugar transporter molecules are suggested to change shape during each transport cycle

between inward and outward conformations (Abramson *et al.*, 2003). This change of shape requires that the transporter moves against the surrounding lipid, *i.e.* the transporter-lipid-complex moves. When temperature decreases and the plasma membrane becomes more viscous the conformational change occurring between inward and outward conformations is probably more tedious.

Temperature-dependence of maltose transport in an ale strain was shown to follow the typical pattern described for integral membrane proteins (Rautio and Londesborough, 2003). Maltose transport activity decreased four-fold when the temperature was decreased from 20°C to 10°C and even a 20-fold decrease was observed when the temperature was decreased further from 10°C to 0°C. There was practically no maltose transport at 0°C in the ale strain studied (Rautio and Londesborough, 2003). However, Guimarães *et al.* (2006) observed that a lager strain was still able to carry maltose at 0°C but at low velocity. Takahashi *et al.* (1997) observed that when the temperature was decreased from 14°C to 8°C, a larger decrease in the utilization rate of maltose and maltotriose was observed in an ale than in a lager strain. In the ale strain, 10% and 30% decreases in the utilization rate of maltose and maltotriose, respectively, were observed whereas in the lager strain utilization rate of both sugars decreased only by 5%. Interestingly, it was also shown that a temperature decrease from 14°C to 8°C didn't have any effect on glucose utilization in either of the strains. Different maltose (maltotriose) utilization patterns observed between ale and lager strains when temperature decreases could be due to differential temperature-dependence of maltose (maltotriose) uptake in ale and lager strains.

As described in the paper III, four brewer's yeast strains, two ale and two lager strains, were studied for temperature-dependence of maltose transport. Brewer's yeasts were harvested during growth on maltose at 24°C and their maltose transport activities were assayed at a temperature range of 20–0°C. At 20°C all four strains had similar maltose transport activities (III, Fig. 2). However, at 15°C there was already difference observed between ale and lager strains in their temperature-dependence and when temperature was reduced further to 10°C this difference was even more pronounced. Below 10°C the change in the Arrhenius plot was steeper for ale than for lager strains and at 0°C the lager strains had about five-fold greater activity than the ale strains. With temperature decrease, a continuous decrease in maltose transport capacity, even if more pronounced below 10°C, was observed (III, Fig. 2). This is in accordance with observations of Beney and Gervais (2001) that there is no sharp phase transition but rather a

continuous change in the membrane fluidity where transporters loose gradually their activity.

Fermentations with ale strains are usually performed near 20°C and thus these temperature-dependence effects described have only minor significance for ale fermentations. Lager strain fermentations are, however, usually carried out at temperatures between 6°C and 14°C. A decrease in maltose uptake rate observed at this temperature range can have relatively large effects on maltose transport capacity during the main fermentation. For maltose (and maltotriose) uptake it would be beneficial if the process temperature during the lager main fermentation is raised from the conventional temperature.

When temperature decreases also affinity for substrate can be affected. However, when K_m and V_{max} changes in maltose transport were followed in an ale strain in respect to temperature decrease (Rautio and Londesborough, 2003) it was observed that K_m remained approximately the same in all temperatures tested and it was V_{max} which changed dramatically (more than 100-fold) when temperature was decreased. Similar temperature-dependence of V_{max} was observed for a lager strain (Rautio and Londesborough, 2003). Other authors report similar results, for example Nedwell (1999) has shown that low temperature diminishes specific affinity (V_{max}/K_m) for substrate uptake. Most probably velocity of transport decreases because conformational change between inward-facing and outward-facing structures is slower when the membrane becomes more viscous.

3.11 Effect of different dominant maltose/maltotriose transporters of ale and lager strains on the temperature-dependence of maltose transport (Paper III)

It has been shown that there are strain-dependent differences among *Saccharomyces* species in the fluidity of membranes (Torija *et al*., 2003). These differences can be due to adaptation of the organism to its environment. In organisms adapted to low temperature environments there tends to be an increased proportion of unsaturated membrane lipids, and a decreased proportion of branched chain lipids compared to species adapted to moderate or high ranges of temperature (Nedwell, 1999). Ale and lager strains have been used in different process temperatures for hundreds of years. Thus, it is possible that the plasma membranes of ale and lager strains have adapted to the different process temperatures

to the extent that also the lipid composition of their membranes has been modified accordingly.

The effect of possible differences between ale and lager strain plasma membrane composition on the temperature-dependency of maltose transport was studied with Integrant 1 strain (III). In this strain the ale type *AGT1* gene is expressed under the control of the *PGK1* promoter in the lager strain background, *i.e.* present in the lager strain plasma membrane. When Integrant 1 strain was grown on glucose it expressed practically only the pure Agt1-type introduced transporter. It has been observed that the Agt1 transporter is the dominant transporter type in ale strains and that ale strains are more temperature-dependent in their maltose transport. Thus, when ale type Agt1 transporter was functionally expressed in the lager strain plasma membrane in Integrant 1 strain, this strain was expected to be as temperature-dependent as ale strains, assuming temperature-dependence is an intrinsic property of the transporter molecule itself (and not a property of the plasma membrane). Very strong temperature-dependence of maltose uptake for the Integrant 1 strain was observed. Its temperature-dependence was even slightly stronger than those observed for ale strains (III, Fig. 2). This implies that differences in temperature-dependence of maltose uptake are not due to the possible plasma membrane differences between ale and lager strains but are instead intrinsic characteristics of the Agt1 transporter itself. Thus, different temperature-dependences of maltose transport by these ale and lager yeasts seem to result from different maltose transporter types present in these strains.

3.12 Temperature-dependence of maltose transport by Mtt1 and Malx1 transporters (Paper III)

The Agt1 protein seems to be particularly sensitive to the physical state of the surrounding lipid, resulting in very low activity at low temperatures. Temperature-dependence of two other maltose transporters Mtt1 and Malx1 were studied separately, with constructs over-expressing either the *MALx1* (99% identical to *MAL31* in the SGD) or *MTT1* genes under the control of the *PGK1* promoter. *MALx1* transformants exhibited strong temperature-dependence. The rates of maltose uptake at 0°C were approximately 2–3% of those observed at 20°C (III, Fig. 3). The Mtt1 transporter instead was observed to exhibit relatively little temperature-dependence. There was even 7.5–9% of the relative activity left when the uptake rates were compared at 0°C and 20°C. Taken together the tem-

perature-dependence of the three transporters was observed to decrease in the order Agt1≥Malx1>Mtt1. These results further confirmed that high temperature-dependence is a property of the transporter protein itself rather than due to an hypothetical difference in the lipid membranes of the strains.

In brewery fermentations when *AGT1* integrant strains are used it must be taken into consideration that the Agt1 transporter is particularly temperature-dependent. In the present study it was observed that the main fermentation temperature of 18°C is enough to confer the positive effect of the integrant strains.

3.13 Effect of energetic status of the yeast cells and glucose stimulation on maltose uptake (Paper III)

Electrochemical proton potential is significantly lowered in starved yeast cells compared to actively metabolizing cells. This is because ATP content and adenylate energy charge (EC) is relatively low in starved cells. When yeast cells growing on fermentable sugar are harvested, washed and suspended in a medium lacking a carbon source, their intracellular adenylate energy charge can decrease. The adenylate energy charge and active transport rates of sugars such as lactose and maltose of such cells can be increased by treatment with glucose for a few minutes immediately before the zero-*trans* sugar uptake assay (Guimarães *et al*., 2008). There might be differences in the energetic status between ale and lager strains and this could explain different temperature-dependences observed. To test this hypothesis, yeast suspensions were treated with glucose immediately before the maltose transport assays. Temperature-dependence of maltose transport was observed to be similarly relieved in both ale and lager stains when glucose stimulation was performed before the uptake rate measurement (III, Fig. 1). Thus, there was a difference between the temperature sensitivities of maltose transport by the lager and ale strains that was not explained by differences in adenylate energy charge.

3.14 Possible reasons for different temperature-dependences between Agt1, Malx1 and Mtt1 transporters (Paper III)

Several possible reasons can be suggested for observed different temperature-dependences between Agt1, Malx1 and Mtt1 transporters. Amino acid sequences

of these transporters differ from each other. In particular, the Agt1 transporter differs from the other two significantly. Agt1p is only 54% identical to Mtt1p and Malx1p, whereas identity between Mtt1p and Malx1p is 90%. Differences in the amino acid sequences can, for example, have effect on α-helix formation leading to different topology of the protein. It is possible that due to different topology, Agt1 and Malx1 transporters exhibit greater shape changes than Mtt1 during the catalytic cycle. Thus, these transporters need to move against the surrounding lipid more and because of this can't work efficiently when the membrane becomes more viscous at low temperatures.

All three transporters, Agt1, Mal31 and Mtt1 are thought to be members of the major facilitator superfamily (MFS) characterized by 12 transmembrane-spanning domains (Nelissen *et al*., 1995; Salema-Oom *et al*., 2005). However, different results have been obtained when the number and location of the transmembrane domains is predicted by different software and computational programs. There are also data that contradict the 12-spanner analysis. For the Agt1 protein, 12 transmembrane domains have usually been suggested (Han *et al*., 1995; Nelissen *et al*., 1995; Smit *et al*., 2008) but in the SGD database only 10 transmembrane domains are predicted for the Agt1p. Also, Malx1 protein has been reported to possess 12 transmembrane domains (Cheng and Michels, 1989; Nelissen *et al*., 1995) but Smit *et al*. (2008) predicted Mal31p would possess 13 transmembrane domains, whereas in the SGD database only 8 transmembrane domains are predicted for this protein. Mtt1p has been suggested to possess 12 transmembrane domains (Salema-Oom *et al*., 2005). It is possible that transmembrane domain number actually differs between these transporters and possibly results in differential temperature-dependence. However, as long as there are no three dimensional models available for these maltose transporters nothing can be confirmed of their transmembrane domain number.

In studies by Han *et al*. (1995) and Salema-Oom *et al*. (2005) (where 12 transmembrane domains for each of these transporters were predicted) it has been shown that amino acid changes between Agt1, Mtt1 and Malx1 proteins are predominantly located in certain transmembrane domains. Transmembrane domains 2, 9 and 11 were found to be the least similar regions between Mtt1 and Mal31 (Salema-Oom *et al*., 1995) as well as between Mal61 and Agt1 transporters (Han *et al*., 1995) (Mal31p and Mal61p have practically identical amino acid sequences). Interactions between certain amino acids of integral membrane proteins and membrane lipids have been shown to be important for stabilization and functionality of membrane proteins. It is possible that these particular trans-

membrane domains, 2, 9 and 11, have specific roles in protein-lipid interactions between the transporter protein and surrounding lipid. Thus, a particular effect on temperature-dependence might be observed when these domains are affected.

Some amino acid residues have been reported to be particularly important in interactions between integral membrane proteins and surrounding lipid. These are amino acids possessing aromatic amphipathic side chains, *i.e.* tryptophan and tyrosine. Another group of important interactions is formed between basic side chains of lysine and arginine and with phosphate groups of lipids (Deol *et al.*, 2004). Amino acid residue analysis was performed on Agt1, Malx1 and Mtt1 proteins (http://www.expasy.ch/tools/protparam.htmlit) and it was observed that in Agt1p there is less of both tryptophan and tyrosine compared to the Mal31 and Mtt1 proteins (6,1% compared to 6,8% and 6,5%, respectively). A similar observation was made when arginine and lysine residue presence was compared between Agt1, Mtt1 and Malx1 proteins (8,4% in Agt1 compared to 9,1% and 9,3% in Mtt1 and Mal31, respectively). This possibly suggests that there are fewer interactions between the Agt1 protein and lipid surrounding it than there is between Malx1 and Mtt1 type proteins and lipids around them. Possibly the less interactions there are between transporter and surrounding lipid the more prone the transporter is to work less well at lower temperatures. Another explanation could be linked to the Agt1 transporter having broadest substrate specificity. It is possible that the central cavity of the Agt1 protein is largest and shape change during the transport cycle is greater than is needed for other transporters with more restricted substrate range.

3.15 Yeast cells have limited capacity to functionally express transporters in their cell membranes (Paper II, III)

Several studies have shown that capacity of the yeast cell to functionally express transporter proteins in the cell membrane is limited (Hopkins *et al.*, 1988; van der Rest *et al.*, 1995b; Opekarová *et al.*, 1993). It was also observed in the present study that the positive effect of over-expressed *AGT1* on maltose/maltotriose uptake is limited. There probably is limitation in the amount of transporters able to localize and/or be functionally active on the plasma membrane. This restriction could be due to several factors. First, the secretory pathway's capacity to deliver the transporters to the plasma membrane may be limited. Secondly, the space in the plasma membrane where transporters are inserted may be lim-

ited. Thirdly, since protein–lipid interactions are important for proper function of transporters, the availability of specific lipids might be the limiting factor.

Because of their hydrophobicity, the membrane proteins are trafficked in a complex with specific lipids within the vesicle membrane (Opekarová *et al*., 1993). Lipid rafts have been shown to function in biosynthetic delivery of proteins to the cell surface. Proteins that are delivered to the plasma membrane in lipid rafts also remain in these rafts after delivery (Hearn *et al*., 2003; Malínská *et al*., 2003; Umebayashi and Nakano, 2003). For this, the presence of an adequate amount of ergosterol and/or sphingolipids (main components of lipid rafts) in the yeast cell is necessary for efficient delivery of transporters through the secretory pathway to the cell surface and stable location there. It has been shown, for example, that Can1p (arginine H^+ symport), Fur4 (uracil permease), Tat2 (high affinity tryptophan permease) and Pma1 (ATPase) delivery is impaired if there is a deficiency in ergosterol and/or sphingolipids in the cell (Hearn *et al*., 2003; Malínská *et al*., 2003; Umebayashi and Nakano, 2003).

In addition to targeting to the plasma membrane, lipid composition has been reported to be important also for determining the correct topological organization of membrane proteins, either during the initial membrane assembly or dynamically after membrane insertion has occurred (Bogdanov *et al*., 2002). Stabilization of the transporters in the membrane after delivery has been shown to be dependent on lipids (Bagnat *et al*., 2000). Moreover, post-assembly reorganization has been observed in the response to lipid environment, *i.e*., transmembrane domains can reorganize in response to the lipid environment (Bogdanov *et al*., 2002). Maltose transporters have been shown to need an adequate amount of ergosterol for their proper function (Guimarães *et al*., 2006). At least catalytic function of maltose transporter molecules in the plasma membrane was shown to be dependent on ergosterol content. Guimarães *et al*. (2006) suggest that ergosterol also possibly has a role in delivery of the maltose transporters to the cell surface.

The capacity of the secretory pathway to deliver transporter proteins to the plasma membrane seems to be mostly affected by the availability of specific lipids, especially ergosterol and sphingolipids. Thus, the secretory pathway may be a limiting factor, particularly at the later phases of fermentations, when there is a deficiency of lipids in the yeast cell. Yeast cells are not able to synthesize unsaturated fatty acids or sterols in anaerobic fermentation conditions after oxygen depletion. During the second half of brewery fermentations, maltose and maltotriose uptake rates are low (II, Table 5). At the late phases of fermentation, in addition to difficulties in delivery of transporters to the surface, also trans-

porters that already exist in the plasma membrane probably work suboptimally because the membranes do not contain sufficient sterols. In particular, the daughter cells formed in the last rounds of cell division may be deficient in sterols because no new sterols can be made (Guimarães, 2008). Thus, during the second half of the fermentation, maltose transport is severely affected. A similar effect is observed also at the early phase of the fermentation if the fresh wort is pitched with sterol-deficient cropped yeast. In this case, yeast cells need time to synthesize lipids needed before the maltose uptake can be initiated.

Precise localization of maltose transporters in the plasma membrane has not been confirmed, *i.e*. whether they are present in lipid rafts. However, since maltose transporters have been shown to be dependent on ergosterol for their efficient function (Guimarães *et al*., 2006), like other transporters localized in the lipid rafts it would seem plausible that they also are located in these microdomains. In addition, H^+ symporters generally seem to be localized to the lipid rafts (Hearn *et al*., 2003; Umebayashi and Nakano, 2003; Malínská *et al*., 2003).

Lipid rafts have been shown to regulate the turnover of transporter proteins in yeast. Grossmann *et al*. (2008) suggest that exclusion of transporter protein from the lipid raft to surrounding membrane leads to endocytosis of the transporter. Thus lipid rafts represent a protective area within the plasma membrane to control turnover of transporters (Grossmann *et al*., 2008). Lipid rafts are small (10–200 nm) microdomains (Pike, 2009) and thus probably constitute only a minor part of the plasma membrane. Space in lipid rafts can be therefore limited. It can be that excess proteins are removed from lipid rafts and exposed to endocytosis. Possibly limited space in the lipid rafts restricts the capacity to functionally express transporter proteins. It is known that maltose transporters are removed from the plasma membrane (by endocytosis) when they are not required, whereas the cytosolic maltase enzyme is not the target for such inactivation (Görts, 1969). Limited space in the plasma membrane could be one possible reason for the phenomenon observed.

3.16 Benefits of non-functional Agt1 transporters for lager strains (Paper III)

Frame shift mutation in the *AGT1* gene leading to the formation of a truncated non-functional protein product is found in all lager strains studied so far. Possibly this change was present already in the original *S. cerevisiae* component forming the lager strain hybrid. Otherwise, frame-shift mutation should have

occurred soon after the hybridization event since it is present in all lager strains studied. This point mutation (that can be regarded as a quite easily reversible mutation) has not been selected against during the evolution of lager strains but instead is nowadays a common feature in these strains. There is probably some reason for preservation of this non-functional form during the evolution. It is possible that "loss" of the Agt1 protein from lager yeast may have been beneficial for lager strains. This might be because the capacity of the yeast cell to functionally express transporters in the plasma membrane is limited (van der Rest *et al.*, 1995b; Hopkins *et al.*, 1988; Opekarová *et al.*, 1993) and thus putative loss of the Agt1 transporters from the plasma membrane would have provided more capacity, for example, for functional expression of Mtt1-type transporters, which have higher activity at low temperatures. Fermentations by lager strains have been traditionally carried out at approximately 10°C and thus strains functionally expressing Mtt1 transporters would have a clear selective advantage in the low temperature conditions where lager yeasts evolved. It is not clear why the apparently defective *AGT1* genes are still present in all tested lager strains. It is possible that although truncated Agt1 cannot transport maltose it has some other important function and the mutated gene has been retained in the genome for this reason. Based on amino acid sequence analysis, truncated Agt1 possess six or seven transmembrane helices (www.ch.embnet.org/software/TMPRED_form .html). However, it has not been shown experimentally whether the truncated Agt1 transporter is able to localize to the plasma membrane.

3.17 Identification of regulatory elements in the *AGT1* promoters of ale and lager strains (Paper IV)

Significant differences in the *AGT1* gene expression levels in ale and lager strains were observed (I). The possibility that *AGT1* genes are differently regulated in these strains due to different structures of their promoters was investigated in study IV. Generally very little is known about the composition of promoters in industrial strains as most of the promoter region studies have been performed with laboratory strains.

AGT1 upstream regions of one ale (A60), two lager (A15 and A24) and one laboratory yeast strain CEN.PK2-1D were sequenced by chromosome walking (IV). It was observed that the upstream sequence of laboratory strain CEN.PK.2-1D was identical to the *AGT1* promoter of S288C strain of *Sccharomyces* Genome Database whereas the upstream sequences of ale and lager strains diverged markedly

from the laboratory strains, *i.e.* the first 315 bp of the *AGT1* upstream sequence (–315 to –1 from the start codon) were highly identical (99%) to the corresponding promoter of the laboratory strains S288C and CEN.PK2-1D, after which the sequences diverged totally from that of the laboratory strains (IV, Fig. 2).

In the *AGT1-MAL12* promoter region of laboratory strains there are three MAL-activator (Levine *et al*., 1992; Sirenko *et al*., 1995) and one Mig1 binding element (Hu *et al*., 1995) present (IV, Fig. 2). This is one Mig1 binding element less than in the conventional *MALx1- MALx2* intergenic region (Levine *et al*., 1992; Sirenko *et al*., 1995; Hu *et al*., 1995) (IV, Fig. 1). Although the first 315 bp upstream from the *AGT1* gene are practically identical between ale, lager and laboratory strains only TATA box is located in this region but not any of the MAL-activator or Mig1 elements (IV, Fig. 2).

The *S. bayanus*-derived *AGT1* upstream sequence of WS34/70 diverged markedly from corresbonding *S. cerevisiae*-derived sequences (only 43% identity) (IV, Table 2). Thus, it is highly unlikely that the primers used in the chromosome walking of the present study would have annealed to the promoter region of the *S. bayanus AGT1* gene. Since lager strains are polyploid (possessing more than one copy of each *S. cerevisiae*- and *S. bayanus*-derived chromosome) (Querol and Bond, 2009) and sister chromosomes probably have diverged during evolution, different forms of *S. cerevisiae*-derived *AGT1*-bearing loci may exist. However, only a single type of *AGT1* promoter sequence was identified in the chromosome walking performed, indicating that there is no such variation between the *S. cerevisiae*-derived sister chromosomes in the *AGT1* loci in the lager strains studied.

To find similar promoter regions for the ale and lager strains in the present study, SGD Fungal Genome Database (http://www.yeastgenome.org/cgi-bin/blast-fungal.pl) and NCBI database were searched using BLASTN limited to different *Saccharomyces* strains. Highly identical *AGT1* upstream sequences were found in three different natural isolates of *S. cerevisiae*; M22, RM11-1a and YPS163 and in *Saccharomyces pastorianus* Weihenstephan 34/70. Less conserved matches found were upstream sequences of the putative *AGT1* genes in *S. paradoxus* NRRL Y-17217 and *S. mikatae* IFO 1815 (IV, Table 2). Multiple sequence alignment of the *AGT1* upstream sequences of the ale and lager of the present study, *S. cerevisiae* strains S288C, RM11-1a, YPS163, and M22, *S. paradoxus* NRRL Y-17217, *S. mikatae* IFO 1815 and *S. pastorianus* WS34/70 strains was constructed (IV, Supplementary Fig. 1). Generally, all promoters showed high levels of similarity in various, rather long, segments but some in-

sertion/deletion changes between the strains were also observed (IV, Table 3). All the upstream sequences included in the analysis contained a TATA-box and several Mig1p and MAL-activator binding sites.

Among the brewer's yeast strains the *AGT1* promoter regions of lager strains A15 and A24 were identical to each other and 97% identical to WS34/70 *AGT1* upstream sequences (*S. cerevisiae*-derived). Moreover promoter regions in ale and lager strains were observed to be otherwise highly identical to each other except for one 22 bp deletion and two 94 and 95 bp insertions in the ale strain compared to the lager strains (IV, Fig. 2). Computational analysis of promoter elements revealed that the promoter regions of ale and lager strains contained several Mig1p and MAL-activator binding sites, *i.e.* ale strain contained seven Mig1p and five MAL-activator binding sites, whereas the corresponding number in the lager strain were two and four (IV, Fig. 2; IV, Supplementary Fig. 1). The extra MAL-activator site in the ale strain promoter may explain the stronger expression of *AGT1* observed in the ale strains compared to the lager strains.

A remarkably long distance between both the MAL-activator and Mig1 binding elements and the *AGT1* coding sequence was observed. The most proximal MAL-activator site was found to be located approximately 830 bps from the start codon of the *AGT1* gene and the distance observed between the Mig1 binding sites and the *AGT1* start codon was approximately 1100 bp. However, the *AGT1* expression was observed to be strongly repressed in these strains when glucose was present (I). This suggests that the Mig1 transcription factors binding to the promoter region of *AGT1* at a considerable distance are functioning as repressors. Furthermore, the earlier studies revealed strong maltose induction of *AGT1* expression in the ale strains (I), indicating that MAL-activators, located more proximal to *AGT1* gene than the Mig1 elements, are also functional. However, MAL-activator sites located further upstream in the ale and lager *AGT1* promoters (>1500 from the start codon) possibly have only minor contribution to the induction. Because of the extra MAL-activator binding site in the ale strains compared to the two binding sites in the lager strain, the promoter of the ale strain has in the 1500 bp promoter region proximal to the *AGT1* gene the same number (three) of MAL-activator binding sites as the well-studied promoter of *AGT1* in the laboratory strain S288C. It is possible that for the strong expression of *AGT1* all three MAL-activator elements are necessary.

Another explanation for the stronger expression of the *AGT1* gene in the ale strain may be linked to telomeric silencing. The role of chromatin remodelling in the regulation of expression of *MAL* genes has been reported (Houghton-Larsen

and Brandt, 2006; Dietvorst and Brandt, 2008). The *MAL* genes were found to be strongly silenced in some strains, whereas in other strains the non-functional COMPASS complex did not cause any changes in the expression of *MAL* genes (Houghton-Larsen and Brandt, 2006). It has also been observed that in a single strain some chromosome ends are more prone to the telomeric silencing than others (Loney *et al*., 2009). It is possible that the different *MAL* loci located at different chromosome ends are not uniformly regulated by telomeric silencing. The earlier expression studies (I) showed that the *MALx1* genes were strongly but the *AGT1* weakly expressed in the lager strains. This suggests that if telomeric silencing is the reason for the low expression of *AGT1* genes in the lager strains, chromosomes possessing other *MAL* loci, however, are not regulated by telomeric silencing.

3.18 Comparison of *AGT1*-bearing loci in *S. cerevisiae*, *S. paradoxus* and *S. mikatae* (Paper IV)

Among the similar sequences found in gene banks ale and lager strain *AGT1* promoter regions were found to be more similar to corresponding regions in natural isolates of *S. cerevisiae*, *i.e.* strains M22, YPS163 and RM11-1a than to *AGT1* promoter regions of laboratory strains. Ale and lager strain *AGT1* promoter sequences were also more similar to the *S. paradoxus* and *S. mikatae AGT1* upstream regions than those of the laboratory strains. The strong similarity between the *AGT1* upstream sequences of *S. paradoxus*, *S. mikatae*, ale and lager yeasts, and the three natural isolates of *S. cerevisiae* prompted us to compare the structures of the *MAL* loci containing *AGT1* in these strains using sequences from gene banks. RM11-1a strain was chosen as a representative of natural isolates of *S. cerevisiae*. The *AGT1*-containing *MAL* loci from *S. cerevisiae* RM11-1a, *S. paradoxus* NRRL Y-17217 and *S. mikatae* IFO 1815 were found to be much more extensive (IV, Fig. 3) than the classical *MAL* locus, with its single copies of the *MALx1*, *MALx2* and *MALx3* genes. At least 9 putative *MAL* loci genes were spread throughout the 32–34 kbp contig regions from *S. paradoxus* and *S. mikatae*, and there are probably more *MAL* genes beyond the borders of the contigs since there were *MAL* ORFs at the borders. There were also multiple putative *MAL* genes in the 22 kbp RM11-1a contig, at least 7, including one at the border of the contig. The laboratory strain S288C contains a putative isomaltase-encoding gene, *YGR287c* (73% identical to *MALx2*) in its *AGT1* bearing locus in addition to the

AGT1, *MAL12* and *MAL13* genes. *YGR287c* was found also in *S. cerevisiae* RM11-1a and the *S. mikatae* and *S. paradoxus* contigs.

The overall sequences of *S. mikatae* contig 789 and *S. paradoxus* contig 294 *MAL* loci were observed to be very similar to each other. There were even three different putative maltose transporter-encoding genes in the same *MAL* locus in the *S. paradoxus* and *S. mikatae* strains. These genes (*MALx1*, *AGT1* and a gene most similar to *MTT1*), relate to the three major maltose/maltotriose transporters known in *S. cerevisiae* (Malx1p, Agt1p and Mtt1p (Salema-Oom *et al.*, 2005; Alves *et al*., 2007)). Evidently, the *MAL* genes had already been multiplied in the locus of the ancestral strain from which the *Saccharomyces sensu stricto* strains derive. Divergence of *S. cerevisiae* from this ancestor and further divergence of laboratory strains from natural *S. cerevisiae* seems to be accompanied by deletion of some of the repeats. The *AGT1*-containing *MAL* locus of *S. cerevisiae* RM11-1a could have derived from the *S. mikatae* and S. *paradoxus* loci by deletion of the sequence between the first and third *MALx3* ORFs (IV, Fig. 3). This deletion would remove only extra copies of the maltose transporter and MAL-activator genes. The *MAL1* locus of *S. cerevisiae* S288C could be derived by a further deletion, this time of the sequence between *AGT1* and the first *MALx2* seen in the RM11-1a contig (IV, Fig. 3). Sequence data from S288C shows that compared to RM11-1a there is a deletion from 317 bp upstream of the *AGT1* ORF until approximately 450 bp upstream of the *MALx2* ORF.

4. Conclusions

Maltose and maltotriose are the two most abundant sugars of wort. Thus, efficiency of their utlization is an important factor defining the overall fermentation efficiency. It has been earlier shown that sugar transport into the yeast cells is the rate limiting step in their utilization and this was further confirmed in the present sudy. Over the last years, efficient uptake of maltose and maltotriose into the yeast cells has become an important issue because of brewer's tendency to move to a greater extent to the utilization of high and very-high-gravity worts where the sugar concentrations have been increased. Yeast cells are usually not able to utilize efficiently and fast such high amounts of sugars and often there is residual maltose and especially maltotriose left after (very) high-gravity wort fermentations. This lowers the efficiency of the process and also impairs the beer flavour. Improved maltose and maltotriose uptake into the yeast cells would make fermentation process more efficient.

Ale and lager strains were shown to differ markedly in maltose and maltotriose transporter types they functionally express in their plasma membranes. In the lager strains Malx1-type transporters were observed to be the dominant transporter type at 20°C, whereas the relatively high activity of maltose transport by lager strains at low temperatures suggests that the Mtt1 transporters, with low temperature-dependence, dominate at low temperatures. In the ale strains Agt1-type transporters were observed to predominate. A common characteristic for the lager strains was that the *AGT1* gene they possessed was defective. This was because of a frame shift mutations leading to premature stop and formation of truncated non-functional protein product. Thus, maltotriose must be carried in the lager strains either by Mphx, Mtt1 or putative SbAgt1 transporters.

It was shown that sugar utilization efficiency during the main fermentation can be improved significantly by using a modified lager strain where the *AGT1* gene has been repaired and put under the control of a strong *PGK1* promoter not

susceptible to glucose repression. The integrant strains constructed fermented wort faster and more completely compared to the host strain. They also produced beer containing more ethanol and less residual maltose and maltotriose. Importantly there was no change in yeast handling performance, *i.e*. propagation, growth and sedimentation behaviour and with little or no change in the level of yeast-derived volatile aroma compounds. Improvement of fermentation performance was more pronounced in the VHG (24°P) wort fermentations compared to those performed in HG (15°P) wort. Time savings in the running time of the main fermentation were significant with the integrant strains. In 15°P wort fermentations, time to reach the highest apparent attenuation level was decreased by 8–20% with integrant strains compared to the host and in VHG wort fermentations even more significant, 11–37%, time savings were obtained. These are economically significant changes, *i.e*. marked increase in annual output from the same size brew house and fermentor facilities can be obtained. This means in practise that the main fermentation, which usually lasts approximately seven days could be reduced to even five days.

There is also an environmental aspect since beer brewed with these integrant strains can be regarded as more environmentally friendly produced beer. This is because brewhouse fermentor facilities needed are decreased, which leads to a decrease in the energy needed for the process, *i.e*. less facilities to be cooled, cleaned, *etc*.

Maltose transport in the ale strains was observed to be more sensitive to decreasing temperature compared to the lager strains. It was observed that the reason for the difference in temperature-dependence between ale and lager strains was not the hypothetical differences between the ale and lager strains plasma membrane compositions but instead was related to different dominant transporter types present in plasma membranes between ale and lager strains. Temperature-dependence of each transporter was observed to decrease in the order Agt1≥Malx1>Mtt1. Different sensitivity to low temperatures between ale and lager strains is most probably due the different environments in which they have evolved, *i.e*. their utilization at different process temperatures for centuries, which possibly lead to development of different dominant roles for different maltose/maltotriose transporters between ale and lager strains. Lager fermentations are traditionally performed at a temperature range of 6–14°C. In lager strains in the present study maltose uptake was observed to be highly temperature-dependent at this temperature range and significantly lower compared to that at 20°C. Thus, rising the lager fermentation temperature from the traditional

temperature near 20°C would have a positive effect on maltose and maltotriose uptake during the fermentation. The Agt1 transporter was observed to be especially temperature-dependent and thus when the integrant strains constructed are used the process temperature must be particularly considered. It was observed that 18°C was high enough to confer the advantages of integrant strains.

Mutation in the *AGT1* gene, leading to the formation of a non-functional product, seems not to be selected against during evolution of lager strains but instead is found in all lager strains studied. This would suggest that non-functional Agt1 transporters must have some beneficial features for lager strains. It has been earlier shown that yeast cells have limited capacity to functionally express transporters in plasma membrane. It is possible that membrane space or the secretion capacity of transporters to the plasma membrane is limited. Thus, “loss” of Agt1 transporters from lager yeast may have increased capacity for functional expression of other maltose/maltotriose transporters. Mtt1 transporter was found to be lager strain-specific among brewer’s yeast strains as well as the least temperature-dependent of the transporters studied. Lager strains expressing Mtt1 type transporters would have clear selective advantage in the low temperature conditions where lager yeast evolved. Possibly rendering the Agt1 non-functional has released either secretory capacity or plasma membrane space or both for Mtt1 transporters.

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Paper I

Characterization and functional analysis of the *MAL* and *MPH* loci for maltose utilization in some ale and lager yeast strains

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Characterization and Functional Analysis of the *MAL* and *MPH* Loci for Maltose Utilization in Some Ale and Lager Yeast Strains

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Maltose and maltotriose are the major sugars in brewer's wort. Brewer's yeasts contain multiple genes for maltose transporters. It is not known which of these express functional transporters. We correlated maltose transport kinetics with the genotypes of some ale and lager yeasts. Maltose transport by two ale strains was strongly inhibited by other α-glucosides, suggesting the use of broad substrate specificity transporters, such as Agt1p. Maltose transport by three lager strains was weakly inhibited by other α-glucosides, suggesting the use of narrow substrate specificity transporters. Hybridization studies showed that all five strains contained complete *MAL1*, *MAL2*, *MAL3*, and *MAL4* loci, except for one ale strain, which lacked a *MAL2* locus. All five strains also contained both *AGT1* (coding a broad specificity α-glucoside transporter) and *MAL11* alleles. *MPH* genes (maltose permease homologues) were present in the lager but not in the ale strains. During growth on maltose, the lager strains expressed *AGT1* at low levels and *MALx1* genes at high levels, whereas the ale strains expressed *AGT1* at high levels and *MALx1* genes at low levels. *MPHx* expression was negligible in all strains. The *AGT1* sequences from the ale strains encoded full-length (616 amino acid) polypeptides, but those from both sequenced lager strains encoded truncated (394 amino acid) polypeptides that are unlikely to be functional transporters. Thus, despite the apparently similar genotypes of these ale and lager strains revealed by hybridization, maltose is predominantly carried by *AGT1*-encoded transporters in the ale strains and by *MALx1*-encoded transporters in the lager strains.

In all-malt brewer's worts, maltose accounts for ca. 60% of the total fermentable sugars and glucose and maltotriose each account for ca. 20%. Adjunct carbohydrates may be added to worts, particularly in so-called high gravity brewing, and often contain relatively more glucose. The sugars are fermented in the order glucose, maltose and maltotriose. This order results because glucose represses the synthesis of maltose and maltotriose transporters and of the α-glucosidases (maltases) that hydrolyze these sugars inside the cell (see references 9 and 14 and references therein). Glucose also inactivates preexisting maltose transporters (16, 21), a process that also occurs when brewer's yeast strains are added to wort (20). Maltose transport limits the rate of maltose fermentation (10, 15, 20). Significant amounts of maltotriose and maltose can remain in beer, lowering the yield of ethanol and causing flavor problems. Ale and lager yeasts appear to differ in their ability to utilize maltotriose, with lager strains utilizing maltotriose faster so that residual maltotriose is more common in ale fermentations (26). Maltose transport is more strongly inhibited by glucose in some ale strains than in some lager strains (5, 20). Maltotriose, sucrose, and trehalose were much stronger inhibitors of maltose transport by ale strains than by lager strains (20).

The five very similar, unlinked *MAL* loci (*MAL1* to *-4* and *MAL6*) each contain up to three different genes: each *MALx1* (where $x = 1$ to 4 or 6) encodes a maltose transporter, *MALx2*, a maltase, and *MALx3*, a transcriptional activator of the other two genes (1, 3, 4, 10, 25). The *MAL* loci each map to the telomeric region of a different chromosome, *MAL1* to chromosome VII, *MAL2* to chromosome III, *MAL3* to chromosome II, *MAL4* to chromosome XI and *MAL6* to chromosome VIII. Strains of *Saccharomyces cerevisiae* can contain maltose transporters encoded by several different *MALx1* genes, as well as *AGT1*, *MPH2*, and *MPH3* genes (see below). The substrate specificities of the different transporters are not well defined. Problems have included obtaining strains with defined, single transporters and obtaining sufficiently pure radioactively labeled substrates. The ability to ferment and grow on different sugars also has been used to indicate the substrate specificity of the maltose transporters, but the interpretation of these data can be ambiguous.

Chang et al. (2) were the first to obtain a yeast strain in which the maltose transporter was encoded by a single defined gene, *MAL61* (2). This strain can transport and grow on maltose and turanose but not on maltotriose, α-methylglucoside or melezitose, suggesting that the Mal61 transporter is specific for maltose and turanose. The other Malx1 transporters are 97% identical to Mal61 and may have similar substrate specificity.

An allele of *MAL11* ("*MAL1g*") (3) with only 57% identity to *MAL11* was characterized by Han et al. (11) and renamed *AGT1* for α-glucoside transporter. Yeast strains carrying the *AGT1* gene on a plasmid can ferment maltotriose, isomaltose, palatinose, and α-methylglucoside in addition to maltose and turanose, suggesting that Agt1 transporters have a relatively broad specificity, including, importantly, maltotriose. Subsequently, the Agt1 transporter was shown to carry at least maltose, maltotriose, sucrose, trehalose, melezitose, and α-methylglucoside, with trehalose the preferred substrate (23).

The hypothesis that Malx1 transporters are highly specific and unable to carry maltotriose has been challenged (7).

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Strains with *MAL31*, *MAL61*, or *AGT1* as the sole maltose transporter gene grew well on maltotriose and transported radiolabeled maltose and maltotriose with maximum velocities close to 40 nmol min^{-1} mg of dry $yeast^{-1}$ and K_m values between 2.7 and 7.2 mM. The Agt1 transporter had a slightly lower K_m for maltotriose (4.0 mM) than for maltose (5.1 mM), while the Mal31 and Mal61 transporters had lower K_m values for maltose than for maltotriose. These results conflict with earlier findings that strains containing only Mal61 maltose transporter cannot grow on maltotriose (2, 11) and that Mal21p does not support H^+-symport activity with maltotriose (24). Day et al. (7) discuss the possibility that small sequence differences between *MALx1* genes from different strains could alter the substrate specificity of the encoded transporters. A further complication is that use of commercially available [^{14}C]maltotriose without further purification can overestimate the rate of maltotriose transport by more than fourfold (8).

The *S. cerevisiae* genome contains two genes, YDL247w on chromosome IV and YJR160c on chromosome X, that are identical to each other, 75% identical to *MAL61* and 53% identical to *AGT1* and have been named, respectively, *MPH2* and *MPH3* (for maltose permease homologues). When introduced on single-copy plasmids to a strain lacking a functional maltose transporter, these genes confer the ability to grow on maltose, maltotriose, α-methylglucoside, and turanose, and to transport radiolabeled maltose and maltotriose (6). K_m values were 4.4 and 7.2 mM for maltose and maltotriose, respectively, and the maximum rate was higher (49 U mg dry $yeast^{-1}$) for maltotriose than for maltose (39 U mg dry $yeast^{-1}$).

Jespersen et al. (12) mapped maltose transporter genes in 30 brewer's yeast strains by hybridization of specific probes to separated chromosomes. There were few differences between the ale strains compared to lager strains that might explain the different kinetics of maltose transport by ale strains and lager strains. In particular, all five ale strains and 22 of 25 lager strains contained *MAL11*, *MAL31*, *MAL41*, and *AGT1* genes. *MAL21* was present in 13 of 25 lager strains but none of the ale strains and *MPH2* was present in 24 of 25 lager strains but only one ale strain. *MAL61* and *MPH3* were not detected in any strain.

The primary objectives of the present study were (i) to determine why maltose transport is strongly inhibited by other α-glucosides in some brewer's strains, but not in others (20), and (ii) to determine why brewer's strains differ in their ability completely to ferment maltose and maltotriose (26). Competition between maltose and maltotriose for transporters could be partially responsible for their incomplete consumption during fermentations of high-gravity worts. Our results show that in some brewer's yeast strains some of the apparently multiple maltose transporter genes do not code functional transporters. This can account for some marked differences between strains in α-glucoside transport and wort fermentation.

MATERIALS AND METHODS

Materials. D-[U-^{14}C]maltose was from Amersham Biosciences (Espoo, Finland). Maltose for uptake experiments (minimum purity, 99%), maltotriose (minimum purity, 95%) and trehalose were from Sigma-Aldrich (Helsinki, Finland). Maltose for growth media and α-methylglucoside (methyl-α-glucopyranoside) were from Fluka (Helsinki, Finland).

Strains. Four industrial lager strains (A15, A24, A64, and A72) and two industrial ale strains (A60 and A179) from VTT's collection were used as typical representatives of strains in current industrial use. Strains A180 and A181 were isolated as single cell clones from A179 and appeared identical to A179. The laboratory strains were S288C, S150-2B, CEN.PK2-1D (VW-1B), and RH144-3A and the chromosome marker strain, YNN295, from Bio-Rad (Espoo, Finland). To determine the Mal-phenotypes, strains were grown in media with 1% yeast extract—2% Difco (Sparks, MD) Bacto peptone (YP) containing 2% maltose for 2 days at 25°C. All strains reached an optical density at 600 nm (OD_{600}) of 10 to 12, except for the three laboratory strains S288C, S150-2B, and RH144-3A, whose OD_{600} remained below 1.4. These three laboratory strains were defined as Mal-negative.

Maltose transport. Yeasts were grown in 100 ml of YP–4% maltose in 250-ml flasks shaken at 150 rpm and 25°C to an OD_{600} of 6 to 12, corresponding to about 2 to 4 mg of dry yeast/ml. Under these conditions residual maltose was between 2 and 0.5%. The yeast were harvested by centrifugation (10 min, 9,000 × *g*, 0°C), washed with ice-cold water and then with ice-cold 0.1 M tartrate–Tris (pH 4.2), and finally suspended in the same buffer to 200 mg of fresh yeast/ml. Zero-*trans* [^{14}C]maltose uptake rates were immediately determined essentially as described by Lucero et al. (17), and 1 U catalyzes the uptake of 1 μmol of maltose min^{-1} (5 mM maltose, pH 4.2, 20°C).

PFGE. Yeast strains were propagated in YP–2% glucose for 2 days at 30°C and then harvested by centrifugation (3,000 × *g*, 5 min, 4°C). Supernatants were decanted, and cells were resuspended in 10 ml of 4°C 50 mM EDTA (pH 8). Cell concentrations were determined by OD_{600} measurements, and 8×10^6 to 60×10^6 cells were placed in each sample plug. Sample plugs were prepared with the CHEF Genomic DNA Plug Kit for Yeast (Bio-Rad). Cells were centrifuged (3 min, 5,000 × *g*, 4°C) and resuspended in the kit's Cell Suspension Buffer. Lyticase was added to a 150-U/ml final concentration, followed immediately by melted 2% Clean Cut agarose to a final concentration of 0.75%. These mixtures were dispensed into molds and allowed to solidify to produce plugs that could be loaded into the sample wells of the pulsed-field gel electrophoresis (PFGE) apparatus. After the agarose solidified, the sample plugs were pushed out of the molds into the kit's lyticase buffer containing 170 U of lyticase/ml. Plugs were incubated in the lyticase solution for 2 h at 37°C. The lyticase buffer was removed, and the plugs were rinsed with sterile water. Plugs were incubated in the kit's proteinase K reaction buffer containing 240 U of proteinase K/ml for 18 h at 50°C. After that the plugs were washed four times in the kit's washing buffer (1 h per wash) with gentle agitation and stored at 4°C in the same buffer.

Sample plugs were loaded into the wells of a 1.0% UltraPure agarose (Bio-Rad) gel. PFGE was performed at 14°C in 0.5× TBE buffer (89 mM Tris, 89 mM boric acid, 2 mM EDTA [pH 8]). A CHEF Mapper XA Pulsed Field Electrophoresis system (Bio-Rad) was used with the following settings: 6 V/cm in a 120° angle, pulse length increasing linearly from 26 to 228 s, and total running time of 22 h 52 min. A commercial chromosome marker preparation from *S. cerevisiae* strain YNN295 (Bio-Rad) was used for molecular mass calibration. After electrophoresis, the gels were stained with ethidium bromide and scanned and quantified with a Typhoon imager (Amersham Biosciences, Espoo, Finland) to estimate the total amount of DNA in each lane.

Chromosome blotting and hybridization. Chromosomes separated by PFGE were partially depurinized by soaking the gels in 0.25 M HCl for 20 min. After that the gels were treated with 0.5 M NaOH–1.5 M NaCl for 30 min at room temperature and neutralized in 1.5 M NaCl–0.5 M Tris buffer (pH 7.5) for 30 min. The DNA was then transferred to a nylon filter (Hybond-N; Amersham Biosciences) by capillary blotting in 20× SSC (3 M NaCl–0.3 M sodium citrate [pH 7.0]). After blotting, DNA was UV cross-linked to the membrane. Prehybridization was performed at 48°C for 2 h in a hybridization mixture containing 5× SSPE (3 M NaCl–0.2 M sodium phosphate–0.1 M EDTA [pH 7.4]), 5× Denhardt solution, 50% formamide, 100 μg of single-strand salmon sperm DNA, and 1 μg of poly(A) DNA/ml. The hybridization mixture was then replaced with a fresh lot containing a labeled DNA probe at 0.5×10^6 cpm/ml. The probes were labeled with ^{32}P by the random primer protocol using Hexalabel Plus DNA Labeling Kit (Fermentas Life Sciences, Hanover, MD). Unless stated otherwise, hybridization was carried out at 42°C overnight. After hybridization, filters were washed twice with 2× SSC at room temperature for 5 min and then in 0.1× SSC–0.1% sodium dodecyl sulfate (SDS) at 65°C for 40 min. Filters were exposed overnight to a phosphorimager screen, which was then scanned and signals quantified with ImageQuant software (Amersham Biosciences). Hybridization signals were normalized to the total amount of DNA in each gel lane before blotting.

Expression studies. Yeasts were pregrown on YP–2% glucose, inoculated into 50 ml of YP containing 2% maltose, 2% glucose, or 1% maltose–1% glucose to give an initial OD_{600} of 0.5 and incubated with shaking (225 rpm) at 24°C in

250-ml flasks. At intervals, samples were withdrawn and centrifuged (1,800 × *g*, 2 min, 4°C). RNA was isolated from the yeast pellets with TRIzol Reagent (Life Technologies, Carlsbad, CA) according to the manufacturer's instructions. Glucose, maltose, and ethanol in the supernatants were analyzed by high-pressure liquid chromatography, using a Waters 2690 Separation Module (Waters Corp., Milford, MA) and a Waters System Module liquid chromatography coupled to a Waters 2414 differential refractometer and Waters 2487 dual wavelength absorbance detector. The columns were a 100-by-7.8-mm Fast Acid column and a 300-by-7.8-mm HPX87H column from Bio-Rad. The columns were equilibrated and run with 2.5 mM H_2SO_4. Elution was at 0.5 ml/min and 60°C.

RNA was isolated by adding 1 ml of TRIzol reagent and 500 μg glass beads to 30 to 50 μg of yeast cells and homogenizing them with a Bead Beater (Howard Industries, Milford, IL) five times for 35 s. Homogenized samples were incubated for 5 min at room temperature (20 to 25°C). Chloroform (0.2 volumes) was added, and the tubes were shaken vigorously by hand for 15 s, incubated for 2 min at room temperature, and centrifuged (12,000 × *g*, 15 min, 4°C). The aqueous phase was collected, and RNA was precipitated by adding a 0.5 volume of isopropanol, followed by incubation for 10 min at room temperature and then centrifugation (12,000 × *g*, 10 min, 4°C). The RNA pellet was washed once with 75% ethanol and then dissolved in water.

RNA samples were fractionated on formaldehyde-agarose gels and blotted onto nitrocellulose filters by capillary blotting in 20× SSC. Filters were prehybridized for 1 h at 42°C in 15 ml of a hybridization solution containing 50% deionized formamide, 10% dextran sulfate, 1 M NaCl, 1% SDS, and 125 μg of herring sperm DNA/ml. After prehybridization, the probe (see Table 2) was added to 0.5×10^6 cpm/ml, and the incubation continued overnight. Probes were labeled with ^{32}P by using the Hex Label Plus DNA Labeling kit (Fermentas Life Sciences). To correct for variations in loading, SYBR Green II (BMA)-stained gels were scanned with a Typhoon fluorescence imager before blotting and rRNA bands were quantified. Blots were washed once in 5× SSPE for 15 min at 42°C, twice in 1× SSPE–0.1% SDS for 15 min at 42°C and twice in 0.1× SSC–0.1% SDS for 15 min at 42°C. Blots were visualized by mounting them on a phosphorimager screen overnight (unless stated otherwise) and scanned and quantified with a Typhoon phosphorimager and ImageQuant software.

Sequencing of *AGT1* genes. *AGT1* gene sequences of each strain were amplified by PCR with the specific primers *AGT1* frw (5′-ATGAAAAATATCATT TCATTGGT-3′) and *AGT1* rev (5′-TTAACATTTATCAGCTGCATTT-3′) from both ends of the gene. Genomic DNA of four brewer's strains (A15, A24, A60, and A179) was used as the template. The PCR-generated fragments were cloned by using the TOPO TA cloning kit (Invitrogen, Carlsbad, CA) and propagated in *Escherichia coli*. Plasmid DNA was isolated from independent *E. coli* clones and sequenced. Whole gene sequences were obtained with eight sequencing primers; universal M13 Forward and Reverse primers to sequence the start and end of the pCR-TOPO plasmid-ligated *AGT1* gene and six internal primers from the coding strand. In addition to these clones when the whole gene was sequenced, an additional 20 independent clones from each strain were sequenced with only one of the internal sequencing primers (Sekv4, 5′-AAA GCAGATTGAATTGAC-3′). This primer, starting at nucleotide 1011, gave a readable sequence from approximately nucleotides 1150 to 1500, which includes the region where the genes from some strains appeared to have a frame shift. The model 3100 Genetic Analyzer sequencer was used (Applied Biosystems, Foster, CA). Multiple alignments were performed with the Multalin program at http://prodes.toulouse.inra.fr/multalin/multalin.html.

RESULTS

We characterized the maltose uptake kinetics of several brewer's yeast strains and then determined the (apparent) *MAL* genotypes of these strains by hybridization studies. Because brewer's strains are aneuploid and, in the case of lager strains, alloploid (13), we attempted to quantitate the *MALx1* gene doses in these strains. We then examined the expression levels of the *MALx1*, *AGT1*, and *MPHx* genes and compared the *AGT1* gene sequences in ale and lager strains.

Inhibition of maltose transport by trehalose and maltotriose. Trehalose is the preferred substrate of the Agt1 transporter but is not a substrate for the Malx1 and Mphx transporters (6, 23). Therefore, trehalose is expected to inhibit competitively maltose transport by the Agt1 transporters but not necessarily to affect transport by the Malx1 and Mph1 transporters. Maltose transport in both ale strains was strongly inhibited (50% at 10 mM trehalose and over 80% at 100 mM trehalose). For ale strain A60, inhibition leveled off at ~82% (1/V = 5.5 in Fig. 1), suggesting that inhibition is partial, with ~18% of the maltose transport capacity insensitive to trehalose. For ale strain A179, inhibition was 88% (1/V = 8.3) and still rising at 100 mM trehalose, suggesting that a smaller fraction (<10%) of the maltose transport capacity is insensitive to trehalose. All three lager strains were less sensitive to trehalose (Fig. 1). For A24 and A64, inhibition reached 30 and 55% (1/V values of 1.4 and 1.8, respectively) at 100 mM trehalose, whereas maltose transport by lager strain A15 was not affected by up to 100 mM trehalose. This result suggests that maltose-grown A15 does not express maltose transporters capable of carrying trehalose, whereas in A24 and A64 these transporters can account for 30 to 50% of the maltose transport capacity at 5 mM maltose.

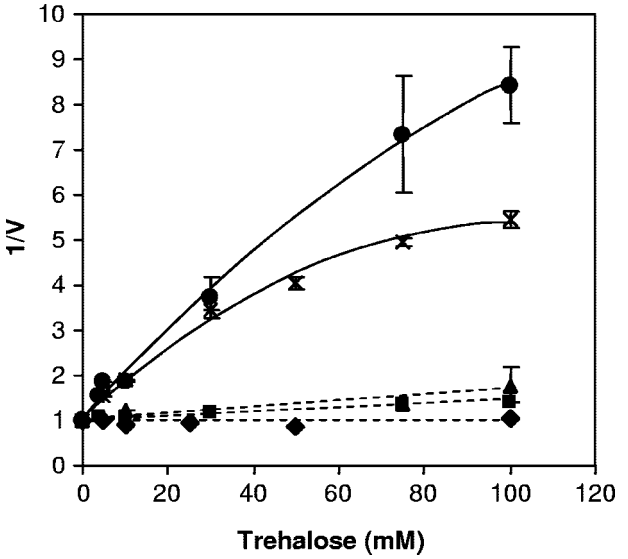

FIG. 1. Inhibition of maltose transport by trehalose. Rates of zero-*trans* uptake of maltose were measured at 5 mM [^{14}C]maltose in the presence of the indicated concentrations of trehalose. For each strain, the rate in the absence of trehalose was set at 1.00. The figure shows the reciprocal rates (1/V) plotted against trehalose concentration for two ale yeasts, A60 (×) and A179 (●) and three lager yeasts: A15 (◆), A24 (■), and A64 (▲). Error bars show the range between two or three independent experiments.

Also maltotriose and α-methylglucoside strongly inhibited maltose transport by both ale strains but only weakly inhibited transport by the lager strains (Table 1). Maltotriose is a substrate of Agt1, Mphx, and Mal31 transporters, and α-methylglucoside is a substrate of Agt1 and Mphx but not of the Mal31 transporter (see reference 6).

These kinetic results suggest that when these brewer's strains are grown on maltose, high-specificity maltose transporters, such as Malx1p, account for 0 to 15% of the maltose transport capacity of ale strains but for 40 to 80% of the maltose transport capacity of lager strains. Correspondingly, low-specificity transporters, such as Agt1p and Mphp, are predicted to be important in the ale strains.

TABLE 1. Inhibition by maltotriose and α-methylglucoside of [^{14}C]maltose transport[a]

Inhibition type	Inhibition with strain:				
	A15	A24	A64	A60	A179
Avg inhibition by maltotriose (%) ± SD	12 ± 7	16 ± 11	24 ± 4	66 ± 3	59 ± 25
Avg inhibition by α-methyglucoside (%) ± range	23 ± 3	10 ± 10	13	74 ± 3	41

[a] Transport of 5 mM [^{14}C]maltose was measured with the indicated strains (grown on maltose) in the presence or absence of 50 mM maltotriose or 170 mM α-methylglucoside. Where errors are shown, results for maltotriose are averages ± standard deviations (n = 3 to 6) and results for α-methylglucoside are averages of duplicates ± ranges.

***MAL* genotypes of the strains.** To test this hypothesis, we first identified which *MAL* loci were present in the strains. At low DNA loads, PFGE resolved 15 chromosome bands from the marker yeast strain, YNN295 (Fig. 2). Chromosomes VII and XV were presumed to comigrate as a duplex. Chromosome IV, faint in Fig. 2, is clearly visible in the brewer's yeasts at higher DNA loads (Fig. 3A). The four laboratory strains all differed from each other and from the marker strain in the size of one or more chromosomes. For the brewer's strains, greater differences appeared, including bands not present in the marker strain. Bands at or near the positions of chromosomes that often carry *MAL* loci—i.e., chromosomes VII, III, II, XI, and VIII—could be tentatively identified, although chromosome III was smaller in the lager strains than in the YNN295 marker strain. Chromosome III could not be identified in the ale strains based on mobility alone. Blots of these PFGE gels were hybridized sequentially with specific probes for the transporter, maltase, and activator genes at *MAL* loci (Table 2). Previously sequenced *MALx1* genes are 97% identical, and the *MAL61* probe is expected to hybridize to all of them. In some *MAL1* loci the transporter gene is the *AGT1* allele instead of *MAL11* (11), and we used a specific *AGT1* probe to detect this allele. *MALx2* genes are 97% identical and the *MAL62* probe is expected to hybridize to all of them. There is greater variation among *MALx3* genes: *MAL13*, *MAL23*, and *MAL43* are 91 to 94% identical to *MAL63*, but *MAL33* and an allele of *MAL13*, found in *MAL1* loci containing *AGT1* and referred to in the present study as *MAL13*(*AGT1*), share only ca. 75% identity to each other and to other *MALx3* sequences. We used specific probes [*MAL33* and *MAL13(AGT1)*] to detect these two genes.

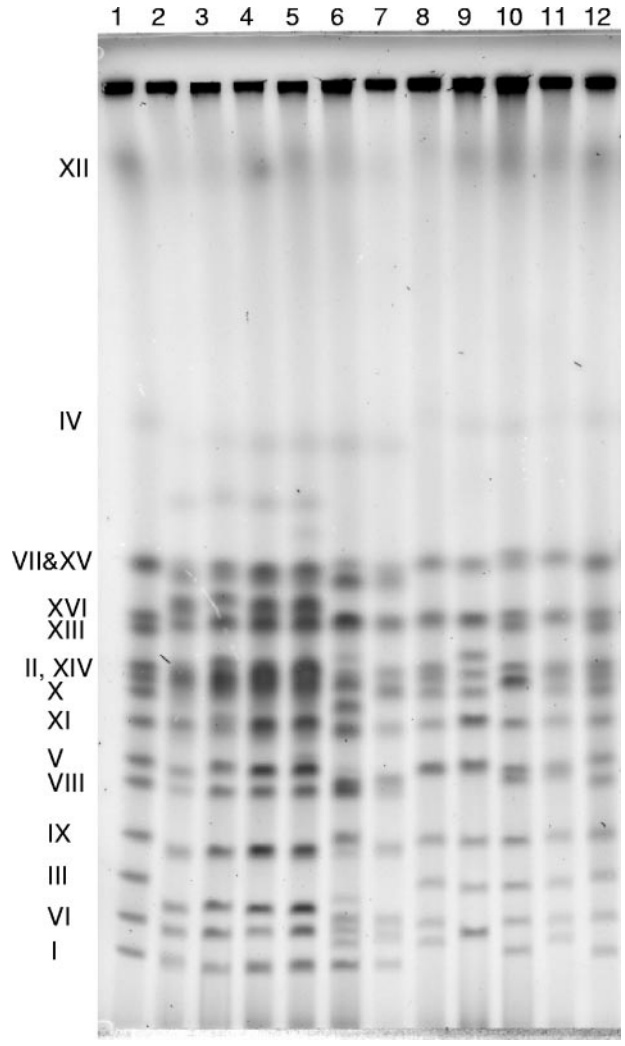

FIG. 2. PFGE separation of chromosomes from laboratory and brewer's strains. The gels were loaded with 8×10^6 cells/lane of the brewer's strains and 30×10^6 cells/lane of the laboratory strains. Strains were as follows: lane 1, chromosome marker strain YNN295; lane 2, A15; lane 3, A24; lane 4, A64; lane 5, A72; lane 6, A60; lane 7, A179; lane 8, S288C; lane 9, S150-2B; lane 10, CEN.PK2-1D; lane 11, RH144-3A; lane 12, chromosome marker strain YNN295. Chromosomes are identified on the left: chromosomes VII and XV are not resolved; chromosome II travels immediately above chromosome XIV.

The *MAL61* and *MAL62* probes hybridized in many lanes at the expected positions of the VII/XV duplex and chromosomes II, XI, and III, which carry, respectively, *MAL1*, *MAL3*, *MAL4*, and *MAL2* loci (Fig. 3D). The *AGT1* and *MAL13(AGT1)* probes hybridized to the VII/XV duplex of all of the strains in the present study, suggesting that they all carried (on chromosome VII) *MAL1* loci containing the *AGT1* allele of the transporter gene and the *MAL13(AGT1)* allele of the activator gene. These probes did not hybridize to any other chromosomes. The *MAL61*, *MAL62*, and *MAL63* probes also bound to the VII/XV duplex of all six brewer's strains (Fig. 3D, E, and F). This pattern suggests that the brewer's strains each contained at least two kinds of chromosome VII, one with a *MAL1* locus containing the *AGT1* and *MAL13*(*AGT1*) alleles and another with a *MAL1* locus containing *MAL11* and *MAL13* alleles. In contrast, *MAL61* and *MAL63* did not bind at this duplex in the laboratory strains (*MAL62* did; Fig. 3E), suggesting that the single copy of chromosome VII in the (haploid) laboratory strains contained only the *AGT1* and *AGT1(MAL13)* alleles.

Three lager strains (A24, A64, and A72), one ale strain (A60) and one laboratory strain (CEN.PK2-1D) contained

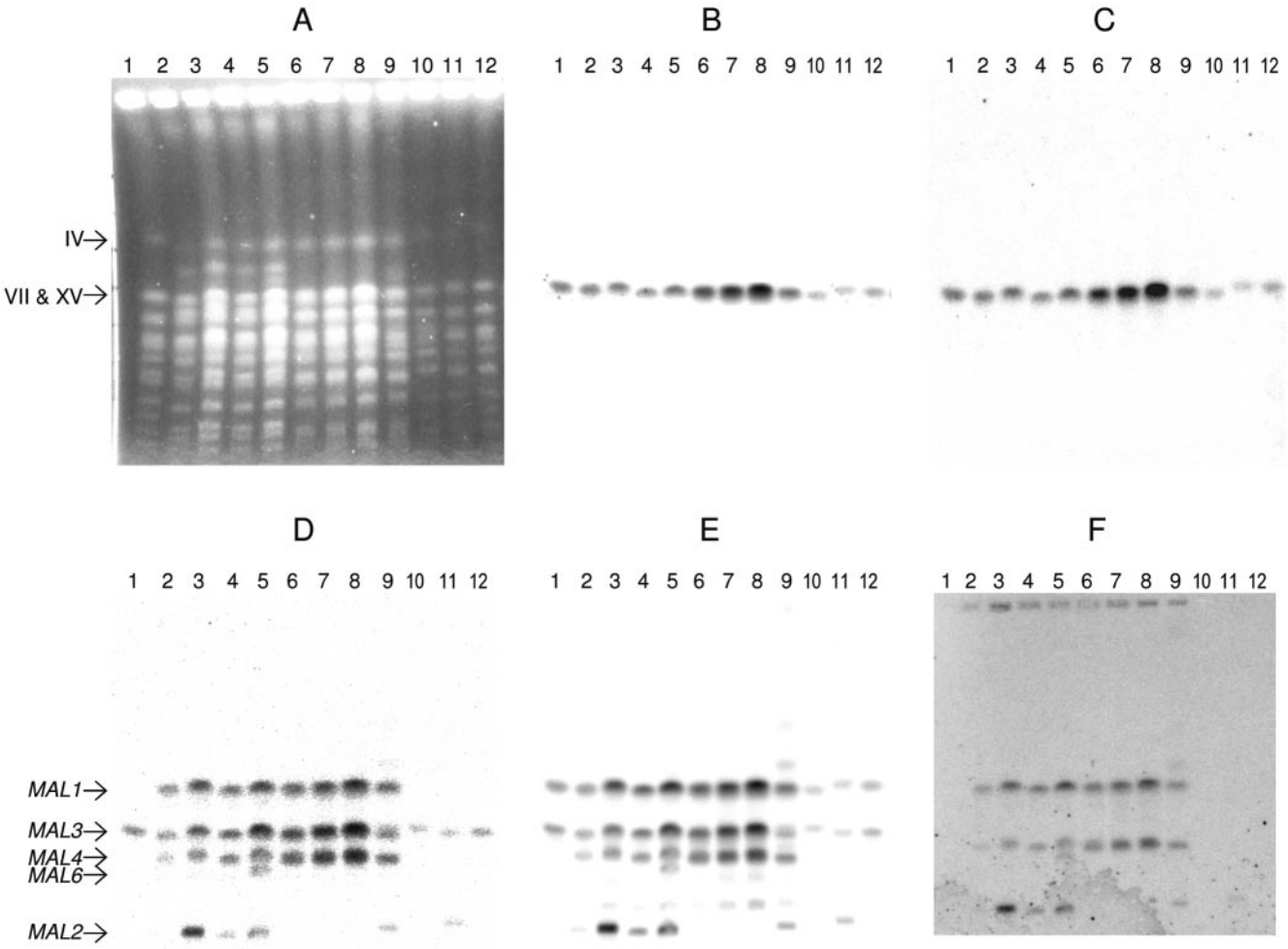

FIG. 3. Detection of *MAL* loci. A PFGE gel was loaded with about 30×10^6 cells/lane. Strains were as follows: lane 1, Marker YNN 295; lane 2, A15; lane 3, A24; lane 4, A64; lane 5 A72; lane 6, A179; lane 7, A180; lane 8, A181; lane 9, A60; lane 10, S150-2B; lane 11, CEN.PK2-1D; and lane 12, RH144-3A. (A) Separated chromosomes stained with ethidium bromide. Chromosome IV and the duplex VII/XV are indicated. The gel was then blotted, and the blot was hybridized with the following probes: *AGT1* (B), *MAL13(AGT1)* (C), *MAL61* (D), *MAL62* (E), and *MAL63* (F). The *MAL* loci are identified on the left of panel D. The image for panel F was darkened to make the bands more visible.

complete *MAL2* loci on chromosome III. Chromosome III is possibly missing from the other ale strain (see A179 in Fig. 2). The CEN.PK2-1D strain is known to have a *MAL2-8^c* locus (22).

The *MAL61* and *MAL62* probes both bound to chromosome II of all strains, suggesting that all strains carry *MAL3* loci with transporter and maltase genes (Fig. 3D and E). As expected, the *MAL63* probe did not bind to chromosome II (Fig. 3F), which is consistent with the low identity (75%) between *MAL63* and *MAL33*. However, the *MAL33* probe bound to chromosome II of all strains (data not shown), indicating that these *MAL3* loci are complete, i.e., they contain transporter, maltase, and activator genes.

In all of the brewer's strains, but none of the laboratory strains, the *MAL61*, *MAL62*, and *MAL63* probes hybridized to chromosome XI, suggesting that the brewer's strains all carried complete *MAL4* loci.

A72 was the only strain where the *MAL61*, *MAL62*, and *MAL63* probes all hybridized to chromosome VIII, which carries the *MAL6* locus. For all of the other strains the hybridization of the *MAL61* probe to chromosome VIII was very faint, and hybridization of *MAL62* and *MAL63* probes could not be detected. None of the 30 brewer's yeasts studied by Jespersen et al. (12) contained a *MAL6* locus.

Weak hybridization was sometimes observed to other chromosomes. For example, with ale strain A60, the *MAL61*, *MAL62* and *MAL63* probes hybridized to a chromosome of ~1.3 Mbp (Fig. 3D, E and F). Binding of a *MAL61* probe to an ~1.3 Mbp chromosome also was observed by Jespersen et al. (12) in a lager yeast. For all brewer's strains, the *MAL62* probe hybridized weakly to chromosome IX (immediately above the *MAL22* bands in Fig. 3E).

Deduced differences in the *MAL* genotypes (Table 3) between the five brewer's strains were few. In particular, ale strain A60 was identical to lager strains A15, A24, and A64 except for the weakly observed *MAL* hybridization to the ~1.3-Mbp chromosome of A60. Ale strain A179, and its derivatives, A180 and A181, were the only brewer's strains lacking a *MAL2* locus. A72 was the only strain with a complete *MAL6* locus. The maltose-negative laboratory strains S150-2B, RH144-3A,

TABLE 2. PCR primers used to make probes

Gene	Direction[a]	Primer sequence	Sequence detected[b]
AGT1[c]	F	5′-TTGCTTTACAATGGATTTGGC-3′	842–1828
	R	5′-CTCGCTGTTTTATGCTTGAGG-3′	
MAL13(AGT1)[d]	F	5′-GACTTTAACTAAGCAAACATGC-3′	3–580
	R	5′-CGTTCGATATTTGTGCAAAGCT-3′	
MAL61[c]	F	5′-GGAGCCTTTCTATGCCCTGC-3′	361–1140
	R	5′-TAATGATGCACCACAGGAGC-3′	
MAL62	F	5′-GCGTTGATGCTATTTGGGTT-3′	155–933
	R	5′-GAAAAATGGCGAGGTACCAA-3′	
MAL63	F	5′-GTATTGCGAAACAGTCTTGC-3′	5–886
	R	5′-CATCGACACAGTTAGTAGCC-3′	
MAL33	F	5′-ATAGCTCCACCTCAGCCAGA-3′	772–1313
	R	5′-TGATTGCAATGTTTCAGGGA-3′	
MPHx[e]	F	5′-TCCGTGGATCTTGTTGGAAA-3′	505–1294
	R	5′-GTCCAAAAGCGTAAAGGTCA-3′	

[a] F, forward; R, reverse.
[b] The numbering is from the first nucleotide of the translational start.
[c] *AGT1* and *MAL61* primers were the same as in reference 12.
[d] Primers were designed with the *Saccharomyces* Genome Database primer design program (http://seq.yeastgenome.org/cgi-bin/web-primer).
[e] *MPHx* primers were the same as in reference 6.

and YNN295 had complete *MAL1* [with *AGT1* and *MAL13 (AGT1)* alleles] and *MAL3* loci. Evidently, their *MAL1* and *MAL3* loci, even collectively, are insufficient for a maltose-positive phenotype. The maltose-positive laboratory strain, CEN.PK2-1D, had a genotype similar to the maltose negative strains plus a complete *MAL2* locus.

Quantitative analysis of *MAL61* and *MAL62* hybridization signals. Brewer's yeasts are aneuploid. The copy number of each chromosome can vary between strains, and the same cell can carry different versions of each chromosome, including those that sometimes carry *MAL* loci. These differences will change the strengths of the hybridization signals. We normalized the intensity of the hybridization signals obtained with *MAL61* and *MAL62* probes at each *MAL* locus relative to the intensities obtained at the *MAL3* locus (Table 4). For each locus of strain A15, the signal strengths normalized in this way were similar for both probes, suggesting that in each *MAL* locus of strain A15 the ratio of *MALx1* and *MALx2* genes is the same as that in its *MAL3* locus. Since the *MAL61* and *MAL62* probes gave the same result, the different signal intensities probably reflect different copy numbers of the *MAL* loci in A15 (*MAL1* > *MAL3* > *MAL4* > *MAL2* > *MAL6*) rather than differences in sequence similarity to the probes.

For the other yeasts (Table 4), the normalized signals at *MAL1* and *MAL2* loci were markedly stronger with the *MAL62* probe than with the *MAL61* probe (see Discussion). Neverthe-

TABLE 3. *MAL* and *MPH* genotypes of the studied strains[a]

Locus	Genotype for strain(s):						
	S150-2B, RH144-3A, YNN295	CEN.PK2-1D	A60	A179, A180, A181	A15	A24, A64	A72
MAL1	*AGT1 MAL12 MAL13(A)*[b]	*AGT1 MAL12 MAL13(A)*[b]	*AGT1 MAL11 MAL12 MAL13 MAL13(A)*[b]	*AGT1 MAL11 MAL12 MAL13 MAL13(A)*[b]	*AGT1 MAL11 MAL12 MAL13 MAL13(A)*[b]	*AGT1 MAL11 MAL12 MAL13 MAL13(A)*[b]	*AGT1 MAL11 MAL12 MAL13 MAL13(A)*[b]
MAL2[c]		*MAL21-23*	*MAL21-23*		*MAL21-23*	*MAL21-23*	*ML21-23*
MAL3[c]	*MAL31-33*	*MAL31-33*	*MAL31-33*	*MAL31-33*	*MAL31-33*	*MAL31-33*	*MAL31-33*
MAL4[c]			*MAL41-43*	*MAL41-43*	*MAL41-43*	*MAL41-43*	*MAL41-43*
MAL6			(*MAL61*)[d]	(*MAL61*)[d]	(*MAL61*)[d]	(*MAL61*)[d]	*MAL61* *MAL62* *MAL63*
Other *MAL* loci	Chr IX[e]	Chr IX[e]	Chr IX[e] 1.3 Mbp[f]	Chr IX[e]	Chr IX[e]	Chr IX[e]	Chr IX[e]
MPHx	(*MPH2*)[d] *MPH3*	(*MPH2*)[d] *MPH3*				*MPH2*	*MPH2*
Other *MPH* loci					Chr VII/XV		

[a] No entry means that the gene(s) was not detected in this yeast.
[b] *MAL13(A)* indicates the *MAL13(AGT1)* allele.
[c] The *MAL2*, *MAL3*, and *MAL4* loci either were completely absent or contained all three genes *MALx1*, *MALx2*, and *MALx3*, shown as, e.g., *MAL21-23*.
[d] Parentheses indicate that the hybridization detected was very weak.
[e] The *MAL62* probe (but not other probes) bound weakly near chromosome IX in all strains, stronger in brewer's strains.
[f] In A60 *MAL61*, *MAL62*, and *MAL63* probes bound weakly to a chromosome of ~1.3 Mbp.

TABLE 4. Hybridization intensities of *MAL61* and *MAL62* probes at other *MAL* loci compared to *MAL3*[a]

Strain	Hybridization intensity of *MAL61* and *MAL62* probes for *MAL* loci 1, 2, 4, and 6							
	MAL1		*MAL2*		*MAL4*		*MAL6*	
	MAL61	*MAL62*	*MAL61*	*MAL62*	*MAL61*	*MAL62*	*MAL61*	*MAL62*
A15	131	146	15	22	54	63	8	0
A24	118	164	133	183	61	60	10	0
A64	87	143	28	50	66	73	8	0
A72	75	115	17	54	35	54	20	18
A60	127	222	35	57	112	138	0	0
A179	80	119	0	0	78	84	0	0

[a] A PFGE gel blot was hybridized sequentially to *MAL61* and *MAL62* probes. Band intensities were normalized by setting the signals for each probe to 100 at the *MAL3* locus.

less, there was qualitative agreement between the two probes at all loci and some strain-specific differences in loci strengths were evident. A24 differed from other strains by having the strongest signal strengths at *MAL2*, suggesting that *MAL2* is the most abundant *MAL* locus in this strain and that in A24 several copies of chromosome III carry a *MAL* locus. The other strains had weak signals at *MAL2*, suggesting that in these strains probably only one (or, for A179, no) copy of chromosome III carries a *MAL* locus. The signal strength at *MAL4* was weakest in A72, suggesting that in this strain fewest copies of chromosome XI carry a *MAL* locus. The lager strains all had more copies of *MAL3* loci than *MAL4* loci, whereas for the ale strains (A60 and A179) hybridization to *MAL3* and *MAL4* were similar.

To confirm that these observations were not experimental artifacts of the particular filter probed, three independent filters were probed with *MAL61* and the relative intensities of hybridization at different *MAL* loci compared (Fig. 4). The hybridization patterns were similar to those of Table 4. In particular, the stronger *MAL21* signal with A24 than with the other yeasts and the stronger *MAL41* signals with ale strains than lager strains were reproducible between gels.

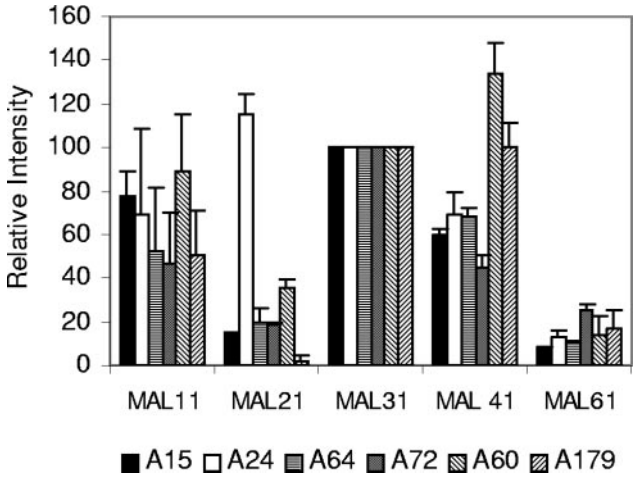

FIG. 4. *MAL61* hybridization band intensities. Blots of three independent PFGE gels with different amounts of DNA (8×10^6 to 30×10^6 cells/lane) were hybridized with the *MAL61* probe. Band intensities were normalized by setting *MAL31* on each blot to 100. The bars represent the average relative intensity of each *MALx1*, and the error bars represent the standard deviations.

***MPH* genotypes.** PFGE blots also were hybridized with the *MPHx* probe, which recognizes the *MPH2* and *MPH3* sequences found on chromosomes IV and X. The *MPHx* probe did not bind strongly, and long exposure times were needed to identify the hybridization sites (Fig. 5). Laboratory strains S150-2B, CEN.PK-1D, and RH144-3A (and also YNN295, not shown in Fig. 5) hybridized the *MPHx* probe to chromosome X and, rather weakly, to chromosome IV. The much weaker hybridization to chromosome IV observed with these haploid strains may indicate that the *MPH2* genes, located on chromosome IV, have low sequence similarity to the probe. However, chromosome IV stained only weakly with ethidium bromide in our PFGE gels (Fig. 2 and 3A) and the *MPHx* probe also hybridized to the sample slot (Fig. 5). Thus, chromosome IV

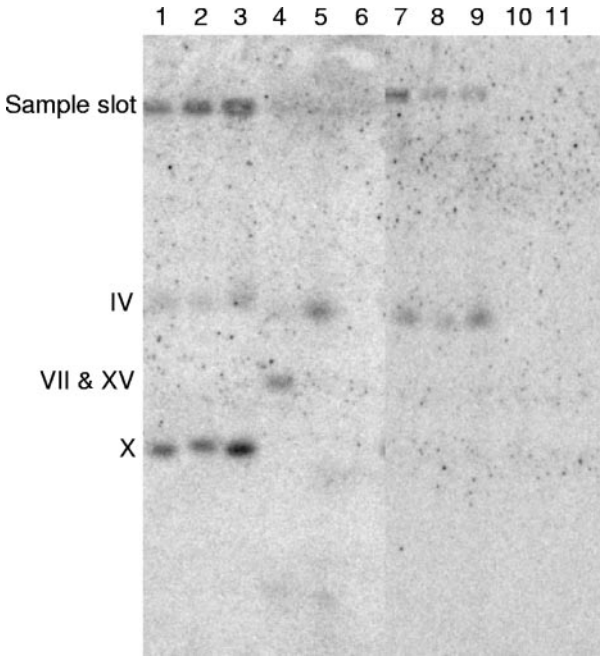

FIG. 5. PFGE filters probed with *MPHx*. The two filters (lanes 1 to 6 and 7 to 11, respectively) were probed with *MPHx* and then exposed for 3 days. The samples were as follows: lane 1, S150-2B; lane 2, CEN.PK2-1D; lane 3, RH144-3A; lane 4, A15; lane 5, A64; lane 6, A60; lane 7, A24; lane 8, A64; lane 9, A72; lane 10, A179; and lane 11, A180. The positions of chromosomes IV and X, the duplex VII/XV, and the sample slot are shown.

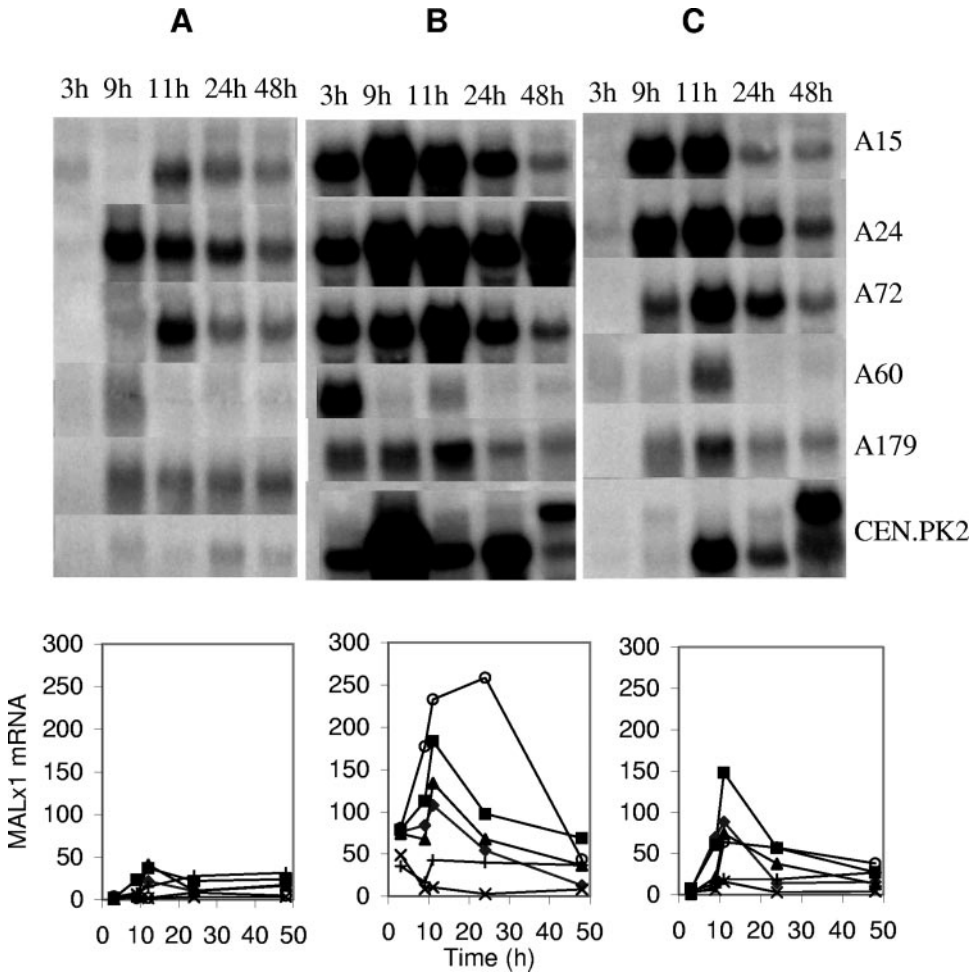

FIG. 6. Expression of *MALx1* during growths of three lager strains, two ale strains, and a maltose-positive laboratory strain on 2% glucose (A), 2% maltose (B), and 1% glucose–1% maltose (C). The upper panels show blots probed with *MAL61*. The lower panels show densitometric quantitation with normalization to *TDH1*. The normalized mRNA amounts are expressed in the same arbitrary units. The results from a single experiment are shown. Similar results were obtained in replicate growths on 2% maltose and 2% glucose. The strains are A15 (◆), A24 (■), A60 (×), A72 (▲), A179 (+), and CEN.PK2-1D (○).

may have been partially trapped in the slot instead of migrating through the gel.

The brewer's strains showed three different behaviors (Fig. 5). The ale strains, A60 and A179 (and A180), did not hybridize with the *MPHx* probe and presumably lack *MPHx* sequences. For lager strains A24, A64, and A72, the *MPHx* probe hybridized to chromosome IV and the sample slot, but not to chromosome X. Thus, these three strains carry *MPH2* but not *MPH3*. With lager strain A15, the *MPHx* probe hybridized to the VII/XV duplex but not to chromosomes X or IV (the faint darkening near chromosome IV in Fig. 5, lane 4, is an artifact not seen on other filters). A15 is the strain from which our *MPHx* probe was cloned. This PCR was repeated and the sequences of the two independent probes were identical to the corresponding sequence of *MPH* in the *Saccharomyces* Genome Database (http://www.yeastgenome.org). Thus, A15 contains at least 790 bp of *MPH* sequence, and the hybridization signal locates this sequence to the chromosome VII/XV duplex.

Expression of maltose transporter genes during shake flask cultivations. Six yeast strains were grown on YP–2% glucose, YP–2% maltose, or YP–1% glucose–1% maltose to test whether their maltose transporter genes were expressed. Glucose and maltose (and maltotriose, which we did not study) are the sugars of practical importance in brewery fermentations. In all cases, glucose was exhausted at 9 to 11 h, maltose was exhausted by 25 h, and ethanol peaked at about 10 h and was completely reconsumed by 33 h (data not shown). The incubations were continued up to 48 h.

We probed the Northern filters with *MAL61* (which recognizes all *MALx1* genes), *AGT1*, *MPHx* and, as a reference, *TDH1* probes (*TDH1* encodes isoenzyme 1 of glyceraldehyde-3-phosphate dehydrogenase). Similar results were obtained with normalization to *TDH1* or rRNA (data not shown). During and after growth on glucose, expression of *MALx1* was low (less than 50 arbitrary units) for all strains (Fig. 6). During growth on 2% maltose, the maltose-positive laboratory strain CEN.PK2-1D and all three lager strains had rapidly increasing

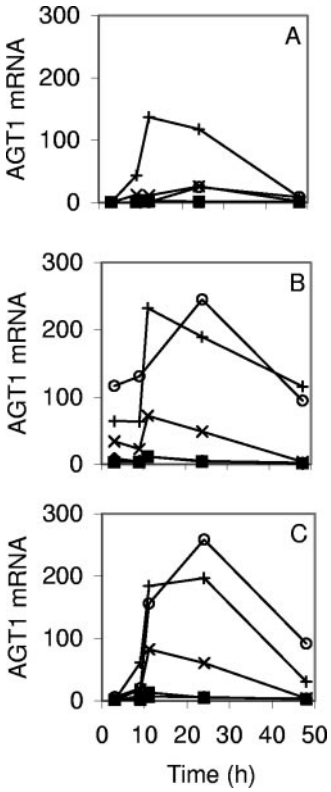

FIG. 7. Expression of *AGT1* during growths on 2% glucose (A), 2% maltose (B), and 1% glucose–1% maltose (C). The mRNA levels are expressed in the same arbitrary units in panels A, B, and C. Similar results were obtained in replicate growths on 2% maltose and 2% glucose. The strains are A15 (◆), A24 (■), A60 (×), A72 (▲), A179 (+), and CEN.PK2-1D (○).

levels of *MALx1* messengers, reaching 100 to 250 arbitrary units (AU). However, expression remained low in both ale strains. During growth on the 1% glucose–1% maltose mixture, expression of *MALx1* by the lager strains and by CEN.PK2-1D was delayed until 9 or 11 h (glucose had decreased to between 0.4 and 0.1% at 9 h; data not shown) and then rose to 60 to 150 AU. Again, the ale strains showed low expression (under 30 AU). Thus, only the lager strains and CEN.PK expressed *MALx1* genes strongly, and their expression was repressed by glucose and required maltose.

Figure 7 contains normalized plots of *AGT1* expression during the same experiments. The lager strains did not express significant amounts of *AGT1* under any of these growth conditions (normalized signals were below 13 AU). Ale strain A179 expressed *AGT1* abundantly in all three media (140 to 240 AU), although on 2% glucose and 1% glucose–1% maltose expression was delayed until 9 h, by which time glucose had decreased to 0.4 and 0.1%, respectively. Ale strain A60 had a pattern qualitatively similar to that of A179, but the levels of *AGT1* messenger were lower (reaching 26 to 80 AU). Although the *AGT1* genes of the ale strains were repressed by glucose, those of A179, at least, did not require maltose for induction.

Compared to *MALx1* and *AGT1*, expression of the *MPHx* genes was very low for all strains in all growth conditions (data not shown). To obtain signals, the probed filters were exposed for 3 days instead of overnight. The highest level of apparent expression was with strain A179, which does not contain *MPHx* genes (Fig. 5 and Table 3). We assume the weak signals detected from these overexposed filters reflect cross-reactivity to a non-*MPH* messenger.

TABLE 5. Sequencing of *AGT1* genes from ale and lager strains

Strain	No. of PCRs[a]	No. of independent clones[b]	No. of sequences	
			616-amino-acid protein[c]	394-amino-acid polypeptide[d]
Whole gene sequences				
A15	3	5	1	4
A24	2	3	0	3
A60	3	5	5	0
A179	2	3	2	1
Nucleotides 1153 to 1500				
A15	3	22	1	21
A24	2	20	0	20
A60	4	18	17	1
A179	4	21	21	0

[a] The number of independent PCRs.
[b] The number of independent clones sequenced.
[c] The number of sequences encoding full length, 616-amino-acid proteins.
[d] The number of sequences encoding the truncated, 394-amino-acid polypeptide.

Sequence of *AGT1* in two ale and two lager strains. *AGT1* genes were amplified from four brewer's yeasts in three (A15), two (A24), three (A60), and two (A179) independent PCRs, ligated into the pCR-TOPO plasmid and independent clones isolated and sequenced (Tables 5 and 6). Of the eight whole gene sequences from the two ale strains (A60 and A179), seven encoded full-length, 616-amino-acid proteins, similar to the sequence in the *Saccharomyces* Genome Database (SGD; http://www.yeastgenome.org). *AGT1* is entered as *MAL11* because it is the allele of *MAL11* present in the S288C strain of the SGD. However, one sequence (from A179) had a stop codon (TGA) at nucleotides 1183 to 1185, leading to a truncated, 394-amino-acid sequence. For the two lager strains, seven of eight whole gene sequences encoded this truncated polypeptide, but one (from A15) encoded the full-length protein. The nucleotide change leading to the stop codon was the insertion of an extra T at position 1183, which results in a stretch of eight consecutive T's instead of seven, the TGA stop codon at positions 1183 to 1185, and a frameshift in the rest of the nucleotide sequence.

These results might mean that both A15 and A179 contain both correct and frameshifted versions of *AGT1*. Alternatively, A15 may contain only the frameshifted version and A179 only the correct version, but during PCR or sequencing reactions the sequences of seven or eight T's were occasionally misread. To distinguish these hypotheses, we cloned and sequenced ~20 independent pCR-TOPO clones containing the portion from nucleotides 1153 to 1500 of the *AGT1* gene from each strain. For the two ale strains, 38 of 39 sequences encoded full-length proteins (Table 5), strongly suggesting that the single occurrence of the frameshifted sequence represents a PCR or sequencing error rather than the presence of both correct and defective versions of *AGT1* in these ale strains. Con-

TABLE 6. Amino acid changes between the proteins coded by the *AGT1* genes in the *Saccharomyces* Genome Database and the ale and lager strains

TMD[b]	Amino acid[a]					
	Position	SGD	A60	A179	A15	A24
	40	D	N	N	N	N
	45	K	R	R		
	59	T		I		
	74	V			M	M
	78	M	T	T		
	80	A	T	T		
	83	D			E	E
	102	K	I	I		
	128	S	N	N	N	N
I (164–184)	164	L	Q	Q	Q	Q
	168	M	T	T		
	175	T	P	P		
	198	I	V	V		
II (202–222)	215	I	V	V	V	V
	219	I	M	M	M	M
	226	S	G	G	G	G
	228	A	T	T	T	T
III (241–266)	276	G			D	D
IV (284–311)	333	V	I	I		
	359	N	D	D	D	D
	375	T			A	A
	381	S	T	T	T	T
	385	V	C	C	C	C
	392	Y		C		
	395	E			Stop[c]	Stop[c]
V (405–427)	409	L	V	V	V	V
VI (432–459)	448	I	V/I[d]	I/V[d]	V	V
	459	S	G	G		G
VII (461–489)	488	A	T	T		
VIII (504–532)	509	L		I		
	548	V	A			
	556	T		S		
	595	A		D		

[a] Amino acids are shown at positions where the SGD and brewer's yeast protein sequences differ; no entry means the amino acid is the same as in the SGD.

[b] The positions of transmembrane domains (TMDs) indicated in SGD are shown.

[c] The sequences of the lager strains' hypothetical proteins after the Stop codons were obtained by correcting the frameshift.

[d] Residue 448 was the same as in the SGD in 3 of 13 A60 sequences and 10 of 17 A179 sequences.

versely, 41 of 42 sequences from the two lager strains contain the extra T, strongly suggesting that all copies of *AGT1* in these strains are defective. The *AGT1* sequences from all four strains are deposited in the NCBI databases as DQ091763 (A60), DQ091764 (A179), DQ091765 (A15), and DQ091766 (A24).

DISCUSSION

The inhibition studies (Fig. 1 and Table 1) indicate that maltose transport by the two studied ale strains was mainly mediated by broad specificity α-glucoside transporters that also can carry trehalose, maltotriose, and other α-glucosides, whereas transport by the three lager strains used mainly transporters with higher specificity for maltose. Indeed, maltose transport by the A15 lager strain was not detectably (±7%) inhibited by 100 mM trehalose. Agt1p is the only transporter known to carry both maltose and trehalose (6, 23). We therefore expected that the Agt1 transporter would be absent from A15 and of minor importance in the other lager strains but would be the dominant maltose transporter of the ale strains.

Based on hybridization studies (Fig. 3), all of our brewer's strains contained both *MALx1* and *AGT1* genes, as has been previously reported for other brewer's yeasts (12). During growth on maltose, *AGT1* genes were strongly expressed in both ale strains and weakly expressed in the three lager strains, whereas *MALx1* genes were expressed strongly in the lager strains and weakly expressed in the ale strains (Fig. 6 and 7). The *AGT1* sequences from the ale strains encoded full-length proteins expected to be active transporters (a property that has been confirmed by overexpression of these *AGT1* genes in laboratory yeasts; V. Vidgren, M.-L. Vehkomäki, L. Ruohonen, and J. Londesborough, unpublished results). In contrast, *AGT1* sequences from the two tested lager strains encoded truncated polypeptides that are unlikely to be functional transporters. Thus, the genotypes determined by hybridization were misleading, and the expression and sequence results support the hypothesis that Agt1 transporters dominate in ale strains and Malx1 transporters dominate in lager strains.

If Agt1 transporters are primarily responsible for maltose uptake in ale strains, then in the final stages of wort fermentations by ale strains maltose competes with an excess of maltotriose for the same transporters. This competition may partially explain why residual maltose (and maltotriose) are more common in ale fermentations than lager fermentations (26). The lack of functional Agt1 transporters in A15 and A24 raises the question of how they transport maltotriose. *MPHx* genes were present in all three lager yeasts, although they were absent from the two ale strains. However, these genes were not expressed in the lager strains during growth on maltose or glucose. Possibly, they are expressed under other conditions, e.g., in the presence of maltotriose. Recently, A15 and three other lager strains have been shown to carry *MTT1* genes, which encode maltose transporters with relatively high activity toward maltotriose (8). The *MTT1* gene is 98% identical to the *Saccharomyces pastorianus mty1* gene (M. Salema-Oom, unpublished; NCBI accession number AJ491328). Another possible maltotriose transporter may be Mal31p. In contrast to early reports that Malx1 transporters cannot carry maltotriose, recent work suggests that Mal31p and Mal61p can carry maltotriose (7) and A15 and A24 both contain *MAL31* genes.

The (hypothetical) proteins encoded by the *AGT1* genes from the two lager strains were almost identical (Table 6). We have no other reason to think that A15 and A24 are closely related, which suggests that there may be other lager strains with similarly defective *AGT1* genes. More sequences are needed to test whether this mutation is a general characteristic of lager strains.

The two ale protein sequences differ at one amino acid residue, 59, in the first 447 amino acids, and at five residues in the remaining 169 amino acids (Table 6). Apparently, two different copies of *AGT1* were cloned from each ale yeast since in 3 of 13 sequences from A60 and 10 of 17 sequences from A179 nucleotide 1342 was A (leading to the same amino acid, I448, as in the SGD) and in the remaining sequences it was G (leading to V448). Thus, these strains probably contain at least three different versions of chromosome VII, one carrying *MAL11* and two carrying different versions of *AGT1*. Approximately 30% of the amino acid changes were in transmem-

brane domains, which account for ~30% of the total amino acid sequence (Table 6), suggesting that there was no bias for or against changes in the transmembrane domains. Altogether, there were 33 amino acid changes between the SGD sequence and the brewer's yeasts' sequences, 13 common to all of the brewer's strains, 5 specific for the lager strains, and 15 specific to one or both ale strains. The lager and ale strains are thus more similar to each other than either is to the laboratory strain, S288C, whose genome provided the SGD. S288C is maltose negative, and it is not known whether its *AGT1* gene encodes a functional transporter.

For all strains except A15 the hybridization signal intensities at the *MAL1* and *MAL2* loci were markedly higher for the *MAL62* probe than the *MAL61* probe (Fig. 4A). Signal strengths were normalized by setting the *MAL31* and *MAL32* signals to 100 for each strain, so this result suggests that the ratio of *MAL11* to *MAL12* genes and of *MAL21* to *MAL22* genes is less than the ratio of *MAL31* to *MAL32* genes. Michels et al. (18) described *MAL3* loci in which tandem partial repeats result in more copies of *MAL31* genes than *MAL32* genes. A tandem repeat could contribute to the unequal labeling by the *MAL61* and *MAL62* probes. However, for the *MAL1* locus, unequal labeling also is expected, because the brewer's yeasts have some *MAL* loci with *AGT1* sequences, which do not bind the *MAL61* probe but are accompanied by *MAL12* genes that do bind the *MAL62* probe. Based on this argument the apparently equal binding of *MAL61* and *MAL62* probes to the *MAL1* locus of strain A15 is unexpected and suggests that this strain may carry (defective) *AGT1* sequences that are not accompanied by *MAL12* sequences.

The ale strains differed from one another kinetically in that trehalose inhibition approached a limiting value of ~82% for A60 but was possibly complete for A179 (Fig. 1). A60 possesses an intact *MAL2* locus that is missing in A179. Mal21p may, therefore, be the trehalose-insensitive maltose transporter of A60. All three maltose-negative laboratory strains lacked *MAL2*, whereas the maltose-positive laboratory strain, CEN.PK2-1D, possessed this locus (Table 3). Although expression of *MALx1* genes was low in both ale strains (Fig. 6) *MALx1* was expressed in A60 during early growth on maltose. The lager strains also differed among themselves. Maltose transport by A15 was completely insensitive to inhibition by trehalose, whereas maltose transport by A24 (which also contains a defective *AGT1* gene) and A64 (whose *AGT1* gene was not sequenced) was reduced by 30 or 55%, respectively, at 100 mM trehalose. Thus, A24 and A64 must have a maltose transporter that either carries or is inhibited by trehalose, and, at least for A24, it cannot be Agt1p. Previously studied Malx1 and Mphx transporters do not carry trehalose (6, 7). Mtt1 (see above), which is present in some lager strains but is possibly defective in A15 (8) is another candidate protein for this function. Another possibility is that A24 and A64 contain maltose transporters that bind and are inhibited by trehalose but do not carry it.

Day et al. (7) found that strains with Mal31p, Mal61p, or Agt1p as the sole maltose transporters transported both [^{14}C]maltotriose and [^{14}C]maltose with similar maximum velocities (40 nmol min^{-1} mg dry mass^{-1}) and K_m values (2.7 to 7.2 mM) for both sugars. These results imply that 50 mM maltotriose should strongly inhibit (by ~70%) the uptake of 5 mM [^{14}C]maltose by each of these three transporters, but we found inhibitions of only 12 to 24% (Table 1) for our lager strains, in which Malx1 transporters were dominant. Each of these lager strains contains several *MALx1* genes, including *MAL31* and, for A72, *MAL61* (Table 3), but we do not know which of these genes are functional. Even if *MAL31* and *MAL61* are functional in these strains, they may not be identical to the genes used by Day et al. (7). A change altering only one amino acid might abolish activity toward maltose or maltotriose or greatly change the relative K_m values.

When more sequences from brewing strains are available, it will become clear whether most lager strains have defective *AGT1* genes and most ale strains have *AGT1* genes that differ from the sequence in the *Saccharomyces* Genome Database and resemble those of A60 and A179. Our results suggest that to understand fully the transport of maltose and other α-glucosides by brewer's strains it may be necessary to sequence each strain's transporter genes and kinetically characterize the transporters they encode. Some of these redundant genes are defective, but little is known about how small sequence changes may affect the activity and substrate specificity of the encoded transporters.

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Paper II

Improved fermentation performance of a lager yeast after repair of its *AGT1* maltose and maltotriose transporter genes

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Improved Fermentation Performance of a Lager Yeast after Repair of Its *AGT1* Maltose and Maltotriose Transporter Genes[▽]†

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The use of more concentrated, so-called high-gravity and very-high-gravity (VHG) brewer's worts for the manufacture of beer has economic and environmental advantages. However, many current strains of brewer's yeasts ferment VHG worts slowly and incompletely, leaving undesirably large amounts of maltose and especially maltotriose in the final beers. α-Glucosides are transported into *Saccharomyces* yeasts by several transporters, including Agt1, which is a good carrier of both maltose and maltotriose. The *AGT1* genes of brewer's ale yeast strains encode functional transporters, but the *AGT1* genes of the lager strains studied contain a premature stop codon and do not encode functional transporters. In the present work, one or more copies of the *AGT1* gene of a lager strain were repaired with DNA sequence from an ale strain and put under the control of a constitutive promoter. Compared to the untransformed strain, the transformants with repaired *AGT1* had higher maltose transport activity, especially after growth on glucose (which represses endogenous α-glucoside transporter genes) and higher ratios of maltotriose transport activity to maltose transport activity. They fermented VHG (24° Plato) wort faster and more completely, producing beers containing more ethanol and less residual maltose and maltotriose. The growth and sedimentation behaviors of the transformants were similar to those of the untransformed strain, as were the profiles of yeast-derived volatile aroma compounds in the beers.

The main fermentable sugars in brewer's wort are maltose (ca. 60% of the total), maltotriose (ca. 25%), and glucose (ca. 15%). In traditional brewery fermentations, worts of about 11° Plato (°P) are used, corresponding to a total fermentable sugar concentration of about 80 g · liter^{-1}. Many modern breweries ferment high-gravity worts (15 to 17°P), and there are efforts to raise the concentration to 25°P, corresponding to a total sugar concentration of about 200 g · liter^{-1}. Industrial use of such very-high-gravity (VHG) worts is attractive because it offers increased production capacity from the same-size brew house and fermentation facilities, decreased energy consumption, and decreased labor, cleaning, and effluent costs (34, 35).

Whereas glucose, which is used first, is transported into yeast cells by facilitated diffusion, the α-glucosides maltose and maltotriose are carried by proton symporters (2, 26, 39). Maltose transport seems to have a high level of control over the fermentation rate. Thus, during the early and middle stages of fermentation of brewer's wort by a lager yeast, the specific rate of maltose consumption was the same as the specific zero-*trans* maltose uptake rate measured off line with each day's yeast in each day's wort spiked with [^{14}C]maltose (27). Furthermore, introducing a constitutive *MAL61* (maltose transporter) gene into a brewer's yeast on a multicopy plasmid accelerated the fermentation of high-gravity worts (17). Maltotriose is the last sugar to be used in brewing fermentations, and significant amounts of residual maltotriose sometimes remain in beer, causing economic losses (lower yield of ethanol on wort carbohydrate) and possibly undesirable organoleptic effects. The problem of residual sugars in beer is more serious when high-gravity and VHG worts are used. Some, but not all, maltose transporters can also carry maltotriose. The *MALx1* genes (x = 1 to 4 and 6) encode transporters that carry maltose efficiently but are generally believed to have little or no activity toward maltotriose (1, 3, 13, 30), although substantial activity toward maltotriose was reported by Day et al. (4). Some yeast strains contain a gene 57% identical to *MAL11* that is usually known as *AGT1* but is recorded in the *Saccharomyces* Genome Database (SGDB) as *MAL11*. The Agt1 transporter has relatively high activity toward maltotriose, as well as maltose (13), and similar K_m values (4 to 5 mM) for these two substrates (4). Alves et al. (1) found that the specific deletion of *AGT1* from several *Saccharomyces cerevisiae* strains also containing at least one *MALx1* gene (*MAL21*, *MAL31*, and/or *MAL41*) abolished their ability to transport maltotriose but did not decrease their maltose transport activity. These results supported the belief that the Mal21, Mal31, and Mal41 transporters cannot carry maltotriose, though it remains possible that there are differences between Malx1 transporters from different strains. The same group has also shown (33) that overexpression of *AGT1* on a multicopy plasmid in an industrial yeast strain with a very limited ability to ferment maltotriose provided the strain with increased maltotriose uptake activity and the ability to ferment maltotriose efficiently. In 2005, a novel kind of α-glucoside transporter was independently found by two groups (6, 30) in some industrial strains of brewer's, baker's, and distiller's yeasts. These transporters are coded by *MTT1* (also called *MTY1*) genes, which are 90 and 54% identical to the *MAL31* and *AGT1* genes, respectively. The Mtt1 transporters have

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† Dedicated to the memory of Isabel Spencer-Martins, a yeast scientist who made a great contribution to our knowledge of sugar transport.

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TABLE 1. PCR primers used in this study

Name[a]	Primer sequence[b]	Sequence(s) detected[c]
ChrWalk1oligo1	5′-GGCACTATCCTTTTTCCCTTC-3′	127 to 147 of *AGT1* gene
ChrWalk1nested	5′-CATTTTTTGAGGCAGCCTTC-3′	32 to 52 of *AGT1* gene
ChrWalk2oligo2	5′-TTAAAATTGGGTACACTC-3′	−146 to −163 of *AGT1* promoter
ChrWalk2nested	5′-AGTACGCATCAACGGAGT-3′	−220 to −237 of *AGT1* promoter
AGT1-F	5′-CGAGATCTCGATGAAAAATATCATTTCATTGGT-3′	1 to 23 of *AGT1* gene
AGT1-R	5′-GCAGATCTGCTTAACATTTATCAGCTGCATTT-3′	1831 to 1851 of *AGT1* gene (ale), 1832 to 1852 of *AGT1* gene (lager)
AGT1Sek1	5′-GAAATGAAGCTAACAGCG-3′	251 to 268 of *AGT1* gene
AGT1Sek2	5′-GATGATTGGTTTGCAAAT-3′	501 to 518 of *AGT1* gene
AGT1Sek3	5′-TGTTGGTTATTTGGTCAA-3′	751 to 768 of *AGT1* gene
AGT1Sek4	5′-AAAGCAGATTGAATTGAC-3′	1011 to 1028 of *AGT1* gene
AGT1Sek5	5′-GGGTACACTTTGCTCCTG-3′	1251 to 1268 of *AGT1* gene (ale), 1252 to 1269 of *AGT1* gene (lager)
AGT1Sek6	5′-TGCTGGCCCGTATTTGCT-3′	1502 to 1519 of *AGT1* gene (ale), 1503 to 1520 of *AGT1* gene (lager)
AGT1p-705F	5′-CG*GAATTC****CAGCGG***CAAGTCAAGAGAAGAT GGAAC-3′	−684 to −705 of *AGT1* promoter
AGT1p-387F	5′-CG*GAATTC****CAGCGG***GCGAGGAACAAG GTTTTTTTC-3′	−367 to −387 of *AGT1* promoter
AGT1p-1R	5′-CG*GAATTC*CGATTATAATATTTTTTTAGTTGT-3′	−1 to −22 of *AGT1* promoter
AGT1 Southern F	5′-TTGCTTTACAATGGATTTGGC-3′	842 to 862 of *AGT1* gene
AGT1 Southern R	5′-CTCGCTGTTTTATGCTTGAGG-3′	1805 to 1825 of *AGT1* gene
IntegScreenAGT1	5′-GCCTAAATATTTGCCTTTGGG-3′	−342 to −362 of A15 *AGT1* promoter
IntegScreenA15AGT1	5′-CGTTCATCTCATTAATCAT-3′	238 to 219 of A15 *AGT1* gene
IntegScreenA60AGT1	5′-TGTTCGTCTCATTAATCAC-3′	238 to 219 of A60 *AGT1* gene
IntegScreenPGK1	5′-GCTTCCAATTTCGTCACACA-3′	−411 to −431 of *PGK1* promoter

[a] F, forward; R, reverse.
[b] BglII restriction sites are underlined, EcoRI sites are underlined and italicized, and MspA1I sites are in bold italics.
[c] The numbering is from the first nucleotide of the translational start.

high activity toward maltotriose and are the only known α-glucoside transporters with lower K_m values for maltotriose than for maltose (30).

Before the discovery of the *MTT1* genes, Vidgren et al. (36) sequenced *AGT1* genes from two apparently unrelated lager strains and two apparently unrelated ale strains of brewer's yeast. Surprisingly, at that time (because other maltotriose transporters were not known), the *AGT1* genes from the lager strains contained an insertion of one nucleotide, resulting in a premature stop codon, and encoded a truncated, nonfunctional 394-amino-acid polypeptide, whereas those from the ale strains encoded full-length 616-amino-acid transporters. This premature stop codon was later shown (37) to be present in *AGT1* genes from all eight of the lager strains tested but was not in any of the four ale strains tested, whereas *MTT1* genes were present in all of the lager strains tested but in none of the ale strains tested.

In the present work, we have tested whether lager fermentations can be accelerated and residual maltotriose levels decreased by repairing the defective *AGT1* genes of lager strains with appropriate DNA sequences from ale strains. Furthermore, the *MALx1* and *AGT1* genes are repressed by glucose and induced by α-glucosides (9, 16, 19, 25), so that replacing the native *AGT1* promoter with a constitutive *S. cerevisiae* promoter might also increase α-glucoside transport activity and accelerate wort fermentations. The objectives of the present work were to confirm that α-glucoside transport has a high level of control over the rate and extent of wort fermentation and to create a genetically modified lager yeast strain that has improved fermentation performance but contains only *Saccharomyces* DNA.

MATERIALS AND METHODS

Materials. Maltose syrup (Cerestar C Sweet M 015S8) was from Suomen Sokeri (Jokioinen, Finland). [U-^{14}C]maltose was Amersham CFB182 from GE Healthcare Ltd. (Bucks, United Kingdom). [U-^{14}C]maltotriose was ARC627 from American Radiolabeled Chemicals, Inc. (St. Louis, MO), and was repurified before use (6). Nucleotides, enzymes, and antimycin A were from Sigma-Aldrich (Helsinki, Finland) or Roche (Espoo, Finland).

Strains and vectors. Strain A63015 (A15) is a lager yeast from the VTT Culture Collection. Like other lager strains, it is alloploid and probably aneuploid. It contains at least two, probably nonidentical, copies of chromosome VII carrying *MAL1* loci containing both *AGT1* and *MAL11* (36). Strain A60 is an ale yeast. Working stocks were suspensions containing 200 mg of fresh yeast mass · ml of 30% glycerol^{-1} stored in ca. 1-ml portions at −80°C. Plasmid pKX34, for selection of G418 resistance, was kindly provided by C. Lang-Hinrichs (18).

Sequencing of the *AGT1* promoter of lager strain A15. Ligation-mediated PCR amplification was based on the work of Mueller and Wold (24). Portions (50 ng) of strain A15 total chromosomal DNA were digested with blunt-end-generating restriction enzyme DraI or HaeIII for 1 h at 37°C. The restriction enzymes were then removed with the Qiagen QIAquick PCR purification kit, and DNA was eluted in 30 μl of elution buffer (Qiagen). Digested DNA was ligated in a linker mixture (24) containing the PCR linker I (5′-GCGGTGACCCGGGAGATCT GAATTC-3′) and PCR linker II (5′-GAATTCAGATCT-3′) primers with T4 DNA ligase for 3 h at room temperature. After ligation, ligase and primer nucleotides were removed with the Qiagen QIAquick PCR purification kit and DNA was eluted with 50 μl of elution buffer. The first PCR was performed with 5 μl of the ligation mixture, 5 μl of PCR linker I primer (1 pmol/μl), and 5 μl of a specific primer (ChrWalk1oligo1) from the *AGT1* gene (10 pmol/μl; the specific primers used in this study are listed in Table 1). The PCR conditions were 94°C for 3 min; 30 cycles of 94°C for 1 min, 60°C for 2 min, and 72°C for 2 min; and 72°C for 10 min. For the second PCR, 1 to 10 μl of a 1/50 dilution of the previous PCR product, 2.5 μl of the PCR linker I primer (1 pmol/μl), and 2.5 μl of a nested primer (ChrWalk1nested; Table 1) from the *AGT1* gene (10 pmol/μl) were used. Conditions for the second PCR were the same as for the first, except that an annealing temperature of 55°C was used. PCR fragments were resolved on 1% agarose gels. In chromosome walking round 1, ca. 500- and 800-nucleotide fragments were obtained with HaeIII-digested DNA as the template and ca. 500- and 350-nucleotide fragments were obtained with DraI-digested DNA as the

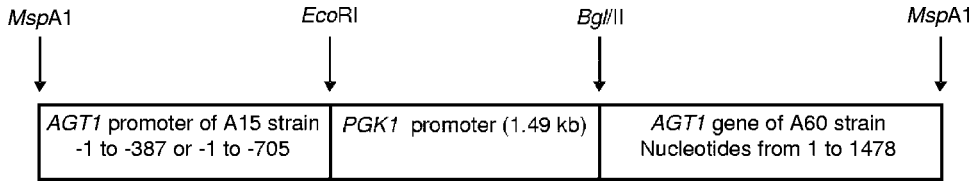

FIG. 1. Diagram of the integration cassettes used in this study. The short and long cassettes contain, respectively, 387 and 705 nucleotides of the *AGT1* promoter from strain A15.

template. The PCR fragments were resolved on 1% preparative agarose gels, cut out, and purified with a Qiagen gel purification kit. The larger fragment of each pair was sequenced directly, without cloning, by using the AGT1p-1R primer (Table 1).

For the second round of chromosome walking, the specific and nested primers were planned according to the sequences obtained from the first round. Specific primer ChrWalk2oligo2 and nested primer ChrWalk2nested were used. Otherwise, chromosome walking was performed as in the first round except that annealing temperatures of 53 and 58°C were used with the specific and nested primers, respectively. After the secondary (nested) PCR, the PCR fragments were resolved on 1% agarose gels. With DraI-digested DNA as the template, fragments of ca. 250 nucleotides and a faint, slightly smaller band were formed. With HaeIII-digested DNA, fragments of ca. 400 and 700 nucleotides were obtained. The 700-bp HaeIII fragments were cloned into the TOPO vector, and two independent clones were sequenced with universal M13 forward and reverse primers. Sequencing was done with the model 3100 Genetic Analyzer sequencer (Applied Biosystems, Foster City, CA).

Integration cassettes. Integration cassettes were constructed in the pBluescript II SK(−) vector (Stratagene, La Jolla, CA). The 1.49-kbp *PGK1* promoter and 0.37-kbp *PGK1* terminator cassette originating from expression vector pMA91 (23) in the YEplac195 multicopy vector (10, 29) was the source of the promoter-terminator cassette. There is a BglII site located between the *PGK1* promoter and terminator. The promoter-terminator cassette was detached with HindIII from the YEplac195 vector and ligated into the pBluescript II SK(−) vector at its HindIII site.

A gene that encodes a functional (36) Agt1 maltose-maltotriose transporter was amplified by PCR with DNA from the ale strain, A60, as the template. PCR primers AGT1-F and AGT1-R (Table 1), for the sense and antisense directions, respectively, were used. To facilitate the next cloning step, BglII restriction sites (underlined in Table 1) were introduced by PCR. The PCR product was cloned into a pCR-TOPO vector (Invitrogen, Espoo, Finland), and the sequence of the *AGT1* gene was verified by using eight sequencing primers, i.e., universal M13 forward and reverse primers, to sequence the start and end of the pCR-TOPO plasmid-ligated *AGT1* gene and six internal primers (AGT1Sek1 to AGT1Sek6; Table 1) from the coding strand. The *AGT1* gene was then excised from the pCR-TOPO plasmid with the BglII enzyme and ligated between the *PGK1* promoter and terminator at the BglII site in the pBluescript II SK(−) plasmid described above.

AGT1 promoter fragments of two different lengths were amplified by PCR with DNA from lager strain A15 as the template. Two primer pairs were used: AGT1p-705F and AGT1p-1R to amplify an *AGT1* promoter fragment from −705 to −1 and AGT1p-387F and AGT1p-1R to amplify an *AGT1* promoter fragment from −387 to −1. To facilitate the next cloning steps and the final detachment of the integration cassette, EcoRI and MspA1I restriction sites were introduced by PCR into the 5′ ends of these fragments and an EcoRI site was introduced into their 3′ ends, as shown in Table 1.

The *AGT1* promoter fragments were ligated to pCR-TOPO plasmids, and their sequences were verified. The promoter fragments were excised from the pCR-TOPO plasmids with EcoRI and separately ligated into the pBluescript II SK(−) vector already possessing the *PGK1* promoter-*AGT1*(A60)-*PGK1* terminator cassette. Ligation was carried out at the EcoRI site, which is located next to the 5′ end of the *PGK1* promoter. Thus, short and long integration cassettes were constructed with, respectively, 387- and 705-nucleotide *AGT1* promoter fragments flanking the *PGK1* promoter on the 5′ side. The cassettes were excised from the pBluescript II SK(−) vector by using MspA1I. It detaches the 3.37-kbp (possessing 387 bp of the *AGT1* promoter flank) and 3.70-kbp (possessing 705 bp of the *AGT1* promoter flank) integration cassettes. Detachment takes place at the 5′ end of the ligated *AGT1* promoter fragments at the newly introduced MspA1I site and on the other side of the cassette at the MspA1I site located in the *AGT1* gene at nucleotide 1478; i.e., the cassettes lack the 3′-terminal 373 nucleotides of the *AGT1* open reading frame (ORF).

Transformation of A15 and selection of *AGT1*-expressing transformants. The A15 lager strain was transformed with the short or long integration cassette together with plasmid pKX34, which contains the kanamycin marker gene giving resistance to the antibiotic G418. A15 cells were inoculated into 2% yeast extract–1% peptone medium (YP) containing 2% glucose, grown overnight at 30°C to 2×10^7 cells/ml (optical density at 600 nm [OD_{600}] of about 1), and transformed by the lithium acetate transformation procedure (11, 14). Transformed cells were resuspended in 1 ml of YP–2% glucose and incubated for 17 h at 30°C with shaking (250 rpm). The cells were pelleted for 15 s and resuspended in 1.0 ml of sterile water. Portions of 500 μl were spread on selective plates of YP–2% glucose containing G418 (200 μg/ml) or YP–2% maltotriose containing antimycin A (3 μg/ml) and G418 (200 μg/ml). The plates were incubated at 30°C for up to 6 days. Transformation frequencies, determined by counting the colonies appearing on the YP–2% glucose–G418 plates, were around 50 to 110 transformants/μg of total transforming DNA (cassette plus selection plasmid DNA).

Southern analyses. Chromosomal DNA was restricted with EcoRI, XbaI, or XmnI. Hybridization was performed with a 984-bp *AGT1* probe prepared with primers AGT1 Southern F and AGT1 Southern R (Table 1). According to the sequence of SGDB laboratory strain S288C, EcoRI cutting sites are 2,425 bp upstream and 1,993 bp downstream from the *AGT1* ORF, respectively, so that an EcoRI fragment of 6,270 bp was expected (the defective *AGT1* ORF of strain A15 is 1,852 bp). A 6.3-kb fragment was detected, indicating that EcoRI sites are in similar locations in the A15 and S288C strains. When integration of the cassette has taken place, a new EcoRI site is introduced at the beginning of the *PGK1* promoter (Fig. 1) and an EcoRI fragment of 5.3 kb is expected. XbaI restriction was used to investigate whether more than one cassette copy integrated into the *AGT1* locus. The SGDB gives XbaI sites 1,753 bp upstream and 1,244 bp downstream of the *AGT1* ORF, so that native loci should give a 4.8-kb fragment (recognized by the above-described *AGT1* probe). The cassette contains no XbaI site, so that after integration of one cassette copy a 6.3-kb fragment is expected, whereas multiple integration will give a larger fragment. XmnI sites are 1,528 bp upstream and 683 bp downstream of the *AGT1* ORF (according to the SGDB), and there is also an XmnI site at position −592 of the *PGK1* promoter in the cassette. XmnI restriction is therefore expected to give a 4.1-kb fragment from the native *AGT1* locus and a 3.1-kb fragment (recognized by the *AGT1* probe) and a 2.4-kb fragment (not recognized) after a single integration event. Multiple tandem integration would give different results, depending on the orientations and sizes of the different cassette copies.

DNA from agarose gels was blotted onto nylon filters (Hybond N; Amersham Biosciences, Espoo, Finland) by standard procedures (31). Probes were labeled with digoxigenin-11-dUTP (Roche, Germany), and hybridization signals were detected with the chemiluminescent (CSPD; Roche, Germany) or colorimetric (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside–nitroblue tetrazolium; Promega, Madison, WI) method by using alkaline phosphatase activity according to the manufacturer's instructions (Roche, Germany).

PCR analyses. PCR was performed by standard procedures. The primers used are listed in Table 1. To verify the integration of the cassette, two PCRs were used. In the first, primers binding to the *AGT1* promoter and the *AGT1* ORF were used. Specific primers which differed from each other in three nucleotides were used for the A15 and A60 *AGT1* ORFs, respectively. The IntegScreenAGT1 primer, which binds to the *AGT1* promoter, and the IntegScreenA15AGT1 primer are expected to produce a 0.6-kb fragment when endogenous *AGT1* is present. The IntegScreenAGT1 and IntegScreenA60AGT1 primers are expected to produce a 2.1-kb fragment when the intact cassette has been inserted.

In the other PCR, a primer in the *PGK1* promoter (IntegScreenPGK1; Table

1) and a primer in the *AGT1* ORF 3′ to the integration cassette (AGT1 Southern R) were used. With these primers, a 2.3-kb PCR fragment is expected after integration of the cassette as planned into the *AGT1* locus of A15 and no product is expected from A15 DNA.

To confirm that integration had occurred 3′ to the frameshift of the *AGT1* gene of A15, a PCR was performed with the IntegScreenPGK1 and AGT1 Southern R primers. The PCR products were cloned into the pCR-TOPO vector (Invitrogen, Espoo, Finland) and sequenced with the AGT1Sek3, AGT1Sek4, and AGT1Sek5 primers (Table 1).

Transcription analyses. Strain A15 and integrants 1, 2, and 14 were pregrown in YP containing 20 g · liter^{-1} glucose (duplicate 50-ml portions in 250-ml flasks) for 30 h at 24°C to OD_{600}s between 16 and 18 and then diluted into YP containing 20 g · liter^{-1} glucose, maltose, or maltotriose (duplicate 100-ml portions in 500-ml flasks) to an initial OD_{600} of 0.5 and shaken at 18°C. Samples were taken when about half of the initial sugar was consumed (at 13 h) and at stationary phase (36 h). Samples containing 50 to 150 mg of fresh yeast were quickly filtered, and the yeast was washed with RNase-free water, frozen in liquid nitrogen, and stored at −80°C as previously described (28). The cells were then lysed, and specific mRNAs were quantitated by TRAC analyses (28); for the mRNA-specific, fluor-labeled probes used, see Table 3. Probes were designed and TRAC assays were performed by PlexPress (Helsinki, Finland).

Genetic stability. A15 and integrants 1, 2, and 14 (after curing of plasmid pKX34) were repeatedly grown in YP containing 20 g · liter^{-1} glucose at 24°C. Each cycle, the yeasts were grown for about 24 h to an OD_{600} of about 12 (or on weekends for 72 h to an OD_{600} of about 21) and then diluted into fresh medium to an OD_{600} of 0.05. After 14 cycles (corresponding to 110 to 112 cell divisions), appropriate dilutions were spread on agar plates containing YP and either 20 g · liter^{-1} glucose or 20 g · liter^{-1} maltotriose and 200 mg · liter^{-1} antimycin A. Plates were incubated at 24°C, and the colonies were counted daily. Ten colonies of each strain were transferred from the glucose plates to liquid YP containing 20 g · liter^{-1} glucose and grown into early stationary phase, and DNA was extracted and analyzed.

Maltose and maltotriose transport assays. Zero-*trans* rates of α-glucoside uptake were determined with 5 mM substrate in 0.1 M tartrate–Tris (pH 4.2, 20°C) as described earlier (21, 32), with minor modifications (12). One unit catalyzes the uptake of 1 μmol of maltose (or maltotriose) per min under these conditions.

Cell viability. Cell viabilities were determined by staining with methylene blue in phosphate buffer, pH 4.6, according to Analytica-Microbiologica-EBC method 3.2.1.1 (7).

Tall-tube wort fermentations. Two fermentation series, A and B, were performed 10 months apart. 25°P worts were made in the VTT pilot brewery from malt with high-maltose syrup as an adjunct (accounting for 40% of the total extract) and contained glucose at 24 g · liter^{-1}, maltose at 110 g · liter^{-1}, maltotriose at 42 g · liter^{-1}, total fermentable sugars at 180 g · liter^{-1}, free amino nitrogen at 380 mg · liter^{-1}, zinc at 0.2 mg · liter^{-1}, magnesium at 180 mg · liter^{-1}, and calcium at 60 mg · liter^{-1}. The pH was 5.2. A 16°P wort was made by diluting 25°P wort with water containing enough $ZnSO_4$ to give a final zinc concentration of 0.2 mg · liter^{-1} in series A and 0.4 mg · liter^{-1} in series B. In series B, extra $ZnSO_4$ was also added to the 25°P wort to give a final Zn concentration of 0.4 mg · liter^{-1}. The 16 and 25°P worts were oxygenated immediately before use to 10 and 12 mg of oxygen · liter^{-1}, respectively.

Pitching yeasts were grown and fermentations were performed essentially as described earlier (27), except that main yeast growths and 24°P fermentations were at 18°C. The precultures of 100 ml of autoclaved YP containing 4% maltose in 250-ml Erlenmeyer flasks were inoculated with 500 μl of glycerol stock (100 mg yeast) and grown overnight at 24°C to an OD_{600} between 6 and 10 (series A) or 20 (series B). For main growths, 3-liter lots of 16°P wort in 5-liter Erlenmeyer flasks (series A) or 1.2-liter lots in 3-liter Erlenmeyer flasks (series B) were inoculated with 50 ml of preculture and shaken on an orbital shaker at 18°C for 2 days and the stationary-phase culture was then allowed to settle at 0°C for 16 to 24 h. Most of the supernatant was decanted, and the settled yeast was mixed into a smooth slurry and diluted with supernatant to 20 g of centrifuged yeast mass/100 g of slurry. Static fermentations were carried out in stainless steel tall tubes designed to mimic industrial cylindroconical fermentors. Yeast was pitched into 2.0 liters of the 16 and 25°P worts at 10°C to concentrations of 5.0 and 8.0 g of centrifuged yeast mass · liter^{-1}, respectively, equivalent to about 20 and 32 million cells · ml^{-1}. Dilution of worts with the yeast slurry led to calculated extracts of 15 and 24°P immediately after pitching. Samples (about 30 ml) withdrawn daily through sampling ports 23 cm above the cones were centrifuged, the pellets were washed twice with water, and their dry mass was determined overnight at 105°C. The pH of the supernatants was measured, and after degassing, their densities were determined with an Anton Paar DMA58 density meter.

At the end of the B fermentation series, yeast was cropped from some of the tall tubes. To do this with minimal exposure to air, the entire contents of each tall tube were transferred to a 2-liter screw-cap glass bottle and kept at 0°C for 2 days. During this time, the yeast settled. Most of the supernatant beer was decanted, and the settled yeast was mixed into a slurry, diluted with its own beer to 20 g of centrifuged mass/100 g of slurry, and pitched into fresh wort as described above.

Chemical analyses of beers. The ethanol concentrations of the final beers were determined by quantitative distillation according to Analytica-EBC method 9.2.1 (8). Volatile aroma compounds were measured by gas chromatography as previously described (38), and residual sugars were measured by high-performance liquid chromatography (Waters, Milford, MA).

Fermentation calculations. Apparent extracts were calculated from the densities by Analytica-EBC method 9.4 (8). "Extract" is a measure of the sum of fermentable sugars and nonfermentable soluble carbohydrate in wort: a solution with an extract of *x*°P has the same density as a water solution containing *x* g of sucrose in 100 g of solution. "Apparent extracts" measured during fermentations are uncorrected for the effect of ethanol on the density. Apparent attenuations are the difference between the original extract and the current apparent extract divided by the original extract ([original extract − current apparent extract]/original extract). The apparent attenuation limit is the apparent attenuation measured after exhaustive fermentation with excess yeast and is a measure of the total amount of fermentable carbohydrate in a wort.

Nucleotide sequence accession number. The sequence of the *AGT1* promoter of strain A15 has been deposited under accession no. EU864227 in the NCBI database.

RESULTS

Characterization of transformants. The intention was to repair one or more copies of the defective *AGT1* gene in the alloploid lager strain, A15, and at the same time place the gene(s) under the control of a constitutive *PGK1* promoter of *S. cerevisiae*. Figure 1 shows the two integration cassettes planned to achieve this aim. Between nucleotides −1 and −317, the sequence of the *AGT1* promoter was identical to that of the *MAL11* promoter in the SGDB, except for two changes (A for C at −8 and A for G at −152). However, between −318 and −807, the level of identity fell to 35%.

Lager strain A15 was cotransformed with each of these integration cassettes together with plasmid pKX34, which confers resistance to G418. After transformation, the cells were plated onto either YP containing 20 g of glucose · liter^{-1} and G418 (200 mg · liter^{-1}) or YP containing 20 g of maltotriose · liter^{-1}, antimycin A (3 mg · liter^{-1}), and G418 (200 mg · liter^{-1}). The rationale for the selection procedure was that pregrowth on glucose represses endogenous genes for α-glucoside transporters, causing long lag phases before growth on maltotriose when respiration is inhibited with antimycin A (6, 20). Transformants containing a constitutively expressed maltotriose transporter gene were expected to start growing sooner. Within 2 days at 30°C, 170 to 470 colonies appeared on the glucose-G418 plates. On the maltotriose-antimycin A-G418 plates, in contrast, only one colony appeared after 2 days and after 6 days the total number of colonies was between 4 and 13% of the number appearing on glucose-G418 (Table 2). There was no clear difference between the long and short forms of the cassette in the number of colonies recovered.

Southern blot analysis with the *AGT1* probe was applied to 36 of the 119 colonies recovered on maltotriose-antimycin A-G418 plates. For 30 colonies, both the 6.3-kb EcoRI fragment expected from the native A15 strain and the 5.3-kb EcoRI fragment expected after the integration of either cassette into the correct (*AGT1*) locus (see Materials and Methods) were

TABLE 2. Selection of transformants on glucose or on maltotriose-antimycin A[a]

Transformation	No. of colonies					
	Glucose, day 2	Maltotriose-antimycin A				
		Day 2	Day 3	Day 4	Day 5	Day 6
1	171			2	7	22
2	473		1	7	14	37
3	434	1	2	8	18	38
4	460			1	4	22

[a] Cells (1×10^8) were transformed with 0.6 μg of pKX34 DNA and (transformation 1) 6.4 or (transformations 2 to 4) 8.0 μg of the short (transformations 1 and 2) or long (transformations 3 and 4) form of the cassette DNA. After transformation, the cells were incubated overnight in YP containing 20 g of glucose · liter^{-1} and washed with water. Half were plated onto YP containing glucose (20 g · liter^{-1}) and G418 (200 mg · liter^{-1}), and half were plated onto YP containing maltotriose (20 g · liter^{-1}), G418 (200 mg · liter^{-1}), and antimycin A (3 mg · liter^{-1}). Plates were incubated at 30°C, and total colonies were counted each day.

detected, so these cells contained both an endogenous *AGT1* gene and a transformed *AGT1* gene with a *PGK1* promoter. Six of the colonies tested showed only the 5.3-kb fragment, so that these cells contained only the transformed *AGT1* gene. These 36 colonies and another 19 were tested by PCR with primers in the *AGT1* promoter and the A60-type *AGT1* ORF, and all gave the 2.1-kb product expected after the insertion of an intact cassette with the *PGK1* promoter between the *AGT1* promoter and ORF (see Materials and Methods). Both primers recognize sequences in the cassette, so this result shows that these cells contained the cassette somewhere in their genomes. For A15, the analogous PCR but with primers in the *AGT1* promoter and the A15-type *AGT1* ORF gave the expected native 0.6-kb fragment. Seven transformant colonies (including integrants 1, 2, and 14) that gave both native and recombinant EcoRI fragments in Southern analyses were also tested with this PCR, and all gave the 0.6-kb fragment, confirming that they contain both native and repaired *AGT1* loci.

For 17 of the above-described 36 clones, another PCR was performed with primers in the *PGK1* promoter and the *AGT1* ORF 3′ to the integration cassette (primers IntegScreenPGK1 and AGT1 Southern R; Table 1). All 17 colonies (including integrants 1, 2, and 14) gave the expected 2.3-kb fragment (see Materials and Methods), verifying that the cassette had integrated as planned into the endogenous *AGT1* locus.

To confirm that integration had occurred 3′ to the frameshift of *AGT1* of A15, 20 independent PCR clones of this 2.3-kb fragment were isolated from seven transformants and sequenced from nucleotide 768. After nucleotide 768, the ORFs of the *AGT1* genes in strains A60 and A15 differ (36) at nucleotides 827, 997, 1123, 1183 (where the frameshift in A15 *AGT1* occurs), 1465 and 1647, whereas the transformation cassettes contained A60 sequence up to nucleotide 1478. In 18 of the 20 independent clones, the variant nucleotides at positions up to and including nucleotide 1183 were derived from the A60 sequence, showing that the second crossover had occurred 3′ to the frameshift in the A15 sequence. The exceptions were one of the seven independent PCR clones from integrant 1 (where the crossover had apparently occurred between nucleotides 827 and 997 and nucleotides 997, 1123, 1183, and 1465 were all derived from A15) and one of the seven independent PCR clones from integrant 14 (where the crossover was between nucleotides 1123 and 1183, but we did not identify nucleotide 1465). In this second case, the A15 nucleotide found at position 1183 might be a PCR error, but for the exceptional clone from integrant 1, this is very unlikely, since four nucleotides within the region of the cassette DNA (997, 1123, 1183, and 1465) were all derived from A15. This suggests that, in addition to at least one copy of repaired *AGT1* where crossover has occurred 3′ to the frameshift, integrant 1 also contains a copy of modified *AGT1* where crossover has occurred 5′ to the frameshift. Nucleotide 1465 was derived from A15 in 6 cases and from A60 in 10 cases (undetermined in 4 cases), showing variability in the crossover site at the 3′ end of the cassette.

Transcriptional analysis and α-glucoside transport capacity of the integrants. Integrant 1 (the first to appear during selection; Table 2), integrant 2, and integrant 14 were chosen for studies of α-glucoside transport and fermentation performance. They were cured of plasmid pKX34 by cultivation in the absence of the antibiotic G418. Single-cell colonies were isolated that were unable to grow in the presence of G418 and therefore presumably lacked the plasmid. To check that these cured integrants contained no bacterial DNA from the plasmid, their chromosomal DNAs were digested with either EcoRI or HincII and Southern blots were probed with, respectively, a ca. 1.2-kb EcoRI fragment or a ca. 3-kb HincII fragment of the pKX34 plasmid. These fragments together cover most of the bacterial DNA in the pKX34 plasmid (see reference 18). No hybridization bands were observed from integrants 1, 2, and 14, but the expected strong signals were seen with linearized plasmid pKX34 (not shown).

These three integrants each yielded both 6.3-kb and 5.3-kb EcoRI fragments and so contained both native and repaired *AGT1* loci. The 5.3-kb bands were markedly more intense than the 6.3-kb bands (Fig. 2), suggesting that each integrant contains more repaired loci than native loci, so that A15 must contain at least three *AGT1* loci. Restriction with XbaI yielded the expected 4.8-kb band from A15. Each integrant gave both a 4.8-kb band and the 6.3-kb band expected after the integration of a single cassette copy at each repaired *AGT1* locus (see Materials and Methods). No larger band was observed, showing that multiple integration had not occurred. The 6.3-kb bands were stronger than the 4.8-kb bands, in agreement with the EcoRI results, again suggesting that A15 must contain at least three *AGT1* loci. Results obtained with XmnI were similar, integrants yielding weaker 4.1-kb bands corresponding to the native loci and stronger 3.1-kb bands corresponding to repaired *AGT1* loci.

To test whether the repaired *AGT1* genes were expressed, strain A15 and integrants 1, 2, and 14 were grown at 18°C in shake flasks in YP containing 200 g · liter^{-1} glucose, maltose, or maltotriose. Figure 3 shows the apparent expression levels of the actin gene *ACT1* (as a control), the maltose transporter genes *MTT1*, *AGT1*, and *MALx1*; and the maltase gene *MALx2* in yeast samples taken after 13 h of growth (at OD_{600}s of 5.3 ± 0.5, 4.2 ± 0.5, and 4.0 ± 0.6 on glucose, maltose, and maltotriose, respectively, and residual glucose at 7.0 ± 0.3 g · liter^{-1}; residual maltose and maltotriose were not measured but were presumably similar) or in stationary phase at 36 h (at an OD_{600} of 23 ± 2). The TRAC probes used are shown in Table 3, and the fluorescence signals from these specific probes are assumed to

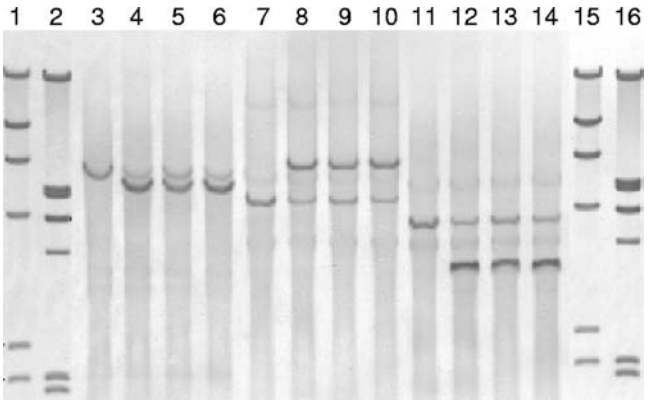

FIG. 2. Southern analyses of strain A15 and integrants 1, 2, and 14. Lanes 1 and 15, molecular weight marker II (23,130, 9,416, 6,557, 4,361, 2,322, and 2,027 bp); lanes 2 and 16, molecular weight marker III (21,226, 5,148, 4,973, 4,268, 3,530, and 2,027 bp); lanes 3, 7, and 11, strain A15; lanes 4, 8, and 12, integrant 1; lanes 5, 9, and 13, integrant 2; lanes 6, 10, and 14, integrant 14. Chromosomal DNA was restricted with EcoRI (lanes 3 to 6), XbaI (lanes 7 to 10), or XmnI (lanes 11 to 14); separated in 0.8% agarose gel; blotted onto a nylon filter; and probed with a 984-bp *AGT1* PCR fragment. The band sizes (in kilobases) predicted from the SGDB sequence of native *AGT1* loci are as follows: EcoRI, 6.3; XbaI, 4.8; XmnI, 4.1. For repaired loci, they are as follows: EcoRI, 5.3; XbaI, 6.3; XmnI, 3.1. Tandem integration of cassette DNA would give a 9.6-kb or larger XbaI band(s).

be proportional to the expression levels. Results in Fig. 3 are normalized to the total amounts of mRNA in each sample. This resulted in similar *ACT1* signals from the different yeasts, so that normalization to *ACT1* levels, instead of total mRNA, would not alter the interpretation of the results. At 13 h, the apparent expression level of the resident *AGT1* gene of A15 was independent of the sugar type and at 36 h it was only slightly higher after growth on maltose or maltotriose than after growth on glucose. Compared to strain A15, all three integrants showed increased expression of *AGT1* (2.6- to 5.8-fold increases at 13 h; 3.8- to 12-fold increases at 36 h). Thus, the repaired *AGT1* genes under the control of *PGK1* promoters were expressed more strongly than the resident gene. In contrast to *AGT1*, the other α-glucoside transporter genes, *MTT1*, *MALx1*, and *MALx2*, were not expressed during growth on glucose and were only weakly expressed in stationary-phase cells after growth on glucose. These genes were expressed during and after growth on maltose and maltotriose at similar levels in strain A15 and the three integrants. Maltose and maltotriose transport activities were measured in cells of each strain harvested at 13 h from one of the two replicate growths on glucose. Strain A15 cells exhibited little or no activity (<0.2 U · g of dry yeast^{-1}), which was expected, as their *AGT1* genes do not encode functional transporters and their other α-glucoside transporter genes were not expressed.

In another experiment, yeast cells were grown on YP containing 20 g of glucose · liter^{-1} at 24°C and harvested at 8.5 ± 0.4 g of glucose · liter^{-1} (strain A15 and integrants 1 and 2) or 10.7 g of glucose · liter^{-1} (integrant 14). The maltose transport capacity of strain A15 was 1.6 U · g of dry yeast^{-1}, whereas integrants 1, 2, and 14 exhibited 10.3, 6.6, and 5.1 U · g of dry yeast^{-1}. Addition of 50 mM unlabeled maltotriose to the assay mixtures inhibited the uptake of 5 mM radiolabeled maltose by 13% for strain A15 but by 59, 50, and 41%, respectively, for integrants 1, 2, and 14. The greater inhibition of the integrants by maltotriose was consistent with their maltose

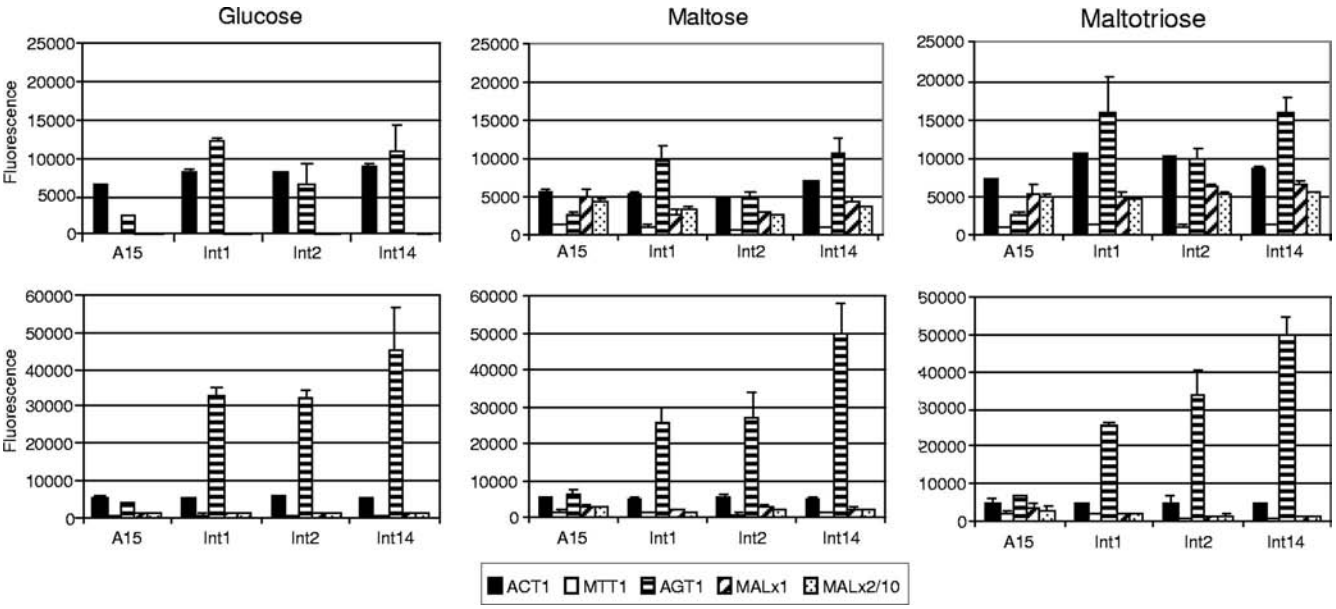

FIG. 3. Expression of *AGT1* and other α-glucoside transporter and maltase genes during batch growth of strain A15 and integrant 1 (Int1), Int2, and Int14 on 20 g · liter^{-1} glucose, maltose, or maltotriose at 18°C. Yeast samples were collected after 13 h (upper row), while sugars were still present at about 7 g · liter^{-1}, or at 36 h (lower row), when cells were in stationary phase. Samples were lysed and analyzed by TRAC with probes specific for *ACT1* (actin control); the transporter genes *MTT1*, *AGT1*, and *MALx1*; and the maltase gene *MALx2*. Shown are the fluorescence signals from specifically bound probes. The signals from the *MALx2* probe were divided by 10 (*MALx2*/10). Results are averages ± ranges of data from two replicate growths of each yeast strain on each sugar.

TABLE 3. TRAC probes used in this study

Gene	Probe sequence	Fluor[a]	Target nucleotides[b]
ACT1	CGGTTTGCATTTCTTGTTCGAAGTCCAAGGCGA	NED	656–688
MTT1	ACTGTTTGTATAGCCAATCCAAATGCGTAAAGGTCAAAC	NED	1278–1316
AGT1	GAGTTTTTCCCTTTCCGAATGGATCAACCAC	HEX	1738–1768
MALx1	GGTTTCTGGTAAATCGACAACAGCCCAAGCTAA	FAM	1615–1647
MALx2	ACCGGGCTTGATCGTGATTCTCGAT	FAM	1033–1057

[a] Fluorescent labels attached to the probes: FAM, 6-carboxyfluorescein; HEX, hexachloro-6-carboxyfluorescein; NED, 2,7′,8′-benzo-5′-fluoro-2′,4,7-trichloro-5-carboxyfluorescein.
[b] Numbering is from the translational start according to the SGDB.

transport activity being caused by the broad-specificity Agt1 transporters (for which maltotriose is necessarily a competitive inhibitor of maltose transport). After growth on YP containing 20 g of maltose · liter^{-1}, strain A15 exhibited relatively high maltose transport activity (18 U · g of dry yeast^{-1}) and integrants 1, 2, and 14 showed only modest (10 to 20%) increases in activity (to, respectively, 20, 21, and 22 U · g of dry yeast^{-1}). However, the transport activity of maltose-grown cells toward maltotriose increased from 3.4 U · g of dry yeast^{-1} for strain A15 to 8.5, 5.1, and 5.4 U · g of dry yeast^{-1}, respectively, for integrants 1, 2, and 14. Thus, compared to the host strain, maltose-grown integrants had an improved capacity to transport maltotriose in particular.

Genetic stability. Strain A15 and integrants 1, 2, and 14 were cycled 14 times through growth on YP containing 20 g of glucose · liter^{-1}. This corresponded to 110 to 112 cell divisions for the different strains. The final yeast suspensions were diluted and spread on nonselective (20 g of glucose · liter^{-1}) and selective (20 g of maltotriose plus 200 mg antimycin A · liter^{-1}) plates. Colonies appearing were counted daily (Table 4). The numbers of colonies on nonselective plates did not increase between 2 and 4 days, and 10 colonies of each strain were picked at random. DNA from these colonies was restricted with EcoRI, and Southern analyses were run as in Fig. 2. The same results were obtained as in Fig. 2; i.e., all 10 A15 colonies gave only the native 6.3-kb band and all 10 colonies of each integrant gave both this band and the 5.3-kb band expected after integration of the cassette DNA (not shown). Thus, the integrant genotypes were stable through 110 generations of nonselective growth. Growth of A15 on the selective plates started after a long lag: no colonies had appeared by day 9, but by day 12 the numbers reached 65 to 88% of the numbers on nonselective plates. In contrast, by day 5 the numbers of integrant colonies on selective plates were 85 to 109% of the numbers on nonselective plates (except 55% for the 25-μl sample of integrant 14). Thus, the phenotype of fast adaptation to growth on maltotriose-antimycin A after growth on glucose was preserved through 110 generations. It is also notable that for integrant 1 (the first to appear during selection; Table 2), the numbers of colonies on selective plates on day 4 were already 58 to 84% of the numbers of colonies on nonselective plates, whereas this proportion was only 19 to 61% for integrants 2 and 14, suggesting that the phenotypic difference between integrant 1 and the others was also preserved.

Wort fermentations with the parent strain and integrants. The cured yeasts were propagated in 16°P wort, and their fermentation performance was tested in static tall-tube fermentors that imitate industrial cylindroconical fermentation tanks. All three integrants fermented both 15°P wort (at 14°C) and 24°P wort (at 18°C) faster and more completely than did the parent, A15, lager strain (Fig. 4 and Table 5; see also Fig. 6), resulting in time savings and increased ethanol yields. For all three fermentation series (one at 15°P, two at 24°P), time savings were estimated as the differences between the time required by A15 and that required by each integrant to reach an apparent attenuation of 80%. The apparent attenuation limits of these worts were close to 86%. Brewers would want to reach an apparent attenuation as close as possible to this limit, but in practice some fermentable sugars always remain in the final beer (and have desired organoleptic effects in some beers). For the two fermentation series (15°P and series B at 24°P) where the control strain, A15, reached a higher apparent attenuation than 80%, we also calculated the time savings at the highest attenuation reached by all of the strains (Table 5).

For the 15°P wort, the differences in final apparent extracts (a measure of residual carbohydrate) were relatively small (0.14 to 0.19°P lower for integrants than for A15) and increases in ethanol yield were close to the experimental error (Table 5).

TABLE 4. Genetic stability of integrants[a]

Strain and vol (μl)	No. of colonies						
	Glucose plates		Maltotriose/antimycin A plates				
	Day 2	Day 4	Day 2	Day 4	Day 5	Day 9	Day 12
A15							
25	57	57	0	0	0	0	50
50	123		0	0	0	0	80
100	213		0	0	0	0	
Integrant 1							
25	45	47	0	26	43		
50	95		0	80	104		
100	170		0		186		
Integrant 2							
25	48	46	0	9	43		
50	109		0	44	99		
100	177		0		150		
Integrant 14							
25	56	58	0	22	31		
50	98		0	60	101		
100	171		0		142		

[a] Each strain was cycled through growth on glucose for 110 to 112 cell divisions and diluted to an OD_{600} of 2.5×10^{-4}, and portions of the indicated volumes were spread onto agar plates containing glucose or maltotriose-antimycin A and incubated at 24°C for up to 12 days. No entry indicates that the colonies were not counted.

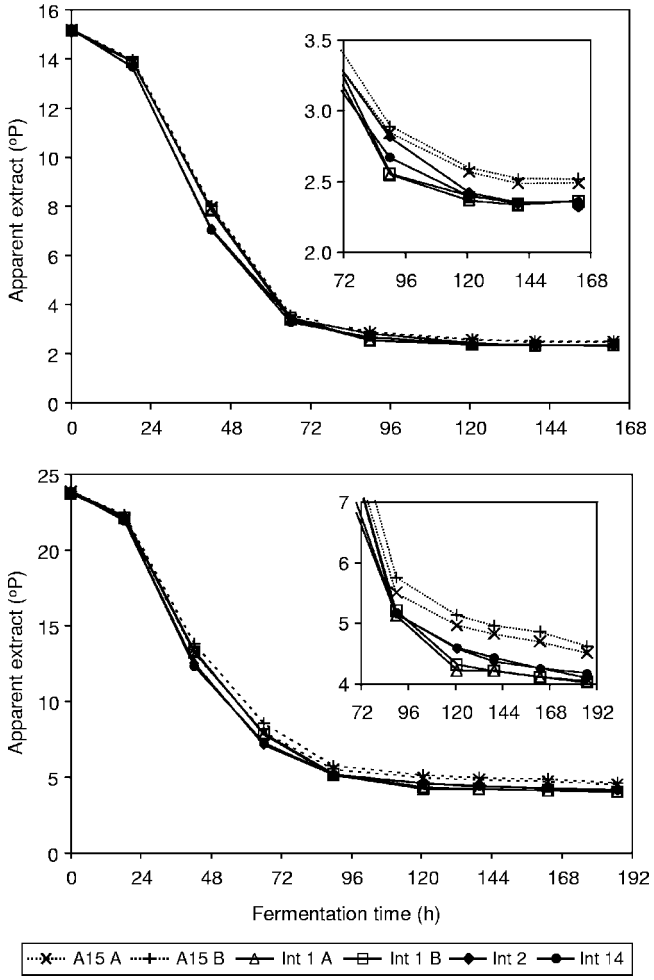

FIG. 4. Attenuation profiles during the fermentation of 15°P (upper panel) and 24°P (lower panel) worts by duplicate growths of strain A15 (A15A, A15B), duplicate growths of integrant 1 (Int 1 A, Int 1 B), and single growths of integrants 2 and 14 (Int 2, Int 14). The 15 and 24°P worts were pitched with, respectively, 5.0 or 8.0 g of fresh yeast mass · liter^{-1} at 10°C, and fermentations were continued at 14°C. The 24°P fermentations were shifted to 18°C at 20 h. Insets show detail during the last 4 or 5 days.

Time savings were also small (2 to 8 h) at an apparent attenuation of 80% but were significant (13 to 32 h, corresponding to 8 to 20% decreases in fermentation time) at 83% apparent attenuation.

For the 24°P worts, larger differences in final apparent extract occurred, 0.40 to 0.53°P in the series A fermentations and 0.25 to 0.39°P in the series B fermentations, which were performed 10 months later (Table 5). Time savings at an apparent attenuation of 80% were 13 to 57 h, corresponding to 10 to 30% decreases in fermentation time. Greater proportional decreases in fermentation time (14 to 37%) were observed in the B series at an apparent attenuation of 81%. Final ethanol production from the 24°P worts rose from 93 g · liter^{-1} for A15 to 95 to 96 g · liter^{-1} for the integrants, and the residual maltose and maltotriose levels were, respectively, about 1.5 and 4 to 7 g · liter^{-1} lower in beers made by the integrants than in those made by A15. Time savings and increases in ethanol production were greatest for integrant 1, which was also the first integrant to appear on the maltotriose-antimycin A plates during the selection of transformants.

Of yeast cells sampled after 20 h from the 24°P fermentations of series A, the integrants exhibited markedly higher maltose transport activity under standard assay conditions (5 mM maltose, pH 4.2, 20°C), but this difference was smaller at 72 h (Table 5) and at 120 and 168 h (data not shown). At 20 h, all of these fermenting worts still contained glucose (with the different yeast strains, the level of glucose fell from 13 ± 3 g · liter^{-1} at 16 h to 3.6 ± 1.0 g · liter^{-1} at 25 h), but it had disappeared by 42 h. In these series A 24°P fermentations, there was little difference between the rates of glucose and maltose consumption by the different yeasts (data not shown) but maltotriose was consumed more rapidly by the integrants. This difference was already evident at 42 h, when maltotriose had fallen from 42 g · liter^{-1} to 32.2 ± 0.7 g · liter^{-1} for strain A15 and 26.7 ± 0.1, 29.2, and 28.1 g · liter^{-1}, respectively, for integrants 1, 2, and 14. By 92 h, maltotriose was at 13.2 ± 0.5 g · liter^{-1} for strain A15 and 5.6 ± 0.3, 9.1, and 10.1 g · liter^{-1} for integrants 1, 2, and 14.

In both 15 and 24°P worts, integrants 1, 2, and 14 grew similarly to strain A15 (Fig. 5). Strain A15 is a so-called "dusty yeast" that sediments poorly at the end of wort fermentations, and this pattern was retained by the integrants. Integrants 2 and 14 sedimented more slowly than strain A15 at the end of fermentation.

During yeast handling, the viability of the integrants was essentially as good as that of strain A15 (Table 5). After propagation, strain A15 had a slightly higher viability (99.4% and 98.6% in series A and B) than the integrants (ca. 97.6 to 98.3%), but all of the strains cropped from the 15°P fermentations at 99% viability. Integrants cropped from the 24°P fermentations at higher viabilities (94.7 to 96.4%) than strain A15 (92.5 ± 2.5%) in series A, but no difference was observed in series B. Final beer pH values were consistently slightly (ca. 0.03 U) lower for the integrants than for strain A15, but these differences were small compared to the differences between beers from 24°P worts (4.29 ± 0.03 for all of the yeast strains) and 15°P worts (3.95 ± 0.03).

Most brewery fermentations are performed with yeast cropped from an earlier fermentation and used again. Figure 6 shows the result obtained when strain A15 and integrant 1 were cropped from a 24°P fermentation and repitched into a new batch of 24°P wort. As expected, tall-tube fermentations of 24°P wort by recycled yeast were much slower than when laboratory-grown yeast was used (this is because the recycled yeast contains smaller amounts of sterols and unsaturated fatty acids and therefore needs more oxygen than can be easily added to 24°P worts under laboratory conditions). However, the difference between strain A15 and integrant 1 was, at least qualitatively, preserved through the cropping and repitching: after 210 h of fermentation, the integrant 1 fermentations had an apparent extract 0.85 ± 0.23°P (mean ± range of duplicates) lower than that of the strain A15 fermentations.

Figure 7 shows the profiles of yeast-derived volatile aroma compounds in beers collected at the end of the series A 24°P fermentations. These esters and secondary alcohols and acetaldehyde are important components of beer flavor. In most cases, differences between the aroma profiles of beers made

TABLE 5. Wort fermentations by A15 and three integrants[a]

Parameter	A15	Integrant 1	Integrant 2	Integrant 14
Series A pitching yeast viability (%)	99.4[c]	98.2 ± 0.2	97.9	98.3
Series A 15°P fermentation				
Final AE (°P)	2.50 ± 0.02	2.36 ± 0.00	2.31	2.36
Extra AE (°P)		0.14	0.19	0.14
Time to 80% AA (AE = 3.0°P) (h)	83.1 ± 1.5	76.7 ± 0.9[b]	81.1	75.2
Time saved at 80% AA (h)		6.4	2.0	7.9
Time to 83% AA (AE = 2.6°P) (h)	121 ± 2	89 ± 0[f]	108	100
Time saved at 83% AA (h)		32	13	21
Final ethanol concn (g · liter^{-1})	57.3 ± 0.2	57.7 ± 0.2	57.7	57.1
Crop viability (%)	99.0 ± 0.2	99.2 ± 0.1	99.0	99.2
Series A 24°P fermentation				
Transport at 20 h (U · g DY^{-1})[d]	12.3 ± 0.2	17.2 ± 1.7	18.2	20.9
Transport at 72 h (U · g DY^{-1})[d]	6.7 ± 0.7	7.2 ± 0.8	7.9	7.7
Final AE (°P)	4.57 ± 0.05	4.04 ± 0.01	4.10	4.17
Extra AE (°P)		0.53	0.47	0.40
Time to 80% AA (AE = 4.8°P) (h)	161 ± 11	104 ± 2[b]	110	111
Time saved at 80% AA (h)		57	51	50
Final ethanol concn (g · liter^{-1})	93.3 ± 0.3	95.4 ± 0.1[b]	95.6	95.0
Final maltose concn (g · liter^{-1})	2.7[e]	1.1 ± 0.0	1.2	1.2
Final maltotriose concn (g · liter^{-1})	7.1[e]	0.0 ± 0.0	3.2	3.4
Crop viability (%)	92.5 ± 2.5	96.4 ± 1.6	95.3	94.7
Series B 24°P fermentation				
Pitching yeast viability (%)	98.6 ± 0.4	97.6 ± 0.2	98.3	97.7
Final AE (°P)	4.41 ± 0.03	4.02 ± 0.00	4.02 ± 0.01	4.16 ± 0.02
Extra AE (°P)		0.39	0.39	0.25
Time to 80% AA (AE = 4.8°P) (h)	126 ± 4	88 ± 1[f]	98 ± 2	113 ± 1
Time saved at 80% AA (h)		38	28	13
Time to 81% AA (AE = 4.6°P) (h)	154 ± 3	97 ± 5[b]	112 ± 2	133 ± 4
Time saved at 81% AA (h)		57	42	21
Final ethanol concn (g · liter^{-1})	93.1 ± 1.1	95.2 ± 0.8[b]	95.4 ± 0.4	94.9 ± 0.6
Crop viability (%)	96.0 ± 0.3	95.7 ± 1.1	95.0	96.3

[a] Series A fermentations are shown in Fig. 2 and 3, and series B fermentations for A15 and integrant 1 are shown in Fig. 6. AE, apparent extract; AA, apparent attenuation. Final AE, ethanol concentration, crop viability, and (where shown) maltose and maltotriose concentrations were measured after 165, 187, and 187 h, respectively, in the series A 15°P, series A 24°P, and series B 24°P fermentations. For A15 and integrant 1, results are means ± ranges from duplicate fermentations pitched with independently grown lots of yeast. For integrants 2 and 14, results are from single fermentations or, where shown, averages ± ranges of duplicate fermentations pitched with the same lot of yeast.

[b] The difference between integrant 1 and A15 duplicates is significant at $P < 0.1$ (two-tailed paired t test).

[c] Viability was measured for only one of the duplicate A15 growths.

[d] Transport shows the maltose transport activity as units per gram of dry yeast (DY) measured in yeast sampled at 20 and 72 h.

[e] Final sugar concentrations were measured in only one A15 duplicate fermentation; assays of both duplicates 18 h earlier gave 2.9 ± 0.1 and 8.1 ± 0.4 g of maltotriose · liter^{-1}.

[f] The difference between integrant 1 and A15 duplicates is significant at $P \leq 0.05$ (two-tailed paired t test).

with the integrants and strain A15 were small. However, integrants 1 and 2 produced ca. 30% more 3-methylbutyl acetate, which gives a banana-like flavor generally considered beneficial (taste threshold, 0.6 to 6 mg · liter^{-1}). Integrant 14 apparently produced 2.5-fold as much acetaldehyde as strain A15. Acetaldehyde (apple-like flavor) at this level would be undesirable in a lager beer.

DISCUSSION

Vidgren et al. (36, 37) found that all eight of the lager strains tested contained defective *AGT1* genes that cannot encode functional maltose and maltotriose transporters because of an extra thymidine residue at nucleotide 1183 which introduces a premature stop codon before the last four transmembrane domains. The present work shows that correcting this sequence defect and, at the same time, placing the *AGT1* genes under the control of a constitutive promoter increases the transport activity of lager strain A15 toward maltose and, especially, maltotriose and improves its fermentation performance in high-gravity and VHG worts. Transformants of strain A15 (integrants 1, 2, and 14) engineered in this way fermented 15 and 24°P worts faster and more completely than did strain A15, producing beers containing more ethanol and lower levels of residual maltose and maltotriose. This result confirms earlier findings (17, 27) that the rate of α-glucoside uptake is a major factor limiting the rate of wort fermentation. The size of the changes was economically significant (Table 5): primary fermentation times for 15 and 24°P worts were decreased by 8 to 20% and 11 to 37%, respectively, which represents a marked increase in annual output from the same-size brew house and fermentor facilities, and for 25°P worts the final ethanol concentrations were increased by 2% (from about 93 to 95 g of ethanol · liter^{-1}), giving a similar increase in yield from raw materials. Residual maltose and maltotriose in the final beers were markedly decreased (Table 5). These improvements were

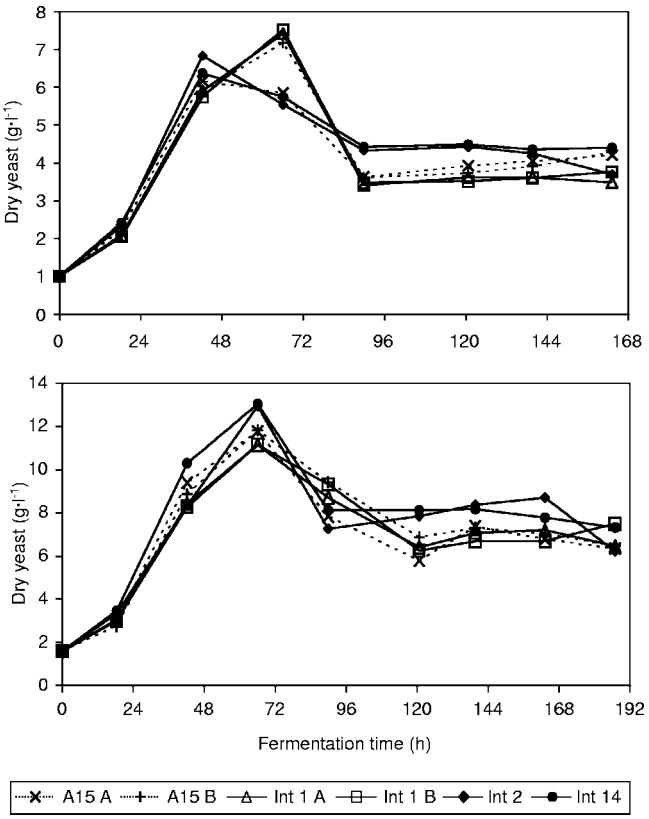

FIG. 5. Yeast in suspension during the fermentations shown in Fig. 4 of 15°P (upper panel) and 24°P (lower panel) worts by strain A15 and integrant 1 (Int 1), Int 2, and Int 14. The plots show the increases in yeast mass in suspension caused by growth during the first 2 to 3 days, followed by decreases as the yeast sediments in the static fermentations. Experimental details are the same as in the legend to Fig. 4.

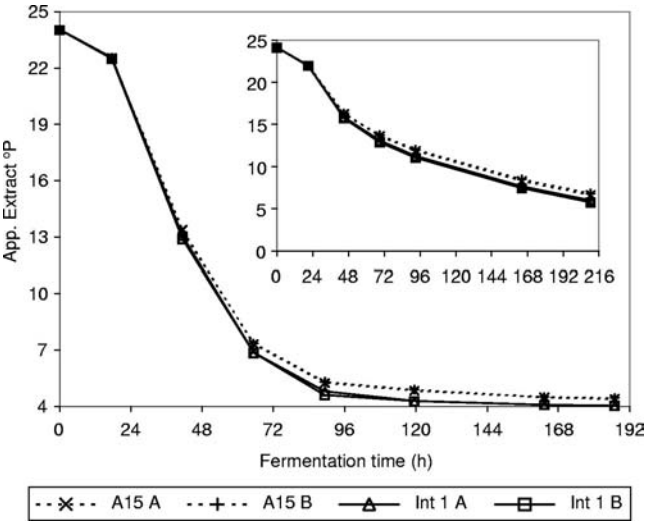

FIG. 6. Effect of cropping and repitching strain A15 and integrant 1. Duplicate growths of strain A15 (A15A, A15B) and integrant 1 (Int 1 A, Int 1 B) were pitched at 8.0 g of fresh yeast mass · liter^{-1} into 24°P wort, each fermentation being performed in duplicate. At 89 h, one of each duplicate pair was stopped and the yeast was cropped as described in Materials and Methods. The attenuation profile of the other duplicate is shown in the main part of the figure. The cropped yeasts were again pitched at 8.0 g of fresh yeast mass · liter^{-1} into 24°P wort, and the attenuation profiles of these fermentations are shown in the inset. App., apparent.

obtained without a change in yeast handling performance (propagation, growth, and sedimentation behavior; Table 5 and Fig. 5) and with little or no change in the levels of yeast-derived volatile aroma compounds (Fig. 7). For at least one transformant (integrant 1), improved fermentation performance compared to the parent strain was still observed when both yeast strains were recycled from one fermentation to the next (Fig. 6), as in normal brewery practice.

The integrants are not expected to contain any non-*Saccharomyces* DNA, and Southern analyses did not reveal any integration of bacterial DNA from the marker plasmid, pKX34, into the chromosomal DNA of integrant 1, 2, or 14. This test cannot exclude the adventitious integration of bacterial sequences too short for hybridization with the probes used. In any case, because the integrants were constructed by methods involving the use of restriction enzymes, it is probably a legal requirement in many countries that beers manufactured with these recombinant yeast strains (or analogous strains constructed by the same method from other brewer's yeast strains) must be labeled to indicate the use of genetically modified organisms.

During selection after transformation, integrant 1 already formed an observable colony on day 2, before integrants 2 and 14 (Table 2). Faster adaptation of integrant 1 to growth on maltotriose in the presence of antimycin was retained after nonselective growth of integrants 1, 2, and 14 through 110 generations (Table 4), so this appears to be a stable phenotype. After growth on glucose, integrant 1 had markedly higher maltose transport activity than integrants 2 and 14 and after growth on maltose it had markedly higher maltotriose transport activity than integrants 2 and 14. Integrant 1 showed somewhat greater improvements in VHG wort fermentation

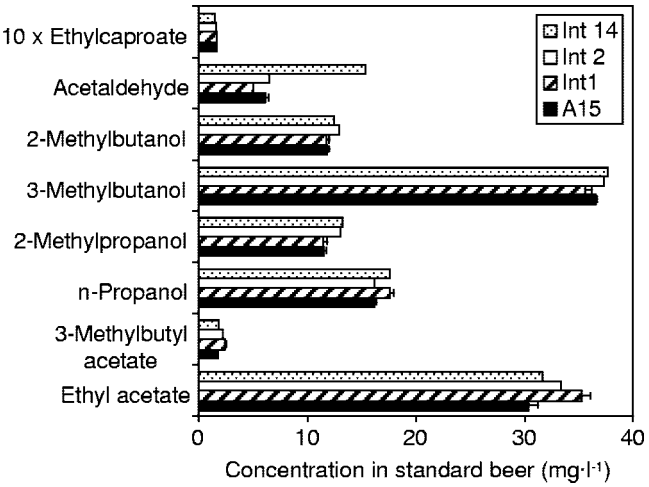

FIG. 7. Volatile aroma compounds in beers from series A 24°P fermentations. Results are normalized to a standard beer containing 35 g of ethanol · liter^{-1}. Error bars for integrant (Int) 1 and strain A15 show the range between the average and highest values for duplicate fermentations with independently grown yeast lots.

behavior than integrants 2 and 14 (Table 5). All three integrants contained both native and recombinant versions of *AGT1* and apparently more copies of the recombinant version than the native version (Fig. 2). Furthermore, none of these integrants contained tandem insertions of the cassette DNA (Fig. 2). Thus, whereas the physiological properties of these three integrants during the selection procedure and during wort fermentation seemed to correlate with their α-glucoside transport activities after growth on glucose or maltose, these transport activities did not, apparently, correlate with the number of repaired copies of *AGT1*. We have no clear explanation for the superior performance of integrant 1 compared to that of integrants 2 and 14. We cannot exclude the possibility that some of the selected clones, including integrant 1, carry other mutations in addition to repair of one or more copies of *AGT1*. Furthermore, if, as seems likely, strain A15 has at least three nonidentical chromosomes VII, the repaired *AGT1* genes of integrants 1, 2, and 14 may be on different versions of chromosome VII and these versions may exhibit different transcription behavior, perhaps resulting from different chromatin structures. *MAL* loci are located in telomeric regions that are known to be sensitive to chromatin modifications (15). It may also be relevant that sequencing of seven independent 2.3-kb PCR fragments extending from the new *PGK1* promoter to nucleotide 1825 of the *AGT1* ORF of integrant 1 suggested that this integrant contains a copy of *AGT1* driven by the *PGK1* promoter but still containing the premature stop codon at nucleotide 1183, in addition to two or more copies of repaired *AGT1*. However, there is evidence that the truncated Agt1 protein encoded by the native *AGT1* gene of strain A15 is not itself an active transporter (37).

Transcription analyses (Fig. 3) showed that integration of the cassette DNA did not alter the expression of other maltose transporter genes (*MTT1* and *MALx1*) or the maltase gene (*MALx2*) but caused increased expression of *AGT1* in integrants 1, 2, and 14 compared to that in strain A15 both during batch growth in the presence of glucose, maltose, or maltotriose and in the subsequent stationary phase. Possible differences between integrants in the expression level of *AGT1* were observed (expression was lower in integrant 2 than in integrants 1 and 14 in samples harvested at about 7 g of sugar · liter^{-1} and higher in integrant 14 than in integrants 1 and 2 in stationary-phase samples) but were close to the experimental error. A low level of expression of *AGT1* was observed in strain A15, which was independent of the sugar used (glucose, maltose, or maltotriose). In earlier work, expression of *AGT1* could not be detected in strain A15 by Northern analysis during batch growth on glucose, maltose, or glucose-maltose mixtures (36). With a different TRAC probe (complementary to nucleotides 1358 to 1388 of the *AGT1* ORF), constant but weak expression of *AGT1* was detected during wort fermentations by another lager strain (28).

After growth on glucose (which represses the endogenous α-glucoside transporter genes), the integrants showed an absolute increase in maltose transport capacity, compared to that of strain A15, of up to 8 U · g of dry yeast^{-1} (for integrant 1), but after growth on maltose (which induced the endogenous genes), the absolute increase was smaller (only 2 U · g of dry yeast^{-1} for integrant 1). Also during wort fermentations, although higher maltose transport activity was observed in integrants than in strain A15 at 20 h, while glucose was still present, the absolute difference between integrants and the parent strain was smaller later in the fermentation (Table 5). Several mechanisms intervene between α-glucoside transporter genes and α-glucoside transport activity. According to the classical model (9, 16, 19, 25), these genes are induced by maltose and maltotriose and repressed by glucose in laboratory yeasts growing at relatively low sugar concentrations (ca. 20 g · liter^{-1}), and similar observations have been reported for lager strains (including A15) under such conditions (see, e.g., reference 36). However, Rautio et al. (28) found that during VHG wort fermentations by lager strains, expression of the *MALx1*, *MALx2*, and *MTT1* genes started to be induced while glucose concentrations were still high (25 g · liter^{-1}), reached a maximum at about the time maltose started to be used, and then rapidly decreased while maltose and maltotriose levels were still high. The cause of this decrease in expression is not known. The recombinant *AGT1* genes in integrants 1, 2, and 14 are driven by the constitutive *PGK1* promoter, and so *AGT1* mRNA is expected to be expressed in the presence of glucose. With glucose-grown cells harvested at about 7 g of glucose · liter^{-1}, we found much higher expression of *AGT1* in integrants 1, 2, and 14 than in host strain A15 (but no expression of *MTT1*, *MALx1*, or *MALx2* in any strain; Fig. 3). In laboratory strains, maltose transporters are subject to glucose-triggered catabolite inactivation and this proteolytic process is related to the presence of PEST sequences in the N-terminal cytoplasmic domains of some maltose transporters (22). Such sequences are not found in Agt1 and Mphx transporters (5). However, nothing is known about how catabolite inactivation affects the stability of Agt1 transporters in brewer's yeast strains. It is also not known how the expression of these recombinant genes might impact on mechanisms regulating the expression of the endogenous genes, such as the above-mentioned decrease in the expression of these genes while maltose and maltotriose are still present. A further relevant factor is that space in the plasma membrane is limited, so that when the recombinant *AGT1* genes and endogenous transporter genes are simultaneously expressed, the transporters synthesized may compete with each other for membrane space. In this case, some molecules of endogenous maltose transporters might be replaced by Agt1 molecules without much increase in the total number of maltose transporter molecules per unit of membrane. This hypothesis is supported by the finding that after growth on YP containing 20 g · liter^{-1} maltose, there was only a modest (ca. 10% for integrant 1) increase in maltose transport activity but a larger increase in maltotriose transport activity (2.5-fold for integrant 1), which is consistent with the replacement of transporters with high specificity for maltose (e.g., Malx1 transporters) by the broader-specificity Agt1 transporters. Similar findings have been reported by other workers: overexpression of *AGT1* increased maltotriose transport activity threefold but slightly decreased maltose transport activity (33), whereas specific deletion of *AGT1* abolished maltotriose transport activity but caused a nearly 20% increase in maltose transport activity (1). To improve our understanding of how to increase transport activities (which is relevant to most biotechnological operations), it is evidently necessary to learn more about the processes that may limit the successful insertion of transporters into the plasma

membrane, including possible competition between different proteins for space in the membrane.

The selection strategy (growth of glucose-repressed cells on maltotriose in the presence of the respiratory inhibitor antimycin A) was exceptionally effective; e.g., all 36 selected clones that were tested contained a *PGK1* promoter between the promoter and ORF of the *AGT1* gene. Both lager and baker's yeast strains have difficulty adapting from growth on glucose to growth on maltotriose when respiration is inhibited (20, 40), and for strain A15 this difficulty seems to be greater than for some other lager strains (6). In principle, the integration cassettes used to transform lager strain A15 should work in the same way with other lager strains, since all of the lager strains studied contain the same premature stop codon in the ORFs of their *AGT1* genes (36, 37) and both of the lager strains studied have identical *AGT1* promoter sequences (V. Vidgren, M. Kankainen, J. Londesborough, and L. Ruohonen, unpublished results). The *AGT1* promoters of ale yeasts are essentially identical to those of lager yeasts from nucleotide −1 to nucleotide −564 (Vidgren et al., unpublished), so that transformation of ale yeasts with the short cassette is expected to put their *AGT1* genes (which do not contain the premature stop codons found in lager strains) under the control of the constitutive *PGK1* promoter. This tactic might increase the maltose and maltotriose transport capacity and fermentation performance of ale yeasts, but this has not yet been tested.

This work was started before the discovery of the *MTT1* genes. These genes encode α-glucoside transporters, for which maltotriose is the preferred substrate rather than maltose (30). They have been found in all of the lager yeasts tested but not, so far, in ale yeasts (30, 37). The Mtt1 transporters function better than Agt1 transporters at the low temperatures (≤10°C) characteristic of traditional lager fermentations (37), whereas in present industrial practice lager fermentations with high-gravity worts are often conducted at higher temperatures (14 −18°C), as was also done in this work. The success of the present work, however, suggests that putting also the *MTT1* genes of lager strains under the control of constitutive promoters would be likely to improve their fermentation performance.

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Paper III

The temperature dependence of maltose transport in ale and lager strains of brewer's yeast

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RESEARCH ARTICLE

The temperature dependence of maltose transport in ale and lager strains of brewer's yeast

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Abstract

Lager beers are traditionally made at lower temperatures (6–14 °C) than ales (15–25 °C). At low temperatures, lager strains (*Saccharomyces pastorianus*) ferment faster than ale strains (*Saccharomyces cerevisiae*). Two lager and two ale strains had similar maltose transport activities at 20 °C, but at 0 °C the lager strains had fivefold greater activity. *AGT1*, *MTT1* and *MALx1* are major maltose transporter genes. In nine tested lager strains, the *AGT1* genes contained premature stop codons. None of five tested ale strains had this defect. All tested lager strains, but no ale strain, contained *MTT1* genes. When functional *AGT1* from an ale strain was expressed in a lager strain, the resultant maltose transport activity had the high temperature dependence characteristic of ale yeasts. Lager yeast *MTT1* and *MALx1* genes were expressed in a maltose-negative laboratory strain of *S. cerevisiae*. The resultant Mtt1 transport activity had low temperature dependence and the Malx1 activity had high temperature dependence. Faster fermentation at low temperature by lager strains than ale strains may result from their different maltose transporters. The loss of Agt1 transporters during the evolution of lager strains may have provided plasma membrane space for the Mtt1 transporters that perform better at a low temperature.

Introduction

Among different beer types, ales are made by fermentation of brewer's wort at 15–25 °C, whereas lagers are traditionally made by fermentation at lower temperatures, 6–14 °C (Bamforth, 1998). Brewer's yeasts can be divided into ale strains (which cannot use melibiose) and lager strains (which can). Ale strains usually grow and ferment poorly at temperatures below about 12 °C, whereas lager strains perform well down to at least 7 °C (Walsh & Martin, 1977). Ale strains have been used for thousands of years, but it has been suggested that lager strains probably originated in low-temperature wort fermentations in Bavaria a few hundred years ago (see, e.g. Hornsey, 2003).

Most ale strains are thought to be varieties of *Saccharomyces cerevisiae* (Hammond, 1993; Tornai-Lehoczki & Dlauchy, 2000; Kobi *et al.*, 2004). However, it has been shown recently that they include strains (e.g. isolates from Trappist beers) that are hybrids between *S. cerevisiae* and *Saccharomyces kudriavzevii* (Gonzalez *et al.*, 2008) and some other ale strains previously classified as *S. cerevisiae* may be hybrids (Querol & Bond, 2009). Lager strains have been variously described as *Saccharomyces carlsbergensis*, *Saccharomyces uvarum* and *Saccharomyces pastorianus* (Hammond, 1993). They have alloploid genomes and are hybrids of two species: *S. cerevisiae* and *Saccharomyces bayanus* (Naumova *et al.*, 2005; Caesar *et al.*, 2007; Dunn & Sherlock, 2008). The hybrids are thought to have been selected during low-temperature wort fermentations. The cryophilic nature of lager yeasts probably derives from the *S. bayanus* partner (Sato *et al.*, 2002). The complete sequencing of lager strain WS34/70 confirmed its hybrid nature: 36 chromosomes were found, 16 of *S. cerevisiae* type, 12 of *S. bayanus* type and 8 chimeric (Nakao *et al.*, 2009). The presence of the chimeric chromosomes (part *S. cerevisiae*, part *S. bayanus*) suggested posthybridizational reorganization. Dunn & Sherlock (2008) also reported posthybridizational reorganization of lager strain chromosomes, and divided lager strains into two groups, originating from two separate hybridization events between *S. cerevisiae* and *S. bayanus*. They propose that distinct, but similar, *S. cerevisiae* strains were involved in the two events, and that hybridization was

followed by the loss of a large portion of the *S. cerevisiae* genome from Group 1, whereas in Group 2, the loss of *S. cerevisiae* genes was much smaller.

The α-glucosides maltose and maltotriose together account for about 80% of the total fermentable sugars in brewer's wort. Efficient assimilation of these sugars is essential for fast and complete wort fermentations. All yeast α-glucoside transport systems characterized so far are H^+-symporters that are driven by the electrochemical proton gradient across the plasma membrane. The active transport of maltose and maltotriose across the plasma membrane is a major rate-limiting step in the fermentation of wort (Kodama *et al.*, 1995; Rautio & Londesborough, 2003; Stambuk *et al.*, 2006).

Several kinds of genes for α-glucoside transporters are found in *Saccharomyces* yeasts. *MALx1* genes ($x = 1$–4 and 6) occur in five unlinked *MAL* (maltose) loci (*MAL1*–*MAL4* and *MAL6*). Most studies (e.g. Han *et al.*, 1995; Salema-Oom *et al.*, 2005; Alves *et al.*, 2008) indicate that the Malx1 transporters are narrowly specific for maltose (K_m ~3 mM) and turanose, but activity towards maltotriose has been claimed (Day *et al.*, 2002a). The identical *MPH2* and *MPH3* (**m**altose **p**ermease **h**omologue) occur on different chromosomes and encode transporters able to carry maltose (K_m ~4 mM), maltotriose (K_m ~7 mM), α-methylglucoside and turanose (Day *et al.*, 2002b). *AGT1* (**α**-**g**lucoside **t**ransporter) encodes the α-glucoside transporter with the widest substrate specificity reported so far (Han *et al.*, 1995). The Agt1 transporter can carry trehalose and sucrose (K_m ~8 mM) as well as maltose, maltotriose and α-methylglucoside (K_m 20–35 mM) (Stambuk & de Araujo, 2001). A gene called *MTT1* (**mt**y-like **t**ransporter; Dietvorst *et al.*, 2005) or *MTY1* (**m**altotriose **t**ransport in **y**east; Salema-Oom *et al.*, 2005) encodes a transporter that has a higher affinity for maltotriose (K_m ~20 mM) than for maltose (K_m ~70 mM) and can also carry trehalose and possibly turanose (Salema-Oom *et al.*, 2005). Nakao *et al.* (2009) have recently sequenced the genome of the lager strain, Weihenstephan 34/70. They found a gene, LBYG13187, that they believe to be the *S. bayanus* counterpart of the *S. cerevisiae AGT1* because its closest homology was 79% identity to the *AGT1* sequence in the *Saccharomyces* genome database (SGDB, where *AGT1* is referred to as *MAL11*). Here, we call this gene *Sb-AGT1*, although nothing is known yet about the substrate specificity or other properties of the transporter it encodes.

Several authors have compared maltose transport by individual ale and lager yeast strains. For example, Crumplen *et al.* (1996) found that glucose more strongly inhibited maltose transport by an ale strain than that by a lager strain. Rautio & Londesborough (2003) found that trehalose and sucrose (a substrate of only Agt1 among known maltose transporters) strongly inhibited maltose transport by an ale strain, but only weakly inhibited maltose transport by a lager strain. Vidgren *et al.* (2005) reported that α-methyl glucoside (a substrate of only Agt1 and Mphx transporters) inhibited maltose transport into two ale strains by 41–74%, but inhibited maltose transport into three lager strains by only 10–23%. Taken together, these results suggested that the dominant maltose transporters of the ale strains studied had a broader specificity than those of lager strains, and were probably Agt1 proteins.

However, hybridization studies showed that all the ale and lager strains tested contained *AGT1* and several *MALx1* genes (Jespersen *et al.*, 1999; Vidgren *et al.*, 2005). This apparent discrepancy was partially resolved by the finding (Vidgren *et al.*, 2005) that the *AGT1* genes of two lager strains contained premature stop codons. The same defect has been found in other lager strains, but not in ale strains (Vidgren *et al.*, 2007; Nakao *et al.*, 2009). *MTT1* genes were found in all four lager strains examined by Dietvorst *et al.* (2005). *MPHx* sequences were found (usually on chromosome IV, corresponding to *MPH2*) in some, but not all lager strains and less frequently in ale strains (Jespersen *et al.*, 1999; Vidgren *et al.*, 2005). The expression of *MPHx* genes seems to be strain specific. Expression was very low in several lager strains growing on maltose or glucose (Vidgren *et al.*, 2005), but Gibson *et al.* (2008) and James *et al.* (2003) found a stronger expression of *MPHx* during wort fermentations with other lager strains.

The rates of most enzyme-catalysed reactions approximately double for each 10 °C increase in temperature. However, reactions catalysed by integral membrane proteins usually exhibit nonlinear Arrhenius plots with increased temperature dependence at lower temperatures, where the structure of the membrane lipids changes from a more fluid liquid-crystalline phase to a more rigid gel phase. This phase transition depends on the membrane lipid composition (e.g. the presence and type of sterols and fatty acids) and on external factors, such as the osmotic pressure (Guyot *et al.*, 2006).

Rautio & Londesborough (2003) found strong temperature dependence for maltose transport (*c.* 70-fold between 0 and 20 °C) for an ale strain. However, Guimarães *et al.* (2006) reported a markedly smaller temperature dependence (*c.* 11-fold) for a lager strain. The apparent predominance of Agt1 transporters in ale strains, but not in lager strains, suggested that the different temperature dependencies of maltose transport (and, therefore, the fermentation rate) in ale and lager strains might reflect differences in the properties of their maltose transporters. This article presents evidence supporting this hypothesis. We report (1) the distribution of *MTT1*, defective *S. cerevisiae*-derived *AGT1* and *S. bayanus*-derived *AGT1* genes among ale and lager strains and (2) the temperature dependence of maltose transport into ale and lager strains and into genetically engineered yeasts expressing *AGT1*, *MALx1* and *MTT1*

maltose transporter genes from particular brewer's yeast strains. A preliminary report of some of this work has been given (Vidgren *et al.*, 2007).

Materials and methods

Materials

U-^{14}C maltose was from Amersham Biosciences (Espoo, Finland). Maltose for uptake experiments (minimum purity, 99%) and trehalose were from Sigma-Aldrich (Helsinki, Finland), and maltotriose was from MP Biomedicals (Solon, OH). Maltose and glucose for growth media were from Fluka (Helsinki, Finland). G418 was from Invitrogen (Espoo, Finland).

Strains

The industrial strains used in this work are listed in Table 1. CMBS-33 was kindly provided by J.M. Thevelein (Katholieke Universiteit, Leuven, Belgium) and WS34/70 was from the Weihenstephan Brewery (Freising, Germany). The other strains were from the VTT Culture Collection. The frequently used strains A-63015, A-66024, A-75060 and A-10179 are hereafter referred to as A15, A24, A60 and A179, respectively. Two laboratory strains, CEN.PK2-1D (VW-1B; maltose-positive) and S150-2B (maltose-negative), were also used.

Table 1. Distribution of *AGT1*, *Sb-AGT1* and *MTT1* genes in some industrial yeasts

Strain	Origin	*AGT1*	*Sb-AGT1**	*MTT1*
Lager strains				
A-60012	Weihenstephan 1	D	P	P
A-62013	Weihenstephan 294	D	P	P
A-63015 (A15)	Nordic brewery	D	P	P
A-66024 (A24)	Nordic brewery	D	P	P
A-82064	Nordic brewery	D	P	P
A-85072	Nordic brewery	D	P	P
A-95143	Nordic brewery	D	P	P
WS34/70	Weihenstephan	D	P	P
CMB33	Belgium	D	P	P
Ale strains				
A-10179 (A179)	UK brewery	P	M	M
A-60055	NCYC 1200	P		M
A-60056	NCYC 240	P		M
A-75060 (A60)	Nordic brewery	P	M	M
A-93116	NCYC 1087	P		M
Baker's yeasts				
B-62001	Nordic baker's yeast	P		P
B-62003	Nordic baker's yeast	P		P
Distiller's yeasts				
C-72051	Nordic distillery	P		P
C-77076	Nordic distillery	M		M
C-91180A	Nordic distillery	M		M

*Sb-AGT1 indicates a gene with 79% identity to *AGT1* recently discovered in WS34/70 by Nakao *et al*. (2009).
D, defective, frame shift mutation; P, present; M, missing.

PCR analyses

Primers are shown in Table 2. PCR reactions were performed using standard procedures. To test for the presence of *MTT1* genes, total chromosomal DNA from each strain was used as a template with MTT1 Frw and MTT1 Rev primers to generate a 247-bp fragment. To test for the presence of *S. cerevisiae*-type *AGT1* genes, the primers AGT1 Frw and AGT1 Rev were used to generate 986-bp fragments. These fragments (842–1828 of the *AGT1* ORF) include the frame shift and premature stop codon (starting at nucleotide 1183) described previously (Vidgren *et al.*, 2005) in lager strains A15 and A24. They were cloned to a pCR-TOPO vector (Invitrogen) and sequenced using the AGT1Sekv4 primer to test for the frame shift. The putative *S. bayanus*-type *AGT1* gene (LBYG13187; Nakao *et al.*, 2009) was also studied. Most of the sequence of this gene has been published (Nakao *et al.*, 2009) and the 5′-terminal 400-bp sequence was kindly provided by Dr Y. Nakao. Total chromosomal DNA from several brewer's yeast strains and laboratory strain CEN.PK2-1D was used as a template with AGT1bay_Cl_Frw and AGT1bay_Cl_Rev primers to generate 1833-bp fragments corresponding to the complete ORF and stop codon. The fragments obtained were cloned into the pCR-TOPO vector and their sequences were determined using AGT1baySekv1–AGT1baySekv3 primers and universal M13 forward and reverse primers, which bind close to the cloning sites of pCR-TOPO.

Laboratory strains bearing an ale or a lager strain *AGT1* gene in a multicopy plasmid

The *AGT1* genes from lager strain A15 and ale strains A60 and A179 were cloned by PCR using AGT1-F and AGT1-R primers. The sequences of these clones were verified as described earlier (Vidgren *et al.*, 2009). AGT1-F and AGT1-R primers bear BglII restriction sites, which facilitated the next cloning step, i.e., the ligation of the PCR fragments to YEplac195 multicopy vectors (Gietz & Sugino, 1988) at the BglII site between the *PGK1* promoter and terminator. In addition, the *KanMX* cassette (Wach *et al.*, 1994) was introduced into the YEplac195 plasmid at the multiple cloning site to confer resistance to G418. The laboratory strain S150-2B was transformed with these YEplac195-*PGK1-AGT1-KanMX* constructs or with the empty YEplac195-*KanMX* plasmid as a control. The lithium acetate transformation procedure (Gietz *et al.*, 1992) was used and transformants were selected using G418 selection.

Table 2. PCR primers

Name	Primer sequence*	Sequence detected†
MTT1_Cl_Frw	5′-CGAGATCTCGATGAAGGGATTATCCTCATT-3′	1–20 of *MTT1*
MTT1_Cl_Rev	5′-CGAGATCTCGTCATTTGTTCACAACAGATGG-3′	1828–1848 of *MTT1*
MTT1Sekv1	5′-CTTTGAATAGCAATACAG-3′	404–421 of *MTT1*
MTT1Sekv2	5′-AGAACTAGGATATAAGCT-3′	801–818 of *MTT1*
MTT1Sekv3	5′-TATCCAATATTGTCTTGG′3′	1212–1229 of *MTT1*
MTT1Sekv4	5′-GGTTATGTTTTGCCACTC-3′	1601–1618 of *MTT1*
MTT1Frw	5′-TTGGTAGGTTTGACCTTTAC-3′	1271–1290 of *MTT1*
MTT1Rev	5′-AGATGCCATATTATATGCGT-3′	1499–1518 of *MTT1*
AGT1Frw	5′-TTGCTTTACAATGGATTTGGC-3′	842–862 of *AGT1*
AGT1Rev	5′-CTCGCTGTTTTATGCTTGAGG-3′	1808–1828 of AGT1
AGT1Sekv4	5′-AAAGCAGATTGAATTGAC-3′	1011–1028 of *AGT1*
AGT1bay_Cl_Frw	5′-CGAGATCTCGATGAAAAATATACTTTCGCTGG-3′	1–22 of *Sb-AGT1*
AGT1bay_Cl_Rev	5′-GCAGATCTCGTCATAACGCCTGTTGACTCG-3′	1814–1833 of *Sb-AGT1*
AGT1baySekv1	5′-CCTACGATATCACTTCTC-3′	443–460 of *Sb-AGT1*
AGT1baySekv2	5′-CGCCTTACAATGGATCTG-3′	843–860 of *Sb-AGT1*
AGT1baySekv3	5′-ACGCTTGGTTCCTGGGTA-3′	1255–1272 of *Sb-AGT1*

*BglII restriction sites are underlined.

†The numbering is from the first nucleotide of the translational start. *Sb-AGT1* refers to the putative *Saccharomyces bayanus*-derived counterpart of *AGT1* (Nakao *et al.*, 2009).

Laboratory strains bearing a lager strain *MTT1* or *MALx1* gene in a multicopy plasmid

MTT1 and *MALx1* genes were cloned from lager strain A15 by PCR with standard procedures using MTT1 Cl Frw and MTT1 Cl Rev primers, which contain BglII sites. Because the sequences of the *MTT1* and *MALx1* ORFs are identical to each other at both their starts and their ends, both genes were obtained with these primers. The PCR products were cloned into the pCR-TOPO vector and their sequences were determined using MTT1Sekv1–MTT1Sekv4 primers and universal M13 forward and reverse primers, which bind near the cloning site of pCR-TOPO. From the nine sequenced clones, four were $>99\%$ identical to the *MTT1* sequence reported by Dietvorst *et al.* (2005) and five were $>98\%$ identical to the *MAL31* type sequence in the SGDB. Clone 1 was 100% identical to the *MTT1* sequence of Dietvorst *et al.* (2005) and was chosen to represent *MTT1*. Clone 2 was 99% identical to the *MAL31* sequence in the SGDB and was chosen to represent *MALx*1. They were excised from the pCR-TOPO plasmid using the BglII enzyme and ligated between the *PGK1* promoter and terminator at the BglII site in the YEplac195 multicopy vector. The laboratory strain S150-2B was transformed with either the YEplac195-*PGK1-MTT1* or the YEplac195-*PGK1-MALx1* construct using the lithium acetate transformation procedure (Gietz *et al.*, 1992).

Construction of a lager yeast with an integrated, ale yeast-type *AGT1* gene

Construction of Integrant 1 has been described previously (Vidgren *et al.*, 2009). Briefly, the defective *AGT1* gene in the lager strain A15 (with a premature stop codon at nucleotide 1183) was repaired using an integration cassette containing nucleotides 1–1478 of the *AGT1* ORF from ale strain A60 functionally fused to a *PGK1* promoter and flanked on the 5′-side by the *AGT1* promoter sequence (-1 to -705). The ORF of the repaired gene has the ale yeast sequence from nucleotide 1 to somewhere between 1183 and 1478 (i.e. the frame shift and premature stop codon are removed), followed by the lager yeast sequence to the end of the *AGT1* gene, and it is under the control of a *PGK1* promoter.

Maltose transport assays

For maltose transport studies, native ale and lager strains were grown in YP (10 g yeast extract and 20 g peptone L^{-1}) containing 40 g maltose L^{-1}. YP-40 g glucose L^{-1} was used for the growth of Integrant 1, so that its endogenous maltose transporters were repressed. YP-40 g glucose L^{-1} supplemented with G418 (200 mg L^{-1}) was used for S150-2B derivatives transformed with a YEplac195-*KanMX* plasmid (with or without an *AGT1* gene). S150-2B derivatives transformed with YEplac195 plasmids lacking *KanMX* (and with or without an *MTT1* or *MALx1*) were grown in a synthetic complete medium (Sherman *et al.*, 1983) lacking uracil and containing 20 g glucose L^{-1}. Yeasts were grown in 100 mL of medium in 250-mL Erlenmeyer flasks at 150 r.p.m. and 24 °C. They were usually harvested at an $OD_{600\,nm}$ between 4 and 7 (i.e. at 2 ± 1 mg dry yeast mL^{-1}) while sugar was still present, but were grown into the stationary phase when so stated. After centrifugation (10 min, 9000 ***g***, 0 °C), the yeast pellets were washed with ice-cold water and then with ice-cold 0.1 M tartrate-Tris (pH 4.2) and finally suspended in

the same buffer to 200 mg of fresh yeast mL^{-1}. For standard assays, about 1-mL portions of yeast suspension were equilibrated for 10 min to assay temperature (0–20 °C) in a water bath. Zero-*trans* [^{14}C]-maltose uptake rates were then determined at 5 mM maltose (unless stated otherwise) as described (Guimarães *et al.*, 2006). Reactions were started by adding 40 μL of yeast suspension to 20 μL containing 15 mM [^{14}C]-maltose (about 1000 c.p.m. $nmol^{-1}$) and any inhibitors specified in the text. Reactions were stopped after 10–300 s by addition of 10 mL ice-cold water and immediate filtration through a (prewashed) HVLP membrane (Millipore). The membrane was rinsed with another 10 mL of ice-cold water and transferred to a scintillation cocktail and the radioactivity in the trapped yeast was counted. To ensure linearity with respect to time, two reaction times, *t* and 2*t*, were used, each in duplicate. Reaction times were chosen according to the yeast and temperature, so that the reaction rate calculated from the 2*t* assays was at least 90% of that calculated from the *t* assays.

Stimulation of maltose transport by glucose

Where indicated, yeast suspensions were treated with glucose immediately before the maltose transport assays as described by Guimarães *et al.* (2008). The yeast suspension was mixed with 0.1 volume of 0.28 M glucose, incubated for 8 min at 20 °C and then maltose transport was assayed as described above. For the study of Fig. 1, the glucose-treated yeast was first assayed at 20 °C, then immediately transferred to an ice-water bath and assayed at 0 °C after 15 min and again after 36 min. The yeast suspension was then immediately returned to a 20 °C bath and assayed after a further 15 min.

Results

Functionality and distribution of *S. cerevisiae*-type *AGT1* genes among yeast strains

The *AGT1* maltose transporter genes in two ale strains (A60 and A179) differ slightly from the sequence in the SGDB, but encode full-length proteins, whereas those in two lager strains (A15 and A24) encode truncated, 394 amino acid polypeptides because of a frame shift and a premature stop codon at nucleotide 1183 (Vidgren *et al.*, 2005). To determine the functionality of these genes, the laboratory strain S150-2B (maltose-negative) was transformed with plasmids containing *AGT1* genes from A15, A60 or A179 under *PGK1* promoters or with the empty plasmid. Transformants were grown on glucose, harvested in the early stationary phase and assayed for maltose transport. Transformation with the empty plasmid or with *AGT1* from lager strain A15 did not increase transport activity. Transformation with *AGT1* from ale strains increased the maltose transport activity from $< 0.2\,U\,g^{-1}$ dry yeast (control plasmid) to $13.1 \pm 0.4\,U\,g^{-1}$ dry yeast (*AGT1* from A60; mean ± SD, $n = 3$) or $10.0\,U\,g^{-1}$ dry yeast (*AGT1* from A179). Maltose transport by transformants carrying *AGT1* genes from A60 or A179 was strongly inhibited by 50 mM maltotriose (85% and 79%, respectively) and 75 mM trehalose (94% and 85%, respectively), which is characteristic of maltose transport by the broad specificity Agt1 transporter. Between 0.5 and 55 mM maltose, the transporter encoded by *AGT1* from A60 exhibited a single K_m of 1.5 mM maltose, which is lower than that (5–10 mM) estimated by Han *et al.* (1995) and much lower than that reported (18 mM) by Stambuk & de Araujo (2001). These results show that the *AGT1* genes from these two ale strains encode functional, broad-specificity

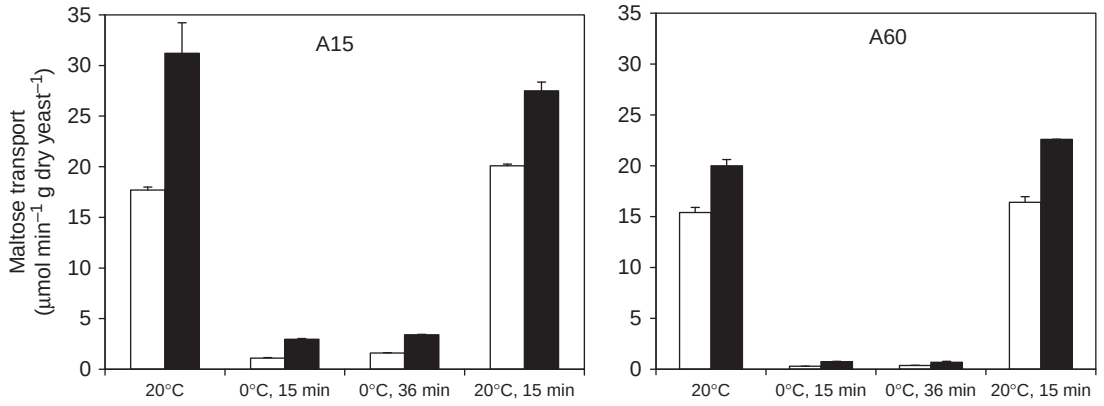

Fig. 1. Temperature dependence of maltose transport by lager (A15) and ale (A60) strains. Yeasts were harvested during growth on maltose at 24 °C and their maltose transport activities were assayed at 20 and 0 °C. For standard assays (white columns), the yeasts were equilibrated to 20 °C for 8 min and then assayed at 20 °C, transferred to 0 °C and assayed at 0 °C after 15 and 36 min and then returned to 20 °C and reassayed at 20 °C after 15 min. For glucose-activated assays (black columns), the same procedure was used, except that after 4 min at 20 °C, glucose was added to 28 mM and incubation was continued for 8 min before the first 20 °C assay. Results are averages ± ranges of duplicate assays.

α-glucoside transporters, whereas the defective *AGT1* gene from lager strain A15 does not encode a functional maltose transporter.

The distribution of *AGT1* genes in different kinds of industrial yeasts was studied by PCR (Table 1). All nine lager strains studied contained *S. cerevisiae*-type *AGT1* genes with the same defect as strains A15 and A24. All five ale strains, both baker's strains and one of the three distiller's strains studied contained *AGT1* genes without this defect. The other two distiller's strains lacked *AGT1*. It can be concluded that this particular *AGT1* gene mutation, producing a premature stop codon, is characteristic of lager strains. These studied lager strains are not, to our knowledge, more closely related to each other than are lager strains in general.

Distribution of an *S. bayanus*-type *AGT1* gene in brewer's yeast strains

The *Sb-AGT1* gene (Nakao *et al.*, 2009) is only 79% identical at the nucleotide level to *AGT1* from *S. cerevisiae*, and so might not be revealed by earlier Southern hybridization and PCR studies using probes and primers designed for *AGT1* from *S. cerevisiae* (Jespersen *et al.*, 1999; Vidgren *et al.*, 2005). Using primers designed for *Sb-AGT1*, we found this gene in all the lager strains studied, but not in either studied ale strain (Table 1) or in the maltose-positive laboratory strain CEN.PK2-1D. In two tested lager strains, A15 and A24, the sequence of the *Sb-AGT1* gene was 100% identical to that reported by Nakao *et al.* (2009) for *Sb-AGT1* of WS34/70, which encodes a polypeptide of 610 amino acids.

Distribution of *MTT1* genes in ale and lager strains

MTT1 genes have earlier been demonstrated in lager strains PYCC4457 (the type strain of *S. carlsbergensis*) (Salema-Oom *et al.*, 2005) and A15, CMBS33, OG2252 and WS34/70 (Dietvorst *et al.*, 2005). We found *MTT1* in all nine tested lager yeast strains (including three of the above-mentioned ones), but not in any of the five ale strains (Table 1). An *MTT1* gene was also present in both tested baker's strains and in one of the three distiller's strain, the same that also contained an *AGT1* gene.

The temperature dependence of maltose uptake by brewer's yeast strains

Brewer's yeasts were harvested during growth on maltose at 24 °C and their maltose transport activities were assayed at different temperatures. For lager strain A15, the activity measured in the standard way at 0 °C was 9.3 ± 0.9% of that at 20 °C, whereas for ale strain A60, the activity at 0 °C was 2.1 ± 0.1% of that at 20 °C (Fig. 1, open columns).

Maltose transport is active and depends on the transmembrane electrochemical potential. When yeast cells growing on fermentable sugar are harvested, washed and suspended in a medium lacking a carbon source, their intracellular adenylate energy charge (and therefore their membrane potential) can decrease. The adenylate energy charge and maltose transport rates of such cells can be increased by treatment with glucose for a few minutes immediately before the zero-*trans* maltose uptake assay (Guimarães *et al.*, 2008). This activation with glucose increased the maltose transport activity of both A15 and A60, but did not eliminate the difference in temperature sensitivity between the two yeasts. For glucose-activated A15, the maltose transport rate at 0 °C was 10.9 ± 1.4% of that at 20 °C, and for glucose-activated A60, it was 3.4 ± 0.3% (Fig. 1, black columns). These results showed that there was a difference between the temperature sensitivities of maltose transport by the lager and ale strains that could not be explained by differences in adenylate energy charge. In further work, standard assays, without glucose activation, were used.

When harvested during growth on maltose, two lager strains and two ale strains had similar maltose transport activities at 20 °C, but the activities of both ale strains were markedly more temperature dependent than those of the lager strains (Fig. 2). At 20 °C, the difference between the lager and ale strains was not significant (in μmol min^{-1} g^{-1} dry yeast, 20.3 ± 3.5 for the lager strains and 19.2 ± 5.9 for the ale strains; means ± SDs, $n = 5$, $P > 0.72$ (two-tail Student's *t*-test). However, at 0 °C, the lager strains had about

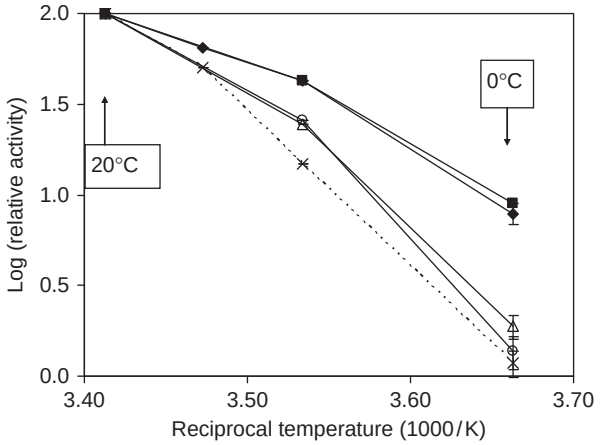

Fig. 2. Arrhenius plots of maltose transport by the lager strains A15(◆) and A24(■), ale strains A179(○) and A60(Δ) and the strain Integrant 1 (×). For each data set, rates are expressed as percentages of the rate at 20 °C. Absolute rates (μmol min^{-1} g^{-1} dry yeast at 20 °C) varied between 12 and 27 for A15, A24, A60 and A179 and between 2 and 4 for Integrant 1. Results at 0 °C are means ± SDs for A15 ($n = 4$) and A60 ($n = 3$) and means ± ranges of independent duplicates for A179 and Integrant 1.

fivefold greater activity than the ale strains and the difference was highly significant. Maltose transport activities at 0 °C in μmol min^{-1} g^{-1} dry yeast were 1.7 ± 0.4 (8.4% of the 20 °C activity) for the lager strains and 0.31 ± 0.05 (1.6% of the 20 °C activity) for the ale strains (means ± SDs, $n = 5$; $P < 0.002$). The relatively smaller activities of the ale strains were also evident at 10 °C.

The temperature dependence of an Agt1-type transporter

Integrant 1 is a derivative of lager strain A15 containing a chimeric *AGT1* in place of the defective native *AGT1* of A15. The chimera consists of nucleotides 1 to *x* (where *x* is between 1183 and 1478) of an *AGT1* gene from ale strain A60 and nucleotides *x*+1 to 1848 of the native *AGT1* of strain A15, driven by a *PGK1* promoter (Vidgren *et al.*, 2009). It encodes a functional, 616 amino acid Agt1 transporter, with the same amino acid sequence as Agt1 of strain A60, because after nucleotide *x*, the ale and lager versions of *AGT1* encode the same amino acid sequence (Vidgren *et al.*, 2005). Compared with A15, Integrant 1 has considerably increased maltose and maltotriose transport activity during growth on glucose (when A15 has negligible activities) and slightly increased maltose transport activity, but considerably increased maltotriose transport activity during growth on maltose (Vidgren *et al.*, 2009). Thus, Integrant 1 produces a functioning Agt1 transporter in a lager yeast background. When grown on glucose, this Agt1 is expected to be the only maltose transporter present (because glucose-grown A15 lacks maltose transport activity). The temperature dependence of maltose transport by glucose-grown Integrant 1 was much greater than that of the lager strains and at least as great as that of the ale strains (Fig. 2).

The temperature dependence of Malx1 and Mtt1 transporters

MALx1 (99% identical to *MAL31* in the SGDB) and *MTT1* (100% identical to the sequence reported by Dietvorst *et al.*, 2005) were cloned from lager strain A15. The maltose-negative laboratory yeast, S150-2B, was transformed with plasmids containing these genes under the control of *PGK1* promoters. Untransformed S150-2B had negligible maltose transport activity (< 0.2 μmol min^{-1} g^{-1} dry yeast at 20 °C). *MALx1* transformants had high activity (55 μmol min^{-1} g^{-1} dry yeast) during growth on glucose and lower activity (6 μmol min^{-1} g^{-1} dry yeast) in the stationary phase. Both growing- and stationary-phase transformants exhibited strong temperature dependence (Fig. 3). The rates at 0 °C compared with 20 °C were 2.8 ± 0.4% (mean ± SD, $n = 5$) for growing cells and 1.9 ± 0.6% (mean - range; $n = 2$) for stationary-phase cells.

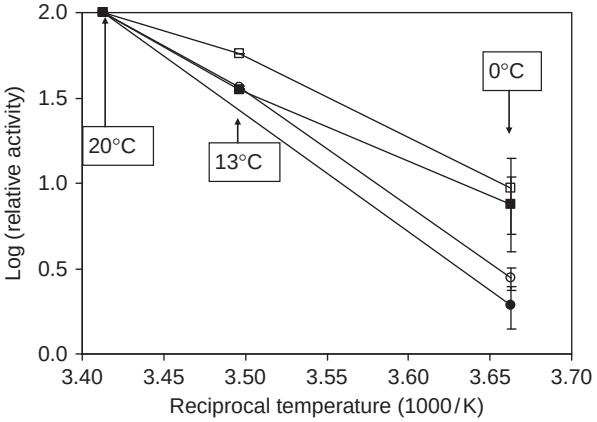

Fig. 3. Arrhenius plots of maltose transport by Mtt1 and Malx1 transporters. Maltose transport was measured using S150-2B transformed with *MTT1* (□, ■) or *MALx1* (○, ●). The maltose concentration in the transport assay was 5 mM (□, ○, ●) or 50 mM (■) and transformants were harvested during growth on glucose (at $OD_{600\,nm}$ of 3–5; □, ■, ○) or in the stationary phase ($OD_{600\,nm}$ of 10; ●). Rates at 13 and 0 °C are expressed as percentages of the rate at 20 °C. Values at 0 °C are (□, ○) means ± SDs ($n = 4$ or 5) or (■, ●) means ± ranges of duplicate experiments.

Compared with *MALx1* transformants, both growing and stationary-phase *MTT1* transformants exhibited lower maltose transport activity at 20 °C, about 1.0 μmol min^{-1} g^{-1} dry yeast at 5 mM maltose and 6.5 μmol min^{-1} g^{-1} dry yeast at 50 mM maltose. Mtt1 is reported to have a high K_m for maltose (60–90 mM, Salema-Oom *et al.*, 2005; 40 mM, Multanen, 2008). At both maltose concentrations, the transport activity exhibited relatively small temperature dependence (Fig. 3). Activities at 0 °C compared with 20 °C were 9.4 ± 4.4% (5 mM; mean ± SD, $n = 4$) and 7.5 ± 2.5% (50 mM; mean ± range, $n = 2$). The absolute maltose transport activity at 50 mM maltose (6.5 μmol min^{-1} g^{-1} dry yeast) was similar to that at 5 mM maltose of stationary-phase cells transformed with *MALx1* (6 μmol min^{-1} g^{-1} dry yeast). Thus, the marked differences in temperature dependence between cells transformed with *MALx1* and cells transformed with *MTT1* are not explained by differences in their absolute maltose transport activities.

Discussion

Because Agt1 is the only maltose transporter known to accept both trehalose and α-methyl glucoside as substrates (Day *et al.*, 2002b; Salema-Oom *et al.*, 2005), the strong inhibition of maltose transport by both trehalose and α-methyl-glucoside in ale strains, but not lager strains (Rautio & Londesborough, 2003; Vidgren *et al.*, 2005), suggests that Agt1 transporters are the dominant maltose transporters in ale strains, but not lager strains. Vidgren *et al.* (2005) showed that two lager strains, A15 and A24, contain *AGT1*

genes with a premature stop codon starting at nucleotide 1183. Here, we show that the *AGT1* from A15 does not encode a functional maltose transporter. Nakao *et al.* (2009) also found this premature stop codon in an *AGT1* gene in a third lager strain, WS34/70. We extend these results to show that the premature stop codon at 1183 is present in the *S. cerevisiae*-type *AGT1* genes of all nine tested lager strains, but is absent from all five tested ale strains. Because the premature stop codon is caused by an easily reversible point mutation, then if the Agt1 transporter were advantageous to lager strains in their normal habitat, one would expect to find lager strains in which this reversal has occurred. The observations that reversal has not occurred in any of the nine tested lager strains, whereas the mutation causing the premature stop codon was not present in any of the five ale strains, suggest that there has been selection pressure in favour of inactivation of *AGT1* during the evolution of lager strains, but not during the evolution of ale strains. The main difference between the conditions under which ale and lager strains have evolved is the lower temperature of lager fermentations. *MTT1* genes were present in all nine lager strains, but in none of the five ale strains. This suggests that the replacement of Agt1 transporters by Mtt1 transporters is an important difference between lager and ale strains, probably related to the lower temperature of lager fermentations.

We show that maltose transport is more strongly temperature dependent in two tested ale strains than in two tested lager strains (the yeasts were grown at 24 °C and then assayed at different temperatures). At 20 °C, all four strains had similar maltose transport activity, but at 0 °C, the ale strains showed about fivefold smaller activities. When single maltose transporters were studied, using genetically engineered strains, their temperature dependence decreased in the order Agt1 ≥ Malx1 > Mtt1. The temperature dependence of Mtt1 (in a laboratory strain) was similar to that of maltose transport by lager yeasts. An ale-type Agt1 transporter working in a lager yeast (Integrant 1) had the high temperature dependence of maltose transport observed for ale yeasts (Fig. 2). This suggests that the different temperature dependencies of maltose transport by ale and lager yeasts result from the different maltose transporters present in these yeasts rather than, for example, a hypothetical difference in the lipid composition of their plasma membranes. Thus, the Agt1 transporter in Integrant 1 had been inserted into a lager yeast membrane, but still exhibited high temperature dependence, which therefore was a property of the transporter protein itself rather than a property of the lipid membrane of ale strains.

We do not yet know what differences between Agt1 and Mtt1 transporters account for their different temperature dependencies. Membrane proteins are sensitive to membrane lipid composition and dynamics. They can have specific lipid requirements for their optimal activity (Opekarová & Tanner, 2003), correct orientation of transmembrane helices (Bogdanov *et al.*, 2002), targeting to the yeast plasma membrane (Umebayashi & Nakano, 2003; Toulmay & Schneiter, 2007) and stable localization in the plasma membrane (Mitsui *et al.*, 2009). The high temperature dependence of reactions catalysed by enzymes embedded in lipid membranes may result from work carried out by the enzyme on surrounding lipid as a result of changes in protein shape during the catalytic cycle (Londesborough, 1980). Thus, one possibility is that Agt1 exhibits greater shape changes than Mtt1 during the catalytic cycle, and so performs more work on the surrounding lipid membrane. Nakao *et al.* (2009) found seven α-glucoside transporter genes in the genome of lager yeast WS34/70, two of which, *S. bayanus*-derived *MALx1* and *S. cerevisiae*-derived *AGT1*, encoded truncated proteins. They also noted an increase in the copy number of *MTT1*, which was present on both the *S. bayanus* and the *S. cerevisiae* versions of chromosome VII. These results are consistent with the suggestion that in lager strains, Mtt1 transporters have become more important at the expense of Agt1 and Malx1 transporters. Nakao *et al.* (2009) located a *MAL31* gene to *S. cerevisiae* chromosome II (Chr. Sc-II) and an *MPH2* gene to Chr. Sc-IV. These loci were earlier observed in most, but not all, studied lager strains by hybridization of specific probes to chromosome blots (Jespersen *et al.*, 1999; Vidgren *et al.*, 2005). These hybridization studies found binding of a *MAL61*-probe (expected to recognize all *MALx1* genes) to Chr. VII from both lager and ale strains, whereas Nakao *et al.* (2009) found an *MTT1* gene on both the *S. cerevisiae* and the *S. bayanus* versions of Chr. VII from lager strain WS34/70. *MTT1* is 91% identical to *MALx1*, and so probably in the lager strains, the *MAL61*-probe bound to *MTT1* genes, which were not known at the time of these hybridization studies. Nakao *et al.* (2009) found a truncated *S. cerevisiae*-derived *AGT1* gene, but did not locate the gene. Presumably, this is the *AGT1* detected on Chr. VII in both hybridization studies. Nakao *et al.* (2009) also reported an *S. bayanus*-derived *AGT1* (*Sb-AGT1*) on Chr. Sb-XV-VIII and a truncated, *S. bayanus*-derived *MAL31* on Chr. Sb-V. Neither of these genes was noted in the hybridization studies, which used probes based on *S. cerevisiae* sequences. Our present results show that *Sb-AGT1* is present in all eight studied lager strains, but, as expected, in neither of the two ale strains studied. The sequence of the *Sb-AGT1* in strains A15 and A24 was identical to that reported by Nakao *et al.* (2009). No information is available on the catalytic properties of the transporter encoded by this gene (it is only 79% identical to the *AGT1* from *S. cerevisiae*).

Both hybridization studies detected *MAL21* and *MAL41* genes on Chr. III and Chr. XI, respectively. Such genes were not observed by Nakao *et al.* (2009) in Weihenstephan

34/70. The estimated sequence coverage was 95.8%, and so one or both genes might be in the unsequenced 4.2%. Alternatively, Weihenstephan 34/70 may lack these genes. Hybridization indicated that *MAL21* was lacking from 13 of the 25 studied lager strains and *MAL41* was lacking from one lager strain (Jespersen *et al.*, 1999). Both our present results and those of Nakao *et al.* (2009) show that in lager strains, the number of different functional α-glucoside transporters is less than the number of different α-glucoside transporter (pseudo)genes. Inactivation of genes encoding less suitable transporters seems to be one way in which lager strains have evolved for low-temperature fermentations.

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Paper IV

Identification of regulatory elements in the *AGT1* promoter of ale and lager strains of brewer´s yeast

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IDENTIFICATION OF REGULATORY ELEMENTS IN THE *AGT1* PROMOTER OF ALE AND LAGER STRAINS OF BREWER'S YEAST

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Running title
Regulatory elements in *AGT1* promoters of ale and lager strains

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ABSTRACT

Agt1 is an interesting α-glucoside transporter for the brewing industry as it efficiently transports maltotriose, a sugar often remaining partly unused during beer fermentation. It has been shown that on maltose the expression level of *AGT1* is much higher in ale strains than in lager strains, and that glucose represses the expression particularly in the ale strains. In the present study the regulatory elements of the *AGT1* promoter of one ale and two lager strains were identified by computational methods. Promoter regions up to 1.9 kbp of the *AGT1* gene were sequenced from the three brewer's yeast strains and the laboratory yeast strain CEN.PK-1D. The promoter sequence of the laboratory strain was identical to the *AGT1* promoter of S288C strain of *Saccharomyces* Genome Database, whereas the promoter sequences of the industrial strains diverged markedly from the S288C strain. The *AGT1* promoter regions of the ale and lager strains were on most parts identical to each other except for one 22 bp deletion and two 94 and 95 bp insertions in the ale strain. Computational analyses of promoter elements revealed that the promoter sequences contained several Mig1p- and MAL-activator binding sites, as was expected. However, some of the Mig1p and MAL-activator binding sites were located on the two insertions of the ale strain, and thus offer a plausible explanation to the different expression pattern of the *AGT1* gene in the ale strains. Additionally, telomeric silencing as a possible means of regulation of *AGT1* expression is discussed.

INTRODUCTION

The genome of the yeast *Saccharomyces cerevisiae* contains from one to five *MAL* loci that are located in the telomeric regions of its different chromosomes. The *MAL* locus of *S. cerevisiae* has been described as a highly constant genomic structure consisting of three genes that are all necessary for maltose utilization (Charron *et al*., 1989). A 0.9 kbp intergenic region between the maltose transporter-encoding *MALx1* gene and maltase-encoding *MALx2* gene forms a divergent promoter from which *MALx1* and *MALx2* are transcribed to opposite directions. The third gene, *MALx3*, is located centromeric to the *MALx1-MALx2* gene pair and it encodes an activator protein needed for efficient expression of the two other *MAL* genes in the locus. The structure of this *MAL* locus is shown in Fig 1. Some *MAL* loci are known to possess extra copies of one or more of the *MAL* genes (Charron *et al*., 1989; Michels *et al*., 1992). In some yeast strains another α-glucoside transporter encoding gene, *AGT1*, replaces *MALx1* in the *MAL1* locus. Agt1 transporters differ from Malx1 transporters by their wider substrate range (Han *et al*., 1995; Salema-Oom *et al*., 2005) including their ability to carry maltotriose.

For the brewing industry, a particularly interesting function of the Agt1 transporter is its ability to efficiently transport maltotriose, as incomplete fermentation of maltotriose (which accounts for about 15–20 % by mass of the fermentable sugars in wort) is a common problem in brewing fermentations. Indeed, practically all brewing strains screened harbour the *AGT1* gene (Jespersen et al. 1999; Vidgren *et al*., 2005). Although the *AGT1* gene was found both in ale (*S. cerevisiae*) and lager (*S. pastorianus*) strains, it encodes a truncated non-functional protein in the lager strains (Vidgren *et al*., 2005; Nakao *et al*., 2009; Vidgren *et al.*, 2010).

Ale and lager strains also markedly differed in their *AGT1* gene expression patterns when grown on glucose (repressing), maltose (inducing) or a mixture of maltose and glucose. In all these three media the expression of the *AGT1* gene was very weak in the lager strains, but strong in the

ale strains (Vidgren *et al*., 2005). It was also clearly seen that glucose repressed the expression of *AGT1* and maltose induced it in both ale and lager strains (Vidgren *et al*., 2005). The difference in the *AGT1* expression levels between the ale and lager strains might be due to faster decay of the mRNA encoding the non-functional protein product in the lager strains. Another possibility is that the expression of the MAL-activator encoding gene or the action of the activator itself is different in the ale and lager strains. The latter could be due, for example, to a specific mutation in the MAL-activator protein. It has been shown that alterations of certain amino acids in the activation domain of Mal33 affect the expression of *MAL31* and *MAL32* genes (Higgins *et al*., 1999). A third possibility, the one discussed in the present paper, is that the *AGT1* genes are differently regulated in these strains due to different structures of their promoters.

The *MAL61-62* divergent promoter region originating from *Saccharomyces pastorianus* has been studied in detail (Levine *et al*., 1992; Hu *et al*., 1995; Sirenko *et al*., 1995). The region is 99% identical to the *MAL31-32* intergenic region of the laboratory strain S288C (SGD *Saccharomyces* Genome Database) implying the conserved nature of the region. The *MAL61-62* and *MAL31-32* divergent promoter regions are 874 and 875 bp long, respectively. As shown in Figure 1 they contain two symmetrically oriented TATA boxes near to the start of each of the two genes (Hong and Marmur, 1987), three binding sites for the MAL-activator (Levine *et al*., 1992; Sirenko *et al*., 1995) and two consensus sequences for the binding of the Mig1 transcription factor (Hu *et al*., 1995). Maltose induction is controlled by the MAL-activator elements (Levine *et al*.,1992; Yao *et al*., 1994) and repression by glucose is partly controlled through the Mig1 binding sites and partly through the regulation of expression of the MAL-activator encoding gene by Mig1 (Hu *et al*., 1995). Associations between Mig1 and *MALx3* genes have been seen in genome-wide analyses (ChIP-on-chip) (Harbison *et al*., 2004; Mukherjee *et al*., 2004).

Bell and co-workers identified a 147 bp repeat element in the divergent promoter region of *MALx1* and *MALx2* of baker's yeast. The repeat element was found as a single copy in *S. pastorianus* derived *MAL61-MAL62* and laboratory strain derived *MAL31-MAL32* divergent promoters, but as up to five copies in some *MALx1-MALx2* divergent promoters of baker's yeast. The copy number of the repeat element correlated to the expression of *MALx1* gene with decrease in expression with increasing copy number of the repeat element (Bell *et al*., 1997). Evidence of the role of chromatin remodelling and structure on the regulation of expression of *MAL* genes also exist. A microarray study revealed that depletion of histone H4, a modification that reduces nucleosome content, caused in addition to the other transcriptomic changes a more than a 10-fold de-repression of *AGT1* and *MAL12* (Wyrick *et al*., 1999). *MALx1* and *MALx2* genes were shown to be repressed by telomeric silencing mediated by the COMPASS complex during late stages of fermentation (Houghton-Larsen and Brandt, 2006).

In the laboratory strain S288C the *AGT1* gene resides in the *MAL1* locus instead of *MALx1*. Between *AGT1* and *MALx2* genes there is a 785 bp intergenic region that forms a divergent promoter for the two genes. The sequence between *AGT1-MAL12* is only partly identical to the conventional *MALx1* and *MALx2* intergenic region. The first 470 bps upstream of the *MAL12* are identical to the conventional *MALx1-MALx2* intergenic region whereas the remaining 315 bps are not. The sequence divergence starts in the 147 bp repeat element in such a way that 47 out of the 147 bp of the repeat element are located in the homologous region. However, most of the regulatory elements are located in the identical region between *MALx1- MALx2* and *AGT1-MALx2* divergent

promoters, and therefore the same *cis*-elements are found in each of the divergent promoters, except for one Mig1 binding element, which is missing in the *AGT1-MALx2* promoter region.

Very little is known about the structures of promoters in industrial strains as most studies have been performed with laboratory strains. Thus, the aim of the present study was to investigate the transcriptional control of *AGT1* and the *cis*-elements involved in the regulation particularly in the industrial brewer's strains. The promoter region of *AGT1* of two lager strains and one ale strain were sequenced and promoter element analyses were performed. This enabled us to conclude that the different expression levels of *AGT1* genes in the ale and lager strains studied under similar conditions may indeed be related to differences in their highly similar promoters.

MATERIALS AND METHODS

Strains

Two industrial lager strains A-63015 and A-66024, one industrial ale strain A-75060 hereafter referred to as A15 and A24 and A60, respectively, from the VTT Culture Collection and a laboratory strain CEN.PK2-1D (VW-1B) were used. The strains were cultivated in YP-glucose medium (1% yeast extract, 2% peptone, 2% glucose).

Chromosome walking

Total chromosomal DNA was isolated as previously described (Ausubel *et al*., 1998). Ligation-mediated PCR amplification was based on the work of Mueller and Wold (1989). 50 ng of total chromosomal DNA of strains A15, A24, A60 and CEN.PK2-1D was digested with the blunt-end-generating restriction enzymes *Dra*I, *Hae*III, *Alu*I or *Pvu*II for 1 h at 37°C. The restriction enzymes were removed with the Qiagen QIAquick PCR purification kit (Qiagen, Helsinki, Finland) and DNA was eluted in 30 µl Elution Buffer (Qiagen). Digested DNA was ligated in a linker mixture containing PCR linker I (5'-GCGGTGACCCGGGAGATCTGAATTC-3') and PCRlinker II (5'-GAATTCAGATCT-3') primers with T4 DNA ligase for 3h at room temperature. After the ligation, ligase and primer nucleotides were removed with the Qiagen QIAquick PCR purification kit and DNA was eluted with 50 µl Elution Buffer.

The first PCR reaction was performed with 5 µl of the ligation mixture, 5 µl PCR linker I primer (1 pmol/µl) and 5 µl of specific primer for the first round of chromosome walking ChrWalk1oligo1 from the *AGT1* gene (10 pmol/µl); the specific primers used in this study are listed in Table 1. The PCR reaction was performed as follows: 94°C for 3 min, 30 x (94°C for 1 min, 60, 53 or 58 °C for 2 min and 72 °C for 2 min) and 72 °C for 10 min. Annealing temperatures for the specific primers were different in the three rounds of chromosome walking; 60, 53 and 58 °C for the first (ChrWalkoligo1), second (ChrWalk2oligo1) and third (ChrWalk3oligo1) round, respectively. For the second PCR, 1 to 10 µl of a 1/50 dilution of the previous PCR product, 2.5 µl of the PCR linker I primer (1 pmol/µl) and 2.5 µl of a specific nested primer (ChrWalk1nested; ChrWalk2nested and ChrWalk3nested) were used (Table 1). Conditions for the second PCR were the same as for the first, except that annealing temperatures of 55, 58 and 59 °C were used, respectively, for primers ChrWalk1nested, ChrWalk2nested and ChrWalk3nested. PCR fragments were resolved on 1% agarose gels. In chromosome walking round I, fragments were seen only after digestion of total chromosomal DNA with *Dra*I, *Hae*III and *Alu*I; chromosomal DNA digested with *Pvu*II did not produce any fragments. With *Hae*III-digested DNA from strains A15 and A60 as

templates, 750 bp fragments were formed. With *Dra*I- and *Alu*I-digested DNA from these strains as templates 500 bp and 350 bp fragments, respectively, were formed. With *Hae*III-digested DNA from strain CEN.PK2-1D as template a 1000 bp fragment was produced. PCR fragments were resolved on a 1% preparative agarose gel. Fragments were cut out and purified with Qiagen's gel purification kit and cloned to the pCR-TOPO vector (Invitrogen, Espoo, Finland).

For the second round of chromosome walking, new specific primers were planned according to the sequence obtained from the first round. Specific primers ChrWalk2oligo1 and nested primer ChrWalk2nested were used (Table 1). Otherwise chromosome walking was performed identically to the first round. After the secondary (nested) PCR reaction, the PCR fragments were resolved on 1% agarose gels. Fragments were seen only when *Dra*I- or *Hae*III-digested total chromosomal DNA was used as a template. With *Dra*I-digested DNA as a template, a fragment of 250 bp was formed from strains A15, A24 and A60, and a fragment of 700 bp from strain CEN.PK2-1D. With *Hae*III-digested DNA, fragments of 700 bp were obtained from all the strains. Fragments were cloned to the pCR-TOPO vector.

For the third round of chromosome walking, specific primers (ChrWalk3oligo1 and ChrWalk3nested) were generated according to the sequences obtained from the second round of the chromosome walking. Otherwise chromosome walking was performed identically to the earlier rounds. With *Dra*I-digested DNA from strains A15, A24 and A60 as templates fragments of 1000 bp (1200 bp for strain A60) were formed. No fragments were obtained from the laboratory strain CEN.PK2-1D with these specific primers. With *Pvu*II, *Hae*III or *Alu*I-digested DNA as templates no fragments were obtained from any of the strains. Fragments obtained from *Dra*I-digested DNA were resolved on 1% preparative agarose gels, purified with Qiagen's gel purification kit and cloned to a pCR-TOPO vector.

Sequencing

Fragments generated in the three rounds of chromosome walking were cloned to pCR-TOPO vectors by using the TOPO TA cloning kit (Invitrogen, Espoo, Finland) and propagated in *Escherichia coli*. Plasmid DNA was isolated from different *E. coli* clones and two independent clones were sequenced for each fragment. The *AGT1* promoter fragments ligated to pCR-TOPO vector were sequenced by universal M13 Forward and Reverse primers. The model 3100 Genetic Analyzer sequencer was used (Applied Biosystems, Foster, CA). The sequences of the *AGT1* promoters of A15, A24 and A60 lager strains were deposited into the NCBI database under accession numbers GI 194740076 (A15), GI 194740077 (A24) and GI 194740078 (A60), respectively.

Computational analyses

AGT1 promoter sequences homologous to the ale and lager strain sequences used in the present study were obtained by using BLASTN (Altschul *et al.*, 1997) against the genomic database limited to available *Saccharomyces* strains. In the first BLASTN search, the *AGT1* gene sequence of the strain A15 (GI: 68989458) was used as a query and matches having an alignment length of ≥500 bp and sequence identity of ≥70% were retained.

Matches were further evaluated by retrieving their upstream sequences (~2000 bp) and constructing pair-wise alignments using EMBOSS-tools (Rice *et al.*, 2000). In the second BLASTN-search, the lager and ale promoter sequences were used and matches were retained when

having an alignment length of ≥ 500 bp. Matches were extended to 2000 bp when necessary, and pooled together with the promoter set of the first search. Multiple sequence alignments were constructed using DIALIGN-TX and MUSCLE (Subramanian *et al*., 2008; Edgar, 2004). Alignments were checked and improved manually by trimming flanking sequences and non-homologous regions. Binding elements of the promoters of the ale and lager strains were identified using the tools of SCPD and Yeastract databases (Zhu and Zhang, 1999; Teixeira *et al*., 2006).

RESULTS

Analysis of the upstream sequences of the ale and lager *AGT1* genes

Three rounds of chromosome walking were carried out with the chromosomal DNA of the ale strain A60 and the lager strains A15 and A24. 1906 bp of sequence in A60 and 1740 bp of sequence in A15 and A24 upstream of the *AGT1* gene were obtained. In the laboratory strain CEN.PK2-1D 801 bp of sequence was obtained with two rounds of chromosome walking. The *AGT1-MAL12* promoter region of the laboratory strain was 100 % identical to the corresponding region of S288C strain for the entire divergent promoter region and the sequence identity continued into the maltase gene region. In contrast, the upstream region of the *AGT1* gene in the ale and lager strains differed markedly from the corresponding region of the laboratory strains. The first 315 bp of the *AGT1* promoter (-315 to -1 of promoter) were almost identical (99 %) to the corresponding promoter of the laboratory strains S288C and CEN.PK2-1D after which the sequences diverged totally from the laboratory strain sequence.

The *AGT1* promoter regions of the lager strains A15 and A24 were 100 % identical to each other in the entire region sequenced (-1 to -1740 bp) but differed from the corresponding promoter of the ale strain A60 (Fig. 2). Compared to the *AGT1* promoters of the lager strains the promoter of the ale strain had a deletion of 22 nucleotides and two insertions. These insertions were 94 bp and 95 bp long and although being approximately the same size they were not otherwise similar. In the laboratory strain CEN.PK2-1D the maltase gene *MAL12* was found at the expected location 785 bp upstream from the *AGT1* gene start codon (Fig. 2). In contrast, the maltase gene was not found in the ale and lager strains in spite of the three rounds of chromosome walking and up to 1906 bp of the upstream sequence identified. To see if there are some other genes located in the upstream sequence of the *AGT1* genes of the brewer's strains studied, the sequenced regions were analysed for open reading frames (ORFs) using NCBI ORF finder (http://www.ncbi.nlm.nih.gov/gorf/gorf.html). Two ORFs longer than 300 bp were found in the upstream regions in both the ale and lager strains. A BLAST search against SGD Fungal Genomes Database (http://www.yeastgenome.org/cgi-bin/blast-fungal.pl) was performed with these ORFs but no significant matches were found.

Homologous *AGT1* promoter sequences present in gene banks

To find homologous promoter regions for the ale and lager strains SGD Fungal Genome Database (http://www.yeastgenome.org/cgi-bin/blast-fungal.pl) and NCBI databases limited to different *Saccharomyces* strains were searched using BLASTN. These searches yielded four highly identical and four less-conserved matches for the *AGT1* promoters that are shown in Table 2. High identity to four strains was found with the *AGT1* promoter sequences obtained from the lager strains (Table 2). These strains were *Saccharomyces pastorianus* Weihenstephan 34/70 and three different strains of

S. cerevisiae; M22, RM11-1a and YPS163. Of these, RM11-1a is a haploid derivative of Bb32, a natural isolate from a Californian vineyard (*Saccharomyces cerevisiae* RM11-1a Sequencing Project, Broad Institute of Harvard and MIT (http://www.broad.mit.edu)). *S. cerevisiae* strains M22 and YPS163 are also natural isolates of *S. cerevisiae*; YPS163 was isolated from an oak tree and M22 from a vineyard in Italy (Doniger *et al*., 2008).

The less conserved *Saccharomyces sensu stricto AGT1* gene promoter sequences were discovered by using the *AGT1* coding sequence of lager strain A15 as a reference. Several significantly similar (80–82% identity) *AGT1* gene sequences were found and their corresponding promoter regions were retrieved and compared to that of the lager strain. The promoter regions of the *AGT1* genes had diverged more than the coding sequences of the *AGT1* gene, and were only 42–61 % identical to the *AGT1* promoter region of the lager strain. Among the sequences found, the upstream sequences of the *AGT1* genes in *S. paradoxus* NRRL Y-17217 contig 294, *S. mikatae* IFO 1815 contig 789 and *S. mikatae* IFO 1815 YM4906 contig 2883 showed the highest identities (approximately 60 %) to the corresponding sequence of the lager strain. The lager strain WS34/70 has two types of *AGT1* genes and consequently two types of promoters, one derived from the *S. cerevisiae* and the other from the *S. bayanus* component of this hybrid strain. The *S. bayanus*-derived *AGT1* promoter sequence of WS34/70 is included in Table 2. The sequence divergence (only 43 % identity) detected between the *S. cerevisiae*- and *S. bayanus* -derived *AGT1* promoter sequences makes it highly unlikely that the primers used in the chromosome walking of the present study would have annealed to the promoter region of the *S. bayanus AGT1* gene.

Analysis of promoter elements by computational methods

The homologous *AGT1* promoter sequences were aligned (Supplementary Fig. 1) to search for common promoter elements. The alignment highlights that the promoter regions of the *AGT1* gene in the strains analysed (lager A15 (this study), ale A60 (this study), *S. cerevisiae* RM11-1a, M22, YPS163 and S288C, *S. paradoxus* NRRL Y-17217, *S. mikatae* IFO 1815 and IFO YM4906 and *S. pastorianus* WS34/70) are similar and that some insertions and deletions between the strains seem to be the most remarkable differences. Especially, the first insertion (-774 to -867) in the ale strain seems to be unique to this particular strain. In addition to the alignment-based approaches, regulatory elements were searched for in each promoter using SCPD and Yeastract databases (Zhu and Zhang, 1999; Teixeira *et al*., 2006).

Multiple hits of the binding sites of Mal63p and Mig1p were identified in both strains. The ale strain contained seven Mig1p and five Mal63p binding sites whereas the corresponding numbers in the lager strain were two and four (Fig. 2). Interestingly, two of the additional Mig1 binding sites in the ale strain compared to the lager strain were not conserved in any of the other sequences analysed, and three of the additional sites were conserved in only some of the strains (Supplementary Fig. 1). In addition, the extra MAL-activator site in the ale strain was not present in any of the other strains analysed (Supplementary Fig. 1)

Comparison of *AGT1* bearing loci in *S. cerevisiae, S. paradoxus* and *S. mikatae*

The *AGT1* promoter sequences of the two *S. cerevisiae* laboratory strains, S288C and CEN.PK2-1D(VW-1B), differed significantly from the corresponding sequences of three natural isolates of *S. cerevisiae*, *i.e.*, strains M22, YPS163 and RM11-1a. The *AGT1* promoters of these natural isolates were very similar to each other (94-97 % identity). The *AGT1* promoters of the three lager and one

ale strains were also very similar (97–99 % if the 94 and 95 bp insertions in *AGT1* promoters of the ale strain are excluded) to the corresponding sequences of these natural isolates (see Supplementary Fig 1). Moreover, the *AGT1* promoter regions of these natural isolates of *S. cerevisiae* were found to be more similar to corresponding *AGT1* promoter regions found in *S. paradoxus* and *S. mikatae* species than to laboratory strains of *S. cerevisiae* (Supplementary Fig. 1; Table 3). The strong similarity between the *AGT1* promoters of *S. paradoxus, S. mikatae,* these four brewing yeasts, and the three natural isolates of *S. cerevisiae* prompted us to compare the structures of the *MAL* loci containing *AGT1* in these strains using sequences from gene banks. The loci analysed were the *MAL1* locus of the laboratory strain, S288C, contig 1.18 of the natural isolate RM11-1a of *S. cerevisiae,* contig 294 of *S. paradoxus* strain NRR Y-17217 and contig 789 of *S. mikatae* IFO 1815 strain.

These *AGT1*-containing *MAL* loci from *S. cerevisiae* RM11-1a, *S. paradoxus* NRR Y-17217 and *S. mikatae* IFO 1815 were much more extensive (Fig. 3) than the classical *MAL* locus found in S288C, with its single copies of the *MALx1*, *MALx2* and *MALx3* genes. At least 9 putative *MAL* loci genes were spread throughout the 32–34 kbp contig regions from *S. paradoxus* and *S. mikatae*, and there are probably more *MAL* genes beyond the borders of the contigs since there were *MAL* ORFs at the borders. There were also multiple putative *MAL* genes in the 22 kbp RM11-1a contig, at least 7, including one at the border of the contig. The laboratory strain S288C contains a putative isomaltase-encoding gene, *YGR287c* (73% identical to *MALx2*) in addition to the classic *MAL11, MAL12* and *MAL13* genes. *YGR287c* was found also in *S. cerevisiae* RM11-1a and the *S. mikatae* and *S. paradoxus* contigs. In *S. paradoxus* and *S. mikatae* contigs there was also an ORF with closest observed similarity (88% in *S. paradoxus* and 84 % identity in *S. mikatae)* to the maltose/maltotriose transporter-encoding *MTT1* gene (Salema-Oom *et al*., 2005; Dietvorst *et al*., 2005).

DISCUSSION

Lager strains are known to be polyalloploid hybrids of *S. cerevisiae* and *S. bayanus* strains (Nakao *et al*., 2009), whereas ale strains are usually described to be closely related to *S. cerevisiae* strains (Tornai-Lehoczki and Dlauchy, 2000). The almost completely sequenced lager strain WS34/70 has been reported to possess two *AGT1* genes in its genome, one derived from *S. cerevisiae* and the other from *S. bayanus* (Nakao *et al*., 2009). The *AGT1* promoter sequence from the two lager strains in the present study was virtually identical to the *S. cerevisiae*-derived *AGT1* promoter region of WS34/70 and only 43% identical to the *S. bayanus*-derived *AGT1* promoter sequences. Because of this significant sequence divergence, only *S. cerevisiae*-derived *AGT1* sequences were detected in the chromosome walking of the present study. Since lager strains are polyploid (possessing more than one copy of each *S. cerevisiae*- and *S. bayanus*-derived chromosome) (Querol and Bond, 2009) and sister chromosomes probably have diverged during evolution, different forms of *S. cerevisiae*-derived *AGT1*-bearing loci may exist. However, only a single type of *AGT1* promoter sequence was identified in the chromosome walking performed, indicating that there is no such variation between the *S. cerevisiae*-derived sister chromosomes in the *AGT1* loci in the lager strains studied.

The *AGT1*-containing *MAL* loci in three *Saccharomyces sensu stricto* species *i.e. S. mikatae, S. paradoxus* and natural isolate of *S. cerevisiae* RM11-1a were far more complicated than the classical *MAL* locus usually described in laboratory strains. There are few reports concerning the

organization of *MAL* locus in the genetically more complex industrial strains and natural isolates. The overall sequences of *S. mikatae* contig 789 and *S. paradoxus contig* 294 *MAL* loci were observed to be very similar to each other. There were even three different putative maltose transporter-encoding genes in the same *MAL* locus in the *S. paradoxus* and *S. mikatae* strains. These genes (*MALx1*, *AGT1* and a gene most similar to *MTT1*), relate to the three major maltose/maltotriose transporters known in *S. cerevisiae* (Malx1p, Agt1p and Mtt1p (Salema-Oom *et al.*, 2005; Alves *et al*., 2007)). Evidently the *MAL* genes had already been multiplied in the locus of the ancestral strain from which these *Saccharomyces sensu stricto* strains derive. Divergence of *S. cerevisiae* from this ancestor and further divergence of laboratory strains from natural *S. cerevisiae* seems to have been accompanied by deletion of some of these repeats. The *AGT1*-containing *MAL* locus of *S. cerevisiae* RM11-1a could have derived from the *S. mikatae* and S. *paradoxus* loci by deletion of the sequence between the first and third *MALx3* ORFs (Fig. 3). This deletion would remove only extra copies of the maltose transporter and MAL-activator genes. The *MAL1* locus of *S. cerevisiae* S288C could be derived by a further deletion, this time of the sequence between *AGT1* and the first *MALx2* seen in the RM11-1a contig (Fig. 3). Sequence data from S288C shows that compared to RM11-1a there is a deletion from 317 bp upstream of the *AGT1* ORF until approximately 450 bp upstream of the *MALx2* ORF.

When the multiple sequence alignment of the *AGT1* promoters of the ale (this study), lager (this study), *S. cerevisiae* S288C, RM11-1a, YPS163, and M22, *S. paradoxus* NRRL Y-17217 (contig 294), *S. mikatae* IFO 1815 YM4906 (contig 2883), *S. mikatae* IFO 1815 (contig 789) and *S. pastorianus* WS34/70 strains was constructed, all promoters showed high levels of homology in various, rather long, segments. These identical regions were analysed and shown to contain a TATA-box and several Mig1 and MAL-activator binding sites.

Mig1p binding sites (GGAAATACGGG) were found with the matrix search tool provided by the SCPD (Zhu and Zhang, 1999). At least one Mig1 binding element was found in the *AGT1* promoters of all strains studied. However, there was significant variation in the number of Mig1 binding elements found in each strain. A60 ale strain possessed a higher number of Mig1 elements, seven all together, than other strains, whereas in the *AGT1* promoter of the lager strains there were only two Mig1 elements. *S. cerevisiae* strains possessed four Mig1 elements and *S. mikatae* and *S. paradoxus* strains only one Mig1 element (Supplementary Fig. 1).

Also the MAL-activator binding sites (MGCNNNNNNNNNMGS where M=adenine or cytosine, S =guanine or cytosine and N=any of the four nucleotides (Teixeira *et al*., 2006; Sirenko *et al*., 1995) occurred in varying numbers. The highest number of MAL-activator elements, five, was found in the *AGT1* promoter of ale strain A60, whereas there were four binding sites in the *AGT1* promoters of the lager strains A15 and WS34/70 as well as in the *S. cerevisiae* strains. In *S. paradoxus* and *S. mikatae* strains there were no MAL-activator elements. The extra MAL-activator site, located at the beginning of the 94 bp insertion (-778 upstream of the start codon), in the ale strain promoter may explain the stronger expression of *AGT1* observed in the ale strains compared to the lager strains (Vidgren *et al*., 2005). The extra MAL-activator binding site in A60 strain is practically identical to the adjacent MAL-activator site, which is common to both ale and lager strains (Supplementary Fig. 1). Thus, the reason for a stronger induction of the *AGT1* gene expression in A60 strains is not likely due to differences of individual MAL-activator sites, *e.g.* higher affinity of the MAL-activator, but rather to the greater number of MAL-activator sites present.

The long distance between both the MAL-activator and Mig1 binding elements and the *AGT1* coding sequence (the most proximal MAL-activator site is located approximately -830 bps from the start codon of the *AGT1* gene) raises some questions about their effectiveness in regulation. Bell *et al*. (1997) have shown that when the distance is increased between MAL-activator sites and the start of the *MALx1* gene in the *MALx1-MALx2* divergent promoter region, the induction of the expression of *MALx1* gene is significantly decreased. Promoter regions used in the studies of Bell *et al*. were from a baker's yeast strain. It was shown that when the distance between the MAL-activator and *MALx1* gene increased to 800–1000 bp only very moderate expression remained. In the present study the distance observed between the Mig1 binding sites and the *AGT1* start codon is even greater (approximately 1100 bp). However, the *AGT1* expression was strongly repressed in these strains when glucose was present (Vidgren *et al*., 2005). This suggests that the Mig1 transcription factors binding to the promoter region of *AGT1* at a considerable distance are functioning as repressors. Furthermore, the earlier studies revealed strong maltose induction of *AGT1* expression in the ale strains, indicating that MAL-activators, located more proximal to *AGT1* gene than the Mig1 elements, are also functional. However, MAL-activator sites located further upstream in the ale and lager *AGT1* promoters (> -1500 from the start codon) probably have only minor contribution to the induction. Because of the extra MAL-activator binding site in the ale strains compared to the two binding sites in the lager strain, the promoter of the ale strain has in the 1500 bp promoter region proximal to the *AGT1* gene the same number (three) of MAL-activator binding sites as the well-studied promoter of *AGT1* in the laboratory strain S288C. It is possible that for the strong expression of *AGT1* all three MAL-activator elements are necessary.

Another explanation for the stronger expression of the *AGT1* gene in the ale strain may be linked to telomeric silencing. The role of chromatin remodelling in the regulation of expression of *MAL* genes has been reported (Houghton-Larsen and Brandt, 2006; Dietvorst and Brandt, 2008). All *MAL* loci are located near the telomeres, and their expression may become repressed by epigenetic regulation known as telomeric silencing, *i.e.* variation in the chromatin structure near the telomeres leading to the silencing of genes located in this region (Pryde and Louis, 1999; Loney *et al*., 2009). It has been observed that telomeric silencing does not occur uniformly but there is significant variation between different strains (Pryde and Louis, 1999). A specific complex consisting of several subunits, the COMPASS complex is known to be involved in telomeric silencing in yeast cells (Miller *et al*., 2001). In a study where the COMPASS complex was rendered non-functional, strain-dependent differences in the telomeric silencing of *MAL* genes were observed. The *MAL* genes were found to be strongly silenced in some strains, whereas in other strains the non-functional COMPASS complex did not cause any changes in the expression of *MAL* genes (Houghton-Larsen and Brandt, 2006). It has also been observed that in a single strain some chromosome ends are more prone to the telomeric silencing than others (Loney *et al*., 2009). Thus it is possible that the different *MAL* loci, located at different chromosome ends, are not uniformly regulated by telomeric silencing. The earlier expression studies (Vidgren *et al*., 2005) showed that the *MALx1* genes were strongly but the *AGT1* weakly expressed in the lager strains. This suggests that if telomeric silencing is the reason for the low expression of *AGT1* genes in the lager strains, chromosomes possessing other *MAL* loci, however, are not regulated by telomeric silencing. A link between the chromatin structure and the *MAL1* locus was also observed in a microarray study where the nucleosome content was reduced (by depletion of histone H4) resulting in reduced genome packaging. This in turn caused over a 10-fold de-repression of *AGT1* and *MAL12* expression in a *S. cerevisiae* laboratory strain (Wyrick *et al*., 1999).

In summary, it may well be that the differences observed in the *AGT1* gene expression between the ale and lager strains are related to the differences in the silencing of *MAL1* locus between the ale and lager yeast strains. Functional studies of MAL-activator elements present in the *AGT1* promoters of brewer's yeast strains, as well as studying the COMPASS complex mediated silencing of *MAL* genes in these specific strains, are needed to further elucidate the regulation of the expression of the *AGT1* gene.

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Table 1. Specific oligos used in Chromosome walking rounds from 1 to 3.

	Name of the oligo	Sequence	Binding site[a]
Specific oligo Round 1	ChrWalk1oligo1	5'-GGCACTATCCTTTTTCCCTTC-3'	127 to 147 of *AGT1* gene
Nested oligo Round1	ChrWalk1nested	5'-CATTTTTTGAGGCAGCCTTC-3'	52 to 33 of *AGT1* gene
Specific oligo Round 2	ChrWalk2oligo1	5'-TTAAAATTGGGTACACTC'3'	-146 to -163 of AGT1 promoter
Nested oligo Round 2	ChrWalk2nested	5'-AGTACGCATCAACGGAGT'3'	-220 to -237 of *AGT1* promoter
Specific oligo Round 3	ChrWalk3oligo1	5'-TTATGCCACAACGTCCAAGA-3'	-604 to -623 of *AGT1* promoter (A15 and A24) -582 to -601 of *AGT1* promoter (A60 strain)
Nested oligo Round 3	ChrWalk3nested	5'-CCATCTTCTCTTGACTTGCT-3'	-687 to -706 of *AGT1* promoter (A15 and A24) -665 to -684 of *AGT1* promoter (A60 strain)

[a] Sequence numbering is from the first nucleotide of the translational start. In rounds 1 and 2 the binding sites of oligos are same in all three strains A15, A24 and A60. In round 3 binding sites differ due to the 22 bp long deletion in A60 strain at position -565 to -586.

Table 2. Comparison of *AGT1* promoter sequences (first 950 bp from -1 to -950).

	Lager A15	*S. mikatae* contig 789	*S. paradoxus* contig 294	*S. cerevisiae* S288C	*S. cerevisiae* YPS163	*S. cerevisiae* M22	*S. cerevisiae* RM11-1a	lager WS34/70 **Sc** *AGT1* prom counterpart
lager WS34/70 ***Sb*** *AGT1* promoter counterpart contig 14.2	44	44	43	43	44	46	45	43
lager WS34/70 ***Sc*** *AGT1* promoter counterpart contig 201.2	97	60	59	55	97	94	97	
S. cerevisiae RM11-1a contig 1.18	99	61	61	58	97	99		
S. cerevisiae M22 contig88.3	98	61	60	59				
S. cerevisiae YPS163 contig 107.6	98	60	60	58				
S. cerevisiae S288C (SGD)	57	45	47					
S. paradoxus NRRL Y-17217 contig 294	61	85						
S. mikatae IFO1815 contig 789	61							

Numbers shown are % identities when corresponding promoters were compared pair-wise. *Sb AGT1* and *Sc AGT1* promoters refer to *S. bayanus* and *S.cerevisiae* derived *AGT1* promoters, respectively. WS34/70 Sc *AGT1* promoter sequence obtained was missing first 50 bp (from -1 to -50 in respect to ATG start codon). Pair-wise comparison was restricted in this case to -50 to -950 of *AGT1* promoter. *S. cerevisiae* M22 promoter sequence obtained was 789 bp and identity comparisons was performed to this region.

Table 3. Major differences between the sequences of *AGT1* promoters[1].

	A ***541-562***	***B*** ***811-904***	***C*** ***1134-1228***	***D*** ***1287-1309***	***E*** ***1512-1529***	**F** **1640-1659**
Ale strain (A60)	Missing	94 bp	95 bp	Missing	Missing	18 bp
Lager strains (A15, A24, WS34/70)	22 bp	Missing	Missing	Missing	Missing	18 bp
Sc YPS163	Missing	Missing	NS	NS	Missing	18 bp
Sc RM11-1a	22 bp	Missing	75 bp	Missing	Missing	18 bp
Sc M22	22 bp	Missing	79 bp	Missing	Missing	18 bp
S. paradoxus (NRRLY-17217)	Missing	Missing	81 bp	23 bp	18 bp	Missing
S. mikatae (IFO 1815)	Missing	Missing	82 bp	23 bp	18 bp	Missing

[1]The numbering is from the start of the longest consensus promoter of A60 ale strain as shown in Fig. 1 of the supplementary material. At the indicated positions sequence is either almost completely missing or represented by the indicated number of base pairs (bp). NS, not sequenced.

Figure Legends

Fig 1. Structure of the *MAL3* locus of *S. cerevisiae* S288C. Three genes are located at the *MAL3* locus: a MAL-activator gene (*MAL33*), a maltose permease gene (*MAL31*) and a maltase gene (*MAL32*). Divergent promoter region of *MAL31* and *MAL32* genes is shown in detail. Known promoter elements are two TATA boxes, two Mig1 binding sites (Hu 1995), three MAL-activator binding sites (Hong and Marmur 1987) and 147 bp repeat element (Bell 1997).

Fig 2. Sequence comparison of ale A60, lager A15, lager A24 and laboratory strain CEN.PK2-1D *AGT1* promoter regions. Cross-lines indicate highly homologous (>99% identity) regions. M=sequence missing compared to corresponding ale/lager sequence.

Fig 3. *S. cerevisiae* S288C Chr VII, *S. cerevisiae* RM11-1a contig 1.18, *S. paradoxus* NRR Y-17217 contig 294 and *S. mikatae* IFO 1815 contig 789 putative *MAL* genes.

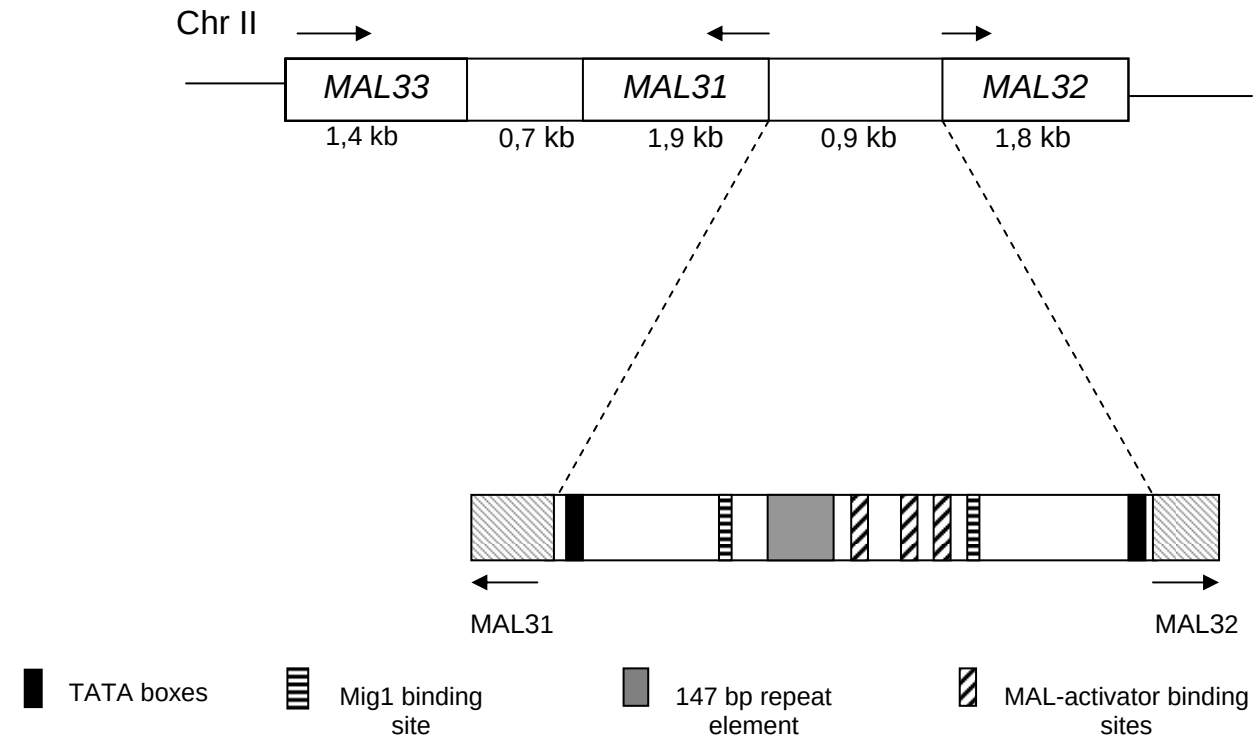

Chr II
MAL33
MAL31
MAL32
1,4 kb
0,7 kb
1,9 kb
0,9 kb
1,8 kb
MAL31
MAL32
TATA boxes
Mig1 binding site
147 bp repeat element
MAL-activator binding sites

Fig 1.

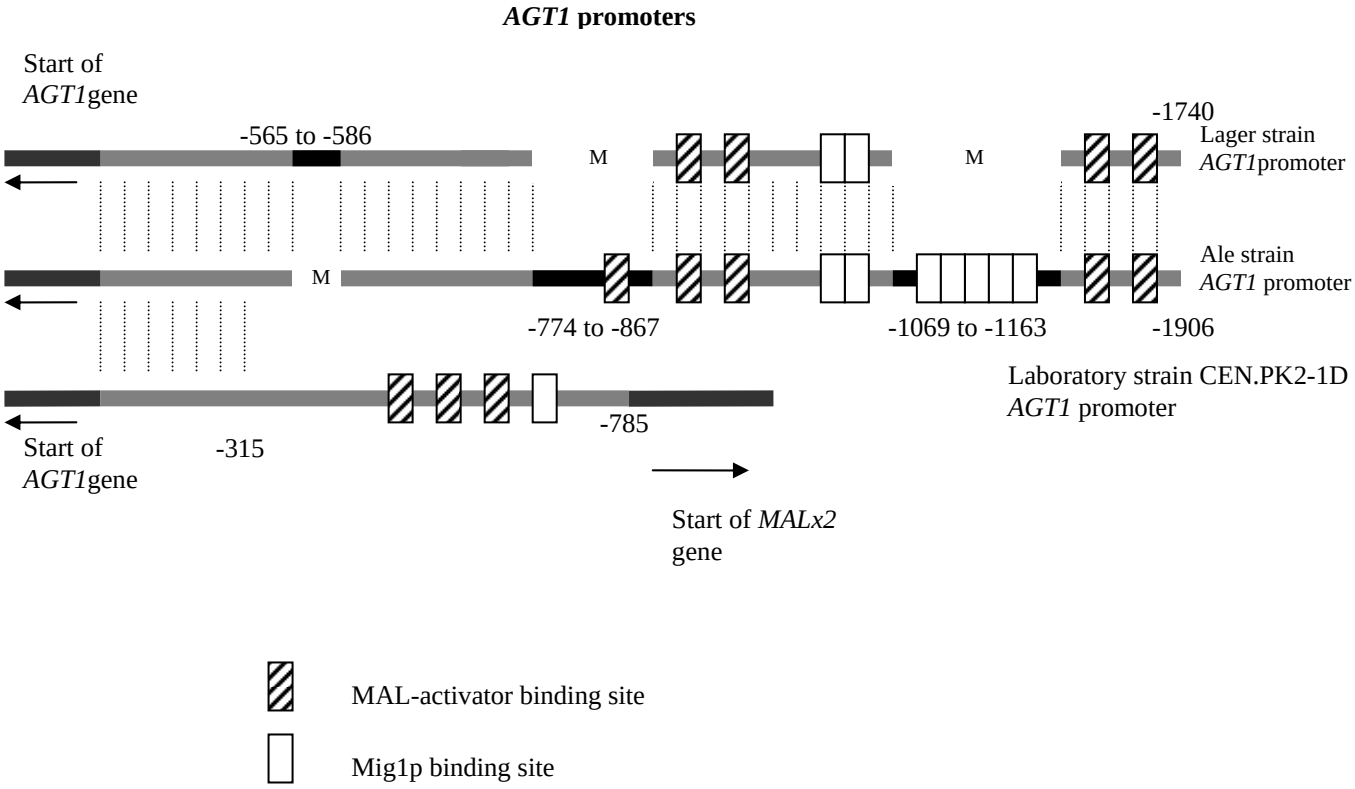
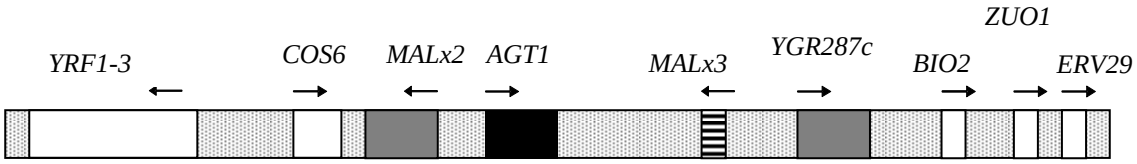
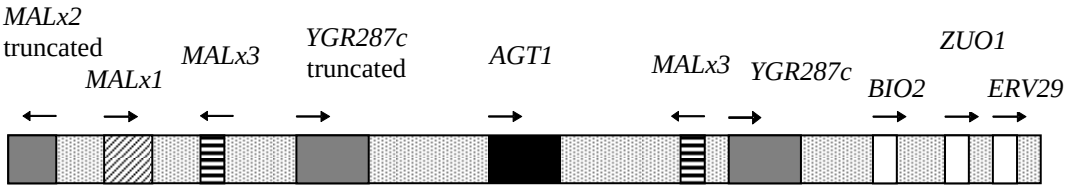

AGT1 promoters
Start of *AGT1*gene
-565 to -586
M
M
-1740
Lager strain *AGT1*promoter
M
Ale strain *AGT1* promoter
-774 to -867
-1069 to -1163
-1906
Laboratory strain CEN.PK2-1D *AGT1* promoter
-785
Start of *AGT1*gene
-315
Start of *MALx2* gene
MAL-activator binding site
Mig1p binding site

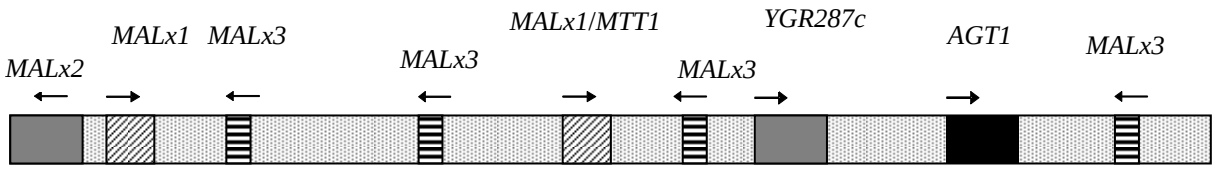
Fig 2.



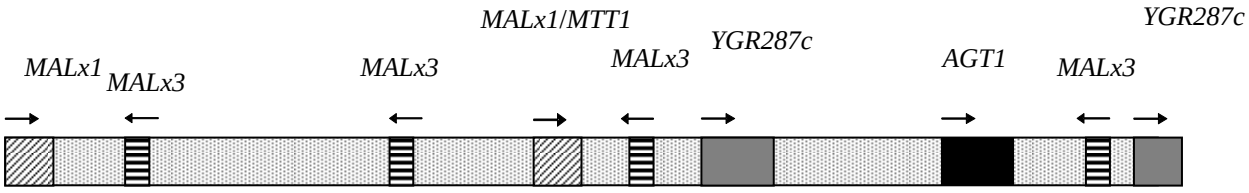
***S. cerevisiae* S288C Chr VII**



***S. cerevisiae* RM11-1a Contig 1.18**



***S. paradoxus* contig 294**



***S. mikatae* contig 789**

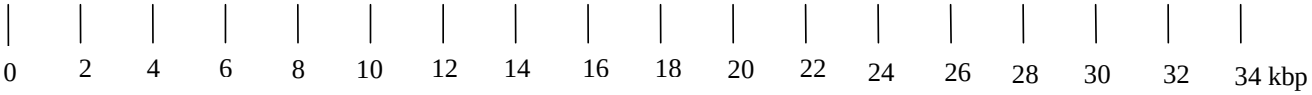
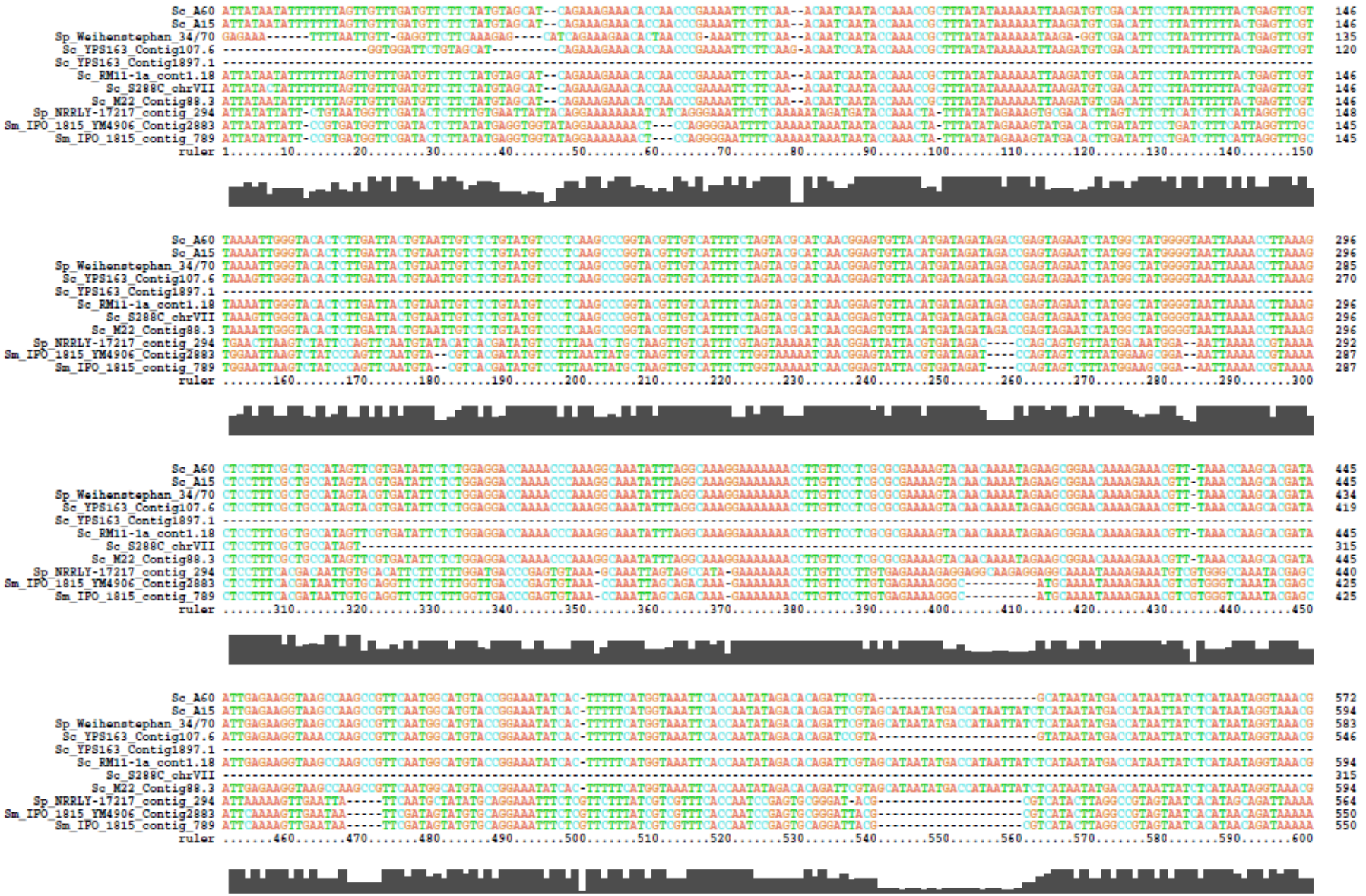
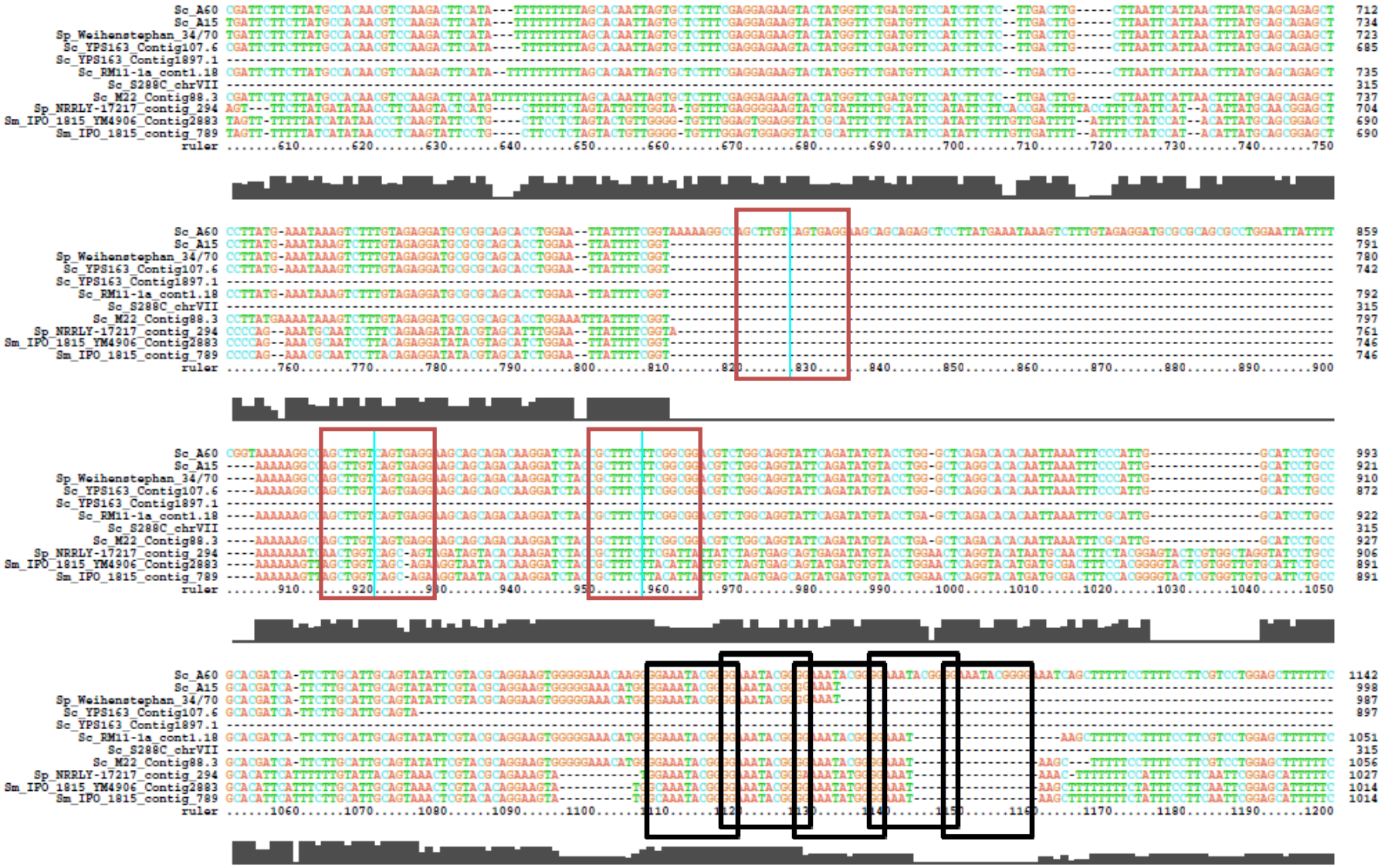
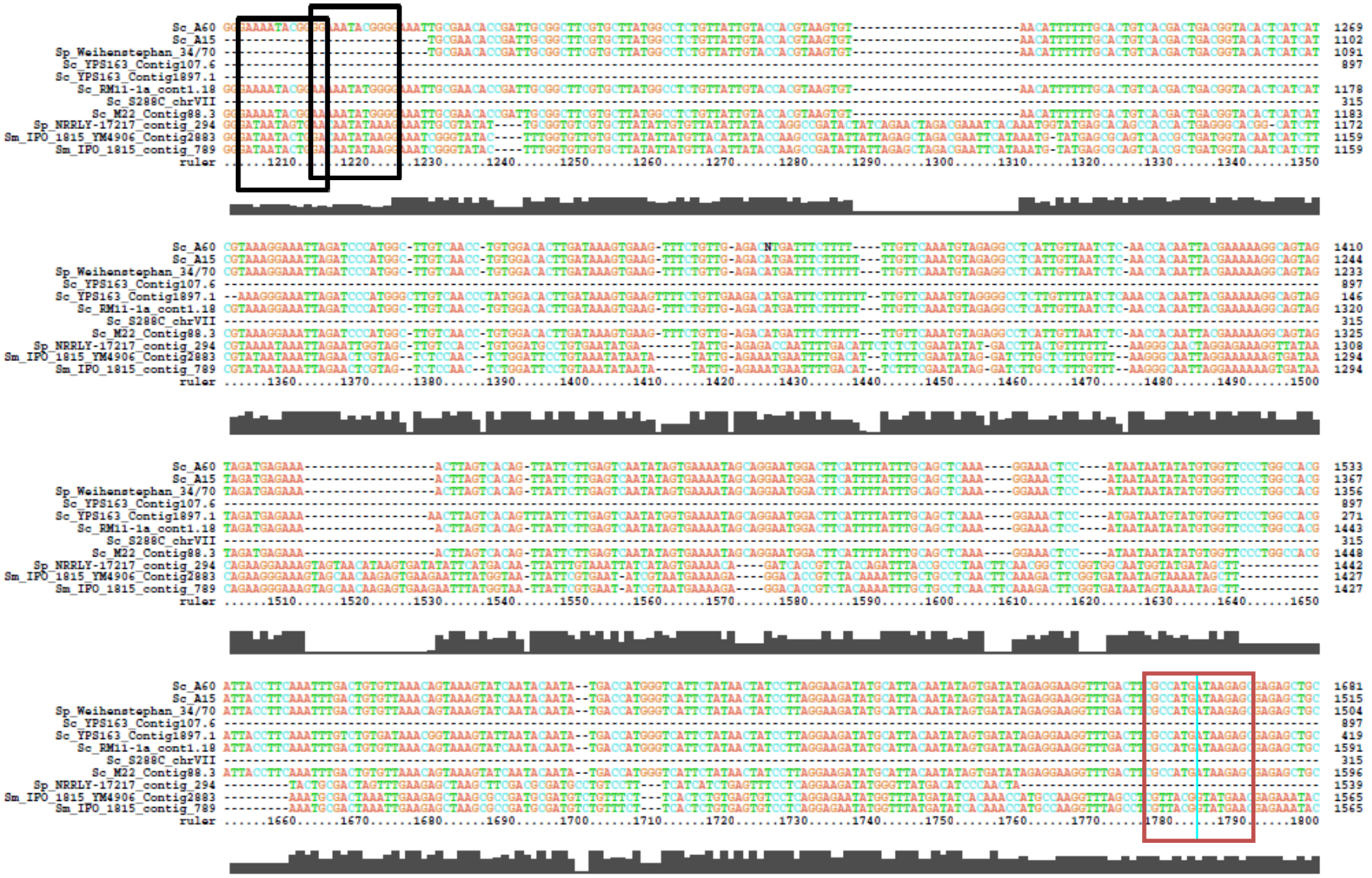


Fig 3.


Sc_A60
Sc_A15
Sp_Weihenstephan_34/70
Sc_YPS163_Contig107.6
Sc_YPS163_Contig1897.1
Sc_RM11-1a_cont1.18
Sc_S288C_chrVII
Sc_M22_Contig88.3
Sp_NRRLY-17217_contig_294
Sm_IPO_1815_YM4906_Contig2883
Sm_IPO_1815_contig_789
ruler


Sc_A60
Sc_A15
Sp_Weihenstephan_34/70
Sc_YPS163_Contig107.6
Sc_YPS163_Contig1897.1
Sc_RM11-1a_cont1.18
Sc_S288C_chrVII
Sc_M22_Contig88.3
Sp_NRRLY-17217_contig_294
Sm_IPO_1815_YM4906_Contig2883
Sm_IPO_1815_contig_789
ruler


Sc_A60
Sc_A15
Sp_Weihenstephan_34/70
Sc_YPS163_Contig107.6
Sc_YPS163_Contig1897.1
Sc_RM11-1a_cont1.18
Sc_S288C_chrVII
Sc_M22_Contig88.3
Sp_NRRLY-17217_contig_294
Sm_IPO_1815_YM4906_Contig2883
Sm_IPO_1815_contig_789
ruler

Supplementary Fig 1. Alignment of A60 ale and A15 lager strain *AGT1* promoter sequences to homologous sequences found in gene banks (SGD Fungal Genome Database and NCBI database limited to different *S. cerevisiae* strains). Alignment was carried out by MUSCLE multiple alignment tool (http://www.ebi.ac.uk/Tools/muscle/index.html). MAL-activator binding site locations ($MGC(N)_9MGS$ M=adenine or cytosine, S=guanine or cytosine, N=any nucleotide (Teixeira *et al*. 2006) are shown as red boxes. Mig1 binding site locations (CGAAATACGGG) (Zhu *et al*. 1999) are shown as black boxes. Sequences start -1 relative to the start codon of each *AGT1* gene (ruler 1 stands for -1 relative to the start codon of each *AGT1* gene). Height of the black bar below the ruler represents the sequence identity at each position.

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Virve Vidgren

Title

Maltose and maltotriose transport into ale and lager brewer´s yeast strains

Abstract

Maltose and maltotriose are the two most abundant sugars in brewer's wort, and thus brewer's yeast's ability to utilize them efficiently is of major importance in the brewing process. The increasing tendency to utilize high and very-high-gravity worts containing increased concentrations of maltose and maltotriose renders the need for efficient transport of these sugars even more pronounced. Residual maltose and especially maltotriose are quite often present especially after high and very-high-gravity fermentations. Sugar uptake capacity has been shown to be the rate limiting factor for maltose and maltotriose utilization. The main aim of the present study was to find novel ways to improve maltose and maltotriose utilization during the main fermentation. Maltose and maltotriose uptake characteristics of several ale and lager strains were studied. Genotype determination of the genes needed for maltose and maltotriose utilization was performed. Maltose uptake inhibition studies were performed to reveal the dominant transporter types actually functioning in each of the strains. Temperature-dependence of maltose transport was studied for ale and for lager strains as well as for each of the single sugar transporter proteins Agt1p, Malx1p and Mtt1p. The *AGT1* promoter regions of one ale and two lager strains were sequenced by chromosome walking and the promoter elements were searched for using computational methods.

The results showed that ale and lager strains predominantly use different maltose and maltotriose transporter types for maltose and maltotriose uptake. Agt1 transporter was found to be the dominant maltose/maltotriose transporter in the ale strains whereas Malx1 and Mtt- type transporters dominated in the lager strains. All lager strains studied were found to possess a non-functional Agt1 transporter. The ale strains were observed to be more sensitive to temperature decrease in their maltose uptake compared to the lager strains. Single transporters were observed to differ in their sensitivity to temperature decrease and their temperature-dependence was shown to decrease in the order Agt1≥Malx1>Mtt1. The different temperature-dependence between the ale and lager strains was observed to be due to the different dominant maltose/maltotriose transporters ale and lager strains possessed. The *AGT1* promoter regions of ale and lager strains were found to differ markedly from the corresponding regions of laboratory strains. The ale strain was found to possess an extra MAL-activator binding site compared to the lager strains. Improved maltose and maltotriose uptake capacity was obtained with a modified lager strain where the *AGT1* gene was repaired and put under the control of a strong promoter. Modified strains fermented wort faster and more completely, producing beers containing more ethanol and less residual maltose and maltotriose. Significant savings in the main fermentation time were obtained when modified strains were used. In high-gravity wort fermentations 8–20% and in very-high-gravity wort fermentations even 11–37% time savings were obtained. These are economically significant changes and would cause a marked increase in annual output from the same-size of brewhouse and fermentor facilities.

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Maltose and maltotriose are the two most abundant sugars in brewer's wort, and thus brewer's yeast's ability to utilize them efficiently is important. Residual maltose and especially maltotriose are often present especially after high and very-high-gravity fermentations and this lowers the efficiency of fermentation. In the present work maltose and maltotriose uptake characteristics in several ale and lager strains were studied. The results showed that ale and lager strains predominantly use different transporter types for the uptake of these sugars. The Agt1 transporter was found to be the dominant maltose/maltotriose transporter in the ale strains whereas Malx1 and Mtt1 type transporters dominated in the lager strains. All lager strains studied were found to possess a non-functional Agt1 transporter. Compared to lager strains the ale strains were observed to be more sensitive in their maltose uptake to temperature decrease due to the different dominant transporters ale and lager strains possessed. The temperature-dependence of single transporters was shown to decrease in the order Agt1 ≥ Malx1 > Mtt1. Improved maltose and maltotriose uptake capacity was obtained with a modified lager strain where the *AGT1* gene was repaired and put under the control of a strong promoter. Modified strains fermented wort faster and more completely, producing beers containing more ethanol and less residual maltose and maltotriose. Significant savings in the main fermentation time were obtained when modified strains were used.

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