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Cover: Cells of budding yeast *Saccharomyces cerevisiae*
Designed by Helma van Doorn and Peter Hock

Printed by: Ridderprint, Ridderkerk, The Netherlands

ISBN number: 90-5335-086-1

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Proefschrift

ter verkrijging van
de graad van Doctor aan de Universiteit Leiden,
op gezag van de Rector Magnificus Dr. D. D. Breimer,
hoogleraar in de faculteit der Wiskunde en
Natuurwetenschappen en die der Geneeskunde,
volgens besluit van het College voor Promoties
te verdedigen op dinsdag 9 mei 2006
klokke 14.15 uur

door

Judith Dietvorst

geboren te Eindhoven in 1977

Promotiecommissie

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“Maltotriose utilization of lager yeast strains in high-gravity brewing”
by Judith Dietvorst

Contents

Chapter 1

General Introduction

Introduction

Saccharomyces cerevisiae is commonly known as 'brewers yeast' or 'bakers yeast' due to its ability to ferment sugars from rice, wheat, barley, and corn to produce alcoholic beverages or carbon dioxide for the leavening of dough. This characteristic fermentation ability has been used for millennia in baking and brewing and much effort has been put into the development of fermentation technology and strain improvement. Due to this long history of application in the production of consumable products, *S. cerevisiae* has been classified as a GRAS organism (Generally Regarded As Safe). Nowadays *S. cerevisiae* is also used for other commercial, biotechnological applications like the production of fuel ethanol from waste materials [289], or the production of heterologous proteins [189,238]. Furthermore, *S. cerevisiae* has been intensively studied as a model organism for research in biochemistry and genetics due to its unicellular eukaryotic nature. *S. cerevisiae* is susceptible to genetic modifications by recombinant DNA technology, which have been further facilitated by the availability of the complete sequence of its genome [24,83].

Brewer's yeasts are divided into two major groups, the top-fermenting ale yeasts and the bottom-fermenting lager strains. Traditionally, fermentations with ale yeasts are performed at room temperature and result in beers with a characteristic fruity aroma. In the case of lager yeasts, fermentation temperature is lower, usually between 8 °C and 12 °C, and therefore fermentations take longer (approximately 2 weeks) compared to fermentations with ale yeasts (4 up to 6 days) [197]. Although ales are very common in Britain, Germany, the United States, and Belgium, lager is the most commonly-consumed beer in the world.

Ale and lager strains exhibit very different genomic properties. Lager yeasts are allopolyploid, containing chromosomes derived from two or more different species. One parental strain is *S. cerevisiae* and for the second strain homology was reported with *S. monacensis* [20,95], *S. bayanus* [252] and *S. pastorianus* [121]. In contrast, ale yeasts are closely related to the *S. cerevisiae* laboratory strain S288c [130]. In paragraph 1.1 additional differences between ale, lager and laboratory yeast strains will be discussed in detail. The subjects discussed in the other paragraphs of this chapter, mostly deal with findings in laboratory strains unless specified differently.

The yeast strain used by a particular brewery is strongly associated with the taste and characteristics of the desired beer. The consistent production of flavour and aroma metabolites is an essential property of an industrial brewing yeast strain. As a consequence a rather conservative attitude towards yeast strain improvement is maintained in the brewer's world. However, higher productivity of the brewery is required to survive within a strongly competitive economy. High-gravity brewing (HGB) is a modern development in the brewing industry in which the fermentations are performed at much higher wort concentrations i. e. 18 or even 24 ° Plato (°P) versus the 12 °P (circa 90-95 g sugar per liter) in traditional brewing processes. In the following part of the introduction several aspects of HGB are discussed. Paragraph 1.2 gives general information on the brewing process and discusses the advantages and disadvantages of the HGB technology. This information will show the importance of the yeast stress responses. Much research focused on the different stresses as

well as the stress response of *S. cerevisiae* as described in paragraphs 1.3 and 1.4. Paragraph 1.5 deals with the fermentable sugars present in wort and the consumption of these sugars by *S. cerevisiae* yeast strains. Efficient beer fermentation requires the rapid and complete utilization of these wort-sugars. Paragraph 1.6 provides information on the Crabtree-effect, one of the reasons for incomplete or so-called "stuck" fermentations in HGB. In paragraph 1.7 the glucose control mechanisms in *S. cerevisiae* are discussed, focusing on regulation of the metabolism of sugars in wort. Maltotriose utilization in fermentation is highlighted in paragraph 1.8 since it is the second most abundant sugar present in wort and often left in beer due to incomplete fermentation. Paragraph 1.9 deals with the use of classical genetic techniques in brewer's strains. The outline of this thesis is described in paragraph 1.10.

1.1 Brewer's strains

Taxonomy

There are at least one thousand strains of the species *Saccharomyces cerevisiae*. These strains include brewing, baking, wine, distilling and laboratory cultures. The classification of *Saccharomyces* yeasts has always been problematic at the species level due to changing views on taxonomy (reviewed by Rainieri *et al*.) [203]. Nowadays, the *Saccharomyces* genus includes two groups of species, *Saccharomyces* sensu stricto (species closely related to *S. cerevisiae*) and *Saccharomyces* sensu lato (species more distantly related to *S. cerevisiae*). Most industrial yeast strains belong to the sensu stricto group.

Amplified fragment length polymorphism (AFLP) analysis, based on the selective PCR amplification of restriction fragments of genomic DNA [275], was used to characterize *S. cerevisiae, S. bayanus, S. pastorianus* and *S. paradoxus* strains of the sensu stricto group. The strains of *S. pastorianus* (bottom-fermenting yeast) showed relatively high percentages of shared fragments with the strains of *S. cerevisiae* and *S. bayanus*, confirming the hybrid nature of these lager yeast strains [11,125].

Ale and lager yeasts

Ale and lager strains can be differentiated not only by their different genomic constitution (as described above) but also by means of their fermentation behavior. The main physiological difference between ale and lager yeasts related to fermentation is the ability of lager yeast to utilize the sugars raffinose (galactose-glucose-fructose) and melibiose (galactose-glucose). Lager yeast strains contain the *MEL* genes and consequently produce the extracellular enzyme α-galactosidase (melibiase) for the hydrolysis of melibiose. Ale yeast strains do not possess the *MEL* genes and consequently do not ferment melibiose and ferment only a third of the trisaccharide raffinose [173]. In addition, flocculation capacity [58] and stress response [29] during fermentation vary greatly between brewer's yeast strains (discussed in more detail in paragraph 1.3).

Yeast strain variation

Laboratory, lager and ale yeast strains vary from one another in their genotype. Genome rearrangements in yeast occur during adaptation to changing environment or any source of cellular stress [1,63,110,156]. Some of these genome changes are observed on nucleotide scale but many changes appear to involve entire genes or chromosomes, resulting in alterations of their copy numbers (reviewed by Kielland-Brandt *et al.*) [125]. Competitive comparative genome hybridization (CCGH) showed that the copy number of *S. cerevisae*-like ORFs in two lager yeast strains may range from one to six [19]. Furthermore, DNA from these two lager yeast strains showed low hybridization to about 56 kb of chromosome XVI from *S. cerevisiae*, indicating the absence of this region of the *S. cerevisiae* genome in the lager strains [19].

In order to distinguish different yeast strains, various methods of DNA analysis are possible such as DNA fingerprinting techniques i. e. random amplified polymorphic DNA (RAPD) analysis [45,150] and amplified fragment length polymorphism (AFLP) analysis [11]. Microarray karyotyping [64] and electrophoretic karyotyping are suitable for analysis of whole chromosomes. Electrophoretic karyotyping is a technique that separates linear chromosomes based on their differences in size (reviewed by Johnston) [119]. The karyotypes of brewing strains and laboratory strains are easily distinguished by Pulsed Field Gel Electrophoresis (PFGE) (Figure 1). Karyotyping is a direct means to provide evidence for the presence of heteroploidy in yeast such as deviations from the basic number of chromosomes [18].

Laboratory strains versus brewer's strains

The availability of procedures for genetic manipulation of the laboratory strain *S. cerevisiae* turned *S. cerevisiae* into one of the best genetically characterized organisms. For multiple reasons, the genetic procedures used with laboratory strains are usually not applicable in brewer's yeast [3]. First, the genetic constitution of brewer's strains is much more complex and diverse due to their usually polyploid (more than twice the haploid number of chromosomes) nature. In addition, brewer's strains may also be aneuploid (chromosome number that is not a multiple of the haploid number since one chromosome set is incomplete) or alloploid (genomes from two different species are present) [125]. Consequently, the copy number of a specific gene is often unknown in most of these brewer's strains and recessive auxotrophic mutations which have been used with their corresponding wild-type genes to select yeast transformants in laboratory strains [219], can not easily be obtained.

Second, brewer's yeast strains reproduce asexually by budding and do not sporulate or do so with a low efficiency. In case of a suitable sporulation frequency, most spores are not viable [116]. This poor sexual performance is explained by the polyploid nature since divergence between homologous sequences impairs chromosome pairing and recombination, required for proper meiosis. Although this characteristic makes conventional genetic analysis with brewer's strains difficult, it contributes to the strain stability of a particular type of yeast. This property enables the brewer to maintain the particular properties of beer [166].

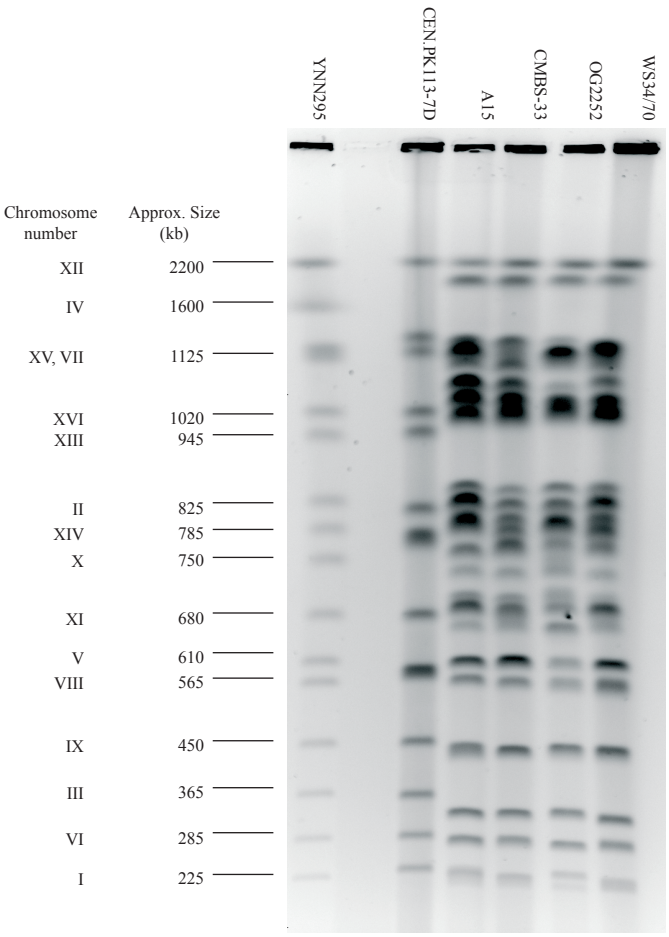

Figure 1. Pulsed Field Gel Electrophoresis (PFGE).
PFGE of one laboratory yeast strain, CEN.PK113-7D, and the four lager yeast strains used in this study (courtesy of L.H.C. Mulder). Chromosomes are separated by length. YNN295 represents a *S. cerevisiae* size marker from BioRad.

1.2 High-gravity brewing

High-gravity brewing is a development in the brewing industry which has been introduced over the past 35 years mainly because of its economical advantages [15]. Gravity refers to the amount of dissolved sugars in wort. In traditional brewing, worts of 11 to 12 degrees Plato (corresponding to about 9 to 10 grams sugar per 100 gram solution) are fermented to beers with 4 to 5 % (v/v) ethanol [40]. In HGB, worts of at least 16 to 18 degrees Plato (° P) are used, resulting in a substantially higher sugar concentration of the fermentation broth and in a higher final alcohol volume [207]. After fermentation and lagering, the beer is diluted to obtain beer with the desired alcohol concentration [23].

The traditional brewing process versus HGB

Fundamentally beer is the product of the alcoholic fermentation by yeast of extracts of malted barley (dried, germinated grains). Both malt and yeast contribute substantially to the character of the beer [13]. The starch in barley is enclosed in cell wall and proteins and these are removed in the malting process, leaving soluble starch. In the malting process, nutrients are generated for yeast development and enzymes are synthesized or activated [13]. Cereal starch is broken down into fermentable sugars and non-fermentable dextrins during a heat treatment (mashing). The resulting sweet syrupy liquid is known as wort. The most widely used method to prepare wort with a higher sugar concentration for HGB has been to supplement worts with sugar syrup adjuncts [207]. The resulting changes in the carbon to nitrogen ratios, due to dilution of the available nitrogen in wort, might result in different flavour profiles. According to Peddie, lowering the nitrogen content by addition of sugar syrups reduces the formation of metabolites like acetyl CoA, resulting in lower ester production [192].

The actual fermentation starts when yeast is mixed with the wort. Although fermentation is an anaerobic process, wort is usually oxygenated at the start of fermentation. Yeast requires this oxygen to synthesize sterols and unsaturated fatty acids for cell membrane biosynthesis and yeast growth [202,220]. In high-gravity worts when syrups are added, oxygen availability is diminished further due to the decreasing solubility of oxygen with increasing wort gravity [12]. Insufficient aerated wort will result in poor yeast growth, a slow fermentation rate and poor sugar consumption (attenuation) [40].

During the fermentation, a decrease in gravity occurs as the fermentable carbohydrates are converted into alcohol. The resulting increase in alcohol is accompanied by the excretion of carbon dioxide and heat, a drop in pH and the production of flavor compounds e. g. aldehydes, esters, diacetyl, dimethyl sulfide. Esters are considered the most important aroma compounds in beer and in general contribute a fruity flavor in beer. The formation of esters is catalyzed by alcohol acetyl transferase (AATase) in an enzymatic reaction between ac(et)yl-CoA and an alcohol.

The means of controlling ester synthesis during beer fermentations are very limited (reviewed by Verstrepen *et al*.) [274]. The use of high-gravity worts leads to disproportionate amounts of the esters ethyl acetate (solvent-like aroma) and isoamyl acetate (fruity banana aroma) [161].

At the start of fermentation the pH is just over 5 and drops to around pH 4 at the end of fermentation. This pH decrease is due to the production of organic acids and the consumption of buffering compounds like basic amino acids and phosphates [49]. A higher final pH is observed when high-gravity worts are used in the fermentation [253].

At the end of the fermentation, lager yeast cells flocculate and form clumps and settle out to the bottom of the fermentor whereas ale yeasts rise to the surface during fermentation creating a thick yeast "head" [84]. The yeast slurry is separated from the "green beer" and stored until used to start a new fermentation (re-pitching) or discarded. The next step in the brewing process is the maturation of the "green beer" (lagering) and involves one of four general schemes: traditional lagering, bottle conditioning, casking and accelerated lagering. Maturation of the beer involves secondary fermentation of remaining fermentable extract by small amounts of yeast. This secondary fermentation occurs at a reduced rate controlled by low temperatures. The low temperatures also aid in settling the remaining yeast and precipitating haze-forming material, i. e. protein/ polyphenol complexes [239]. The carbon dioxide that is produced dissolves in the beer and reduces byproduct formation. For traditional lagering (widely used for lagers), the green beer is moved to the maturation tank for two up till three weeks. Accelerated lagering takes usually between two and five days at temperatures of about 10 ° C. After maturation the beer is filtered to remove the remaining yeast and packaged in bottles, cans or kegs [84]. Bottle conditioning and casking (mostly used for ales) involve secondary fermentation and clarification in the bottle or cask, respectively, induced by addition of yeast and sugar to the beer. In the case of HGB, the beer is diluted with cool carbonated water after maturation and prior to filtering and packaging, to a prescribed original gravity or a desired alcohol concentration.

Advantages and disadvantages of high-gravity brewing

The use of the high-gravity brewing process instead of traditional brewing has a number of advantages for the brewer. HGB enhances the brewery's capacity due to maximal use of equipment, reduced labor costs, lower energy costs, increased plant efficiency and increased ethanol yields per unit of fermentable extract [7,91]. The higher ethanol concentration in HGB promotes precipitation of polyphenol/ protein material resulting in beer with a reduced haze formation and increased microbiological stability [282]. A smoother taste of the beer produced in HGB according to a taste panel has also been reported [91].

The current limit in HGB is 16 to 18 ° P [40,258]. Attempts to use wort of more than 18 degrees Plato, in a process referred to as very-high-gravity brewing (VHGB) [155] resulted in a reduced fermentation rate [52,191] and loss of yeast viability [36,196]. These negative effects are introduced due to the many stress conditions yeasts encounter during the (V)HGB process, like high osmolarity, high ethanol levels and nutrient limitation. As a consequence of this poor fermentation performance and/ or a faster loss in cell viability, the possibility of reusing the yeast for another brew (referred to as re-pitching) is limited. Moreover, maintaining the characteristic flavour and aroma of the beer product is difficult. According to Meilgaard, the concentration of acetate esters in beer made with worts of 20 ° P can be up to 75 % higher compared to 12 ° P worts [161]. Another reported

disadvantage of HGB or VHGB is the lack of foam stability of the resulting beer. The amount of hydrophobic polypeptides, which form the backbone of foam, produced in HGB or VHGB is less compared to the amount present in beer produced in the conventional process [27,48].

1.3 Stress

Stresses during alcoholic fermentation

During beer fermentations yeast is subjected to many adverse conditions such as temperature changes, high pressures, high osmotic values, high ethanol concentrations, nutrient limitations and storage for extended periods of time at low temperatures [165]. These conditions which are necessary for maximum ethanol yield from the fermentable sugars in wort, cause stresses in the yeast.

In the beginning of the fermentation yeast encounters mainly oxidative, heat and, osmotic stress conditions. Oxidative and heat stress are induced when yeast cells are pitched into oxygenated wort after anaerobic storage at low temperatures. Yeast is usually stored at 4 ° C and in case of fermentation with ale yeast, pitched into wort of temperatures between 16 ° C and 24 ° C. Osmotic stress is induced when yeast is exposed to high-gravity worts with a high initial sugar concentration [9]. During, and at the end of the brewing process, yeast encounters nutrient limitations, high ethanol and carbon dioxide levels and a drop in temperature [2,40]. Nutrient limitations are caused by the use of high-gravity worts since decreased oxygen levels lead to lipid deficiencies [202]. Moreover, nitrogen is limited in high-gravity worts due to the addition of syrups [207]. High levels of carbon dioxide are also due to the use of high-gravity worts since CO_2 will escape more slowly from viscous high-gravity worts [258]. These levels result in loss of fermentation capacity and inhibition of growth of the stressed yeast [257]. The high ethanol concentrations at the end of the fermentation, mainly in HGB, cause ethanol stress in the yeast cells [53,198]. Cold stress is induced at the end of fermentation during yeast cropping (removing of settled yeast prior to re-pitching the next brew) and storage [226]. To minimize yeast autolysis, yeast should be stored at low temperatures and re-pitched within three days after cropping.

1.4 Stress response of yeast

The cellular response of *S. cerevisiae* to many environmental stress conditions is the induction of a stress response (SR) which allows adaptation of the yeast cell to its new surroundings [29]. Many of these responses are specific to a given condition and have been extensively studied and reviewed in laboratory strains of *S. cerevisiae* [67,103,148,222]. An example is the Hsf1-mediated heat-shock response to high temperatures, in which a set of stress response genes encoding the heat shock proteins (Hsps) are induced (reviewed by Chatterjee *et al*.) [42]. Hsps act as molecular chaperones to stabilize proteins and reactivate damaged proteins [50]. Another example is the response to osmotic stress. Upon osmotic shock, yeast cells stimulate a mitogen-activated protein (MAP) kinase

cascade, the high osmolarity glycerol (HOG) pathway (reviewed by Hohmann) [103].
In addition to the responses to specific stress conditions, a general stress response mechanism is present. The general stress, or STRE (stress response promoter element) response involves the induction of approximately 200 genes in response to various stress conditions i. e. heat, osmotic and oxidative stress [22,75]. Induction of these 200 genes involves the binding of transcription factors encoded by two homologous and functionally redundant genes, *MSN2* and *MSN4*, to the STRE element (reviewed by Chatterjee *et al*.) [42]. In addition, the yeast activator protein (Yap) family, formed by eight basic-leucine zipper trans-activators, has also been implicated in a variety of stress responses like oxidative, osmotic, drug and heat stress [214,215]. Trehalose accumulation in response to ethanol, heat and osmotic stress, nitrogen limitation, and cold shock [191] revealed its role as stress-protectant and has been extensively reviewed although the actual role of trehalose as stress protectant at the molecular level is not well understood [71,235,256,283]. In HGB at the end of fermentation, much higher levels of trehalose are detected compared to the levels in brewing under normal conditions [191].

Response of yeast to combined stress conditions
During an industrial fermentation process, yeast is exposed simultaneously to multiple stress conditions. In order to obtain a complete view of the cellular processes during an alcoholic fermentation, the response of yeast to these combined stress conditions has also been studied.
In laboratory strains of *S. cerevisiae*, Hsp104 acts as an excellent stress indicator since transcription of *HSP104* is regulated by the transcription factors Msn2/ 4 as well as by Hsf1 [89]. Therefore, the stress response of ale and lager yeast has been characterized and compared to that of laboratory strains, by analyzing the expression of Hsp104 during fermentation of wort [29]. In this study samples were taken throughout the fermentation, heat shocked for one hour and levels of Hsp104 were detected by Western blotting. Results showed that each brewer's strain exhibited its own pattern of Hsp104 expression but none of the strains was as thermotolerant as the laboratory strain. These results confirm the differences between laboratory and brewer's strains, most likely in this case, due to adaptation to growth conditions at lower temperatures. Moreover, in the same study it was shown that the levels of Hsp104 were extremely low at the end of the fermentation. Differences between growth under industrial conditions and growth under laboratory conditions may account for these changes in expression patterns of Hsp104 but may also be a result of the combined stress conditions of the cells late in fermentation [29]. Transcription profiling of two bottom-fermenting lager strains confirmed the decreased transcription of *HSP104* and of other heat shock proteins i. e. cytosolic Hsp70, stress induced Hsp26, mitochondrial chaperones Hsp60 and Hsp10, membrane heat shock protein Hsp30 and Hsp12. Moreover, the transcription factor Hsf1 controlling heat shock gene induction was found to be repressed [114].

Genome-wide expression (transcriptome analysis) of a *S. cerevisiae* laboratory strain and an industrial strain during VHG fermentation confirmed that laboratory and industrial strains behave differently under stress conditions [59]. This study showed that osmotic and ethanol stress responses

occur in both strains, but the laboratory strain was most affected by these conditions. In another study lower tolerance to oxidative stress was found for laboratory strains compared to industrial strains [74]. In addition, it was concluded that different *S. cerevisiae* strains show a wide variability of responses to the different environmental stress conditions which are highly dependent on their genetic and environmental background. Genome-wide expression analysis of lager yeast strains also revealed high expression of genes involved in protein and lipid biosynthesis in the beginning of fermentation [180], and a response of genes involved in the biosynthesis of ergosterol and oxidative stress protection during the initial stages of fermentation [101,114]. Similar induced expression patterns over the initial 24 hours of fermentation were found for the *ERG10* gene, encoding a protein involved in ergosterol biosynthesis and the *TRR1* gene, encoding thioredoxin reductase [101].
Ergosterol is an essential lipid component of yeast membranes and is an important factor in restoring the fermentative capacity of the cell after storage [101] whereas antioxidants thioredoxin and glutathione protect the yeast cell against oxidative stress [38]. These data revealed interaction between ergosterol biosynthesis and the oxidative stress response. This was confirmed by the observation that *erg* mutants producing altered sterols were highly sensitive to oxidative stress-generating compounds [101]. Additionally, genetic analyses in a laboratory strain of *S. cerevisiae* showed that high osmolarity represses the expression of genes involved in the production of sterols (*ERG3*, *ERG6*, *ERG11*, and *ERG25*) and unsaturated fatty acids (*OLE1*) [209]. This apparent osmotic stress effect implies the involvement of ergosterol in osmotolerance, and may therefore be an important factor in HGB techniques.

In studies investigating the transcriptional response of laboratory strains of *S. cerevisiae* to cold, 10 ° C [226] and 4 ° C [169] respectively, induction of transcription of *OLE1* was observed at early stages. The general stress response, trehalose accumulation [112,122] was also observed in these cold-stressed *S. cerevisiae* cells. *OLE1* is encoding fatty acid desaturase which plays a role in maintenance of membrane fluidity and permeability [172]. Thus, regulation of expression of *OLE1* is at least controlled by two stresses, osmotic stress [209] and cold stress [169,226] and illustrates that a significant proportion of genes are controlled by various stresses.

In summary, these studies showed that genome-wide gene expression analysis of brewer's yeast during industrial fermentation processes provides an effective way to identify and monitor the stress responses of these yeasts to multiple important environmental parameters in fermentation.

1.5 Sugar metabolism

The yeast *S. cerevisiae* employs sugars as its main carbon and energy source. Glycolysis is the general pathway for the conversion of glucose to pyruvate, whereby the production of ATP is coupled to the generation of intermediates and NADH. Further energy production can be obtained by respiration of pyruvate. Especially when yeast is cultivated on high sugar concentrations such as present in wort, *S. cerevisiae* has a strong tendency to ferment sugars to ethanol and carbon dioxide. Alcoholic fermentation may occur even under fully aerobic conditions, a phenomenon known as the Crabtree effect (see paragraph 1.6). An overview of sugar metabolism in *S. cerevisiae* is shown in Figure 2.

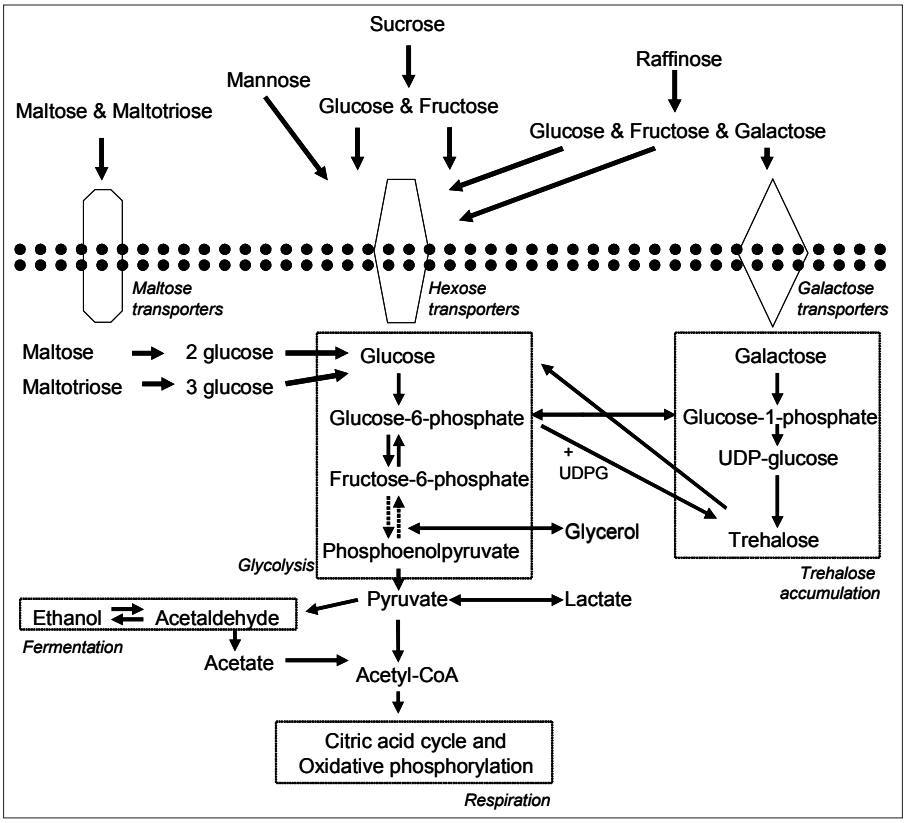

Figure 2. Carbon metabolism in *Saccharomyces cerevisiae*.
An overview of transport of different sugars and their subsequent metabolism. The double dashed arrows represent a series of reactions.

Sugars in wort

Efficient fermentation requires the rapid and complete utilization of the sugars in wort [56,106]. The concentrations of these sugars vary in different worts but maltose is the most abundant fermentable sugar followed by maltotriose. However, some ale yeasts do not ferment maltotriose completely [293]. Other fermentable carbohydrates present in wort are the monosaccharides, fructose and glucose but also mannose and galactose, and the disaccharide sucrose. Dextrins also called amylopectins or α-glucans comprise the bulk of the nonfermentable solids in wort [107] and contribute to the body and mouth feel of beer. The yeast *S. diastaticus* is able to utilize dextrins present in wort due to the presence of the *STA* or *DEX* (starch or dextrin fermentation) genes encoding extracellular amyloglucosidases [136]. Nonfermentable sugars in wort are the β-glucans and pentose sugars like ribose, xylose and arabinose [160]. An example of the concentrations of the various sugars in wort is shown in Table 1.

Table 1. Average carbohydrate levels in wort from an all malt source expressed as a percentage of wort solids [107].

Carbohydrate	% of wort	Description
Maltose	43.9	Disaccharide of two glucose units
Maltotriose	13.6	Trisaccharide of three glucose molecules
Sucrose	4.2	Disaccharide of glucose and fructose
Monosaccharides	9.5	Mainly glucose and fructose, but also galactose and mannose
Dextrins	21.2	Amylopectins, α-glucans
Others	5	Ferment ability depends on yeast strain

Sugar uptake

The limiting factor of maltose and maltotriose metabolism is widely believed to be the transport of these sugars across the plasma membrane by sugar specific carriers [56]. The regulatory mechanisms in yeast ensure an ordered sugar consumption from a medium containing a mixture of sugars [164]. Laboratory strains typically utilize glucose and fructose before the more complex sugars like sucrose. However, during wort fermentation by brewer's yeast sucrose is consumed first, followed by fructose and glucose and then maltose and maltotriose although some overlap occurs [247] (Chapter 4, Table 7).

Sucrose, glucose and fructose

During wort fermentation, the fermentable sugars sucrose, glucose, and fructose are assimilated most rapidly. *S. cerevisiae* cells harbor an extracellular invertase (β-d-fructosidase) encoded by one or several *SUC* genes that hydrolyses sucrose into glucose and fructose [14,37]. Not only the hydrolysis products of sucrose are transported into the yeast cell but also direct uptake of sucrose has been reported [14,171]. Glucose and fructose are the preferred carbon source for *S. cerevisiae* and are taken up by passive, energy-independent facilitated diffusion through glucose (hexose) transporters. Glucose inhibits fructose uptake because the hexose transporters have a higher affinity for glucose than for fructose [273] and glucose can repress the expression of the specific fructose transporter Fsy1 [86,212]. Most brewer's strains are glucophilic, utilizing glucose faster than fructose although some strains consume both monosaccharides at the same rate [164]. Fructose transport in ale yeast strains proceeds exclusively via facilitated diffusion whereas lager yeast strains harbor both the active transport system for fructose uptake as well as the facilitated diffusion system [211].

The monosaccharide mannose is also transported into the yeast cells by hexose transporter (Hxt) proteins. *S. cerevisiae* has 20 genes (*HXT1* to *HXT17, GAL2, SNF3* and *RGT2*) that encode Hxt proteins with high homology concerning their amino acid sequences [133,187]. Transcription of the *HXT1* to *HXT7* genes, encoding the metabolically relevant hexose transporters, is regulated in response to extracellular glucose. The remaining 10 Hxt proteins (Hxt8p to Hxt17p) do not appear to play an important role in glucose uptake since the genes encoding these proteins are expressed only at very low levels [60,187]. *SNF3* and *RGT2* encode plasma membrane proteins involved in glucose sensing to induce expression of specific *HXT* genes [184,185]. After glucose, fructose and mannose are phosphorylated by kinases encoded by *HXK1*, *HXK2* and *GLK1*, they are further metabolized in the glycolytic pathway. A minor sugar of wort, galactose, is transported by the permease encoded by *GAL2* whose expression is regulated by galactose [251,260]. After uptake, galactose is phosphorylated by galactokinase, encoded by *GAL1*, and further converted to glucose-6-phosphate by enzymes encoded by *GAL7*, *GAL10* and *GAL5*.

Maltose and maltotriose

Generally when half of the wort glucose has been taken up the yeast will commence consumption of the α-glucosides maltose and maltotriose, with a slower uptake rate for maltotriose than for maltose [290]. A delay in utilization of maltose and maltotriose can extend the duration of fermentation and may result in incomplete removal due to inhibition by ethanol and the decreased metabolic activity of yeast at the end of fermentation [193].

S. cerevisiae exhibits a shared system of transport for maltotriose and maltose [56]. All α-glucosides are actively transported into yeast cells by a H^+-symport mechanism, which depends on the electrochemical proton gradient across the plasma membrane [270]. For each maltose molecule transported into the cell, one ATP molecule is hydrolyzed [281]. Maltose uptake in *S. cerevisiae* strains requires the presence of at least one of the five highly homologous and unlinked *MAL* loci (*MAL1-4*, *MAL*6). Each locus contains three different genes: *MALx1* encoding a permease, *MALx2* encoding an intracellular maltase or α-glucosidase and *MALx3* encoding a DNA-binding,

maltose-dependent transcriptional activator that specifically controls expression of the *MALx1* and *MALx2* genes [108]. Strains of *S. cerevisiae* may contain α-glucoside transporters encoded by different genes, including *MALx1*, *AGT1* (*MAL11*), *MPH2* and *MPH3*. All these members of the maltose permease family involve active proton transport [242], but differ in substrate range [56] as described in more detail in paragraph 1.8.

The presence of intracellular maltose is necessary for the induction of synthesis of maltase and maltose permease [278]. Under conditions of zero maltose and low glucose, extremely low levels of maltose permease and maltase are produced without expression of *MALx3*. Initial low amounts of maltose are transported into the cell, activating Malx3 protein which increases transcription of *MALx1* and *MALx2* (reviewed by Hazell and Attfield) [96]. The addition of glucose causes a rapid and irreversible loss of maltose transport due to repression of transcription of the maltose permease gene and inactivation of maltose permease [157]. This negative impact of glucose on the metabolism of other sugars is referred to as glucose control and will be described in more detail in paragraph 1.7. After uptake, maltose and maltotriose are broken down into two or three glucose residues respectively, and these glucose molecules are subsequently phosphorylated before entering glycolysis.

1.6 The Crabtree effect

To grow and multiply, yeast is adapted to use organic substances, particularly carbohydrates in the form of sugars. In sugar dissimilation, pyruvate can either be converted into acetyl-CoA, the fuel of the TCA-cycle, or be decarboxylated and reduced to ethanol (Figure 3) [292]. In the latter process, fermentation, only two ATP per glucose are produced whereas in respiration one glucose molecule produces a maximum of 36 ATP molecules. In principle, the oxygen availability will determine whether a yeast respires or ferments pyruvate. Despite fully aerobic conditions some yeasts, including *S. cerevisiae*, may exhibit alcoholic fermentation. This phenomenon is known as the Crabtree effect which enables the yeast to rapidly convert large amounts of sugar before its competitors thus creating a specific niche in its natural environment [57]. The alcohol produced can be consumed later. The molecular mechanism of the Crabtree effect probably results from a multiplicity of factors, including the lack of sufficient capacity of respiration resulting in overflow of pyruvate conversion into ethanol and the regulation of enzyme activities involved in respiration and alcoholic fermentation [267]. Another reason for ethanol formation under aerobic conditions is the presence of high levels of pyruvate decarboxylase, responsible for the conversion of pyruvate into acetaldehyde [271].

The Crabtree effect is one of the reasons for "stuck" fermentations (fermentations that are not complete or do not start at all) in HGB. High glucose levels in wort trigger the yeast to start fermentation, thereby bypassing the respiration stage in which yeast can grow and multiply. This phenomenon is often accompanied by glucose control through a decrease in transcription rate of a number of proteins (catabolite repression) or an accelerated degradation of these proteins (catabolite inactivation).

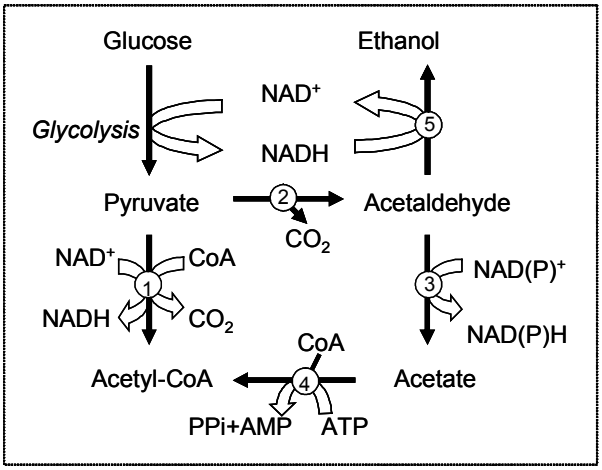

Figure 3. Sugar dissimilation by yeast.
Pyruvate is the key intermediate in sugar dissimilation by yeast. Numbered reactions are catalyzed by the following enzymes: 1 pyruvate dehydrogenase complex; 2 pyruvate decarboxylase; 3 acetaldehyde dehydrogenase; 4 acetyl-CoA synthetase; 5 alcohol dehydrogenase (after Zeeman *et al.* [292]).

1.7 Glucose control in *Saccharomyces cerevisiae*

Catabolite repression and catabolite inactivation

The transcription of genes essential for the catabolism of slowly fermentable sugars i. e. maltose and maltotriose, is repressed when *S. cerevisiae* is cultivated in media such as wort, containing preferred sugars like glucose and sucrose [273]. In addition, transcription of genes for gluconeogenic and mitochondrial functions is repressed (reviewed by Gancedo and Stasyk & Sybirnyi) [73,243]. This catabolite repression is often referred to as glucose repression since glucose is the most repressing sugar and has been extensively studied. While catabolite repression inhibits the synthesis of proteins, the presence of glucose activates a second mechanism, catabolite inactivation [44,104]. Catabolite inactivation is a post-translational degradation process triggered by glucose. Proteolysis of the proteins takes place in the vacuole after internalization by endocytosis [157,210]. Catabolite inactivation affects a variety of proteins, from gluconeogenic enzymes to transport proteins [145].

Catabolite inactivation of the key regulatory gluconeogenetic enzyme fructose-1,6-bisphosphatase (FbPase) in *S. cerevisiae* plays a major role in metabolic adaptation and has been extensively studied [154,206].

Several signaling pathways are involved in the mechanisms of glucose control. The two best investigated and extensively reviewed pathways are the RAS-cyclic-AMP (cAMP) pathway and the main glucose repression pathway [77,126,217]. The RAS-cAMP pathway involves cAMP as second messenger, which in turn activates specific protein kinases. The key elements in the main glucose repression pathway are the sensing hexokinase PII (Hxk2) [98,205], the serine/ threonine protein kinase Snf1 and the DNA binding transcription factor Mig1.

Glucose control has an effect on many metabolic pathways i. e. glycolysis, TCA-cycle, fermentative and respiratory functions and the catabolism of sugars to the level of glucose-6-phosphate (reviewed by Klein *et al*.) [126].

High levels of glucose repress the expression of the *SUC2* gene encoding invertase whereas low levels of glucose are required for maximal transcription of *SUC2* [188]. Mig1 plays a central role in the glucose repression by binding to the *SUC2* promoter and *MIG1* overexpression results in repression of the hydrolysis of extracellular sucrose [31,99,176].

Glucose repression of the *MAL* genes occurs at the transcriptional level, but glucose also stimulates degradation of maltose transporters and inhibits the formation of the active conformation of the transcriptional activator Mal63 (reviewed by Novak *et al*.) [178]. In the presence of maltose the Mal63 protein activates the expression of the genes *MAL61* and *MAL62* and probably that of the *MAL63* gene itself [174]. Transcription of *MAL63* is inhibited by glucose, via Mig1. Removal of a Mig1 binding site in the promoter region of *MAL63* or disruption of *MIG1* increased the levels of Mal63 [109]. Binding sites for Mig1 are also found upstream of the *MAL61* and *MAL62* genes. Hence, in strains where *MAL63* is constitutively expressed, glucose still represses the transcription of the permease Mal61 and the maltase Mal62 [109]. This *MIG1*-dependent mechanism also operates in the presence of maltose. Moreover, maltose permease activity was reduced independent of Mig1, by constitutive activation of the RAS-cAMP pathway [279]. The posttranscriptional events induced by glucose result in the rapid degradation of maltose transporters. Proteolysis of the maltose transporters takes place in the vacuole after internalization by endocytosis [157,210]. Monoubiquitination, i.e. binding of a single ubiquitin molecule to one or more lysine residues of the maltose transporter, signals maximal internalization of this protein [147]. Maltase is not subjected to glucose-induced degradation [87] but hydrolysis of maltose by maltase is competitively inhibited by glucose [236].

The *GAL* genes of *S. cerevisiae* are induced by galactose and repressed by glucose. Glucose repression of the *GAL* genes provides an example of multiple mechanisms collaborating to repress gene expression rapidly and stringently (reviewed by Lohr *et al*.) [142]. Glucose decreases the transcription of *GAL2*, encoding the galactose permease, and even competes for uptake by Gal2 with galactose, thereby decreasing the level of the inducer galactose within the cell (reviewed by Lagunas) [135]. Moreover, catabolite inactivation of the Gal2 permease takes place via ubiquitination, endocytosis and degradation in the vacuole [105]. Glucose also represses *GAL3*, which encodes a

regulatory protein that relieves the inhibition of the transcription activator Gal4 by Gal80 protein [250]. Expression of both *GAL4*, encoding the zinc finger transcription activator, and *GAL1*, encoding a galactokinase is repressed by Mig1 [176]. The transcription of *GAL1*, *GAL2*, *GAL7* and *GAL10* is activated by binding of the transcription activator Gal4. Hence *GAL1* is under dual-level control whereas the other *GAL* genes are under indirect Mig1 control [120,272] .

The sugars fructose and mannose are also able to exert 'glucose repression', but to a lesser extent than glucose [72,255]. The transporter-like proteins, Snf3 and Rgt2 are involved in glucose sensing and in regulation of glucose transport [277]. *MIG1* deletion mutants have shown that the Snf3 and Rgt2 proteins as well as the permease encoded by *HXT4* are subjected to strong Mig1-mediated glucose repression [186]. Moreover, catabolite inactivation of the high-affinity glucose permeases has been described [35,151,204]. The hexose transporters Hxt6 and Hxt7 are degraded in the vacuole after addition of high concentrations of glucose [132].

1.8 Maltotriose utilization in fermentation

Maltotriose is a trisaccharide composed of three glucose molecules. Maltotriose is the second most abundant fermentable sugar in wort and is formed from the breakdown of starch during mashing [107]. A common problem in the brewing industry is a high concentration of yeast fermentable extract in the finished beer. In most instances maltotriose is the main residual sugar [190,247]. Incomplete fermentation leads to a lower ethanol yield and a beer with higher carbohydrate levels and potentially an atypical flavor profile [293]. Therefore, fermentation of maltotriose has received much attention resulting in various explanations. Less efficient fermentation of maltotriose was suggested to be due to poor nutritional supplementation and increased stress in yeast cells used in HGB [40]. Other explanations were the mutation or loss of maltotriose permease during brewing [106] or the conditions experienced during regular fermentation [293]. It was observed that several industrial yeast strains did not ferment maltotriose but only respired it. This observation might explain the incomplete utilization of maltotriose, since in brewing conditions oxygen is completely consumed before maltotriose uptake starts [291]. In contrast, fermentation of maltotriose has been demonstrated for two commercial brewer's strains and a baker's strain of *S. cerevisiae* [143]. Lager yeast strains use maltotriose more rapidly than ale strains [293].

The consumption of maltotriose requires two steps prior to being catabolized via the glycolytic pathway, uptake and hydrolysis into glucose. Most research focused on the first step, maltotriose uptake, since the incomplete utilization of maltotriose in brewing has led to the suggestion of a separate maltotriose uptake system distinct from that for maltose in *S. cerevisiae* [56].

Maltotriose uptake

Maltotriose, like maltose, requires a transporter to enter the cell. There are eight maltose permeases present in *S. cerevisiae* of which five have been characterized: Mal21, Mal31, Mal61, Agt1, Mph2 and Mph3. Attempts to determine the substrate range for the α-glucoside permeases

Mal21, Mal31, Mal61 are inconclusive and often conflicting concerning maltotriose uptake. Radiolabeled maltotriose was used to show that permeases encoded by *MAL31* and *MAL61* were capable of high-affinity transport of maltotriose. This study also showed growth of these yeast strains overexpressing *MAL31* or *MAL61* on the trisaccharide [56]. Other reports indicated that strains only containing Mal61 could not grow on maltotriose [94] and Mal21 could not facilitate H^+ symport activity [241,242]. Recently, other authors reported that only the three α-glucoside permeases encoded by *AGT1*, *MPH2* or *MPH3* are capable of transporting maltotriose [223]. *AGT1* (alpha-glucoside-transporter) encodes a transporter with a substrate range which includes the α-glucosides maltotriose, maltose, isomaltose, melezitose, α-methylglucoside, sucrose, palatinose, trehalose and turanose [94]. Mph2 and Mph3 (maltose-permease-homologue) can transport maltose, maltotriose, α-methylglucoside and turanose as substrates [55].

In the later stages of fermentation, nutritional and environmental stresses are likely to compromise the ability of the yeast cells to ferment any sugar and this might explain why residual sugar can be removed by a second fermentation [293]. As mentioned previously in paragraph 1.5, sucrose and glucose are fermented before maltose and maltotriose. The latter two are widely believed to share the same transport system with maltose as the preferred substrate [56]. Moreover, the uptake rate for maltotriose is slower than for maltose [294]. All these findings together have as result that maltotriose is only utilized in the later stages of a fermentation.

Maltotriose hydrolysis

The second step in the consumption of maltotriose, the breakdown of maltotriose into glucose, received less attention since active maltotriose transport across the plasma membrane, and not intracellular hydrolysis, is the rate-limiting step for fermentation of this sugar [290]. Hydrolysis of maltotriose was compared in two *S. cerevisiae* strains used for brewing, a strain able to ferment maltotriose and a strain unable to ferment the sugar but able to respire maltotriose. The kinetics of maltotriose and maltose hydrolysis by both strains was similar, while the strain that could not ferment maltotriose showed a lower rate of maltotriose transport when compared with the rates of active maltose transport. However, maltotriose and maltose induced synthesis of the same amount of α-glucosidase [290].

Intracellular α-glucosidases or maltases are capable of hydrolyzing terminal, non-reducing 1, 4-linked α-D-glucose residues, as present in maltotriose, with release of α-D-glucose (Figure 4). Synthesis of maltase is regulated by *MALx3*, induced by maltose and repressed by glucose [69] as described in paragraph 1.6. The α-glucosidases from *S. cerevisiae*, encoded by the *MALx2*, *FSP2* and *ROT2* genes, show a higher activity to sucrose and turanose than to maltose [47,194]. Three different α-glucosidases were isolated from a brewer's strain and their substrate specificities were determined. One α-glucosidase, also referred to as isomaltase, showed activity on phenyl-α-glucoside, isomaltose and α-methylglucoside whereas the other two proteins were capable of hydrolyzing phenyl-α-maltoside, turanose, sucrose, maltose, nigerose and maltotriose [153].

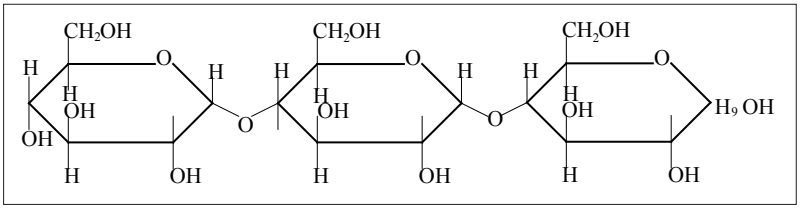

Figure 4. Maltotriose ($C_{18}H_{32}O_{16}$).
Three glucose molecules are joined together via α-1, 4-linkages to form one molecule of maltotriose (O-α -D-glucopyranosyl-(1=> 4)-O-α-D-glucopyranosyl-(1=>4)-D-glucopyranose).

1.9 Genetic techniques for brewer's yeast

Both the lack of selectable genetic markers in brewer's strains and their poor sporulation ability make it difficult to produce and isolate new brewer's strains with overall improved characteristics. A few approaches to overcome these difficulties are described in this paragraph.

Single chromosome-transfer technique
For studying the genetic structure and function of an entire chromosome of a brewer's strain, the single chromosome-transfer technique has been used. This technique makes use of the *kar1-1* mutation and allows the transfer of one or more chromosomes into a strain with the intact *KAR1* gene. *KAR1*, encoding a cell division control protein is involved in nuclear fusion in which two haploid yeast nuclei of opposite mating type, fuse to produce a single diploid nucleus (Figure 5) [218]. In the *kar1-1* mutant fusion of the nuclei of the parent strains after cell fusion is inhibited [46]. However, some heterokaryotic zygotes are formed which contain in addition to the genome of one of the strains one or more chromosomes from the other parent. In crosses of a normal *S. cerevisiae* strain with a *kar1-1* mutant, transfer of genetic information occurs at a low frequency between nuclei [65]. The transfer of specific chromosomes can be achieved when strains are used with appropriate selectable markers. Using this technique, a *S. cerevisiae* strain that carried an extra copy of *S. carlsbergensis* chromosome III, as identified by four markers distributed on both arms (*HIS4*, *LEU2*, *MAT*a, *THR4*), was obtained [177]. More recently the same technique has been used to transfer the *STA2* gene, encoding an extracellular glucoamylase to metabolize starch, from *S. cerevisiae* to the *kar1-1* mutant. Subsequently it was introduced by protoplast fusion in a brewer's strain incapable to use starch [240].

Protoplast fusion
Protoplast fusion is a technique to cross yeast strains that can be used in the genetic modification of brewer's strains since it does not depend on ploidy or mating type [227]. In this technique protoplasts of two yeast strains are mixed and suspended in a fusion agent, usually polyethylene glycol, to

promote protoplast fusion. Subsequently, the fused protoplasts are induced to regenerate their cell walls and recommence division. Using this fusion technique a polyploid strain capable of high ethanol production was obtained by fusion of a flocculant strain with a *S. cerevisiae* sake strain [139]. In other studies protoplast fusion was used to construct a hybrid of *S. cerevisiae* with the ability to utilize starch and to produce a "killer" phenotype. The killer particle prevents contamination by undesirable, sensitive yeasts, and confers immunity from attack by other killer strains. Genes encoding the killer particles and proteins for starch utilization were transferred during this fusion [21]. Although protoplast fusion is an efficient technique, it is not specific enough to modify strains in a predictable manner. The fusion product is often very different from both original fusion partners since many chromosomes are lost after the fusion [246].

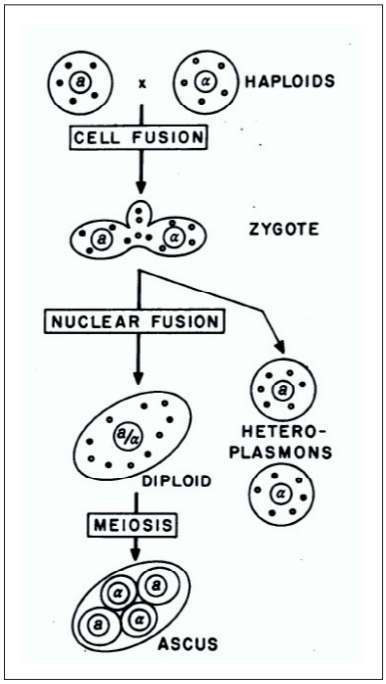

Figure 5. The sexual cycle in *Saccharomyces cerevisiae*. The large circles represent cells and the small ones, which encircle the mating types a and α, represent nuclei. The dots represent mitochondria [46].

Recombinant DNA technology

Recombinant DNA technology makes it possible to modify the genetic composition of brewer's strains without disrupting the many other desirable characteristics of a particular strain. For the transformation of industrial yeast strains, necessary to introduce genes, dominant selectable markers have been developed [85,264] such as resistance to G418 [93], sulfometuronmethyl [41], fluoroacetate [264]

or chloramphenicol [92]. These dominant markers can be used instead of auxotrophic markers, since mutations are not required in the host yeast [3]. The first transformation of both bottom and top fermenting brewer's yeast with a recombinant plasmid was obtained by using the *CUP1* marker for copper resistance [97]. The *KanMX* gene conferring resistance to G418 (a form of gentamycin) has been used widely, in both laboratory and brewer's yeast strains [276]. However, the use of dominant markers has some disadvantages, namely lower transformation efficiency and the appearance of drug-resistant mutants without the marker. Increased drug concentration will reduce the background of non-transformant colonies but also lower the transformation efficiency (reviewed by Akada) [3]. The use of a marker conferring resistance to multiple drugs may reduce this background [5]. The biggest disadvantage of using dominant markers is the introduction of bacterial DNA into the genome of the yeast. Public concern about the safety of genetic engineering forces the brewing companies to refrain from innovations using recombinant DNA technology.

Elimination of unwanted DNA introduced into brewer's strains might be a possibility to partially overcome this problem. The growth-based positive selection for the loss of a marker gene, known as counter-selection [4], has a number of useful applications such as the 'loop-out' of an integrated plasmid containing *TRP1* [259] or Cre-loxP and FLP-FRT systems, in which recombinases induce highly frequent recombination at the target sequences [51,248]. However, most of these counter-selection systems were mainly developed for laboratory yeast and have not yet been applied to industrial strains. An exception is the *ura3*/ 5-FOA system, which has been used in both laboratory strains and brewer's strains. This system uses the 5-fluoro-orotic acid resistance of *ura3* auxotrophs [17].

Another and perhaps better approach to reduce public concern about the safety of genetic engineering might be to focus on self-cloning in which genes of yeast are cloned within the yeast itself (reviewed by Akada) [3]. These self-cloning yeasts do not contain extraneous DNA so there is no risk of drug-resistance gene transfer to pathogenic organisms and no risk of allergic or toxic protein production. The absence of bacterial DNA might facilitate the commercial applications of these yeasts in the brewing industry [8]. A good example of the construction of recombinant industrial yeasts free of bacterial sequences is by directed gene replacement into a nonessential region of the genome [285]. The *MEL1* gene encoding α-galactosidase derived from *S. uvarum* was inserted into a yeast lacking *MEL1* activity to add the capability of utilizing melibiose. A linear fragment containing *MEL1* and *SMR1*, an allele of *ILV2* gene which gives resistance to sulfometuron methyl, was integrated by homologous recombination at the *ILV2* locus [76]. Similarly, the *MET25* gene under control of the constitutive *GAP1* promoter was inserted into the rDNA region of a brewer's strain with the *CUP1* gene as selectable, dominant marker. *MET25* encodes a protein from the methionine biosynthesis pathway which utilizes hydrogen sulfide. The transformants obtained in this study were confirmed to produce ten times less hydrogen sulfide than the parental strain [181].

Strain development for the brewing industry

Despite the development of various techniques for genetic manipulation of brewer's yeast, these would be the last choice to construct new generations of brewer's strains because of the potential impact on sales of beer made with such yeast strains. The isolation of brewer's yeast with the desirable characteristics, like stress-resistance towards the conditions in HGB, directly from the existing yeast culture is preferred. The next choice would be obtaining strains by classical mutagenesis, usually UV and EMS (ethanemethyl sulphonate), and selection. These methods have the advantage that strains with improved properties can be used in the industry without extensive registration procedures. However, recombinant DNA technology will be crucial to define and analyze the properties of new strains and will facilitate the isolation of desired mutants by "classical" methods.

1.10 Outline of this thesis

High-gravity brewing is a technology which allows a higher productivity of the brewery. In this technology more concentrated wort is used, resulting in beer with a higher strength which is subsequently diluted to the desired alcohol content. The yeast strain used by a particular brewer is strongly associated with the final beer product. The current limit in HGB is about 16 to 18 °P since higher gravities lead to aberrant flavour profiles and stuck fermentations due to the exposure of yeast to multiple stress conditions during the fermentation.
A multi-disciplinary European Union project focused on the development of brewer's yeast strains that have a higher resistance to the stress conditions present in high-gravity brewing. Within this project, I focused on the development of molecular biological tools for brewer's strains and on maltotriose utilization in alcoholic fermentation. The results are described in this thesis.
For the rapid identification of genes whose inactivation or overexpression in industrial yeast strains enhances resistance to stress conditions in HGB, a disruption library and an overexpression library were developed. Both libraries contain a dominant marker, selectable in polyploid yeast strains. The detailed construction and evaluation of the quality of these libraries are described in Chapter 2. The possibility of inactivating genes in yeast by means of *in vivo* transposition was analyzed and is also described in Chapter 2. By using the libraries, a maltotriose transporter, encoded by *MTT1*, was identified in lager yeast strains in a selection screen for improved maltotriose utilization. The isolation, identification and characterization of the *MTT1* encoded transporter using uptake assay with labeled maltotriose is described in Chapter 3. Since the isolation of this transporter confirmed that uptake is the limiting factor, I also investigated whether expression of a maltase on the outside of the yeast cell could improve maltotriose utilization. In Chapter 4 the fusion of α-glucosidase encoded by the *MAL32* gene, with the N-terminal secretion signal of Kre1 and the C-terminal domain of either Cwp2 or Flo1 is described. It is demonstrated that α-glucosidase displayed on the cell surface of lager yeast strain A15 was capable of hydrolyzing the α-1, 4-linkages between the glucose subunits of maltotriose under fermentation conditions. Finally, in Chapter 5 a summary and the general discussion of this work are presented.

Chapter 2

Molecular biological tools for industrial yeast strains: Disruption and overexpression libraries *In vivo* transposition

Abstract

High-gravity brewing (HGB) is a development in the brewing industry which uses wort at higher concentration resulting in a substantially higher density of the fermentation broth and in a higher final alcohol volume. The use of the HGB process enhances the brewery's capacity due to reduced energy, labor and capital costs. However, a reduced fermentation rate and loss in yeast viability are introduced due to the environmental stress conditions yeasts encounter during the HGB process. To study the stress-response of industrial yeasts we used several approaches since genetic methods developed for laboratory *Saccharomyces cerevisiae* strains are usually not applicable for industrial yeasts as brewing strains may be polyploid, aneuploid or alloploid with a much more complex and diverse genetic constitution than haploid and diploid laboratory strains. First we selected a dominant marker that could be used in industrial yeast and second we developed a disruption library and an overexpression library containing genomic DNA from four lager strains and a laboratory *S. cerevisiae* strain. Both libraries will allow rapid identification of genes whose inactivation or overexpression enhances stress resistance against the conditions present in HGB. Third we made use of the observation that *S. cerevisiae ade2* mutants form red colonies to investigate whether anti-sense mRNA could inhibit gene expression in *S. cerevisiae*. A fourth approach to study stress related genes was to improve the efficiency of *in vivo* transposition in yeast. We showed that introduction of hyperactive transposase encoded by the corresponding gene with a nuclear localization sequence and transposon DNA into the target yeast cell enhanced the number of random transposon insertion events, but only marginally.

Introduction

High-gravity brewing (HGB) has been introduced into breweries to increase their productivity. HGB uses wort at higher concentration and consequently requires dilution of the obtained beer to the desired alcohol content [15]. A general problem in HGB is the reduced fermentation rate, change in flavor profile of the beer and loss of yeast viability due to several intensified stress factors. Compared to regular brewing, especially the high osmolarity, the high ethanol content, the low extracellular pH, nutrient starvation and high CO_2 levels affect yeast negatively [199]. Improvement of the brewer's yeast itself by increasing the stress-resistance of the industrial yeasts is desired.
Industrial strains are usually polyploid but may also be aneuploid or alloploid [125] with a much more complex and diverse genetic constitution than haploid and diploid laboratory strains. Brewer's yeast strains sporulate poorly and spore germination occurs rarely [116]. Recently, competitive comparative genome hybridisation (CCGH) to *S. cerevisiae* DNA microarrays and quantitative real-time polymerase chain reaction (qRT-PCR) assays were used to examine the copy number of *S. cerevisiae*-like genes [19]. In addition, the entire genome of lager brewing strain Weihenstephan 34/70 (WS34/70) was sequenced (Kodama *et al.,* unpublished). However, the copy number of a specific gene remains still unknown in most of the brewer's strains. Consequently, genetic methods developed for *S. cerevisiae* laboratory strains are not always applicable for industrial yeasts [3]. For example, auxotrophic mutations which have been used with their corresponding wild-type genes to select yeast transformants, can not easily be obtained in polyploid yeast strains [219]. For the transformation of industrial yeast strains dominant drug-resistance markers may be used instead, since mutations are not required in the host yeast [3]. Transformation of industrial yeast strains is an essential tool to apply recombinant DNA technology and therefore a number of dominant drug markers have been developed [85,264]. One of these dominant markers, the *kanMX* gene, has been used in various projects [276]. The *kanMX* gene confers resistance to the aminoglycoside antibiotic G418 (a form of gentamycin). Unlike other commonly used aminoglycoside antibiotics like kanamycin and neomycin, G418 effectively inhibits yeast cell growth [118].

To identify genes involved in stress response in industrial yeast strains we wished to be able to test the effects of overexpression of genes and of mutants with reduced expression or null alleles. The former can be simply achieved by using an overexpression library. To this end a genomic library was constructed containing chromosomal DNA fragments from one laboratory strain and four lager strains present in a multi-copy vector. We used DNA from five different strains to increase the chances of finding relevant genes. A Tn5 transposon provided with a dominant selection marker for *S. cerevisiae* as well as a bacterial selection marker and the strong phosphoglycerate kinase (*PGK1*) promoter was inserted *in vitro* by a transposition reaction. The presence of the dominant marker on the Tn5 transposon is essential since transformation of industrial yeast strains is a required step in the usage of the library. If the transposon is inserted in the promoter region of a gene the *PGK1* promoter will overexpress this particular gene.

Null phenotypes or reduced expression are much more difficult to obtain. In haploid laboratory strains both dominant and recessive null alleles of non-essential genes are easily obtained by homologous integration. Due to the presence of multiple copies of a gene in industrial yeast strains, usually only one copy of the gene is affected and hence only dominant alleles will lead to a phenotype. Moreover, in industrial strains homologous recombination and transformation appear to be less efficient than in laboratory strains. To overcome this problem we attempted different strategies. In a first approach we introduced into the genomic library a similar Tn5 transposon as for the overexpression library but without the *PGK1* promoter. Due to the presence of multiple copies of a mutated gene several effects may occur, including a form of "squelching" by which transcription factors are titrated away from the intact genes, thus lowering their expression [201]. This transcriptional interference, or squelching, is most likely due to competition between activating factors for a productive interaction with limiting transcription factors as described by Gilbert *et al*. and references therein [82]. By using both libraries instead of classical mutagenesis it would be easier to verify the gene of interest due to the presence of unique primer binding sites at each end of the Tn5 transposon for bidirectional sequencing.

In another approach we studied whether anti-sense mRNA can inhibit gene expression. If in the overexpression library described above the *PGK1* promoter is inserted in reverse orientation at the 3'-end of an open reading frame, anti-sense mRNA may be formed. While RNA silencing or RNA interference (RNAi) appears to be absent from *S. cerevisiae* it is a common defense mechanism among eukaryotes against foreign genetic material and cellular mutations [163]. The main actor of RNA silencing is double-stranded RNA (dsRNA) [162] which is synthesized by the enzyme RNA-dependent-RNA polymerase (RdRP) with aberrant RNAs as templates. The next step in RNA silencing is the processing of dsRNAs into short RNA molecules by RNAse-III-endonucleases. Two types of RNAse-III-endonucleases, Drosha and Dicer, are involved in the processing of dsRNAs into three kinds of short RNAs, short-interfering RNAs (siRNAs), repeat-associated short interfering RNAs (rasiRNAs) and microRNAs (miRNAs) respectively [39]. The nuclease Drosha processes dsRNA into miRNA precursors in the nucleus and after transport to the cytoplasm these precursors are processed by the nuclease Dicer into miRNA duplexes of 19-23 nucleotides in length. The miRNA duplexes are first unwound and then assembled into effector complexes referred to as miRNA containing Ribonucleoprotein (miRNP). Finally, single strand miRNAs impair translation by binding via imperfect RNA-RNA interaction to their mRNA target within the 3' Untranslated Region (3'UTR). In addition, some miRNAs are completely complementary to their mRNA target and these induce specific cleavage. Long dsRNAs are directly processed by the nuclease Dicer to yield siRNA and rasiRNA duplexes. The siRNAs get incorporated into the RNA-induced silencing complex (RISC) and, after strand separation by a helicase, the remaining single-stranded RNAs direct the sequence-specific cleavage of single-stranded cytoplasmic RNA.

The rasiRNAs, on the other hand, mediate chromatin condensation and thus play a role in epigenetic changes that result in transcriptional silencing.
In this study we made use of the observation that *S. cerevisiae ade2* mutants form red colonies to investigate whether anti-sense mRNA could inhibit gene expression in the yeast. In *S. cerevisiae ade1* and/ or *ade2* mutations lead to the accumulation of a red pigment resulting in red colonies, whereas wild type *ADE1* and/ or *ADE2* cells form white colonies [262]. The full length *ADE2* gene and two smaller *ADE2* fragments were cloned in reverse orientation behind the *PGK1* promoter in a haploid laboratory strain. The red/white colony color assay was used to investigate inactivation of the *ADE2* gene.

Another tool to identify and isolate stress related genes may be inactivation of genes in yeast by *in vivo* transposition. *In vivo* transposition may generate random transposon insertions after introduction of the transposon and the gene encoding the transposase into the target cell. The advantage of *in vivo* transposition over *in vitro* transposon mutagenesis is that this procedure does not require reintroduction of mutagenized genes into the yeast genome by homologous recombination [221]. This is especially important for brewer's strains, for which homologous recombination is an inefficient process. Moreover, due to cloning of yeast DNA fragments prior to transposon insertion, larger open reading frames are underrepresented in the disruption and overexpression libraries generated in this study. However, in general the frequency of *in vivo* transposition is low [288] due to technical limitations, e.g. expression of transposase (Tnp) in the target host and genetic instability due to expression of Tnp in subsequent generations [54]. To bypass these limitations a technique has been developed in which Tn5 transposase-transposon complexes made *in vitro*, called transposomes, are introduced into the target cell by electroporation [54]. Subsequently, the transposon integrates at random positions in the genome. Tn5 is a bacterial genetic element that transposes via a simple 'cut and paste' mechanism [288]. The components required for this mechanism are: (1) the transposon containing two appropriately spaced and oriented inverted 19 base pair repeat sequences defining the ends of Tn5, called the outside end (OE) sequences [88] and (2) the hyperactive Tnp that contains three mutations, EK54, MA56 and LP372 respectively [88]. This *in vivo* transposition technology can generate transposon insertions in various bacterial species like *Escherichia coli*, *Salmonella typhimurium* and *Proteus vulgaris* with high efficiency of about 10^5 colonies per μg DNA. The technology also works in the yeast *S. cerevisiae* but less efficient: only a few colonies are formed per μg DNA [54]. We hypothesized that this low efficiency might be due to the inability of hyperactive transposase to enter the nucleus in yeast as found for Ty1 integrase which requires a nuclear localization signal (NLS) at the C-terminus for retrotransposition [124]. In addition, nuclear targeting of T-DNA in plants is thought to be mediated by NLS found in two virulence proteins, VirE2 and VirD2 as described by Gelvin and references therein [78]. To test this idea, several plasmids coding for hyperactive Tn5 transposase with or without a NLS were constructed and the effect of the NLS on the transposition efficiency was investigated. Localization of the hyperactive transposase containing a NLS in the nucleus was analyzed using GFP as a reporter protein.

Materials and methods

Strains and media

E. coli XL1-Blue [32] was used for plasmid amplification. Standard methods were used for *E. coli* transformation by heat shock [111]. ElectroMAX™ DH10B™ cells (Invitrogen) were used for transformation by electroporation. *E. coli* was grown in Luria-Bertani (LB) medium [225], when required provided with 40 µg/ ml kanamycin (Boehringer Mannheim), 12.5 µg/ ml tetracycline (Boom bv, Meppel) or 60 µg/ ml ampicillin. Two haploid laboratory *Saccharomyces cerevisiae* strains CEN.PK113-7D (*MATa*), obtained from P. Kötter (J.-W. Goethe Universität, Frankfurt, Germany) and CEN.PK113-3B (*MATa, ura3, his4*), and five lager strains; A15 (kindly supplied by Dr. J. Londesborough, VTT biotechnology, Finland), WS34/70 (Weihenstephan brewery, Freising, Germany), CMBS-33 (kindly supplied by Prof. J. M. Thevelein, KU Leuven, Belgium), LM01 (kindly supplied by Dr. M. C. Walsh) and OG2252 (kindly supplied by Prof. M. C. Kielland-Brandt, Carlsberg Laboratory, Denmark although this strain originates from Alfred Jorgensens Laboratorium, Denmark) were used in this study. Standard lithium acetate methods were used for yeast transformation [80]. Yeast was grown in either YPD [233] or MY [295] medium, supplemented when required, with histidine (20 mg/ l) and uracil (20 mg/ l). G418 (Duchefa) was used at a concentration of 150 µg/ ml or less when indicated. ClonNAT and Hygromycin B (Boehringer Mannheim) were used at the concentrations indicated in the text.

Isolation of yeast chromosomal DNA

Chromosomal DNA was isolated from 2 ml yeast culture grown overnight in YPD. Cells were collected by centrifugation for 1 min at 13,000 rpm. The yeast pellet was washed with 1 ml sterile water, resuspended in freshly made lysis buffer (1 M sorbitol, 0.1 M EDTA (pH 8.0), 0.15 M NaCl) with lyticase (4 mg lyticase/ ml) and incubated for 45 min at 30 °C. After incubation 1600 µl T10E20SDS (10 mM Tris-HCl (pH 8.0), 20 mM EDTA (pH 8.0), 1 % SDS) were added and mixed by inverting the tube. After 5 min at 65 °C, the transparent solution was distributed over two Eppendorf tubes and 1 volume cold phenol/ chloroform (1:1) solution was added. The mixture was vortexed for 5 min before centrifuging it for 5 min at 13,000 rpm. The upper phase was extracted several times with phenol/ chloroform solution till the interphase disappeared completely. Then one volume of chloroform was added to the upper phase. After vortexing for 5 min and centrifugation for 5 min at 13,000 rpm, DNA was precipitated by addition of 0.1 volume 3 M sodium acetate (pH 5.2) and 0.6 volume ice-cold isopropanol. The chromosomal DNA was collected by centrifugation at 13,000 rpm for 15 min, washed with 70 % ethanol, dried under vacuum, dissolved in 30 µl TE (pH 8.0) with RNase, incubated for 1 hr at 37 °C and 30 min at 65 °C and stored at -20 °C.

Table 1. Plasmid features

Plasmid	Features
pRUL409	Genomic library plasmid containing the kanamycin resistance marker, *ARS1* and unique *BamH*I-site for cloning genomic DNA fragments digested with *Sau3A*-I
pMORIS	Plasmid containing the *PGK1* promoter
pRUL401	Plasmid consisting of 4882 base pairs of vector pYES2 (Invitrogen) and containing the *PGK1* promoter from vector pMORIS and the KanMX cassette
pMOD3<R6Kγori/MCS>	EZ-Tn5™ transposon construction vector (Epicentre)
pRUL853	pMOD3<R6Kγori/MCS> (Epicentre) with overexpression transposon containing the KanMX-cassette, Ppgk1 and a tetracycline marker
pRUL854	Transposon construction vector pMOD3<R6Kγori/MCS> (Epicentre) with disruption transposon containing the KanMX-cassette and a tetracycline marker
pRUL1079	Plasmid pRUL409 containing a linker fragment with *Sac*I, *Xma*I, *Sph*I and *Xho*I restriction sites
pRUL1080	Plasmid pRUL1079 containing the KanMX cassette and the *PGK1* promoter upstream the unique *BamH*I-site for cloning the *URA3* gene
pRUL1081	The *URA3* gene from strain CEN.PK113-7D under control of Ppgk1
pRUL739	Plasmid pRUL409 containing the KanMX cassette and the *PGK1* promoter that was generated by PCR with primers PGK1p-A and PGK1p-B, upstream the unique *BamH*I-site for cloning *ADE2* fragments in reversed orientation
pRUL739(2EDA)	The *ADE2* gene from strain CEN.PK113-7D in reversed orientation under control of Ppgk1
pRUL739($ADE_{1.2kb}$)	A 1206 bp *ADE2* fragment from strain CEN.PK113-7D in reversed orientation under control of Ppgk1
pRUL739($ADE_{0.5kb}$)	A 541 bp *ADE2* fragment from strain CEN.PK113-7D in reversed orientation under control of Ppgk1
pGRTEMP2	Vector containing hyperactive Tn5 transposase, kindly provided by Dr. W. S. Reznikoff [88]
pUG35	Vector which allows the N-terminal in frame fusion of the yEGFP3 encoding open reading frame to the gene of interest kindly provided by Prof. J. H. Hegemann
pUG36	Vector which allows the C-terminal in frame fusion of the yEGFP3 encoding open reading frame to the gene of interest kindly provided by Prof. J. H. Hegemann
pUG36(Tnp)	C-terminal fusion between hyperactive Tn5 transposase (Tnp) and GFP in pUG36
pUG35(Tnp)	N-terminal fusion Tnp and GFP in pUG35
TOPO(TnpNLS)	pCR®-Blunt II-TOPO® vector (Invitrogen) with TnpNLS
pUG36(TnpNLS)	C-terminal fusion between hyperactive Tn5 transposase (Tnp) with NLS and GFP

Molecular biological methods and plasmid constructions

Molecular biological techniques were carried out by standard procedures [225]. The QIAprep mini spin kit (Qiagen) was used to isolate plasmid DNA from *E. coli*. The same kit was used to purify plasmid DNA from *S. cerevisiae*, after yeast cells were incubated for 45 minutes at 30 °C with 1 mg/ ml lyticase (Sigma) in buffer P1 (Qiagen). Plasmid features are shown in Table 1. The sequences of oligonucleotides used for the construction of plasmids are shown in Table 2.

Table 2. Oligonucleotides

Name	Sequence (5'=>3')	Tm (°C)
ARSXba	GCT CTA GAGCGG CCG CGT TAT CCC CTG ATT CTG T	60
ARSEco2	CCG GAA TTC GTC GAT GTG CAA AAG CCT AC	60
Tetracycline-FW	GCT CTA GAA GTG CCA CCT GAC GTC TAA G	62
Tetracycline-RV	CGG CCG AGC TCT GAT TCA TTC TGC TAA CCA G	56
Linkerp18a	CTA GAG AGC TCC CCG GGG CAT GCC TCG AGG	-
Linkerp18b	GAT CCC TCG AGG CAT GCC CCG GGG AGC TCT	-
URA3Xho	CCG CTC GAG CAT GTC GAA AGC TAC ATA TAA G	60
URA3BamH	CGG GAT CCG CCA TGA AGC TTT TTC TTT CC	60
PGK1p-A	GCT CTA GAG CAA GTA CCA CTG AGC AG	61
PGK1p-B	CGG GAT CCT GTT TTA TAT TTG TTG TAA AAA	61
ADE2-fw	CGG GAT CCA TGG ATT CTA GAA CAG TTG G	56
ADE2-rv-antisense	GCG GAT CCT TAC TTG TTT TCT AGA TAA GCT	56
ADE2-II-fw-antisense	CGG GAT CCG TTG GAC TTG GAA GCA ATG	56
ADE2-II-rv-antisense	GCG GAT CCC ATT GCT TCC AAG TCC AAC	56
TransposaseNf	CGC GGA TCC ATG ATA ACT TCT GCT CTT CAT CG	64
TransposaseNr	TGC CAA GCT TGC ATG CCT GC	64
TransposaseCr	GAC GTC GAC GAT CTT GAT CCC CTG CGC CA	64
NLSlinkerF	TCG ATC CAA AAA AGA AGA GAA AGG TCG TTG TTA ATA GTC GAC CAG CT	-
NLSlinkerR	CGT CGA CTA TTT AAC AAC GAC CTT TCT CTT CTT TTT TGG	-

PCR amplification

The primers used for PCR are listed in Table 2. PCR amplifications were performed with 50-100 ng genomic DNA or 25-50 ng plasmid DNA. Amplification conditions were: 5 min at 94 ºC, 30 cycles of 1 min 94 ºC, 1 min at 5 ºC below Tm of the primers and 1 min per kb to be amplified at 74 ºC. Followed by a final step of 10 min at 74 ºC. Reactions were performed in a total volume of 50 µl and per reaction 0.5 µl Vent polymerase (New England BioLabs) was used with the buffer supplied. PCR products were analyzed on a 0.7 % agarose gel in 1 x TAE buffer. All PCR products were cloned into the pCR®-Blunt II-TOPO® vector (Invitrogen) before they were recloned in the desired vector.

Plasmids used in genomic, overexpression and disruption libraries

In order to obtain the genomic library, plasmid pRUL409 was constructed by inserting *ARS1,* amplified in a PCR from plasmid pNatCre [244] using the primers "ARSXba" and "ARSEco2" (Table 2), into the *Xba*I and *EcoR*I sites of vector pHSS6 [231], thereby introducing a *Not*I site next to the *EcoR*I sites flanking the ARS. Plasmid pRUL854 containing the disruption transposon, was constructed by inserting the KanMX cassette obtained by digestion of pUG6 [90] with *Bgl*II and *Sac*I into the *BamH*I and *Sac*I sites of the transposon construction vector pMOD3<R6Kγori/MCS> (Epicentre) followed by insertion of the tetracycline marker amplified by PCR from pBR322 [280] using primers "Tetracycline-FW" and "Tetracycline-RV" (Table 2), into the *EcoR*I and *Sac*I sites. Plasmid pRUL401 was constructed by re-ligation of the 4882 bp *Ssp*I fragment of vector pYES2 (Invitrogen) followed by insertion of the *PGK1* promoter from vector pMORIS into the *BamH*I and *EcoR*I sites and insertion of the KanMX cassette from pUG6 [90] into the *BamH*I and *Sac*I sites. For the construction of pRUL853 containing the overexpression transposon, plasmid pRUL401 was digested with *EcoR*I and *Sac*I and the resulting KanMX-Ppgk1 fragment was cloned into vector pMOD3<R6Kγori/MCS> (Epicentre) followed by insertion of the tetracycline marker amplified in a PCR from pBR322 into the *Xba*I and *Sac*I sites of the vector. In order to test the *PGK1* promoter used in the overexpression library, plasmid pRUL1081 was constructed by inserting a linker fragment into the *Xba*I and *BamH*I sites of pRUL409 giving pRUL1079. The linker fragment contained *Sac*I, *Xma*I, *Sph*I and *Xho*I sites and was obtained by boiling a mixture of the two oliglonucleotides, linkerp18a and linkerp18b (Table 2), for 5 minutes and then leaving it overnight in the cooling water bath [213]. The 2273 bp *Sac*I-*Xho*I fragment containing the *PGK1* promoter and the KanMX cassette, from pRUL401, was cloned into the *Sac*I and *Xho*I sites of pRUL1079 to create pRUL1080. Finally, plasmid pRUL1081 was obtained by inserting a PCR amplified *URA3* fragment (primers "URA3Xho" and "URA3BamH") into the *Xho*I and *BamH*I sites of pRUL1080.

Plasmids used for production anti-sense mRNA

Plasmid pRUL739 was constructed by providing plasmid pRUL409 with a KanMX cassette [276] digested from pUG6 by inserting it as a *BamH*I-*Bgl*II fragment into its *BamH*I site and a *PGK1* promoter amplified by PCR from chromosomal DNA of strain CEN.PK113-7D using primers "PGK1p-A" and "PGK1p-B" and inserted into its *Xba*I and *BamH*I sites. Three *ADE2* fragments were amplified by PCR from chromosomal DNA of *S. cerevisiae* strain CEN.PK113-7D using primer-sets "ADE2-fw/ ADE2-rv-antisense", "ADE2-fw/ ADE2-II-reversed" or "ADE2-II-fw-antisense/ ADE2-rv" resulting in 1732, 1206 and 541 base pair fragments, respectively. Plasmid pRUL739 was digested with *BamH*I and dephosphorylated for three hours at 37 °C with 0.5 units Shrimp Alkaline Phosphatase (Promega) to subclone the three *ADE2* fragments in reverse orientation behind the *PGK1* promoter. This resulted in the plasmids pRUL739(2EDA), pRUL739($ADE_{1.2kb}$) and pRUL739($ADE_{0.5kb}$).

Plasmids used for in vivo transposition in yeast

Hyperactive Tn5 transposase (Tnp) was amplified from plasmid pGRTEMP2 [88] by PCR using primers "TransposaseNf" and "TransposaseNr" (Table 2) and inserted into the *BamH*I and *Sal*I sites from vector pUG35 (obtained from Prof. J. H. Hegemann, Institute of Microbiology, University of Düsseldorf, Germany) for an C-terminal fusion with GFP and thereby creating plasmid pUG35(Tnp). Plasmid pUG36(Tnp) was constructed in a similar manner but Tnp was amplified using primers "TransposaseNf" and "TransposaseCr" (Table 2) and inserted into the *BamH*I and *Sal*I sites from vector pUG36 (obtained from Prof. J. H. Hegemann, Institute of Microbiology, University of Düsseldorf, Germany) for a N-terminal fusion with GFP. For the attachment of a nuclear localization signal, Tnp from plasmid pGRTEMP2 [88] was amplified by PCR using the primers "Transposase Nf" and "Transposase Cr" (Table 2) and inserted into the pCR®-Blunt II-TOPO® vector (Invitrogen). An NLS fragment, obtained by boiling a mixture of the two oliglonucleotides "NLSlinkerF" and "NLSlinkerR" (Table 2) for 5 minutes and then leaving it overnight in the cooling waterbath [213], was ligated into the *Sal*I and *Sac*I sites of this vector. The vector pUG36(TnpNLS) was obtained by ligating the TnpNLS fragment digested with *BamH*I and *Sal*I from TOPO(TnpNLS) into the *BamH*I and *Sal*I sites of pUG36.

Construction of the disruption and overexpression libraries

A mixture of approximately equal amounts of chromosomal DNA from lager strains OG2252, WS34/70, CMBS-33, A15 and laboratory *S. cerevisiae* strain CEN.PK113-7D was partially digested with *Sau3A*-I (Figure 1A). The resulting DNA fragments were isolated by agarose electrophoresis and the QIAquick Gel Extraction Kit (QIAGEN). Isolated DNA fragments of 1.5-5 kb were ligated into the *BamH*I site of the commercially dephosphorylated vector pRUL409 (BaseClear labservices). The ligation mixture was electroporated into *E.coli* Electromax DH10B cells (Invitrogen) using standard procedures (Invitrogen) and kanamycin resistant colonies were pooled. Plasmids isolated from the colony-pool formed the genomic plasmid library (Figure 1B). In order to construct the disruption and overexpression libraries, the Tn5 transposon from plasmid pRUL854 for the disruption library and from plasmid pRUL853 for the overexpression library were inserted according to the manufactures instructions, by an *in vitro* transposition reaction (Epicentre Technologies) into the genomic plasmid library (Figure 1C). The Tn5 transposons from both plasmids contained an R6Ky ORI, a tetracycline marker and a KanMX cassette flanked by mosaic ends. The Tn5 transposon of pRUL853 contained the *PGK1* promoter in addition. Plasmids pRUL854 and pRUL853 were digested with *Pvu*II and the released Tn5 transposons were gel purified (QIAquick Gel Extraction Kit, QIAGEN). To enhance transposition efficiency Tn5 transposons were isolated without ethidium bromide staining. Transposon DNA (up to 50 µg/ ml) and target DNA (0.2 µg of the genomic plasmid library) were incubated with 10 µg/ ml hyperactive Tn5 transposase (Epicentre Technologies) in EZ::TN Reaction Buffer (0.5 M Tris-acetate pH 7.5, 1.5 M potassium acetate, 100 mM magnesium acetate, 40 mM spermidine) for 2 hours at 37 °C in a 10 µl reaction volume. The transposition was stopped by addition of 1 µl EZ::TN 10X stopsolution and incubation for 10 minutes at 70 °C. Transposomes (1 µl reaction mix) were electroporated

into *E. coli* DH10B cells (Invitrogen) and kanamycin- and tetracycline- resistant colonies were pooled. Plasmids isolated from the colony-pools formed the disruption and overexpression plasmid libraries, respectively.

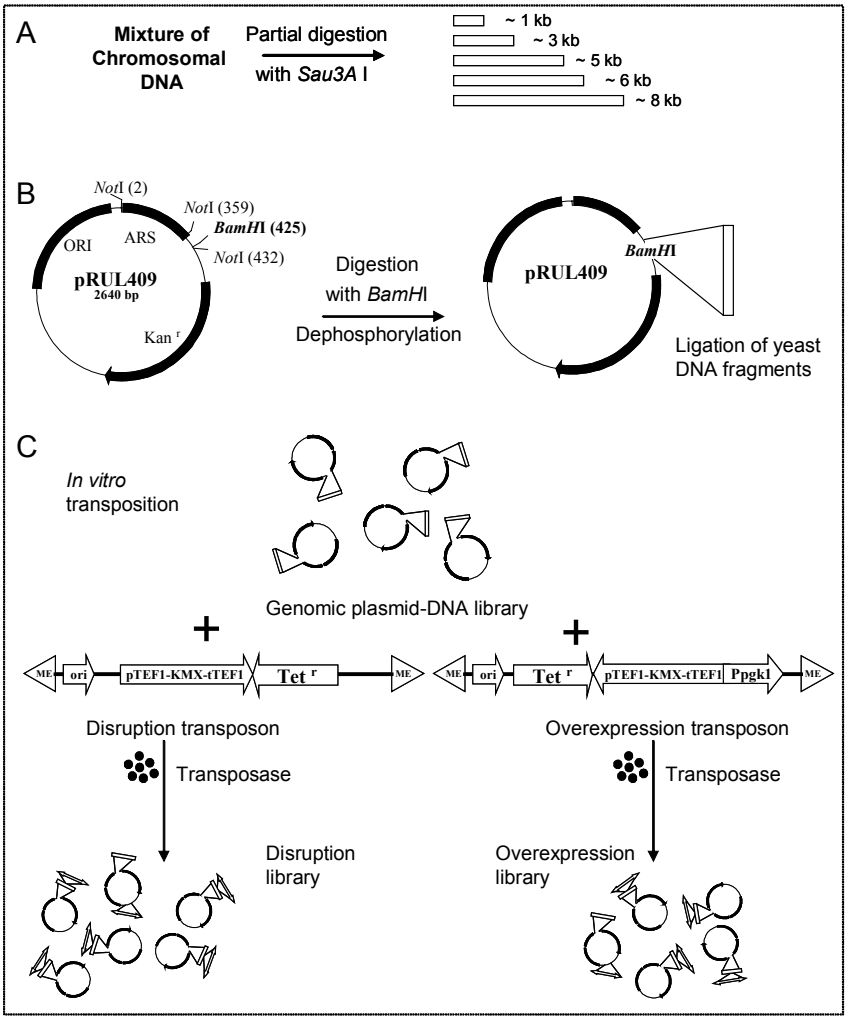

Figure 1. Schematic overview of the construction of the disruption and overexpression libraries. (A) Partial digestion of chromosomal DNA with *Sau3A* I resulting in DNA fragments with variable sizes. (B) Dephosphorylation of pRUL409 digested with *BamH*I and cloning yeast DNA fragments to create the genomic plasmid library. (C) Disruption or overexpression transposon insertion into the genomic plasmid library by an *in vitro* transposition reaction with the Tn5 transposase enzyme to create the disruption and overexpression libraries, respectively.

Colony blotting

For determination of the quality of the genomic libraries, approximately 85,000 bacterial colonies were transferred from the surfaces of agar plates to positively charged nylon membranes (Roche) as described previously [225]. Membranes were placed twice for 2 minutes on Whatman paper saturated with denaturizing solution (0.5 M NaOH, 1.5 M NaCl), 1.5 minutes on Whatman paper saturated with neutralizing solution (1.5 M NaCl, 0.5 M Tris-HCl pH 7.4) and incubated for 2 minutes in neutralizing solution. After the membranes were washed twice in 2 x SSC (0.3 M NaCl, 0.03 M sodium citrate pH 7.0) they were dried and UV-irradiated for 3 minutes for cross-linking of the DNA. The membranes were probed with α-^{32}P dCTP labeled *PGK1* or *ERG1* DNA fragments from strain CEN.PK113-7D [254]. Hybridization was carried out at 62 ºC overnight in hybridization solution (1% blocking reagent (Roche), 2 x SSC, 20 mM sodium pyrophosphate, 1% SDS) plus the labeled DNA probe (25 ng). After hybridization, membranes were washed three times 5 minutes with 2 x SSC/ 0.1 % SDS at room temperature, three times 20 min with 0.1 x SSC/ 0.1 % SDS at 65 ºC and stored in a phosphor screen cassette at room temperature till analysis with the phospho-imager.

RNA extraction and Northern-blot analysis

Tipps, eppendorf tubes and solutions were made RNAse free by autoclaving at 121 ° C for 45 min and hybridization boxes were incubated with 10 % chlorine solution. Total RNA was isolated from yeast by resuspending cell pellets of approximately 10 O.D. units in phenol buffer (400 µl AE buffer (50 mM sodium acetate pH 5.2 and 10 mM EDTA pH 8.0), 40 µl 10 % SDS, 400 µl phenol/ chloroform/ iso-amylalcohol (25:24:1)) and incubated for 5 min at 65 °C [228]. The mixture was stored at least 15 min at -80 °C and centrifuged at 13,000 rpm for 15 min. The aqueous layer was extracted with one volume of phenol/ chloroform/ iso-amylalcohol (25:24:1). After addition of 40 µl 3 M sodium acetate (pH 5.2) and 1 ml 100 % ice cold ethanol, RNA was precipitated for at least 15 min at -20 °C. The RNA was collected by centrifugation at 13,000 rpm for 15 min, washed with 70 % ethanol, dried under vacuum, dissolved in water and stored at -80 °C. Total RNA samples of 10 µg were diluted 1:1 with 100% de-ionised formamide and an equal volume of sample buffer was added. The RNA samples were denaturated by incubation for 10 min at 65 °C and separated on 1% agarose/ 1.5 % formaldehyde gels. Gels were blotted as described [225] to nylon membranes (Boehringer) using a vacuum blotting device. Blots were probed with α-^{32}P dCTP labeled DNA fragments as described for colony blotting.

Microscopy

Images were captured using a Zeiss Axio Plan Imaging microscope, Axio camera MRc5 and a PC with Adobe PhotoShop 8.0. CEN.PK113-3B cells containing pUG36(Tnp), pUG35(Tnp) or pUG36(TnpNLS) were grown in MY medium supplemented with L-histidine for 16 hours at 30 °C. To visualize nuclei, cells in stationary phase were incubated with 0.1 volume of diluted 1:1000 Hoechst 33258 (Invitrogen) for 5 minutes.

Results

Genomic, overexpression and disruption libraries

To be able to identify genes in industrial yeast strains, novel molecular biological tools are needed. For searching genes involved in stress response of industrial *Saccharomyces* strains, an overexpression library and a disruption library were developed. For the construction of these libraries a vector containing a dominant selection marker is required. Selection markers giving resistance to Geneticin (G418), Hygromycin B and, nourseothricin (commercially available as ClonNAT) are available. To investigate whether these selection markers can be used in lager strains, the lager strain LM01 and for comparison the laboratory *S. cerevisiae* strain, CEN.PK113-7D were tested for sensitivity to these three drugs. In the plate assays the lager strain was 25-50 times more sensitive to all three drugs than the laboratory strain (Table 3). Comparison of the sensitivity of both strains to the three drugs indicated minor differences. Therefore based on its successful use in various other projects [62,79,244,284] the KanMX cassette which confers resistance to G418, was selected as the dominant marker in the libraries.

Table 3. Sensitivity to Hygromycin B, G418 and ClonNAT

Drug	Strain	Concentration (µg/ ml)	
		Growth	No growth
Hygromycin B	LM01	0.5	1
	CEN.PK113-7D	40	50
G418	LM01	1	2
	CEN.PK113-7D	40	50
ClonNAT	LM01	0.2	0.5
	CEN.PK113-7D	10	15

Ten fold dilutions of lager strain LM01 and laboratory strain CEN.PK113-7D, grown in YPD till A_{600} of about 1.0, were spotted onto YPD with different concentrations Hygromycin B, G418 and ClonNAT. Plates were incubated at 30 °C for 3 days.

Prior to the construction of the overexpression and disruption libraries, a genomic library was constructed. Genomic DNA of four lager strains (A15, CMBS33, WS34/70 and OG2252) and one laboratory strain (CEN.PK113-7D) was partially digested with *Sau3A*-I and ligated into the dephosphorylated *BamH*I site of the vector pRUL409 (Figure 1A and B). These ligations were electroporated into *E. coli* cells and $6x10^5$ kanamycin resistant colonies were pooled.

The quality of the genomic library was determined by two criteria i. e. restriction analysis and colony blotting. For the restriction analysis, plasmids were isolated from 72 randomly selected colonies from the $6x10^5$ primary transformants of the genomic library and digested with *Not*I. Analysis of the 72 plasmids digested with *Not*I on 0.7 % agarose gel revealed that 50 plasmids contained an insert with an average size of 2.6 kb (Figure 2 and Table 4).

Table 4. Overview of sizes of yeast chromosomal DNA inserts present in the genomic, disruption and overexpression libraries

Insert size (kb)	Number of plasmids		
	Genomic library	Disruption library	Overexpression library
0- 0.9	22	3	9
1.0-1.5	2	5	2
1.6-2.0	17	1	0
2.1-2.5	8	7	2
2.6-3.0	8	1	2
3.1-3.5	10	2	3
3.6-4.0	2	1	3
4.1-4.5	1	0	1
4.6-5.0	0	0	0
5.1-5.5	0	0	0
5.6-6.0	0	0	1
6.0-6.5	2	0	0
6.6-7.0	0	2	2
7.1-7.5	0	1	1
7.6-8.0	0	1	0

Colony blotting was used to check the representation of the *PGK1* and *ERG1* genes in the genomic library. Two times approximately 42,600 bacterial colonies of the library were transferred from agar plates to membranes and hybridized with α-^{32}P dCTP labeled *PGK1* or *ERG1* DNA fragments from strain CEN.PK113-7D. For the *PGK1* probe this resulted in 12 clearly positive spots where 6 could be expected ((number of screened colonies * percentage of plasmids containing an insert * average insert size (bp))/ estimated size of haploid genome (bp): (42,600 * 0.76 * 2.6)/ 14,000)) and for the *ERG1* probe on the same filters 11 clear positive spots were found (data not shown). These data indicated good representation of the genes in the genomic library.

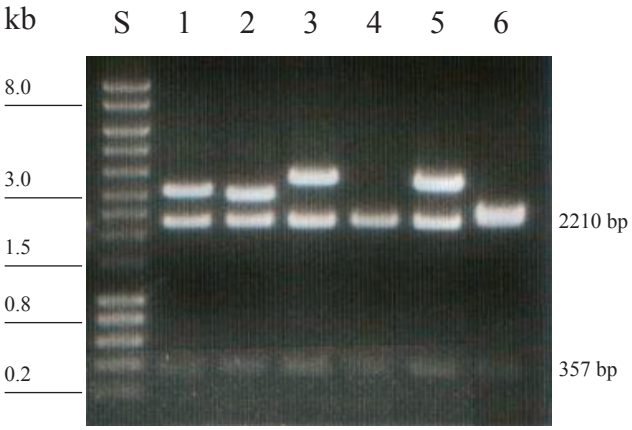

Figure 2. Quality of the genomic library determined by restriction analysis.
Plasmids were isolated from the genomic library and subsequently digested with *Not*I. Digestion mixtures were analysed on a 0.7 % agarose gel with the smartladder (S) (Eurogentec) as a marker. A fragment of 357 bp in size was expected representing the *ARS1* element, and two fragments of 73 bp and 2210 bp in size representing pRUL409 (lane 4). In case a yeast chromosomal DNA fragment was present in the *BamH*I site of pRUL409 one or more extra fragments of variable sizes were expected (lanes 1, 2, 3, 5 and 6). Estimated chromosomal DNA insert sizes in lanes 1, 2, 3, 5 and 6 are 3.1; 3.0; 4.0; 3.9 and 2.3 kb respectively. The figure shown is representative for 10 similar experiments.

For the construction of the disruption library, a Tn5 transposon digested with *Pvu*II from plasmid pRUL854 was inserted *in vitro* by a transposition reaction into a pool of plasmids isolated from the primary transformants of the genomic library (Figure 1C). The Tn5 transposon contained a R6Ky ORI, a tetracycline resistance marker and a KanMX cassette flanked by mosaic ends. Transposition reaction mixtures were electroporated into *E.coli* cells and $4.5x10^5$ kanamycin- and tetracyclin- resistant colonies were pooled.
To check the quality, plasmids were isolated from 24 randomly selected colonies from the $4.5x10^5$ primary transformants of the disruption library. The 24 plasmids were digested with *Hind*III and analysis on an 0.7 % agarose gel revealed that 21 plasmids contained both an insert and a transposon. The average size of the yeast DNA inserts was 3.3 kb. Remarkably this size is larger than that in the genomic library (2.6 kb). An explanation will be proposed in the Discussion section. The remaining three plasmids contained the transposon but lacked a genomic DNA insert of detectable size (Figure 3 and Table 4).

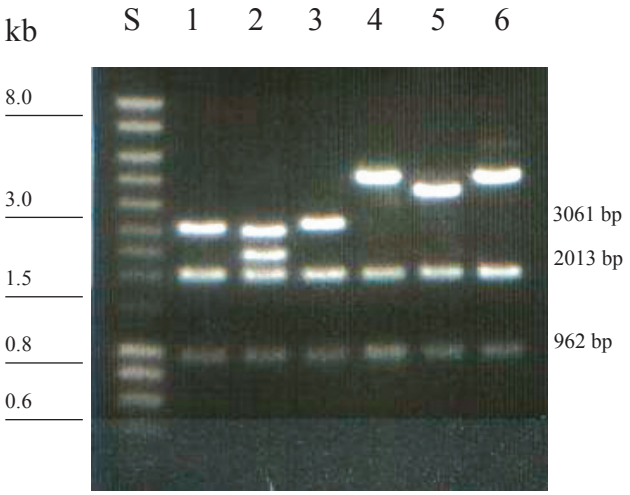

Figure 3. Quality of the disruption library determined by restriction analysis.
Plasmids were isolated from the disruption library and subsequently digested with *Hind*IIII. Digestion mixtures were analysed on a 0.7 % agarose gel with the smartladder (S) (Eurogentec) as a marker. For digestion of pRUL409 containing the disruption transposon but no yeast DNA insert, two fragments of 2013 bp and 962 bp in size, representing transposon DNA, and one fragment of 3061 bp in size, representing vector DNA, were expected (lane 1 and 3). In case a yeast chromosomal DNA fragment was present in the *BamH*I site of pRUL409 one or more extra fragments of variable sizes were expected (lanes 2, 4, 5 and 6). Estimated DNA insert sizes in lanes 2, 4, 5 and 6 are 2.4; 2.2; 1.3; and 2.1 kb respectively. The figure shown is representative for 4 similar experiments.

The overexpression library was constructed similarly as the disruption library but a Tn5 transposon containing a *PGK1* promoter was inserted *in vitro* (Figure 1C). Electroporation of *E .coli* cells with transposition reaction mixtures resulted in $5.1x10^5$ primary transformants. From these primary transformants 32 kanamycin- and tetracycline- resistant colonies were randomly selected for plasmid isolation. The 32 plasmids were digested with *Not*I and analysis on 0.7 % agarose gels showed that 23 plasmids contained an insert of 8.0 kb including the 4.3 kb transposon, thus giving a 3.7 kb yeast chromosomal DNA insert on average (Figure 4 and Table 4). This average insert size is again larger than that in the genomic library (2.6 kb) as will be discussed later.

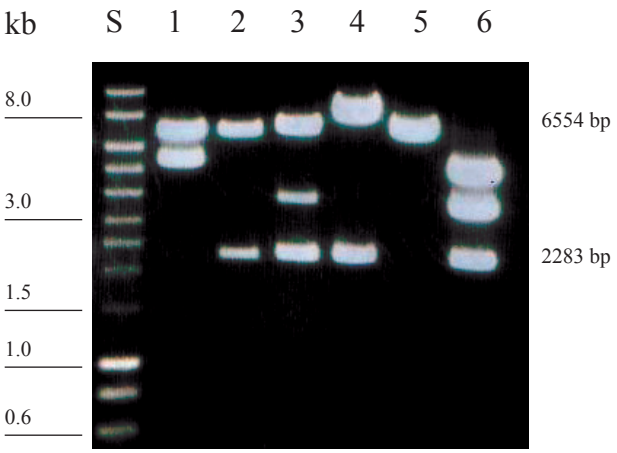

Figure 4. Quality of the overexpression library determined by restriction analysis. Plasmids isolated from the overexpression library were digested with *Not*I. Digestion mixtures were analysed on a 0.7 % agarose gel with the smartladder (S) (Eurogentec) as a marker. Fragments of 357 bp (*ARS1,* not shown) and 6554 bp in size representing pRUL409 with the overexpression transposon were expected when yeast DNA was absent (lane 5). In case a yeast chromosomal DNA fragment was present in the *BamH*I site of pRUL409 and therein the transposon, fragments of 357 bp (*ARS1,* not shown), 2283 bp (the remainder of the vector) and one or more fragments of variable sizes representing the yeast gDNA insert together with the 4271 bp transposon DNA were expected (lanes 2, 3, 5, and 6). If the transposon resided in the vector instead of in the yeast DNA, fragments of 357 bp (*ARS1,* not shown) , 6554 bp (pRUL409 plus transposon) and one or more fragments of variable sizes representing the yeast gDNA insert were expected (lane 1). Estimated DNA insert sizes in lanes 1, 2, 3, 4 and 6 are 5.5; 2.5; 7.0; 3.5 and 4.2 kb respectively. The figure shown is representative for 4 similar experiments.

The constitutive promoter of the *S. cerevisiae* phosphoglycerate kinase gene (Ppgk1) is used for overexpression of the genes cloned into the *BamH*I site in the overexpression library. To determine the actual strength of this promoter (obtained from plasmid pMORIS via PCR) we transformed *S. cerevisiae* wildtype cells with plasmid pRUL1081 carrying the *URA3* gene under control of the *PGK1* promoter. Total RNA was isolated from this transformed strain as well as from the non-transformed wild-type strain. Northern blot analysis showed that transcript levels of *URA3* under control of the *PGK1* promoter in *S. cerevisiae* cells were 1.4 times higher than the transcription levels of the same gene under control of its own promoter (data not shown). The presence of the *URA3* transcript in Ura$^-$ *S. cerevisiae* cells transformed with pRUL1081 confirmed that Ppgk1 was regulating the expression of *URA3* under aerobic growth conditions (data not shown).

Anti-sense mRNA

Anti-sense mRNA is widely used in higher eukaryotes to inhibit gene expression. When the transposon with the *PGK1* promoter from the overexpression library, is inserted into the yeast genome in the reverse orientation at the 3' end of an open reading frame, anti-sense mRNA may be produced. Although *S. cerevisiae* lacks some important genes involved in RNA silencing [6,200], we were nevertheless interested to see whether anti-sense mRNA is able to inhibit gene expression in industrial yeast strains.

To study the possible effect of anti-sense mRNA molecules in yeast we made use of the characteristic phenotype of *ade2* mutants. In *S. cerevisiae, ade1* and/ or *ade2* mutations lead to the accumulation of an intermediate compound in the adenine biosynthesis pathway, P-ribosylaminoimidazole. Cells that are growing aerobically oxidize this compound into a red pigment, resulting in red colonies [262]. Since wild type *ADE1* and/ or *ADE2* cells form white colonies, these red *ade2* mutants are useful because they are simply recognized.

In order to investigate whether the phenotype of *ade2* mutants could be obtained by introducing anti-sense *ADE2* mRNA, the *ADE2* gene was cloned in reverse orientation behind the *PGK1* promoter (Figure 5). In contrast to the *PGK1* promoter used in the overexpression library, the *PGK1* promoter used in this study is amplified directly from chromosomal DNA of *S. cerevisiae* strain CEN.PK113-7D (see Materials and methods section). Subsequently, for the production of anti-sense *ADE2* mRNA *in vivo*, strain CEN.PK113-3B was transformed with this construct (pRUL739(2EDA)) and the empty vector as a control and transformants were selected on YPD containing G418. This resulted in the presence of thousands of only white colonies indicating that the cells produced the Ade2 protein. If *ADE2* anti-sense mRNA had bound to endogenous *ADE2* sense mRNA and translation would have been completely inhibited, red colonies should have appeared on the selection plates.

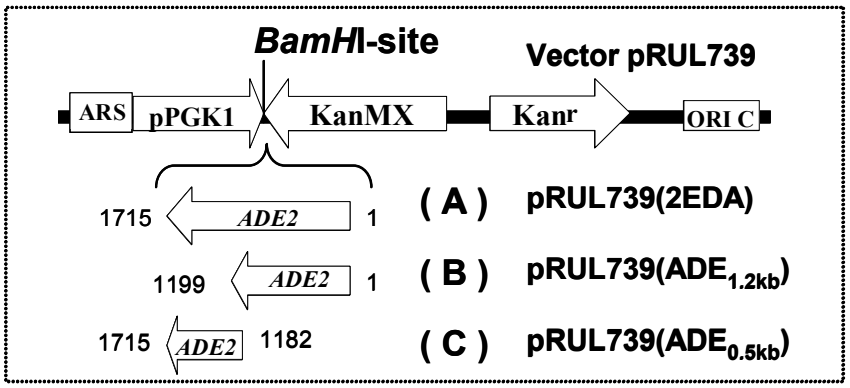

Figure 5. *ADE2* anti-sense constructs.
The entire 1715 bp ORF of *ADE2* (A) and two fragments (B and C) were amplified in a PCR and inserted into the *BamH*I site of the multicopy vector pRUL739. In order to produce high levels of anti-sense mRNA in vivo the *ADE2* fragments were cloned in reverse orientation behind the strong *PGK1* promoter.

Analysis of total cellular RNA extracted from the transformed yeast strains was performed to confirm the presence of anti-sense mRNA. Two different *ADE2* probes were used for hybridization, a 541 bp *BamH*I fragment from pRUL739($ADE_{0.5kb}$) (Figure 5C) and a 1206 bp *BamH*I fragment from pRUL739($ADE_{1.2kb}$) (Figure 5B). Results in Figure 6 show the presence of higher levels of *ADE2* transcript in the CEN.PK113-3B strain carrying the plasmid with full-length *ADE2* in reverse orientation (Figure 6A and B, lane 2) compared to CEN.PK113-3B cells carrying the empty vector only (Figure 6A and B, lane 1) after hybridization with both the 541 bp probe (Figure 6A) and the 1206 bp probe (Figure 6B). This higher level of expression for CEN.PK113-3B containing pRUL739(2EDA) suggests the presence of anti-sense mRNA. However, using this method both sense and anti-sense mRNA can be detected. As it is expected that sense and anti-sense mRNA have a similar length, we cannot discriminate between the two types of mRNA. Therefore two smaller *ADE2* fragments, 541 and 1206 bp respectively were cloned in reverse orientation behind the *PGK1* promoter (Figure 6B and C). Transformation of CEN.PK113-3B cells with these constructs again resulted in only white colonies. Northern blotting analysis revealed similar transcription levels for the CEN.PK113-3B cells carrying pRUL739($ADE_{1.2kb}$) (Figure 6B, lane 3) compared to the cells with pRUL739(2EDA) (Figure 6B, lane 2) when probed with the 1206 bp *ADE2* fragment. If anti-sense mRNA is present, it is expected to have a smaller size. Moreover, CEN.PK113-3B cells carrying pRUL739($ADE_{1.2kb}$) probed with the 541 bp *ADE2* fragment also resulted in the presence of mRNA bands with the same size as endogenous *ADE2* (Figure 6A, lanes 2 and 3) suggesting the presence of sense mRNA instead of anti-sense mRNA. For the CEN.PK113-3B control cells and cells carrying pRUL739($ADE_{0.5kb}$) lower transcription levels were found when probed with both the 541 bp *ADE2* fragment (Figure 6A, lanes 1 and 4) as the 1206 bp *ADE2* fragment (Figure 6B, lanes 1 and 4). Since shorter mRNA bands were expected to hybridize with the 541 bp *ADE2* probe, this result indicates that shorter anti-sense mRNA molecules were absent.

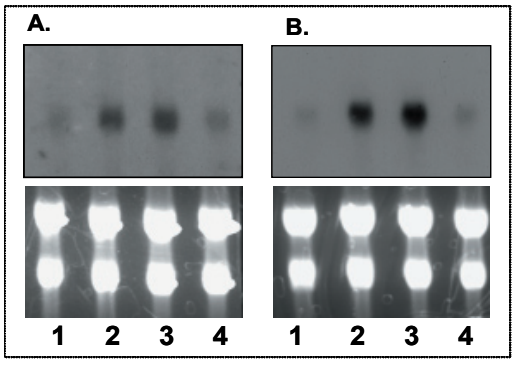

Figure 6. Northern analysis. RNA was extracted from strain CEN.PK113-3B with either pRUL739 (lane 1), pRUL739(2EDA) (lane 2), pRUL739(ADE1.2kb) (lane 3) or pRUL739(ADE0.5kb) (lane 4). Ethidium bromide stained gels are shown at the bottom of each corresponding Northern blot.
A. The blot was probed with the ^{32}P-labeled 541 bp *BamH*I fragment from pRUL739($ADE_{0.5kb}$).
B. The blot was probed with a ^{32}P-labeled 1206 bp *BamH*I fragment from pRUL739($ADE_{1.2kb}$).

In vivo transposition in yeast

The *in vivo* transposition technique in which transposomes are introduced into target cells by electroporation [54] generates less efficiently transposon insertions in *S. cerevisiae* than in bacterial species. This difference in efficiency might be due to the accessibility of the DNA for the transposase enzyme. Yeast DNA is present in a membrane-surrounded nucleus whereas bacterial DNA floats within the cell, usually in a loop or coil. As a consequence, the transposomes have to enter the yeast nucleus before transposon insertion events can occur. To test whether the membrane around the nucleus in yeast is preventing access of the Transposome, several plasmids carrying the gene encoding hyperactive Tn5 transposase [88] with or without a NLS (nuclear localization signal) were constructed. For localization studies, DNA encoding the hyperactive Tn5 transposase (Tnp) enzyme was also cloned in fusion with GFP in pUG35 or pUG36 vectors, giving C- and N-terminal fusions respectively. Microscopy was performed on cells transformed with these constructs. For yeast cells with GFP fused to the C- or N-terminus of the transposase, containing pUG36(Tnp) or pUG35(Tnp) respectively, GFP was observed to be present in strings and dots at the cell periphery (Figure 7A, left panel). In contrast, for cells with Tnp fused to a NLS in addition to GFP, i. e. containing plasmid pUG36(TnpNLS), the GFP signal was found as a single spot in the cell (Figure 7B, left panel). These signals co-localized with the nuclei as shown by staining with Hoechst 33258 (Figure 7A and 7B, middle panels). Hence, the addition of a NLS efficiently targeted Tnp to the nuclei.

To investigate whether the number of transposition events increased as a consequence of the attachment of a nuclear localization sequence (NLS) to Tnp, yeast strains containing plasmid pUG36(Tnp) with or without a NLS were transformed with linear DNA fragments (transposons) containing a KanMX cassette. G418-resistant colonies were selected. The colonies that grew again in the presence of G418 after re-streaking were considered to be G418 resistant due to integration of transposon DNA into the chromosomal DNA. In eight independent experiments the transformation efficiency was still low and variable but in each experiment the number of transposon integrations was higher for the hyperactive Tn5 transposase enzyme with NLS. With NLS the mean and standard deviation was 14 ± 4 events per transformation and without the NLS 6 ± 3 events.

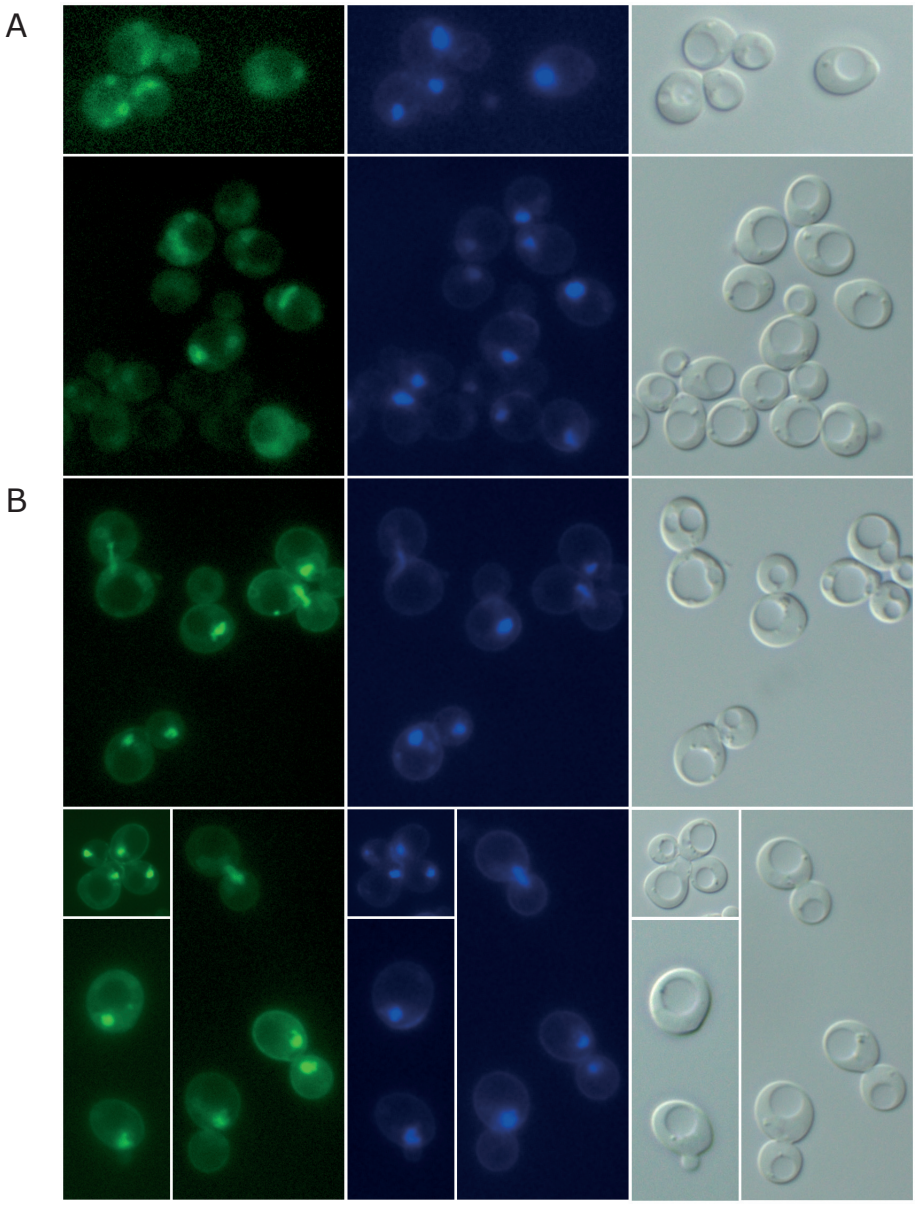

Figure 7. Localization of hyperactive Tn5 transposase (Tnp).
Microscopy was performed on cells containing Tnp-GFP (A) or TnpNLS-GFP (B). Cells were stained with Hoechst to visualize the nuclei as described in the Materials and methods section. Left and middle panels show the GFP and Hoechst signal, respectively. Differential interference contrast (DIC) microscopy pictures are shown to the right of the corresponding fluorescence pictures.

Discussion

In order to identify genes whose inactivation or overexpression enhances stress resistance in industrial yeast strains a disruption library and an overexpression library with the dominant *KanMX* marker were developed (Figure 1).

The quality of the genomic library used to construct both the disruption library and the overexpression library was determined by colony blot analysis and restriction analysis. The data obtained with colony blot analysis indicated a good representation of the genes in the library and restriction analysis revealed the mean insert size and percentage of plasmids containing an insert (2.6 kb and 70 %, respectively). These numbers together with the following formulae, (number of primary transformants * percentage of plasmids containing an insert/ 100 * average insert size in kb) / (5 * size of haploid genome in kb), can be used to estimate the coverage of the library. Assuming the "haploid genome" to be 14,000 kb in size then on average the "haploid genome" of each of the five yeast strains present in the genomic library is covered approximately 16 times ($6x10^5$ * 0.7 * 2.6 / 5 * 14,000).
For the disruption and overexpression libraries, the mean insert size and percentage of plasmids containing an insert were also determined by restriction analysis. Remarkably, the mean insert size of both the disruption and the overexpression library (3.3 kb and 3.7 kb, respectively) and the number of plasmids with an insert (87 and 72 % respectively) were larger than that of the genomic library they originally originated from (2.6 kb and 70 % respectively). A possible explanation could be that in plasmids carrying no or small yeast DNA inserts the transposon integrated more frequently in vector sequences necessary for maintenance in the *E. coli* cells e.g. the kanamycin resistance marker or the origin of replication.
The coverage of both libraries was estimated to be approximately 18 times for the disruption library ($4.5x10^5$ * 0.87 * 3.3 / 5 * 14,000) and approximately 19 times for the overexpression library ($5.1x10^5$ * 0.72 * 3.7 / 5 * 14,000).
The disruption library may be used in two ways (Figure 8). First transformation of the yeast strain of interest with the intact plasmid library since pRUL409, the plasmid in which the libraries are constructed, is a multicopy plasmid. In this way there will be many copies of the disrupted gene present on the plasmid with respect to the number of intact genes present in the yeast genome (Figure 8B). Second, digestion of the plasmid library with *Not*I to isolate linear DNA fragments carrying yeast DNA inserts with a disruption transposon. These linear DNA fragments can subsequently be used for integration by homologous recombination to obtain disruption of a chromosomal copy of the corresponding gene (Figure 8A). In this case one disrupted copy will compete with one (diploid strains) or more (polyploid strains) copies of the intact gene. A disadvantage for this second way to use the disruption library might be the fact that transformation efficiency is 100-1000 times lower when a linear DNA fragment is used than transformation of intact plasmid DNA.

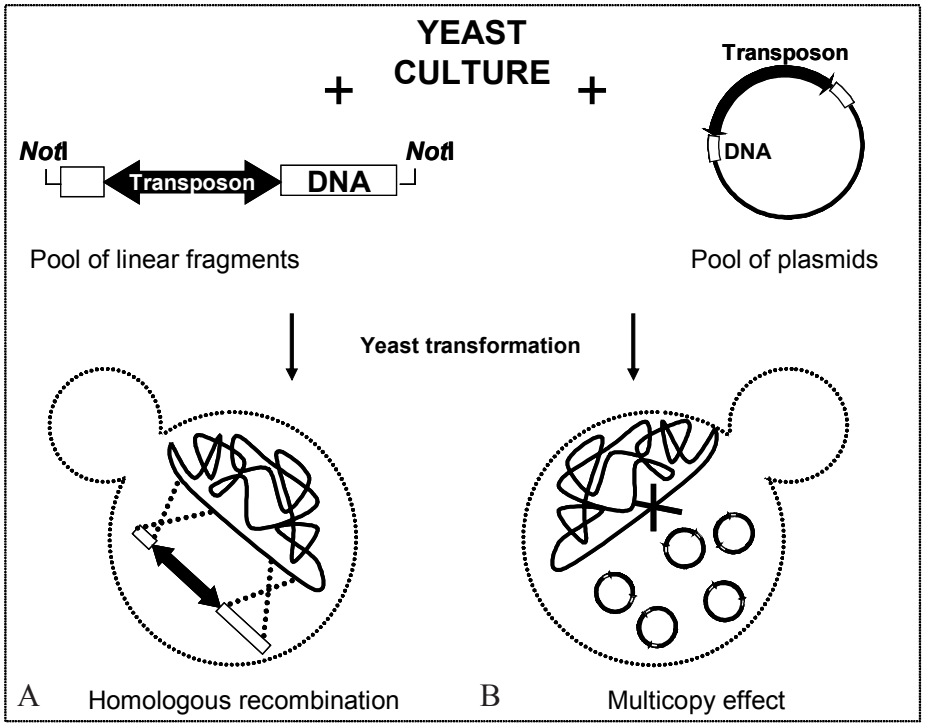

Figure 8. Application of the disruption library.
The disruption library may be used in two ways.
A Transformation of yeast cells with linear DNA fragments in order to achieve gene disruption via homologous recombination. B Transformation of yeast cells with the plasmid DNA library to achieve reduced gene expression via the presence of multiple plasmids with the disrupted gene.

For the usage of the overexpression library it is important to note that the *PGK1* promoter has to be located in the appropriate position of a particular gene to overexpress this gene. Approximately 1 in 280,000 integrants will have the gene of interest under the control of the *PGK1* promoter when assuming a genome of 14 Mb and a window of 100 bp where integration will give a proper transcript. In this way, in the original $3.6x10^5$ transformants with the transposon in the yeast insert ($0.70 * 5.1x10^5$), each gene will be represented 1.3 times under control of the *PGK1* promoter ($3.6x10^5$ / 280,000). In the overexpression library, not all *PGK1*-driven genes will be present in their full-length form on the plasmids. This is not an issue when linear DNA fragments digested from the plasmid library with *Not*I are used for integration into the yeast genome.
The constitutive promoter of the *S. cerevisiae PGK1* gene, used in the overexpression library, was chosen due to its high efficiency under fermentation conditions [138,237]. In contrast, our results showed that the actual strength of the *PGK1* promoter present in our overexpression library is fairly low. In Ura$^-$ *S. cerevisiae* cells transformed with a plasmid carrying the *URA3* gene under

control of Ppgk1, the *URA3* transcript was present, indicating that Ppgk1 was working under aerobic growth conditions. However, transcription of *URA3* under control of the *PGK1* promoter in *S. cerevisiae* wildtype cells was only 1.4 times higher than the expression of the same gene under control of its own promoter. This relatively low expression could be explained by a number of basepair differences we noticed during sequence analysis of the *PGK1* promoter.

For the production of anti-sense mRNA the *PGK1* promoter was also used to regulate transcription of three different *ADE2* fragments. These *ADE2* fragments were cloned in reverse orientation behind a *PGK1* promoter amplified by PCR from chromosomal DNA of CEN.PK113-7D, whereas Ppgk1 used in the overexpression library was obtained from plasmid pMORIS. Northern blotting analysis confirmed the functionality of this *PGK1* promoter present on plasmid pRUL739, and showed that the actual strength of this *PGK1* promoter was much higher (about 70 times higher expression) compared to the one used in the overexpression library (1.4 times higher expression). Interestingly, Northern analysis did not directly show the presence of anti-sense mRNA since all detected mRNA bands had the same size as expected for endogenous *ADE2* mRNA. However, higher transcription levels for CEN.PK113-3B cells carrying pRUL739(2EDA) or pRUL739($ADE_{1.2kb}$) compared to the control cells strongly suggests the presence of anti-sense mRNA *ADE2* molecules. It might be also possible that these anti-sense mRNA *ADE2* molecules induce the transcription of *ADE2*. However, for cells carrying pRUL739($ADE_{0.5kb}$) transcription levels were similar as found for the control cells. This result means that relatively short anti-sense mRNA *ADE2* molecules are absent or have no effect. The fact that red colonies were not obtained in any case means that translation of *ADE2* sense mRNA was not or not sufficiently inhibited. Nevertheless, the observation that transcription of the *ADE2* gene is enhanced after expression of anti-sense RNA is very interesting and worth further investigation.

In literature it is suggested that the absence of the RNA-endonuclease of RNAseIII-like Dicer in *S. cerevisiae* plays a major role in its incapability of silencing through an RNAi mechanism [6,200]. The gene encoding Dicer in fission yeast *Schizosaccharomyces pombe* has been isolated and sequenced, and replacement of this gene by the gene encoding human Dicer partially restored its RNA silencing capacity [200]. It would be interesting to introduce Dicer in *S. cerevisiae* to see whether this would lead to inactivation of genes. While not so useful for laboratory strains this would be a very important and powerful tool for industrial strains.

Recently, the use of so called intrabodies in yeast has been reported [10]. Intrabodies are antibodies that are produced inside the organism and provide a powerful technology to alter the function of the target antigen [149]. This intrabody technology has been applied recently to study the function and intracellular localization of the *S. cerevisiae* Sem1 protein [208]. Further development of this method in yeast would complete the tools to manipulate enzyme activities i.e. mutation at the DNA level, anti-sense RNA for RNA and intrabodies directly at the protein level.

Another powerful tool for the manipulation of enzyme activities via mutation at the DNA level is *in vivo* transposition. This technique would be especially valuable for use in industrial yeast strains since it does not require reintroduction of mutagenized genes into the yeast genome by homologous recombination. As described earlier, transformation efficiency is 100-1000 times

lower when a linear DNA fragment is used than transformation of intact plasmid DNA. Moreover, transformation efficiencies using a dominant marker are 100 times lower for industrial yeast strains than for laboratory strains (this study, data not shown). Thus a technique to manipulate genes and thereby avoiding homologous recombination events would be very valuable for genetic analysis and modification of brewer's yeast strains. *In vivo* transposition in yeast occurs much less efficiently than in bacteria and in this study it was hypothesized that this lower efficiency is due to the presence of a membrane-surrounded nucleus in yeast. We indeed found a consistently higher number of transposon integrations when a NLS sequence was attached to the hyperactive Tn5 transposase expressed in the yeast cells. However, despite the use of a laboratory yeast strain, transformation efficiency was still low and improvement of the efficiency was marginal. A better approach to confirm that attachment of a NLS increases the *in vivo* transposition efficiency in yeast is to use transposomes made *in vitro* with a NLS containing hyperactive transposase. Preliminary results suggest that purification of hyperactive transposase with an attachted NLS is difficult. Moreover, we noticed a background of many small G418 resistant colonies probably arising from abortive transformation. It has been suggested that the use of a marker conferring resistance to multiple drugs such as the *PGKp-YAP1* marker conferring resistance to cerulenin and cycloheximide, might reduce the background of non-transformant colonies [5]. More recent approaches taken for the manipulation of genes such as the use of the Sleeping Beauty transposon [113,141] may help to alleviate some of the other problems described above.

The T-DNA (transferred DNA) of the Ti-plasmid of *Agrobacterium tumefaciens* can be used to introduce modified genes into plants. It has been shown that *A. tumefaciens* can also transfer T-DNA to the yeast *S. cerevisiae* [33,195]. Upon entering the nucleus, the T-DNA sharing homology with the genome preferably integrates by homologous recombination [33] but in the absence of DNA homology, T-DNA is integrated into the genome by illegitimate recombination (IR) as observed in plants [34,263]. Development of methods to enhance IR in yeast would be very useful to provide an alternative tool to manipulate enzyme activities at the DNA level of (industrial) yeast strains.

Acknowledgments

We are grateful to Dennis Krook for his help with the *in vivo* transposition analysis and Bruno Almeida and Debby Lont for their help with the anti-sense mRNA analyses. We thank Karen van Eunen for her help with constructing the transposon-construction-vectors. We wish to thank Dr. W. S. Reznikoff (Department of Biochemistry, University of Wisconsin) for supplying us with plasmid pGRTEMP2 and Prof. Dr. J. H. Hegemann (University of Düsseldorf) for providing plasmids pUG35 and pUG36. We thank Gerda Lamers for her help with microscopy and Raymond Brandt for his extensive technical assistance. This work was funded by the European Union (Contract no. QLK1-CT-2001-01066).

Chapter 3

Maltotriose utilization in lager yeast strains: *MTT1* encodes a maltotriose transporter

Judith Dietvorst, John Londesborough, H. Yde Steensma

Yeast, 2005, 22 (10): 775-788

Abstract

Maltotriose is the second most abundant fermentable sugar in wort and, due to incomplete fermentation, residual maltotriose in beer causes both quality and economic problems in the brewing industry. To identify genes that might improve utilization of maltotriose we developed a library containing genomic DNA from four lager strains and a laboratory *Saccharomyces cerevisiae* strain and isolated transformants that could grow on YP/ 2% maltotriose in the presence of 3 mg/ l of the respiratory inhibitor antimycin A. In this way we found a gene which shared 74 % similarity with *MPH2* and *MPH3*, 62 % similarity with *AGT1* and 91 % similarity with *MAL61* and *MAL31*, all encoding known maltose transporters. Moreover, the gene shared an even higher similarity (98%) with the uncharacterized *Saccharomyces pastorianus mty1* gene (M. Salema-Oom, unpublished, NCBI Accession number AJ491328). Therefore we named the gene *MTT1* (*mty1* like transporter). We showed that the gene was present in four different lager strains but was absent from the laboratory strain CEN.PK113-7D. The ORF in the plasmid isolated from the library lacks 66 base pairs from the 3´-end of *MTT1* but instead contains 54 bp of the vector. We named this ORF *MTT1alt* (NCBI Accession number DQ010174). ^{14}C-Maltose and repurified ^{14}C-maltotriose were used to show that *MTT1* and, especially, *MTT1alt*, encode maltose transporters for which the ratio between activities to maltotriose and maltose is higher than for most known maltose transporters. Introduction of *MTT1* or *MTT1alt* in lager strain A15 raised maltotriose uptake by about 17 % or 105%, respectively.

Introduction

Efficient beer fermentation requires the rapid and complete utilization of the three main fermentable sugars in wort i.e. glucose, maltose and maltotriose [106]. In all-malt brewer's wort 50-60 % of the total sugars are maltose, 15-20 % maltotriose and 10-15 % glucose [290]. After exhaustion of glucose, maltose is the preferred substrate for yeast [56]. Maltotriose, the second most abundant sugar in wort, is utilized only in the later stages of alcoholic fermentation. Incomplete fermentation thus may result in residual maltotriose in beer. This causes both quality (change of flavor) and economic (lower ethanol yield) problems [293]. Therefore, fermentation of maltotriose has received much attention. Maltotriose utilization was compared in the two classes of yeast strains used for brewing, lager and ale yeast strains. Maltotriose uptake from wort by ale yeast was slower than by lager yeast. Residual maltotriose is more common at the end of ale fermentations [293]. Furthermore it is difficult for yeast pre-grown on glucose to adapt to fermentative growth on maltotriose [143,290]. However, most research focused on an essential step in efficient consumption of the wort sugars: their transport into the yeast cell. During the initial stages of the fermentation, glucose not only represses transcription of the genes encoding α-glucosidase and permease responsible for the intracellular hydrolysis and uptake of maltose and maltotriose [35] but also triggers inactivation of pre-existing transporters: catabolite inactivation [28,117].

In S*accharomyces cerevisiae* strains at least one of the five highly homologous and unlinked *MAL* loci is required for maltose utilization [127]. Each locus consists of a permease encoded by *MALx1* [3], a maltase encoded by *MALx2* and a regulator encoded by *MALx3*. Strains of *S. cerevisiae* have been reported to contain additional maltose transporters encoded by *AGT1*, *MPH2* or *MPH3*. *AGT1* (alpha-glucoside-transporter), an allele of *MALx1,* encodes a general α-glucoside transporter that is capable of transporting maltotriose, isomaltose, α-methylglucoside, palatinose, trehalose, and melezitose in addition to maltose and turanose [94]. All but one of the 5 ale and 25 lager strains studied, contained both *MALx1* and *AGT1* genes [115]. The *MPH2* and *MPH3* (maltose-permease-homologue) genes encode active α-glucoside permeases capable of transporting maltose, maltotriose, α-methylglucoside and turanose [56]. Although both maltose and maltotriose have been reported to be substrates for Agt1, Malx1 and Mph2/ Mph3 [55] the reported affinities for maltose are a little higher than for maltotriose. After consumption of glucose, yeast will start the uptake of maltose and maltotriose, with a slower uptake rate for maltotriose than maltose [294].

To identify genes that may effect the utilization of maltotriose we chose to use a genetic approach. However, brewing strains may be polyploid, aneuploid or alloploid [125] with a much more complex and diverse genetic constitution than haploid and diploid laboratory strains. Brewer's yeast strains sporulate poorly and spore germination occurs rarely [116]. Moreover, the copynumber of a specific gene is unknown in these strains. Consequently, standard genetic techniques cannot be applied. For the investigation of maltotriose metabolism in industrial yeast strains we therefore chose to concentrate on genes whose overproduction might have an effect. To increase our chances of

finding a relevant gene, we constructed a library with genomic DNA from four different brewer's strains and one laboratory strain.
We report that by using this library, we identified another sugar transporter able to carry maltotriose. We determined whether the gene encoding this transporter, which we called *MTT1* (*mty1* like transporter), was present in a number of lager strains and showed that introduction of *MTT1* and an altered version of *MTT1* (*MTT1alt*) in lager strain A15 improved growth of this strain on maltotriose.

Materials and methods

Strains and plasmids
E. coli XL1-Blue [32] was used for plasmid amplification. Standard methods were used for *E. coli* transformation [111]. A haploid laboratory strain *S. cerevisiae* CEN.PK113-7D (*MATa*), obtained from P. Kötter (J.-W. Goethe Universität, Frankfurt, Germany) and four lager strains A15 (from the collection of VTT Biotechnology), WS34/70 (from Weihenstephan brewery, Freising, Germany), CMBS-33 (kindly supplied by Prof. J. M. Thevelein, KU Leuven, Belgium) and OG2252 (kindly supplied by Prof. M. C. Kielland-Brandt, Carlsberg Laboratory, Denmark, although this strain originates from Alfred Jorgensens Laboratorium, Denmark) were used in this study. Standard methods were used for yeast transformation [80].

Plasmid pRUL409 was constructed by inserting *ARS1* from plasmid pNatCre [244] into the *Xba*I and *EcoR*I sites of vector pHSS6 [231], thereby introducing a *Not*I site next to the *EcoR*I site flanking the ARS. A KanMX cassette [276] from pUG6 [90] was inserted as a *BamH*I-*Bgl*II fragment into the *BamH*I site of pRUL409 yielding pRUL409(KanMX). This plasmid was digested with *BamH*I and *Xba*I to clone the various *MALx1* and *MTT1* genes. To convert one of the resulting plasmids in this study, #39, from a multicopy plasmid to a single copy plasmid *CEN4* from plasmid YCplac33 [81] was amplified using primers "CEN4SacI" and "CEN4EcoRV" (Table 1) and inserted into the *EcoR*V and *Sac*I sites of plasmid #39. Plasmid pRUL854 was constructed by inserting the KanMX cassette from plasmid pUG6 [90] into the *BamH*I and *Sac*I sites of the transposon construction vector pMOD3<R6Kγori/mcs> (Epicentre Technologies, Madison) and a tetracycline selection marker from pBR322 into the *EcoR*I and *Sac*I sites of the transposon construction vector. The plasmid-transposon border had the following structure: plasmid-CAGctgtctcttatacacatct-transposon, in which the plasmid sequences are uppercase and the transposon mosaic end sequence is lowercase. The *Pvu*II cleavage site used to cleave the transposon from the plasmid is underlined. Restriction enzymes were purchased from Pharmacia and New England BioLabs.
Plasmid stability was determined according to Heus [100] using YPD as non-selective and YPD containing G418 as selective medium.

Media

Yeast cells were grown in YP (Difco peptone 2%, Difco yeast extract 1%) containing 2% D(+)-glucose(Merck), maltose (Merck) or maltotriose (Fluka, 96% pure HPLC). When required G418 (Duchefa) was added at a concentration of 200 µg/ ml, and antimycin A (Fluka) at a concentration of 3 mg/ l. *E. coli* (XL1 blue, DH10B) was grown in LB (Luria Broth) and if necessary kanamycin was added to a concentration of 40 µg/ ml and tetracycline (Roche) to a concentration of 5 µg/ ml. For solid medium 15 g/ l select agar (Gibco) was added to liquid media.

Construction of the disruption library

A mixture of chromosomal DNA isolated from strains OG2252, WS34/70, CMBS-33, A15 and CEN.PK113-7D was partially digested with *Sau3A*-I and used to construct the library. The chromosomal DNA fragments (1 kb up to 8 kb in size) were ligated into the *BamH*I-site of vector pRUL409(KanMX). Plasmid DNA was electroporated into *E. coli* Electromax DH10B cells (Invitrogen) using standard procedures and kanamycin resistant colonies were selected and pooled. A collection of plasmids isolated from the colony-pool formed the "Genomic plasmid library". In order to construct the "Disruption library" the Tn5 transposon from plasmid pRUL854 was inserted *in vitro* by a transposition reaction (Epicentre Technologies) into the genomic plasmid library. The Tn5 transposon contained a R6Ky ORI, a tetracycline marker and a KanMX cassette flanked by mosaic ends. Plasmid pRUL854 was digested with *Pvu*II and the released Tn5 transposon was gel purified (QIAquick Gel Extraction Kit, Qiagen) without ethidium bromide staining. Transposon DNA (up to 50 µg/ ml) and target DNA (0.2 µg of the genomic plasmid library) were incubated with 10 µg/ ml hyperactive Tn5 transposase (Epicentre Technologies) in EZ::TN Reaction Buffer (0.5 M Tris-acetate pH7.5, 1.5 M potassium acetate, 100 mM magnesium acetate, 40 mM spermidine) for 2 hrs at 37 °C in a 10 µl reaction volume. The transposition was stopped by addition of 1 µl EZ::TN 10X stopsolution and incubation for 10 minutes at 70 °C. Transposomes (1 µl reaction mix) were electroporated into *E. coli* DH10B cells (Invitrogen). The quality of both libraries was determined by several criteria i.e. colony blotting and restriction analysis, as has been described in Chapter 2. Here it suffices to state that on average the "haploid genome" of each yeast strain is covered approximately 17 times for the genomic library and approximately 13 times for the disruption library.

Southern blot analysis

Genomic DNA (2.5-5 µg) from strains OG2252, WS34/70, CMBS-33, A15 and CEN.PK113-7D was isolated using phenol-extraction and digested for 16 hrs at 37 °C with 40,000 units *Nco*I or *Xba*I (New England Biolabs) in a 100 µl reaction volume. Digests were precipitated with ethanol, resuspended in 10 µl water and electrophoresed on a 1% agarose gel. The gel was vacuum blotted in blot buffer (0.6 M NaCl, 0.4 M NaOH) onto a positively charged nylon membrane (Roche). The membrane was washed with neutralization buffer (1 M TrisHCl pH5, 2 M NaCl) and 2x SSC. For cross-linking of the DNA the membrane was UV-irradiated for 3 minutes. The blot was probed with an α-^{32}P dCTP labeled 1670 base pairs *MALx1* DNA fragment from strain WS34/70.

Hybridization was carried out at circa 62 ºC overnight in hybridization solution (1% blocking reagent (Roche), 2 x SSC, 20 mM sodium pyrophosphate, 1% SDS) plus the labeled DNA probe (25 ng). After hybridization, filters were washed three times for 5 min with 2 x SSC/ 0.1 % SDS at room temperature and three times with 0.1 x SSC/ 0.1 % SDS at 65 ºC for 20 min.

PCR amplification

The primers used for PCR are listed in Table 1. PCR amplifications were performed with 50-100 ng genomic DNA or 25-50 ng plasmid DNA. Amplification conditions were: 5 min at 94 ºC, 30 cycles of 1 min 94 ºC, 1 min at 5 ºC below Tm of the primers and 1 min per kb to be amplified at 74 ºC. The reaction was concluded by incubation for 10 min at 74 ºC. Reactions were performed in a total volume of 50 µl and per reaction 0.5 µl Vent polymerase (New England BioLabs) was used with the buffer supplied. PCR products were analyzed on a 0.7 % agarose gel in 1 x TAE buffer. All PCR products were cloned into the pCR®-Blunt II-TOPO® vector (Invitrogen) before they were recloned in the desired vector.

Table 1. Primers

Name	Sequence (5'=> 3')	Tm (°C)
MAL31Xba	GC TCT AGA CCG TAT CTA CCT ACT GGC TA	60
MAL31BamH	CGC GGA TCC CTT AAA CCC CGT GTT TAG CG	60
MALx1-fw	AAT TCC CAG AAC AAA TAT GTC	56
MALx1-rv	AAA CTG TAA CTA CAA TTT GGA T	56
Mty1-fw	ACT CCC AAA ATA AGT ACC CT	56
Mty1-rv	AAC AGT AGT TAC AAT AGA TGC	56
#39XbaI	GCT CTA GAT CAC GGA TCT TTA ACA TTA ATT TCT G	60
#39BamHI	CGC GGA TCC AAA TCA TGT TAC CTA C	58
CEN4SacI	CGA GCT CCC TAG GTT ATC TAT GCT GTC TC	64
CEN4EcoRV	CAG ATA TCT GAC GAT AAA ACC GGA AGG AAG	64

Sequences

The sequences of the *MTT1/MALx1* genes from strains CEN.PK113-7D, OG2252, WS34/70 and A15 cloned into vector pRUL409(KanMX) were determined commercially (Baseclear, Leiden, The Netherlands) using the dideoxy method. Sequences have been deposited in NCBI under accession numbers DQ010171 (WS 2.4kb) DQ010173 (WS 2.7kb) from strain WS 34/70 DQ010170 (OG 2.4kb) DQ010172 (OG 2.7kb) from strain OG2252 DQ010169 (CEN 2.4kb) from strain CEN. PK113-7D and DQ010168 (A15 2.4kb). Similarly the sequence of plasmid #39 was determined and deposited in NCBI under accession number DQ010174 (insert#39).

Analysis and purification of [^{14}C]-maltotriose.

[U-^{14}C]-maltotriose (ARC 627) was purchased from American Radiolabeled Chemicals Inc. The purity was determined by adding 1 µl (0.1 µCi) to 250 µl of water containing 200 mg/l each of maltotriose, maltose and glucose and injecting 50 µl of the mixture into an HPLC system consisting of two 7.8 x 300 mm Uhydrogel 120+BioRad HPX-42A+SugarPak columns in series with water as eluent at 0.5 ml/h and 80 °C and Refractive Index detection. Elution times were: maltotriose, 36.5 min; maltose, 40.0 min and glucose, 45.0 min. Fractions of 500 µl were collected between 32 and 47 min, and 200 µl of each fraction was mixed with scintillant and counted.

For purification of ^{14}C-maltotriose, 80 µl of ARC 627 [U-^{14}C]-maltotriose (8µCi) was applied directly to the same HPLC system and the water eluate collected in 250 µl fractions. The radioactivity of individual maltotriose fractions was determined and the purified material was mixed with un-labeled maltotriose to give the required concentration and specific radioactivity.

Transport assays

Yeast cultivated in YP/ 4% maltose or YP/ 4% maltotriose with or without G418 at 25 °C were harvested at the indicated A_{600}, washed quickly with ice-cold water at 0 °C to remove any sugars and resuspended to 200 mg fresh yeast/ ml in 100 mM tartaric acid adjusted to pH 4.2 with Tris base. Zero-*trans* uptake rates of [^{14}C]-maltose (Amersham Life Sciences) and repurified [^{14}C]-maltotriose were immediately determined essentially as described by Lucero [146]. Portions of the yeast cell suspensions were pre-incubated for 5 - 10 min at 20 °C. Then 40 µl of the yeast cell suspensions were added to 20 µl of a solution containing radioactive substrate and any potential inhibitors. Final concentrations were 5 mM [^{14}C]-maltose (approximately 1000 cpm/ nmol) or 5 mM [^{14}C]-maltotriose (300 to 700 cpm/ nmol) with or without 50 mM maltotriose or 50 mM maltose as indicated in Tables 2 and 3. After incubation for 20 sec at 20 °C the reaction was quenched with 10 ml ice-cold water. The mixture was filtered through an HVLP membrane (Millipore) and collected yeast cells were washed with 10 ml ice-cold water. The membrane and yeast were transferred to 3 ml liquid scintillation cocktail and radioactivity was counted. For determination of the amount of radioactivity on the membranes after 0 sec incubation with [^{14}C]-labeled substrates, 10 ml ice-cold water was added first to the substrate and then the yeast, followed by filtration etc. as described above. Reactions were run in duplicate or triplicate. Dry masses of yeast were determined by diluting the yeast suspensions 10-fold with distilled water, centrifuging and drying the pellets overnight at 105 °C. Yeast protein (extracted from all samples with dilute NaOH) varied between 43 and 47 % of yeast dry mass. Results are presented in µmol.min^{-1}(g dry yeast)$^{-1}$.

Results

Isolation of MTT1

The respiratory inhibitor antimycin A is reported either to prevent growth on maltotriose of several *S. cerevisiae* laboratory and lager strains [290] or to introduce very long lag phases before growth on maltotriose [143]. We found that on solid medium antimycin A strongly inhibited growth on maltotriose of lager strain A15 and laboratory strain CEN.PK113-7D, but had less effect on strains WS34/70 and OG2252 (Figure 1). For growth on maltose or glucose as carbon source, the effect of antimycin A was smaller and about the same for all strains (Figure 1). We therefore selected strain A15 for our studies to identify genes involved in maltotriose metabolism.

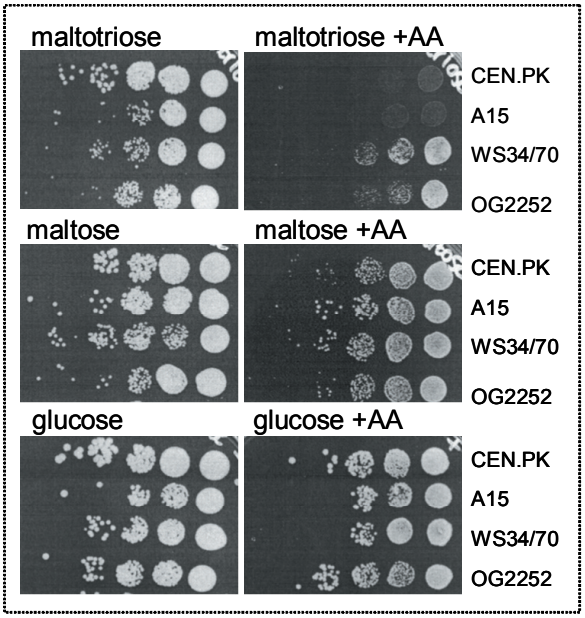

Figure 1. Effect of antimycin A on growth on maltotriose of different yeast strains. Ten fold dilutions of one laboratory strain (CEN.PK113-7D) and three lager strains (A15, WS34/70, OG2252) pre-grown in YP/2% glucose with G418 till A_{600} of about 0.8 were spotted onto YP with 2% of different carbon sources in the absence or presence (+AA) of 3 mg/ l antimycin A. Plates were incubated at 30 °C for 3 days. The figure shown is representative for at least 6 similar experiments.

Strain A15 was transformed with a disruption library containing chromosomal DNA from four lager strains (WS34/70, OG2252, CMBS-33 and A15) and one laboratory strain (CEN.PK113-7D) in the vector pRUL409 as described in the Materials and methods section. The plasmids of this library contain the Tn5 transposon with the R6Ky ORI, a tetracycline marker and a KanMX cassette. Transformants were selected on YP/ 2 % maltotriose plates containing G418 and antimycin A after incubation for 3 days at 30 °C. Of the initially obtained 75 colonies 53 again grew on maltotriose in the presence of antimycin A after re-streaking. Plasmids were isolated from all 53 colonies. After retransformation of A15 with the isolated plasmids only one, named #39, consistently allowed growth of A15 on maltotriose in the presence of antimycin A (Figure 2). The Tn5 transposon resided in the vector part of plasmid #39 resulting in an un-disrupted yeast DNA insert of approximately 2.5 kb in size. Since control strains with the vector provided with a KanMX-cassette did not grow on maltotriose in the presence of antimycin A, the yeast DNA insert must be responsible for this effect (Figure 2).

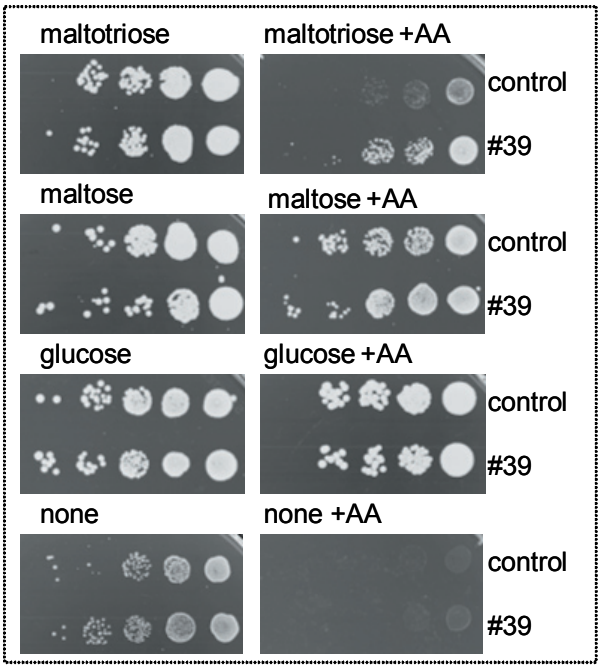

Figure 2. Effect of plasmid #39 on growth of A15.
Ten fold dilutions of A15 strain with plasmid #39 and A15 control strain pre-grown in YP/ 2% glucose plus G418 till A_{600} of about 0.8 were spotted onto YP plus G418 with 2% of different carbon sources in the absence or presence (+AA) of 3 mg/ l antimycin A. Plates were incubated at 30 °C for 3 days. The figure shown is representative for at least 6 similar experiments.

Plasmid #39 is a multicopy plasmid and the observed effect might also be due to the resulting overexpression. However, strain A15 containing a single copy plasmid with the same insert (Materials and methods) again grew on maltotriose in the presence of antimycin A (data not shown), indicating that the presence of a heterologous gene in the insert of plasmid # 39 was responsible for the ability of A15 to grow on maltotriose in the presence of antimycin A. Sequence analysis of the insert showed the presence of a DNA fragment of 2464 base pairs (NCBI Accession number DQ010174) containing 220 base pairs identical to the 3'-end of a *S. cerevisiae* open reading frame with unknown function, YBR298C-A, and the promoter and open reading frame of a gene which shared 74 % similarity with *MPH2* and *MPH3*, 62 % similarity with *AGT1* and 91 % similarity with *MAL61* and *MAL31* from *S. cerevisiae*. Moreover, the open reading frame shared an even higher similarity (98%) with *mty1*, a gene from *Saccharomyces pastorianus* of which to our knowledge only the name and the sequence have been published (M. Salema-Oom, unpublished, NCBI Accession number AJ491328). Compared to the *mty1* gene, the open reading frame on plasmid #39 lacked 66 base pairs of the 3' end of the gene. As the open reading frame is fused to sequences originated from the vector pRUL409, 21 amino acids (KEDLETSVVDEGRSTPSVVNK) from the C-terminus of the *mty1* protein were replaced by 18 amino acids (GNVVAIAAGGELVGMEDL) encoded by the vector sequence before a stop codon. Subcloning showed that the 220 base pairs corresponding to open reading frame YBR298C-A were not responsible for the phenotype. The gene with the *mty1* like open reading frame was named *MTT1* (*mty1*-like transporter) whereas the altered form with the 3-terminal 66 base pairs replaced by 54 base pairs from the vector was named *MTT1alt*.

Presence of MTT1 in various yeast strains

To determine whether *MTT1* and/ or *MALx1* genes were present in the different yeast strains used to construct the library, a PCR using specific primers "MALx1-fw", "MALx1-rv", "Mty1-fw" and "Mty1-rv" (Table 1 and Figure 3a) that discriminate between *MALx1* and *MTT1* was performed. The lager strains WS34/70, OG2252, A15 and CMBS-33 each contained both *MALx1* and *MTT1,* whereas CEN.PK113-7D had only *MALx1* (Figure 3b and c). Transformation of CEN.PK113-7D with *MTT1* or *MTT1alt* containing plasmids did not result in growth of CEN.PK113-7D on maltotriose in the presence of antimycin A (data not shown). We did not explore whether this is caused by differences in transcription, translation or modification of the proteins as a consequence of the differences between a laboratory strain and the lager strain A15. In this respect it is worth noting that the *MTT1* gene probably orginates from the non-*S. cerevisiae* part of the genome of the lager strains.

To further characterize the *MTT1* and *MALx1* genes, Southern blots of chromosomal DNA digested with *Nco*I or *Xba*I were probed with a 1670 bp fragment expected to recognize both *MALx1* and *MTT1*. There are *Nco*I sites at nucleotides 640 and 886 of the *MALx1* ORF and also 1494 bp upstream and 1149 bp downstream of the ORF. We therefore expected complete digestion of chromosomal DNA with *Nco*I to give 2134, 2108 and 246 bp fragments.

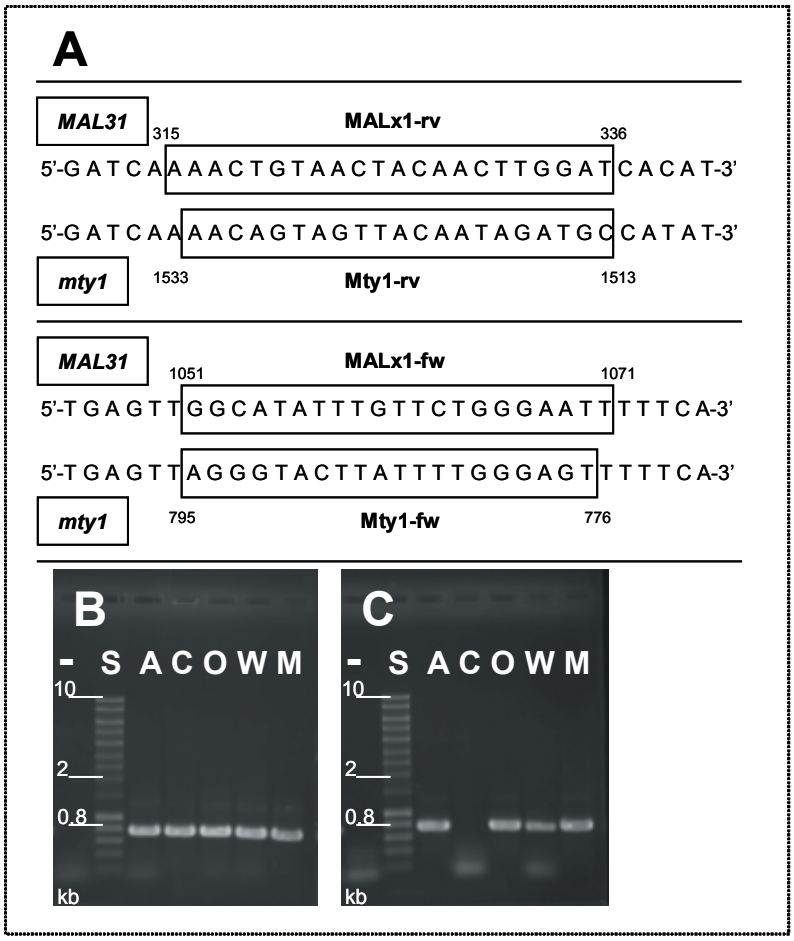

Figure 3. PCR to indicate the presence of *MALx1* and *MTT1* in different yeast strains.
PCR was performed with an annealing temperature of 51 °C and genomic DNA of strains A15 (A), CEN.PK113-7D (C), OG2252 (O), WS34/70 (W) and CMBS-33 (M) as a template. As a negative control of the PCR MilliQ water was added to the reaction instead of template DNA (-). PCR products were analysed on a 0.7 % agarose gel with the smartladder (S) (Eurogentec) as a marker. Fragments of 717 bp in size were expected in both PCR reactions.
A. Binding sites of the primers. Sequences are numbered with respect to the ORF of the *mty1* and *MAL31* genes.
B. PCR to indicate the presence of *MALx1* using primers "MALx1-fw" and "MALx1-rv".
C. PCR to indicate the presence of *MTT1* using primers "Mty1-fw" and "Mty1-rv".

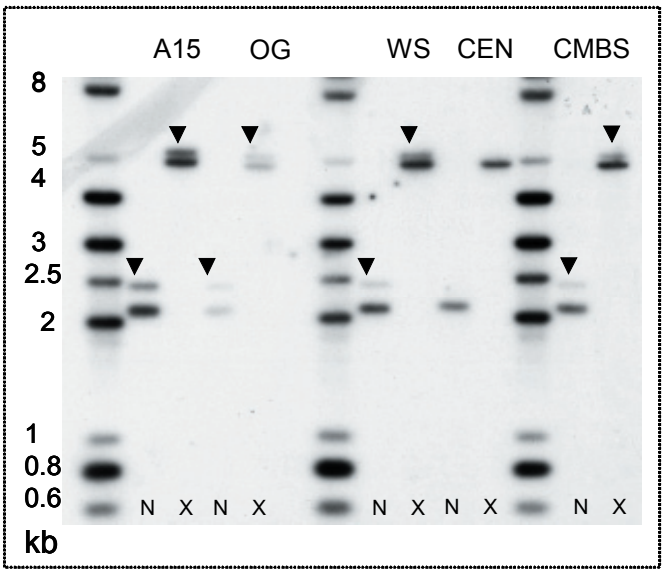

Figure 4. Southern blot analysis.
Genomic DNA of laboratory strain CEN.PK113-7D (CEN) and four lager strains, A15, OG2252 (OG), WS34/70 (WS), CMBS-33 (CMBS), was restricted with *Nco*I (N) or *Xba*I (X) and hybridized to a 1.7 kb DNA fragment of *MAL31* from strain WS34/70. For *Nco*I digested genomic DNA two bands of approximately 2.1 kb were expected and for genomic DNA digested with *Xba*I one band of approximately 4.8 kb. Black arrowheads indicate the presence of additional bands.

In Figure 4, the laboratory strain CEN.PK113-7D yielded a single 2.1 kb band, presumably containing both the 2134 and 2108 fragments. All four lager yeasts gave both this band and an additional, weaker band at about 2.4 kb. Similarly, with *Xba*I all yeasts gave the expected 4.8 kb band with an additional weaker 5.2 kb band in the four lager strains (*Xba*I sites are present 2191 bp upstream and 753 bp downstream of the *MALx1* ORF). The *Nco*I and *Xba*I results both suggest that the lager yeasts have two versions of *MALx1* or *MTT1* genes, one with approximately 0.3 kb extra between the *Nco*I site 1494 bp upstream of the ORF and the *Xba*I site 753 bp downstream of the ORF. Quantification of the intensity of the *Nco*I and *Xba*I bands on the Southern blot suggested a ratio of one copy of the large version for two or three copies of the normal version.

To further study the role of *MTT1* in maltotriose metabolism, *MALx1* and *MTT1* genes from the library strains A15, OG2252, WS34/70 and CEN.PK113-7D were isolated by PCR using the universal primers "MAL31Xba" and "MAL31BamH". This PCR amplifies the sequences between 542 bp upstream and 26 bp downstream of the ORFs and yielded both 2.4 and 2.7 kb products from all lager strains but only the 2.4 kb product from laboratory strain CEN.PK113-7D (data not shown). This suggests the size dichotomoy observed by Southern analysis occurs somewhere between 542 bp upstream and 26 bp downstream of the ORFs. The PCR products were cloned into

pRUL409(KanMX). So far 6 clones have been obtained, one 2.4 kb fragment each from CEN.PK113-7D, A15, WS34/70 and OG2252 and one 2.7 kb fragment each from WS34/70 and OG2252. These were sequenced as described in Materials and methods, and the sequences are deposited in the NCBI under accession numbers DQ010171 (WS 2.4kb), DQ010173 (WS 2.7kb), DQ010170 (OG 2.4kb) DQ010172 (OG 2.7kb), DQ010169 (CEN 2.4kb), and DQ010168 (A15 2.4kb). Sequence analysis showed that the 2.4 kb fragments from CEN.PK113-7D and A15 and the 2.7 kb fragments from WS34/70 and OG2252 each contained *MALx1*, whereas the 2.4 kb fragments from WS34/70 and OG2252 each contained *MTT1*. Detailed comparison of these sequences shows that an extra 291 basepairs were located upstream of the ATG start codon in both 2.7 kb fragments compared to the 2.4 kb fragments (unpublished results). A15 transformants containing these constructs were tested for their ability to start growing rapidly on maltotriose in the presence of antimycin A. Transformants carrying the 2.4 kb *MTT1* versions from WS34/70 or OG2522 grew quickly, whereas transformants carrying the 2.7 kb *MALx1* versions from these strains had a long lag phase (Figure 5). Also transformants with the 2.4 kb versions of *MALx1* from CEN-PK113-7D or A15 did not start growing on maltotriose in the presence of antimycin A quicker than A15 or A15 with the control plasmid (not shown). This suggests a specific role for *MTT1* in the uptake of maltotriose.

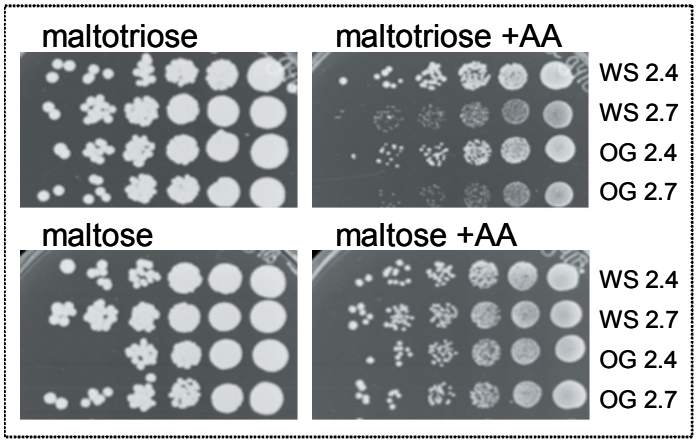

Figure 5. Effect of *MALx1/MTT1* genes on growth of A15.
Ten fold dilutions of A15 strain with plasmids containing the 2.7 kb versions of *MALx1* and the 2.4 kb versions of *MTT1* from lager strains WS34/70 and OG2252 grown in YP/ 2% glucose plus G418 till A_{600} of about 0.8 were spotted onto YP plus G418 with 2% of different carbon sources in the absence or presence (+AA) of 3 mg/ l antimycin A. Plates were incubated at 30 °C for 3 days. The figure shown is a representative for 3 similar experiments.

Maltotriose and maltose uptake assays

To study uptake rates we wished to use labelled maltotriose and maltose. HPLC analysis (see Materials and methods) of two lots of commercial [^{14}C]-maltotriose showed that 84 % of the total label was in maltotriose in lot A and 94 % in lot B. The rest of the label was distributed between putative maltotetraose, maltose, glucose and a compound that travelled a little faster than glucose. For lot A, about 11 % was in maltose and about 3 % in glucose. We compared zero-*trans* uptake rates at 5 mM [^{14}C]- maltotriose (about 500 cpm/ nmol) made with either crude commercial lot A or material repurified as described in Materials and methods. Observed rates for A15 and WS34/70, respectively, were 4.6-fold and 1.8-fold greater with the crude material than with the repurified material. Probably some labelled impurities enter the yeast cells relatively quickly (they are not diluted with unlabelled carrier) causing gross overestimation of maltotriose uptake.

Table 2. Maltose and maltotriose zero-trans uptake rates by strains WS34/70 and A15 harvested in early (A_{600} = 1.5-2) or mid (A_{600} = 4-5)-log phase on YP/ 4% maltose or YP/ 4% maltotriose

	WS34/70		**A15**	
	Early	**Mid**	**Early**	**Mid**
Maltose-grown cells				
Maltose uptake (units/ g dry yeast)[a]	17.0 ± 0.1	14.7 ± 0.5	21.6 ± 1.4	18.3 ± 0.8
Inhibition by maltotriose (%)	17 ± 3	14 ± 5	8 ± 3	6 3
Maltotriose uptake (units/ g dry yeast)	6.4 ± 0.1	6.4 ± 0.7	1.8 ± 0.1	1.6 ± 0.3
Inhibition by maltose (%)	nd	44	Nd	50
Maltotriose uptake/ maltose uptake (%)	38 ± 1	45 ± 7	8.2 ± 0.2	8.6 ± 1.4
Maltotriose-grown cells				
Maltose uptake (units/ g dry yeast)[a]	nd	17.8 ± 0.8	nd	37.3 ± 0.6
Inhibition by maltotriose (%)	nd	18 ± 0.1	nd	9 ± 4
Maltotriose uptake (units/ g dry yeast)	nd	8.1 ± 1.2	nd	4.9 ± 1
Inhibition by maltose (%)	nd	44 ± 1	nd	53 ± 2
Maltotriose uptake/ maltose uptake (%)	nd	46 ± 9	nd	13 ± 3

a Uptake was measured with 5 mM [^{14}C]-maltose or maltotriose and is reported as units (μmol/ min)/ g dry yeast. Inhibition of maltose uptake by 50 mM maltotriose and of maltotriose uptake by 50 mM maltose is also shown. Results are averages of independent duplicate experiments ± the ranges. Nd, not determined.

The lower ratio for WS34/70 than for A15 is probably because the true rate of [^{14}C]-maltotriose uptake is about twice as high for WS34/70 than A15 (see below). We therefore used repurified [^{14}C]-maltotriose for the work reported below.

To elucidate why A15 grows poorly compared to WS34/70 on maltotriose in the presence of antimycin A we compared the uptake activities of the two strains after growth on YP/ 4 % maltose or YP/ 4 % maltotriose. For each strain, yeast cells harvested from maltose in early (A_{600} of 1.5-2) or mid (A_{600} of 4-5) log phase had similar uptake activities (Table 2), as might be expected because residual maltose was still about 2 % at an A_{600} of 5. For maltose-grown cells, maltotriose uptake was nearly four-fold faster by WS34/70 than by A15, whereas maltose uptake rate was slightly faster by A15 than WS34/70. Consequently, the maltotriose uptake rate was about 8.5 % of the maltose uptake rate for A15, but 38 – 44 % for WS34/70. Inhibition of 5 mM [^{14}C]-maltose uptake by 50 mM maltotriose was 14 – 17 % for WS34/70 but only 6 – 8 % for A15. This indicates that, for both yeasts, a large proportion of the maltose uptake capacity is due to transporters that neither carry nor are significantly inhibited by 50 mM maltotriose, and this "maltose-specific" proportion is higher for A15 than for WS34/70.

For cells grown on 4 % maltotriose instead of maltose, slightly higher uptake rates were obtained with WS34/70 for both maltose (+ 13 ± 8 %) and maltotriose (+ 27 %), but for A15 the maltotriose uptake rate increased three-fold and the maltose uptake rate increased two-fold. For A15, the maltotriose uptake rate of maltotriose-grown cells rose to 13 % of the maltose uptake rate whereas for WS34/70 this value remained at 46 %. These results show an unexpected (see Discussion) increase in both maltose- and maltotriose-uptake rates when A15 is grown on maltotriose instead of maltose. However, the maltotriose-uptake rate of maltotriose-grown A15 was still less than that of WS34/70. Results in Table 2 are consistent with the poorer ability of A15 than WS34/70 to grow on maltotriose, although they do not directly explain why this poorer ability is especially noticeable in the presence of antimycin A.

Table 3. Maltose and maltotriose zero-trans uptake rates by strain A15 containing *MTT1* or *MTT1alt* harvested in mid (A_{600} = 4-5)-log phase on YP/ 4% maltose or YP/ 4% maltotriose

	A15 control	**A15** *MTT1alt*	**A15** *MTT1*
Maltose-grown cells			
Maltose uptake (units/ g dry yeast)[a]	18.9 ± 0.3	18.4 ± 0.2	18.9 ± 1.0
Inhibition by maltotriose (%)	11 ± 4	11 ± 4	9 ± 8
Maltotriose uptake (units/ g dry yeast)	1.8 ± 0.1	3.7 ± 0.2	2.1 ± 0.2
Inhibition by maltose (%)	56 ± 2	46 ± 6	52 ± 10
Maltotriose uptake/ maltose uptake (%)	10 ± 1	20 ± 1.4	11 ± 1.9
Maltotriose-grown cells			
Maltose uptake (units/ g dry yeast)[a]	39.8 ± 2.7	28.4 ± 2.0	35.2 ± 0.2
Inhibition by maltotriose (%)	2 ± 8	13 ± 12	5 ± 1
Maltotriose uptake (units/ g dry yeast)	5.4 ± 0.4	5.9 ± 0.1	5.4 ± 0.4
Inhibition by maltose (%)	61 ± 3	58 ± 3	54 ± 10
Maltotriose uptake/ maltose uptake (%)	14 ± 2.2	21 ± 2	15 ± 1.4

Effect of the C-terminal 21 amino acids of Mtt1

Strain A15 containing the original plasmid #39 consistently formed bigger colonies on maltotriose in the presence of antimycin A than did A15 with pRUL409(KanMX) containing *MTT1* genes from the strains WS34/70 or OG2252. Sequence comparison showed that plasmid #39 differs from these two constructs in two ways. First the insert of plasmid #39 misses 66 base pairs at the 3' end of the *MTT1* gene and second it contains the extra 220 base pairs corresponding to ORF YBR298C-A.

To test whether the replacement of the 21 amino acids (KEDLETSVVDEGRSTPSVVNK) at the C-terminus of Mtt1 by 18 amino acids (GNVVAIAAGGELVGMEDL) encoded by vector sequence or the presence of part of ORF YBR298C-A were responsible for the bigger colonies with plasmid #39, several modified *MTT1* and *MALx1* genes were amplified using primers "MAL31Xba", "MAL31BamH", "#39Xba" and "#39BamH". Strain A15 was transformed with the cloned fragments in pRUL409(KanMX) and transformants were tested for the ability to grow on maltotriose in the presence of antimycin A. Neither removal of the 220 base pairs of theYBR298C-A ORF from the original clone #39, yielding plasmid pRUL409KanMX(*MTT1alt*), nor addition of these 220 bp to a plasmid containing *MALx1* from CEN.PK113-7D affected the ability of transformants to grow on maltotriose in the presence of antimycin A. Similarly, no changes were observed when the last 66 base pairs from the *MALx1* gene of CEN.PK113-7B were replaced by 54 base pairs from the vector.

To test whether the replacement of the amino acids coded by these 66 bp altered maltotriose transport, uptake studies were performed on A15 transformants containing the 2.4 kb *MTT1*gene from strain WS34/70 or the originally isolated altered version of *MTT1* (*MTT1alt*). A15 transformants containing plasmids pRUL409(KanMX) (empty vector), pRUL409KanMX(*MTT1alt*) or pRUL409KanMX(*MTT1*) respectively, were grown in YP/ 4% maltose or maltotriose containing G418 at 25 °C and harvested at A_{600} of about 4. Under these conditions approximately 50 % of the cells contained the plasmids (data not shown). Transport assays were performed with both [^{14}C]-maltose and [^{14}C]-maltotriose.

The results in Table 3 showed that all three strains transported maltose at the same rate when grown on maltose. This rate was also similar to that of untransformed strain A15 (Table 2). Inhibition of maltose transport by 50 mM maltotriose was low (about 10 %) for all three transformants, as was also the case for untransformed A15 (6-9 %, Table 2). However, the ratio of activity with maltotriose to maltose was 20 % in the *MTT1alt*-containing strain and 9.5 % in the strain with the empty vector or the non-transformed A15 strain, whereas the ratio in the *MTT1*-containing strain was 11%. It thus appears that replacement of the C-terminal 21 amino acids by 18 amino acids coded by the vector caused a higher maltotriose transport capacity. After growth on maltotriose, maltose and maltotriose transport activity doubled for the A15 strains with the empty vector or *MTT1* gene, as previously found for A15 grown on maltotriose (Table 2). The A15 strain carrying the *MTT1alt* gene showed a smaller (54 %) increase in maltose transport. Moreover, compared to the other strains, the inhibition of maltose transport by 50 mM maltotriose was higher in the *MTT1alt*-containing strain suggesting that a higher proportion of its maltose transporters can carry

also maltotriose. With these maltotriose-grown cells, the ratio of transport activity with maltotriose to that with maltose was again higher (21 %) for cells carrying *MTT1alt* than for those with the empty vector or *MTT1* (14-15 %).

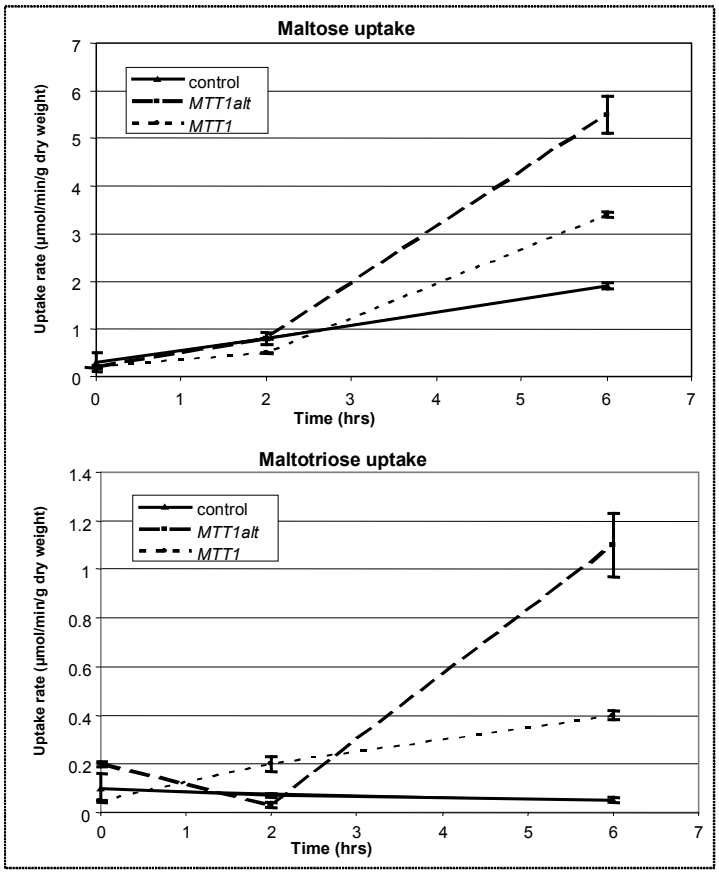

Figure 6. Maltose and maltotriose uptake in A15 strains containing *MTT1alt* and *MTT1*. Strains were grown on YP/ 2% maltotriose plus G418 in the presence of antimycin A. Cells were grown in YP/ 2% glucose plus G418 at 25 °C and harvested at A600 4.0 for transfer to YP/ 2% maltotriose$_{G418}$ with antimycin A (3 mg/ l). Uptake rates were determined at 0, 2 and 6 hrs after transfer. Plotted uptake rates are averages of independent duplicate experiments. Standard deviations are indicated by error bars.

Since the *MTT1* gene was isolated by its ability to improve growth of A15 on maltotriose in the presence of antimycin A, we also studied maltose and maltotriose uptake under these conditions. Yeast strains A15 containing plasmids pRUL409(KanMX) (empty vector), pRUL409KanMX(*MTT1alt*) and pRUL409KanMX(*MTT1*) respectively, were cultivated in YP/ 2 % glucose with G418 to an A_{600} of 4, harvested and diluted to an A_{600} of 2 in YP/ 2 % maltotriose with G418 and containing

antimycin A. Maltose and maltotriose uptake was measured after 0, 2 and 6 hrs of shaking at 25 °C. As can be seen from Figure 6, all three strains had similar transport activities for maltose and maltotriose after the first 2 hrs of incubation in maltotriose containing antimycin A. However, after 6 hrs A15 cells carrying *MTT1alt* exhibited 2.9-fold more maltose uptake and 22-fold more maltotriose uptake then the control cells. For A15 cells carrying *MTT1* maltose uptake was 1.8-fold higher and maltotriose uptake was 8-fold higher than for the control cells. These data confirmed that the *MTT1alt* gene improved the development of maltotriose transport capacity of A15 grown on maltotriose in the presence of antimycin A. They also showed that the replacement of the 66 basepairs at the end of the *MTT1* gene had a strong effect on the transporters when glucose-repressed cells were transferred to maltotriose in the presence of antimycin A.

Discussion

Residual maltotriose in beer is a problem in the brewing industry. In this study we used a library with DNA from four lager strains and one laboratory strain, to identify genes that might improve utilization of maltotriose. In line with reports that attributed poor maltotriose fermentation to limited uptake [56,116,293] we found a new transporter encoded by a gene we named *MTT1* and which we show is a maltose/ maltotriose transporter. First, it has high similarity (74% to 88%) with previously characterized maltose/ maltotriose transporters in *S. cerevisiae*. Second, when introduced into the lager strain A15, it facilitates the growth of this strain on maltotriose in the presence of antimycin A. Third, zero-*trans* maltotriose uptake increased when *MTT1* was introduced into strain A15. It thus appears that we have isolated a gene encoding for a maltose transporter with improved transport capacity for maltotriose.

MTT1 was isolated by giving A15 the ability to start growth on maltotriose in the presence of antimycin A without a long lag phase. Growth curves in YP/ 2 % maltose or YP/ 2 % maltotriose liquid cultures without antimycin A showed similar growth rates for both strains on maltose and slower growth on maltotriose for A15 than for WS34/70 (doubling times were 3.6 ± 0.4 h and 3.0 ± 0.2 h, respectively; data not shown). Because *MTT1* encoded a transporter it was conceivable that the control strain WS34/70, which grows well on maltotriose in the presence of antimycin A, transported maltotriose better than A15. The uptake rates measured for the two lager strains WS34/70 and A15 indeed showed that strain A15 had a weaker ability than WS34/70 to carry maltotriose (Table 2). Inhibition of maltotriose transport by a 10-fold excess of maltose was 44 to 53 % for both strains suggesting that the maltotriose transport capacity of both strains includes transporters that can carry both maltose and maltotriose (Table 2). As well as Agt1, such transporters may include Mphx, Mal31 and Mal61 [55,56]. A15 may contain lower levels of the same transporters as WS34/70 or it may lack functional forms of some particular maltotriose transporter(s) present in WS34/70. Lager strains have complex ploidy and each contain multiple genes for maltose permeases [116]. This makes it difficult to compare the measured uptake rates to published data on

maltose and maltotriose transported by single transporters [55]. Moreover, in our study re-purified [^{14}C]- maltotriose was used. We showed that this re-purification markedly decreased the apparent rate of maltotriose transport in a strain-dependent way.

MTT1 sequences were detected by PCR in all four lager strains, but not in the haploid laboratory strain, CEN.PK113-7D. Two different versions of *MTT1* were found, an altered gene, *MTT1alt*, that we originally cloned from the mixed yeast library and a full length gene, *MTT1*, that was found in the lager strains WS34/70 and OG2252. This latter gene is essentially identical to the *mty1* gene reported by Salema-Oom (unpublished, NCBI Accession number AJ491328). To define the role of these genes in maltotriose transport, we studied maltose and maltotriose transport in A15 transformants carrying multicopy plasmids. Our results confirmed a role for *MTT1* in maltotriose uptake. For A15 transformants grown on maltose, the presence of *MTT1* or, especially, *MTT1alt* raised maltotriose uptake by about 17 % or 105%, respectively (Table 3).

For lager strain WS34/70 only small changes in maltose and maltotriose uptake were observed when cells were grown on maltotriose instead of maltose. However, maltose uptake by A15 doubled and maltotriose uptake increased 3-fold when this lager strain was grown on maltotriose instead of maltose. This change might result if maltotriose is a more efficient inducer than maltose in A15. Another explanation could be a metabolic/ glucose repression model, which proposes that the internal concentration of glucose is lower in A15 during growth on maltotriose than on maltose because its maltotriose transport capacity is markedly lower than its maltose transport capacity. Glucose represses all known maltose and maltotriose transporters, so the lower cytosolic glucose concentration in A15 grown on maltotriose could lead to decreased glucose repression and hence higher levels of the maltose/ maltotriose transporters. For WS34/70, on the other hand, with its higher maltotriose transport capacity, cytosolic glucose concentrations and the corresponding repression levels may be similar during growth on maltotriose or maltose. This model might also explain why the shift from maltose to maltotriose causes a smaller increase in the maltose transport activity of A15 carrying *MTT1alt* compared to A15 carrying either an empty plasmid or *MTT1* (Table 3).

Two lots of commercial ^{14}C-maltotriose were found to be of low radioactive purity (84 % and 94 %). In zero-*trans* uptake assays only very small proportions (usually < 1 %) of substrate enter the cell, so the effect of small amounts of impurities is expected to be high. We found that apparent maltotriose uptake rates obtained with the commercial material were 2 to 5-fold higher than rates obtained with repurified material. Thus, use of commercial material without repurification, or at least checking the purity of each batch, could result in serious over-estimation of the ability of particular transporters to carry maltotriose.

From the PCR experiments with specific primers and the sequences of the cloned genes it was clear that the two lager strains WS34/70 and A15 each contained both *MALx1* and *MTT1* genes. Nevertheless, introduction of the *MTT1* gene from WS34/70 to strain A15, facilitated growth on maltotriose in the presence of antimycin A. This was not a copy number effect because it was also seen with a centromeric plasmid (data not shown). The *MTT1* gene of A15 may be non-functional, but despite several attempts we have not yet succeeded to clone it and confirm this suggestion by sequencing.

The transport data in Table 3 and Figure 6 show that replacing the C-terminal 21 amino acids in the product of *MTT1* by the 18 amino acids in the product of the *MTT1alt* gene has a strong effect on the maltotriose uptake rate by cells carrying these genes. In particular, the ratio of maltotriose/maltose transport was higher for cells carrying *MTT1alt*. These data agree with the agar plate assays which showed a greater ability of *MTT1alt* than *MTT1* to facilitate growth of A15 on maltotriose in the presence of antimycin A. Possible explanations for this difference could be the involvement of the 21 C-terminal amino acids in substrate binding or in stability of the transporter. PEST sequences, rich in proline, glutamate, serine and threonine are involved in the regulated degradation of proteins. A PEST motif has been found in Mal61 [158] and in Mal31 [55], but such a sequence was not found in Mph2, Mph3 or Agt1. While a PEST region spans residues 48-79 of the N-terminal cytoplasmic domain [43] it is not present in the 21 amino acids at the C-terminus of Malx1 or Mtt1.

A protein motif (RSTPS) resembling the 14-3-3 binding motif RS*Xp*S*X*P [170] is present in the 21 C-terminal amino acids of Mtt1. 14-3-3 proteins are involved in many functions including protein localization and activation [269]. The purported binding motif is not present in the 18 amino acids of Mtt1alt that replace the 21 C-terminal amino acids in Mtt1. It is also absent from the C-terminus of Mal61. This is in agreement with the observation that the C-terminus of Mal61 is not involved in glucose-induced ubiquitination, secretion or localization [158]. In line with this idea is that binding of 14-3-3 proteins at the extreme C-terminal end of the *Arabidopsis* plasma membrane H^+-ATPase AHA2 is essential for its activation [70]. However, more work is required to further characterize the *MTT1alt* and *MTT1* genes, preferentially by expressing the genes in a strain that lacks other maltose and maltotriose transporters.

Acknowledgments

We thank Karen van Eunen for her help with constructing the library and Tapani Suortti at VTT for purification of [U-^{14}C]-maltotriose. This work was funded by the European Union (contract no. QLK1-CT-2001-01066).

Chapter 4

Maltotriose utilization in high-gravity brewing: Attachment of the *MAL32* encoded maltase on the outside of yeast cells

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Submitted

Abstract

During high-gravity brewing the fermentation of maltotriose, the second most abundant fermentable sugar in wort, is often incomplete. Poor maltotriose consumption is due to intensified environmental stress conditions during high-gravity fermentation and a lower priority for uptake of the sugar by industrial strains. In this study we investigated whether the use of strains with an α-glucosidase attached to the outside of the cell would reduce residual maltotriose. To this end, the N-terminal leader sequence of Kre1 and the carboxyterminal anchoring domain of either Cwp2 or Flo1 were used for cell surface expression of the maltase gene *MAL32* under transcriptional control of the *PGK1* promoter. We showed that Mal32 displayed on the cell surface of laboratory *Saccharomyces cerevisiae* strains was capable of hydrolysis of α-1, 4-linkages under fermentation conditions, pH 4 or 5 and 10 °C, respectively. Moreover, extracellular hydrolysis of maltotriose by maltase, increased the ability of a strain lacking a functional maltose permease, to grow on maltotriose. Fermentations in two liter tall tubes at 18 °C with an initial wort density of 21.8 °P showed that lager strains expressing Mal32 protein on their cell surface, used maltose and maltotriose faster than the control strain A15 in the early stages of the fermentation. Sugar analysis by HPLC revealed that after 15, 39 and 68 hours of fermentation, more maltose and maltotriose were consumed by the strains expressing Mal32 at their cell surface compared to the control strains. These data show that yeasts expressing maltase on their cell surface might provide a means of conditioning yeast for more complete maltotriose utilization in brewing.

Introduction

In traditional beer fermentations worts are used of 12 degrees Plato (°P) which corresponds to 12 gram sugar per 100 gram solution. These worts yield beers with 5 % ethanol. High-gravity brewing (HGB) is a recent development in the brewing industry in which worts of 18 °P are fermented to produce green beers with a higher alcohol content. In this brewing process fermentation and lagering are followed by dilution to a beer with the desired alcohol concentration. The use of HGB enhances the brewing capacity due to increased production per fermentor. In addition, it reduces the energy demand and the labor and capital costs [7,207]. However, the high osmotic values in HGB and high ethanol concentrations cause environmental stresses, which may reduce the fermentation rate and the viability of the yeast cells. As a consequence, fermentations are often incomplete resulting in residual sugar, especially maltotriose. This results in flavor problems and a lower yield of ethanol.

Wort contains fermentable sugars like glucose, fructose, sucrose, maltose and maltotriose and non-fermentable sugars such as ribose, xylose and arabinose. In all-malt brewer's wort, 50-60% of the total sugars are maltose, 15-20 % maltotriose and 10-15 % glucose [290]. The fermentable sugars are taken up by yeast in a certain order before they are converted. Generally, when half of the wort glucose has been consumed, the yeast will commence uptake of the α-glucosides maltose and maltotriose [290]. All α-glucosides are actively transported into yeast cells by a H^+-symport mechanism, which depends on the electrochemical proton gradient across the plasma membrane [270]. *Saccharomyces cerevisiae* strains contain five loci encoding a maltose permease, Malx1, where x can be 1-4 or 6. In addition three other maltose transporters are present Agt1, Mph2 and Mph3. The various transporters have different substrate specificities [55,94,242]. Maltose and maltotriose have both been reported to be substrates for Malx1, Agt1 and Mph2/Mph3 with maltose as the preferred substrate [56]. This preference for maltose might lead to incomplete utilization of maltotriose during fermentations. To overcome this problem, strains with an efficient maltotriose transporter might be used or an efficient maltotriose transporter might be introduced [61]. Alternatively, when hydrolysis of the trisaccharide takes place outside the yeast cell it will be converted to maltose and glucose, which are rapidly consumed. Secretion of the intracellular enzyme α-glucosidase, capable of the breakdown of maltotriose, into the wort might be a possible application. However, in view of brewer's and consumers demands it might be better to attach α-glucosidase to the yeast cell wall to avoid the presence of additional proteins in the final beer product.

The cell wall of *S. cerevisiae* is a dynamic structure separating the cell from its external environment and also controlling the passage of several macromolecules [30]. It consists of a layered structure made for >95% of equal amounts of glucan and mannoproteins and a small amount of chitin (1-2%) as reviewed extensively [123,128,140]. Chitin is an essential compound since *S. cerevisiae* mutants lacking all three chitin synthases (Chs1, Chs2 and Chs3) are inviable [232]. The inner layer of the cell wall, composed of β-1, 3- and β-1, 6- linked glucose and small amounts of chitin and

mannoproteins, forms the backbone of the cell wall and is linked by covalent bonds to the outer layer of mannoproteins. This outer layer determines the surface properties of the cell and the porosity of the cell wall. Two types of mannoproteins are present in the cell wall (reviewed by Van der Vaart *et al.* and Kondo & Ueda) [131,266]. The first type is loosely associated with the cell wall. These mannoproteins are extractable with sodium dodecyl sulfate (SDS) and therefore called the SDS-extractable mannoproteins. The second type of mannoproteins is the glucanase-extractable mannoproteins. They are released after glucanase digestion of the glucan layer of the cell wall, but not by SDS extraction. Glucanase-extractable mannoproteins like Sed1, Cwp1, Cwp2, Tip1, Tir1/Srp1, agglutinin (Agα1 and Aga1) and flocculin (Flo1) (reviewed by Kondo & Ueda) [131] all have C-terminal regions which are rich in serine and threonine and contain putative glycosyl phosphatidylinositol (GPI) attachment signals [265]. The localization of these GPI-anchored proteins on the cell surface occurs through the secretory pathway (reviewed by Kondo & Ueda) [131]. Based on this knowledge heterologous proteins have been targeted to the yeast cell surface by the addition of an N-terminal secretion signal and a C-terminal GPI-attachment signal, i. e. cell surface display of enzymes such as glucoamylase, cellulase, and lipase [152,167,168].

In the present study, we fused α-glucosidase encoded by the *MAL32* gene, with the N-terminal secretion signal of Kre1 and the C-terminal domain of either Cwp2 or Flo1 [26]. Both fusions were expressed constitutively under transcriptional control of the phosphoglycerate kinase (*PGK1*) promoter. We report that α-glucosidase displayed on the cell surface of lager yeast strain A15 was capable of hydrolyzing the α-1, 4-linkages between the glucose subunits of maltotriose under fermentation conditions.

Materials and methods

Strains and media

Escherichia coli XL1-Blue [32] was used for plasmid amplification. Standard methods were used for *E. coli* transformation [111]. *E. coli* was grown in Luria-Bertani (LB) medium [225] and if necessary ampicillin was added to 60 µg/ml. The yeast strains used in this study are listed in Table 1. CMY1001 and CMY1050 (obtained from C.A. Michels, Queens College and Graduate School of City University of New York Biology Department, Flushing, New York USA) have been described previously [158]. Strain CMY1001 contains a single *MAL1* locus at which the *MAL11* maltose permease gene is replaced by hemagglutinin (HA)-tagged *MAL61*. Strain CMY1050 is a *mal61/HA*-null derivative of CMY1001. Strain GG3206 is a *MAL12* deletion mutant of CMY1001 which was constructed as follows. The upstream primer "leu2disruptieFW" and the downstream primer "leu2disruptieRV" (Table 2) were used to amplify the *LEU2* gene with plasmid pRS315 as the template. The resulting PCR product, which has homology to the *MAL12* sequence at both the 5' and 3' ends of the open reading frame, was then used for one-step gene replacement of *MAL12*. Proper integration was confirmed by Southern blot analysis. Strains GG3203 and GG3204 are

mutants of the CMY1050 strain carrying α-glucosidase encoded by the *MAL32* gene on the cell surface and were constructed as follows. The plasmids pRUL864 or pRUL865 were linearized by *Stu*I digestion and integrated into the *URA3* gene of CMY1050, creating strain GG3203 and GG3204 respectively. Strains GG3207 and GG3208 were created in the same way but the plasmids were integrated at the *URA3* locus of strain GG3206. The same fragments integrated in lager strain A15 yielded the strains GG3209 and GG3210. Growth of the newly constructed strains in YPD with G418 and Southern blotting analysis confirmed proper integration.

Table 1. *Saccharomyces cerevisiae* and lager yeast strains used in this study

Strain	Relevant genotype/ features/ references
CEN.PK113-5D	*MATa ura3-52* haploid laboratory strain. Obtained from P.Kötter (J.-W. Goethe Universität, Frankfurt, Germany)
WS34/70	Lager yeast strain (Weihenstephan brewery, Freising, Germany)
CMY1001	*MAT***a** *MAL61/HA MAL12 MAL13 GAL leu2 ura3-52 lys2-801 ade2-101 trp1-Δ63 his3-200* [157]
CMY1050	*mal61Δ::HIS3* (isogenic to CMY1001) [158]
GG3206	*mal12Δ::LEU2* (isogenic to CMY1001) (this study)
A15	Lager yeast strain. Obtained from J. Londesborough (VTT Biotechnology, Finland)
GG3204	CMY1050 *ura3*::pRUL864 (this study)
GG3205	CMY1050 *ura3*::pRUL865 (this study)
GG3207	GG3206 *ura3*::pRUL864 (this study)
GG3208	GG3206 *ura3*::pRUL865 (this study)
GG3209	A15 *ura3*::pRUL864 (this study)
GG3210	A15 *ura3*::pRUL865 (this study)

Yeasts were grown in either YPD [233] or MY medium [295], supplemented as required, with histidine (20 mg/ l), uracil (20 mg/ l), L-lysine (20 mg/ l), adenine (20 mg/ l), L-leucine (20 mg/ l) or L-tryptophan (20 mg/ l). G418 (Duchefa) was used at a concentration of 150 µg/ ml. Media were solidified by the addition of 1.5% agar (Sphero). Standard methods were used for yeast transformation with both circular and linear plasmid DNA [80].
For the fermentation experiments, the yeast strains were pre-cultured in YP (Oxoid bactopeptone 2 %, Merck yeast extract 1%) with 2% maltose (Sigma). All-malt wort was prepared from blond malt extract extract powder (Pharma Import, Belgium) and 50 µl of a 8.8 mg/ml $ZnSO_4.7H_20$ solution per liter wort was added. After autoclavation for 20 min at 120 ° C, the wort was cooled to room temperature and aerated before inoculation.

Molecular biological methods and plasmid constructions
Molecular biological techniques were carried out by standard protocols [225]. The QIAprep minispin kit (Qiagen) was used to isolate plasmid DNA from *E. coli*. The same kit was used to purify plasmid DNA from *S. cerevisiae*, after yeast cells were incubated for 45 minutes at 30 ° C with 1 mg/ ml lyticase (Sigma) in buffer P1 (Qiagen). Plasmid features are shown in Table 2. The sequences of oligonucleotides used for the construction of plasmids are shown in Table 3.

Table 2. Plasmid features

Plasmid	Features
pMVHIS	Plasmid allowing the galactose inducible expression of 6xHis-tagged proteins in *S. cerevisiae*. The MRGS-6His tag is recognized by Qiagen's $RGSH_4$ antibody [268]
pMVHIS(*MAL32*)	Encodes an N-terminal 6xHis-Mal32p fusion protein. Made by insertion of a fragment that was generated by PCR with primers "MAL32BamHI" and "MAL32SalI" on chromosomal DNA of WS34/70 in the *Bgl*II-*Xho*I sites of pMVHIS
pFB2	Vector encoding the Kre1/HA/Cwp2-fusion protein, obtained from Prof. F. Breinig, University of Saarland, Germany [26]
pFB3	Vector encoding the Kre1/HA/Flo1-fusion protein, obtained from Prof. F. Breinig, University of Saarland, Germany [26]
pRUL1106	Vector pFB2 containing *MAL32* with HA-epitope
pRUL1108	Vector pFB3 containing *MAL32* with HA-epitope
pRS306	Yeast integrative vector with *URA3* marker [234]
pRS306(KanMX)	Plasmid pRS306 with KanMX cassette obtained by digestion of pUG6 [90] with *Bgl*II and *Spe*I into its *BamH*I and *Spe*I sites
pRUL864	Kre1/*MAL32*-HA/Cwp2-fusion under transcriptional control of the *PGK1* promoter amplified in a PCR with primers "PGK1SpeI" and "PGK1SacII" inserted into the *Spe*I and *Sac*II sites of plasmid pRS306(KanMX)
pRUL865	Kre1/*MAL32*-HA/Flo1-fusion under transcriptional control of the *PGK1* promoter amplified in a PCR with primers "PGK1SpeI" and "PGK1SacII" inserted into the *Spe*I and *Sac*II sites of plasmid pRS306(KanMX)

Plasmid construction

For the construction of plasmid pMVHis(*MAL32*) the *MAL32* gene was amplified in a PCR using the primers "MAL32BamHI" and "MAL32SalI" (Table 3) with genomic DNA from lager yeast strain WS34/70 as template DNA. The PCR fragment was inserted into the *Bgl*II and *Xho*I sites from cloning vector pMVHis [268]. The empty pMVHis vector containing an inducible *GAL1* promoter, an *URA3* selection marker and a MRGS-6His tag, recognized by Qiagen's $RGSH_4$ antibody was also used as a control.

The *MAL32* gene was amplified from chromosomal DNA of strain WS34/70 by PCR using primers "MfeI-HA-M32" and "SalI-M32-reversed" (Table 3) and inserted into the *EcoR*I and *Sal*I sites from vector pFB2 or pFB3 (obtained from Prof. F. Breinig, University of Saarland, Germany) [26] under transcriptional control of the *PGK1* promoter. In this way an N-terminal fusion with the secretion signal of Kre1 and a C-terminal fusion with the anchoring domain of either Cwp2 or Flo1 were created, giving plasmids pRUL1106 and pRUL1108, respectively. These fusions were amplified in a PCR with primers "PGK1SpeI" and "PGK1SacII" (Table 3) and inserted into the *Spe*I and *Sac*II sites of plasmid pRS306(KanMX), giving plasmids pRUL864 and pRUL865 respectively. Plasmid pRS306(KanMX) was constructed by inserting the KanMX cassette obtained by digestion of pUG6 [90] with *Bgl*II and *Spe*I into the *BamH*I and *Spe*I sites of vector pRS306 [234].

Polymerase Chain Reaction

The primers used for PCR are listed in Table 3. PCR amplifications were performed with 50-100 ng genomic DNA or 25-50 ng plasmid DNA. Amplification conditions were: 5 min at 94 ºC, 30 cycles of 1 min 94 ºC, 1 min at 5 ºC below the Tm of the primers and 1 min per kb to be amplified at 74 ºC followed by 10 min at 74 ºC. Reactions were performed in a total volume of 50 µl and per reaction 0.5 µl Vent polymerase (New England BioLabs) was used with the buffer supplied. PCR products were analyzed on a 0.7 % agarose gel in 1 x TAE buffer. All PCR products were cloned into the pCR®-Blunt II-TOPO® vector (Invitrogen) before they were recloned in the proper vector.

Table 3. Oligonucleotides

Name	Sequence (5'=>3')	Tm (°C)
MAL32BamHI	CGC GGA TCC ATG ACT ATT TCT GAT CAT CCA G	60
MAL32SalI	ACG CGT CGA CGT CGG CAC TAA TTT TAT TTG AC	60
MfeI-HA-M32	CGC AAT TGT ATC CGT ATG ATG TGC CTG ACT ACG CAA TGA CTA TTT CTG ATC ATC CA	56
SalI-M32-reversed	GCG TCG ACT TTG ACG AGG TAG ATT CTA C	56
PGK1SpeI	GGA CTA GTG CCG ATT TGG GCG CGA ATC C	66
PGK1SacII	TCC CCG CGG CCT TCT CGA AAG CTT TAA CGA AC	66
Leu2disruptieFW	CAT CCA TCT CTT AAG CAG TTT TTT TGA TAA TCT CAA ATG TAC ATC GGG GCG CTA TCG CAC AGA ATC	68
Leu2disruptieRV	GAG GTA GAT TCT ACC TTC CCA TGG TTT CAA AAC TCT GGA GGA AAC TGT GTG GTG CCC TCC TCC TTG	68

Purification of α-glucosidase

For the purification of α-glucosidase encoded by the *MAL32* gene, *S. cerevisiae* strain CEN.PK113-5D was transformed with pMVHis(*MAL32*). The 6 x His-tagged Mal32 protein was isolated from this strain grown in minimal medium with 1 % galactose for 14 hrs at 30 °C till A_{600} between 0.2 and 0.3. The cells were harvested by centrifugation, washed with sterile icecold water and resuspended in PBS buffer (pH 7.0) with proteinase inhibitors (Roche-Boehringer) using 20 µl buffer per O.D. unit per ml culture. Cell suspensions were added to pre-chilled FastPROTEIN™ RED tubes (BIO 101) and homogenized using a FastPrep® Instrument at a speed of 6.0 for 20 seconds. For the release of sufficient amount of protein the tubes were continuously mixed by vortexing for 5 min at 4 °C. The cell extract was cleared by centrifugation in a microcentrifuge for 10 min at 13,000 rpm at 0 °C, mixed with Ni-NTA agarose (QIAGEN) and rotated for 2 hrs at 4 °C. The Ni-NTA agarose was collected on a spin column (Biorad) and washed 10 times with 0.7 ml PBS, followed by washes of 500 µl PBS supplemented with 2, 5 and 10 mM EDTA (pH 8.0), respectively. Bound 6 x His-tagged Mal32 was eluted with 250 µl PBS supplemented with 20 mM EDTA. The washes and elution were carried out at 4 °C.

Western blotting

Aliquots of cell extracts, purified proteins or crude cell wall extracts were separated by 12 % SDS-PAGE [134] and transferred from gel to PVDF membrane (Roche-Boehringer) by semi-dry blotting with a Biorad blotting apparatus for 30 min with 2 mA / cm^2. After blotting, the membranes were washed in TBS (20 mM Tris (pH 8.0), 150 mM NaCl) and incubated overnight at 4 °C in TBS with 1 % blocking reagent (Roche-Boehringer). After o/n incubation the membranes were incubated for 1h with diluted 1:1000 anti-$RGSH_4$ antibody (QIAGEN) in TBS with 0.5 % blocking reagents for detection of 6 x His tagged versions of Mal32 and washed three times with TBST (1 ml Tween 20/ liter TBS). Subsequently, the membranes were incubated for 30 min with diluted 1:7500 Garpo antibody in TBS with 0.5 % blocking reagent and washed three times with TBST and once with TBS. For detection of fusion proteins containing the 9 amino acid HA-peptide, TBS with 5 % blocking reagent (ELK milk powder, Campina Melkuni) was used with 1:1000 diluted Anti-HA-peroxidase antibody (Roche). The BM Chemiluminescence Western Blotting Kit (Roche-Boehringer) was used for immunochemical detection.

Preparation of crude cell wall extracts

Cells grown o/n at 30 °C in YPD were harvested by centrifugation or taken from the -80 °C storage and resuspended in 0.1 M potassium phosphatebuffer (pH 7.0) with proteinase inhibitors (Roche-Boehringer) to a concentration of 20 µl buffer per O.D. unit per ml culture. Cell suspensions were added to pre-chilled FastPROTEIN^{TM}RED tubes (BIO 101) and homogenized using a FastPrep® Instrument at a speed of 6.0 for 20 seconds. The extract was cleared by centrifugation for two times 1 minute at 800 g at 0 °C. For Western analysis, the extract was cleared by centrifugation for two times 1 minute at 500 g instead. The crude cell wall fraction was obtained by centrifugation of the cleared extract for 30 minutes at maximum speed at 0 °C. Prior to use in the colorimetric assay, crude cell extracts were washed with 1 ml 0.1 M potassium phosphate buffer.

α-glucosidase colorimetric assay

To test for α-glucosidase activity, cell extracts were diluted with 0.1 M potassium phosphate buffer (pH 7.0) to a protein concentration of 50 µg/ ml. Protein concentrations were determined using the Bradford assay [25].

Per Eppendorf tube 200 µl of 4 mg 4-nitrophenyl-α-D-glucopyranoside (PNPG) (Aldrich Chem. Co.) per ml 0.1 M potassium phosphate (pH 7.0) was added to 700 µl 0.1 M potassium phosphate buffer containing different amounts of cells, cell extracts or crude cell wall extracts. Reactions were incubated at 30 °C. PNPG substrate contains a similar α-1, 4-linkage as is present in maltose and maltotriose to connect two glucose molecules. Breakage of this α-1, 4-linkage releases 4-nitrophenol resulting in a yellow solution since 4-nitrophenol absorbs at 405 nm. After sufficient color development the time was noted and the reaction was stopped by the addition of 400 µl 1 M Na_2CO_3. The absorbance of the samples at 405 nm was measured. For large numbers of samples microtiter plates (Greiner) were used with per well 75 µl PNPG solution and 100 µl

0.1 M potassium phosphate buffer containing different amounts of cell extract. After sufficient color development the time was noted and the reaction was stopped by the addition of 50 µl 1 M Na_2CO_3. The A_{405} was recorded in a microplate reader model 3550 (Biorad, Hercules, USA).

Glucose determination assay

To measure α-glucosidase activity with maltotriose and maltose as substrate for the Mal32 maltase the liberated glucose was determined using an enzymatic assay (Enzytec ™ D-Glucose) according to the supplier's recommendation. Briefly, Mal32 protein in PBS buffer (pH 7, 5 or 4) was incubated for different time periods in the presence of 0.1 M substrate, maltose or maltotriose, at 30 °C or 10 °C in a total volume of 33 µl. After incubation, 333 µl reagent #1 (powder mixture with triethanolamine buffer (pH 7.6), 80 mg NADP, 190 mg ATP and $MgSO_4$ dissolved in 31 ml distilled water) and 633 µl distilled water were added and the mixture was incubated for 3 min at room temperature. A volume of 6.7 µl reagent #2 (0.7 ml hexokinase (200 U)/ glucose-6-phosphate dehydrogenase (100 U) suspension) was added. After 15 min at room temperature, the absorbance of the NADPH formed was determined at 340 nm and the glucose content calculated using the specific absorbance of NADPH (ε_{340} = 6.3 l x $mmol^{-1}$ x cm^{-1}).

Fermentation in 2 liter tall tubes

Fermentations were carried out essentially as described by Blieck [16]. Yeast cells were grown overnight in 5 ml at 30 °C in a shaker at 200 rpm. The overnight culture was transferred to 50 ml YP/ 2% maltose and incubated for 24 hours at 30 °C in a shaker at 200 rpm. Subsequently, the culture was added to 1000 ml YP/ 2% maltose and grown at 30 °C till early stationary phase. The cells were collected by centrifugation for 5 minutes at 1300 g and counted with a haemacytometer. Yeast was pitched in aerated wort with a pitching rate of 10^6 cells per ml per degree Plato. Fermentations were carried out in 2 liter tall tubes with a height of 72 cm and a diameter of 7.5 cm at 18 °C with an initial wort density of 21.8 °P. Samples for the determination of different parameters were removed 10 cm above the fermenter bottom at the indicated time points until the end of the fermentation. Samples were used directly for analysis or they were washed once with ice-cold water and stored at -80 °C.

Determination of different parameters during the fermentation

Viability was determined by methylene blue counting according to the ECB Analytica method [102] using a haemacytometer. Growth was followed by counting the cells in suspension using a haemacytometer. Wort density (apparent extract) was determined by centrifugation of the samples for 5 minutes at 1300 g at 0 °C and measuring the density of the supernatant using a DMA 35 N density meter (Anton Paar).

To determine the attenuation limit of the wort at the end of the fermentation, a 100 ml sample was transferred to a 300 ml flask with a waterlock and 6 g pressed yeast (Bruggeman) were added. Density of the wort (apparent extract after final attenuation) was determined after 48 hours of stirring at room temperature. The difference in apparent extract (delta apparent extract) was

calculated by subtracting apparent extract after final attenuation from apparent extract determined at the end of fermentation. Sugar concentrations were commercially determined by HPLC analysis (VTT Biotechnology, Finland).

Results

Characterization of the Mal32 maltase

In order to convert maltotriose into maltose and glucose outside the yeast cell, a 6 x His tagged version of Mal32 protein was partly purified as described in the Materials and methods section. Western blotting showed the successful isolation of the protein from strain CEN.PK113-5D containing plasmid pMVHIS(*MAL32*) (Figure 1).

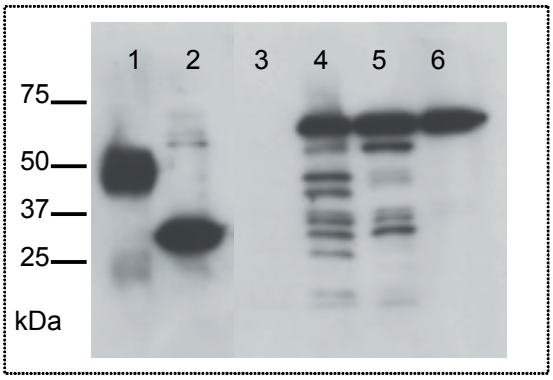

Figure 1. Western analysis.
Cell were grown for 14 hours in minimal medium plus 1% galactose and analyzed directly or used for the preparation of total cell extracts using the FastPrep Instrument and/ or purified using Ni-NTA agarose (QIAGEN). Western analysis of proteins present in cells of strain CEN.PK113-5D + pMVHis (lane 3), or + pMVHis(*MAL32*) (lane 4) are shown. In lane 5 proteins present in total cell extract of strain CEN.PK113-5D + pMVHis(*MAL32*) are shown and the purified Mal32 protein in lane 6. For the Mal32 protein a band of 68 kDa was expected and for the controls, the MW-marker (lane 1) and Bmh1 (lane 2), bands of 45 kDa and 30 kDa, respectively.

The α-glucosidase activity of the purified protein was confirmed by measuring enzyme activity using the PNPG (4-nitrophenyl-α-D-glucopyranoside) colorimetric assay. Unpurified cell extracts from the same strain also contained measurable maltase activity which increased linearly with the extract concentration, whereas similar extracts, containing the same amount of protein, from the strain with the control vector pMVHIS did not (Figure 2). The measured activity in these control extracts was low and independent of the amount of cell extract used in the assay (Figure 2). Based on these results further purification of Mal32 was not attempted. Subsequent analyses were therefore carried out with cell extracts.

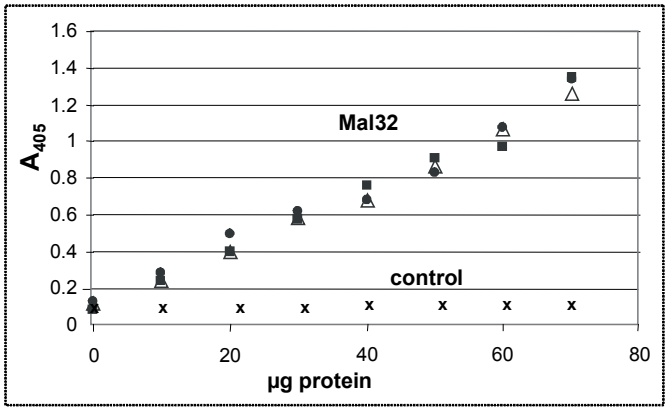

Figure 2. α-glucosidase activity of Mal32 determined in a colorimetric assay.

4-nitrophenyl-α-D-glucopyranoside (PNPG) was used as a substrate for CEN.PK113-5D strains containing Mal32 or the control vector. Total cell extracts were prepared, using the FastPrep Instrument, from strain CEN.PK113-5D with Mal32 (Δ, ■, and ● respectively, each representing an independent experiment) or with the control vector pMVHIS (x). Absorbance at 405 nm was measured after incubation for two hours at 30 °C.

To discriminate between the activity of the enzyme towards maltose and maltotriose we used a different assay in which the released glucose is measured enzymatically. Using this glucose assay the K_m and V_{max} were measured at 30 °C and pH 7 with maltotriose or maltose as substrate. The results in Table 4 clearly show higher K_m and V_{max} values for maltose compared to maltotriose.

Table 4. K_m and V_{max}

Substrate	Conditions	K_m	V_{max}
Maltose	pH 7, 30°C	138 ± 48	1.0 ± 0.2
Maltotriose	pH 7, 30°C	37 ± 3	0.2 ± 0.01

Results are averages of independent triplicate experiments ± their standard deviations.

To determine the feasibility of using this enzyme in brewing we also measured the specific activity under fermentation conditions, pH 4 or 5 and a temperature of 10 °C. The results indicate that Mal32 maltase can break down maltotriose under fermentation conditions, although with a much lower rate than at standard conditions. Activity at pH 5 and 10 °C was only 3 % and at pH 4 and 10 °C 0.7 % of the activity at pH 7 and 30 °C. The results are summarized in Table 5.

Table 5. Specific activities of Mal32 under different conditions

Substrate	Conditions	Substrate concentration (mM)	Specific activity (nmol/min/mg protein)
Maltose	pH 5, 10°C	100	14 ± 0.6 [a]
		150	16 ± 0.5
		200	20 ± 4
		300	25 ± 4
Maltotriose	pH 5, 10°C	100	13 ± 2
		150	15 ± 2
		200	16 ± 2
		300	20 ± 4
Maltose	pH 4, 10°C	100	Below detection
		150	Below detection
		200	Below detection
		300	Below detection
Maltotriose	pH 4, 10°C	100	3 ± 0.3
		150	3 ± 2
		200	5 ± 0.8
		300	6 ± 1
Maltose	pH 7, 30°C	100	1337 ± 156 [b]
		330	2117 ± 32
Maltotriose	pH 7, 30°C	100	554 ± 19
		330	617 ± 52

[a] Results are averages of independent duplicate experiments ± their standard deviations.
[b] Results in these series are of three experiments

Cell surface expression of maltase

For the localization of Mal32 protein outside the cell, Mal32 tagged with the nine amino acid viral epitope hemagglutinin (HA) was subcloned into plasmids pFB2 and pFB3 as described in the Materials and methods section. The fusion protein in plasmid pRUL1106 consists of four parts. First 28 amino acids of Kre1, of which the 26 N-terminal amino acids serve as a leader peptide which is removed by signal peptidase cleavage upon entry into the ER lumen [26]. The 28 Kre1 amino acids are followed by the 9 amino acid HA-peptide fused to the 584 amino acids of Mal32. The final part consists of the C-terminal 71 amino acids of Cwp2, which form a GPI anchor. The entire construct encodes a predicted protein of 692 amino acids with a molecular weight of 76.1 kDa [26].

Plasmid pRUL1108 contains a similar fusion protein in which the C-terminal 104 amino acids of Flo1 replace the C-terminal 71 amino acids of Cwp2. Both fusions were expressed constitutively under control of the phosphoglycerate kinase (*PGK1*) promoter as illustrated in Figure 3.

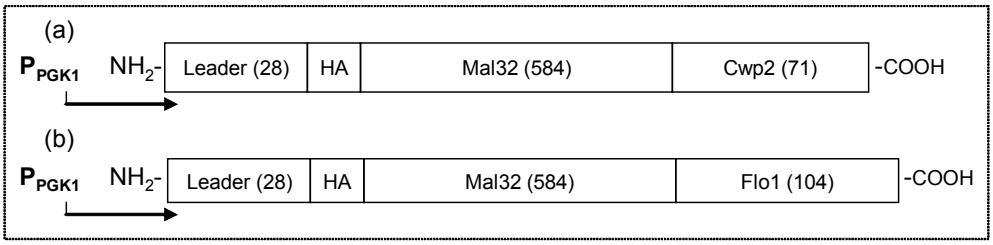

Figure 3. Schematic outline of the constructed cell wall fusion proteins.
The number of amino acids of each part is given in parentheses. Both constructs are under transcriptional control of the yeast *PGK1* promoter and contain the Kre1 signal sequence (Leader) and the hemagglutinin-tag (HA). Construct (a) contains the C-terminal part of Cwp2 derived from pFB2 and construct (b) contains the C-terminal part of Flo1 derived from pFB3 [26].

To test whether the constructs had activity, strain GG3206, a maltase negative mutant of CMY1001 was transformed with the plasmids pRUL1106 and pRUL1108. Transformants were selected on MY plates containing histidine, adenine, lysine and tryptophan after incubation for 3 days at 30 °C and re-streaked on the same selection plates. Maltase activity of the strains was determined in the colorimetric assay. In this assay, cells were incubated with PNPG for circa 3 hours at 30 °C as described in the Materials and methods section. Yeast cells expressing either the pRUL1106 construct or the pRUL1108 construct showed a bright yellow color, whereas yeast cells transformed with the control vectors pFB2 or pFB3 did not (data not shown). The yellow color represents Mal32 maltase activity since there is no endogenous maltase present in strain GG3206. The presence of Mal32 activity of yeast cells containing either the pRUL1106 construct or the pRUL1108 construct allowed growth on YP/ 2% maltose albeit after a very long lag phase. Yeast cells containing the control vector did not grow on YP/ 2% maltose. Growth on glucose as carbon source was not affected and similar for all strains (data not shown).
For biotechnological purposes it is not desirable that the yeast strains contain plasmid DNA due to plasmid instability. Therefore both fusion-constructs were recloned in the vector pRS306(KanMX) as described in the Materials and methods section. The resulting plasmids pRUL864 and pRUL865 were linearized by *Stu*I digestion and integrated at the *URA3* loci of lager strain A15 and of the laboratory strains CMY1050 and GG3206 for control experiments. Proper integration was confirmed by Southern blot analysis and the ability of the yeast strains to grow on YPD in the presence of G418 (data not shown).
Initially, cells of the *mal12*::*LEU2* mutant GG3206 with or without the integrated plasmids were grown in YPD to exponential or stationary phase. After harvesting, maltase activity was determined by incubating the cells in PNPG (4-nitrophenyl-α-D-glucopyranoside) substrate in

phosphate buffer for two hours at 30 °C. Breakage of the α-1, 4-linkage present in PNPG was determined by measuring the absorbance at 405 nm. The measured A_{405} was significantly higher for GG3206 cells with the integrated plasmids than for the control cells. Since strain GG3206 has no endogenous maltase activity, the absorbance measured at 405 nm must be a result of the newly integrated *MAL32* gene. A typical example of these experiments is shown in Figure 4A. To exclude the possibility that cell lysis or secretion of Mal32 were responsible for the detected extracellular activity, supernatants of the different cells were incubated with PNPG as well. In these experiments no activity was measured indicating that Mal32 is attached to the yeast cells (Figure 4B).

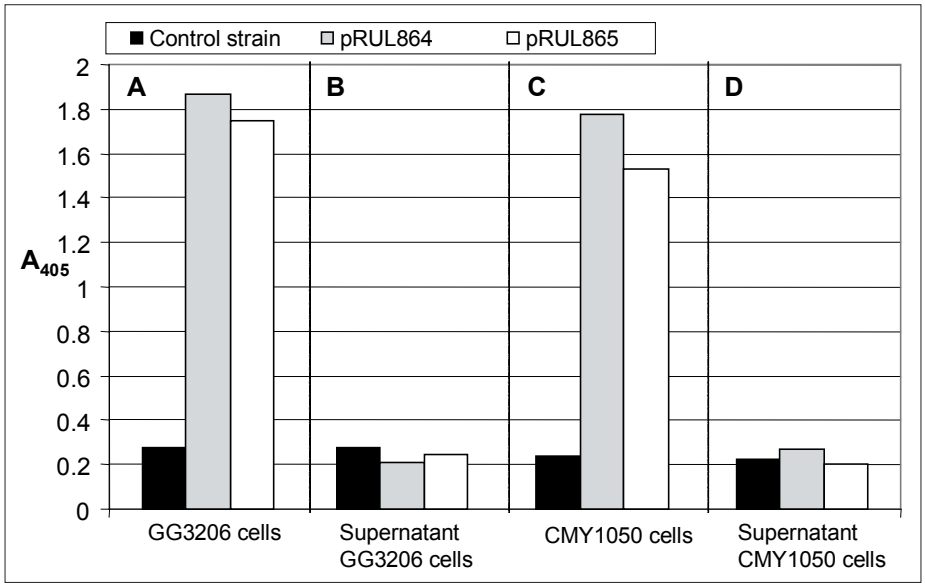

Figure 4. Maltase activity in strains GG3206 and CMY1050 expressing Mal32 on the cell surface.
After growth, the maltase activity was determined using the PNPG method, as described in Materials and Methods, with the cells (A and C) and with the supernatants (B and D). The absorbance (A_{405}) after two hours incubation at 30 °C is shown for strain GG3206 (A) and strain CMY1050 (C) in black or with integrated plasmids pRUL864 (C-terminal part of Cwp2 attached to Mal32 protein), grey, or pRUL865 (C-terminal part of Flo1 attached to Mal32 protein), white, and for the supernatants of the various cells (B and D).

To confirm that hydrolysis of PNPG occurred outside the yeast cells, similar experiments were performed with cells of CMY1050. Strain CMY1050 is a *mal61/HA* null derivative of CMY1001 and thus lacks a functional maltose permease. After incubation of the CMY1050 cells in phosphate buffer with PNPG for 2 hours at 30 °C, substantial activity was found for cells with the integrated plasmids. In contrast, no activity was measured when CMY1050 control cells without an

integrated plasmid were used. These results indicated that uptake of PNPG substrate by Mal61 did not play a role and thus confirmed Mal32 activity outside the yeast cells (Figure 4C). The absence of activity in the supernatants confirmed that the observed activity of the cells was not caused by lysis or secretion (Figure 4D). These experiments clearly show that the maltase is located at the outside of the cells.

To further characterize the presence of Mal32 on the cell surface, growth on maltotriose of strains CMY1001, CMY1050, GG3204 and GG3205 was studied. We found that on solid medium the presence of Mal32 attached to the cell wall with either the localization signal from Cwp2 (GG3204) or Flo1 (GG3205) increased the ability for strain CMY1050 to grow on maltotriose. Growth on maltose or glucose as carbon source was the same for all strains (Figure 5). These results confirm the colorimetric assays and indicate that Mal32 present on the cell surface of laboratory strain GG3206 is able to break down maltotriose into maltose and glucose.

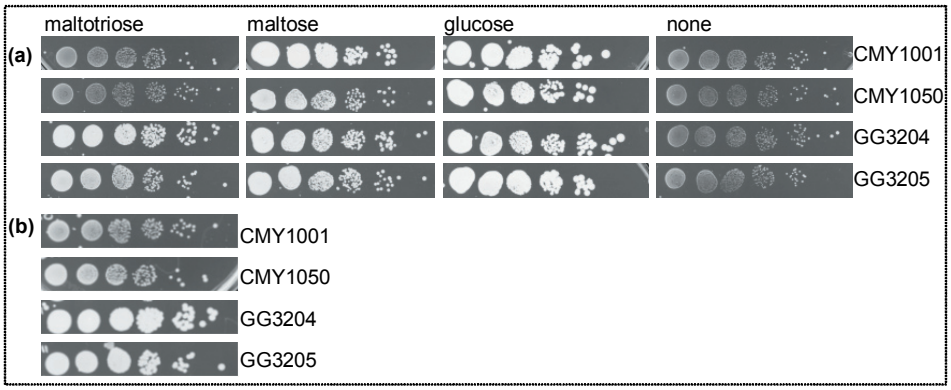

Figure 5. Effect of Mal32 localized on the cell surface on growth on maltotriose of different yeast strains.
Ten-fold dilutions of strains CMY1001 (HA-tagged *MAL61*), CMY1050 (mal61/HA), GG3204 (CMY1050 expressing Mal32 on the cell surface via Cwp2 localization signal) and GG3205 (CMY1050 expressing Mal32 on the cell surface via Flo1 localization signal) pre-grown in YP/ 2% glucose to A_{600} of about 0.8 were spotted onto YP with 2% of different carbon sources. The plates were incubated at 30 °C for 3 days (a) or 5 days (b). The figure shown is representative for two similar experiments.

To confirm Mal32 localization on the cell surface of lager yeast strain A15 and its maltase activity under laboratory conditions, first crude extracts of the cell wall from strains GG3209, GG3210 and A15 were prepared as described in Materials and methods. Subsequently, the extracts were used in the colorimetric assay to determine maltase activity in the crude membrane/ cell wall fractions. Incubation times at 30 °C with PNPG substrate varied from two up to four hours in three experiments but the measured absorbance at 405 nm was at least doubled in all cases, for extracts prepared from strains GG3209 or GG3210 compared to those of the control strain A15 (data not shown). These results indicate that also in lager strain A15 the Mal32 was directed to the cell surface and able to hydrolyze the α-1, 4-linkage present in PNPG substrate.

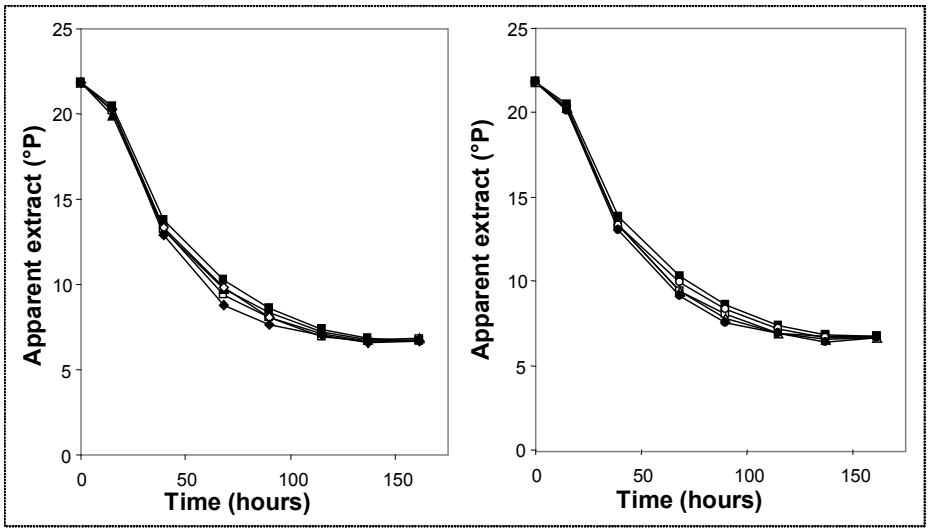

Figure 6. Fermentation profiles during alcoholic fermentations in 2 liter tall tubes at 18 °C.
In the left panel the profiles of the control strain A15 (■ and □) and strain GG3209 (A15 *ura3*:: pRUL864) (▲, ◇ and ♦) are shown and in the right panel the same profiles of the control strain A15 (■ and □) and strain GG3210 (A15 *ura3*::pRUL865) (□, ● and ○). Yeast was inoculated in aerated wort of 21.8 °P with a pitching rate of 10^6 cells/ ml/ °P.

Extracellular maltase activity during fermentation

As strains GG3209 and GG3210, expressing Mal32 maltase on their cell surface, showed more maltase activity than the control strain A15, we decided to follow their fermentation behavior under very high gravity conditions in 2 liter tall tubes at 18 °C. Nine independent fermentations were started with an initial wort density of 21.8 °P. The fermentations of strains GG3209 and GG3210 were performed in triplicate and fermentation of the control strain A15 was performed in duplo, as one fermentation did not start. The fermentation profiles are shown in Figure 6, expressed as the differences in apparent extract (density of the wort) as a function of time.

The differences in apparent extract indicate the amount of fermentable sugar consumed during fermentation and show a similar profile for all strains. However, the apparent attenuation (percentage of sugars fermented from the total amount of fermentable sugars in wort) of the different strains revealed differences in the beginning of the fermentation (Table 6). At 15, 39 and 68 hours the average apparent attenuation showed to be higher for strains GG3209 and GG3210 compared to the control strain A15, although these differences are not statistically significant with the current number of repetitions of the experiment.

The viability during fermentation, measured by methylene blue staining, was higher than 95 % for all strains (Figure 7a) and the amount of cells in suspension, as a parameter for growth, was similar for all strains throughout the fermentation (Figure 7b). These data indicate that the slightly faster

drop in total sugars observed for strains GG3209 and GG3210 at 15, 39 and 68 hours is not due to differences in growth or viability of the cells.

Table 6. Average changes in apparent extract and apparent attenuation

Time (hours)	Strain	Apparent extract (°P)	Apparent attenuation [c] (%)
0 (start)	--	21.8	0
15	A15	20.3 ± 0.2	6.7 ± 0.9 [a]
	GG3209	20.1 ± 0.2	7.6 ± 0.9 [b]
	GG3210	20.2 ±0.1	7.4 ± 0.5 [b]
39	A15	13.5 ± 0.4	37.9 ± 1.7
	GG3209	13.1 ± 0.2	39.7 ± 0.9
	GG3210	13.2 ± 0.2	39.4 ± 0.7
68	A15	9.9 ± 0.6	54.8 ± 2.9
	GG3209	9.4 ± 0.6	56.7 ± 2.6
	GG3210	9.5 ± 0.4	56.3 ± 1.9
90	A15	8.4 ± 0.4	61.7 ± 1.6
	GG3209	8.0 ± 0.4	63.2 ± 1.8
	GG3210	7.9 ± 0.4	63.9 ± 1.8
115	A15	7.2 ± 0.4	67.2 ± 1.6
	GG3209	7.1 ± 0.1	67.4 ± 0.5
	GG3210	7.0 ± 0.2	67.9 ± 0.8
137	A15	6.8 ± 0.1	69.0 ± 0.3
	GG3209	6.7 ± 0.1	69.5 ± 0.5
	GG3210	6.5 ± 0.1	70.0 ± 0.7
161	A15	6.7 ± 0	69.1 ± 0.2
	GG3209	6.7 ± 0.1	69.2 ± 0.3
	GG3210	6.7 ± 0	69.5 ± 0.1

[a] Results are averages of independent duplicate experiments ± the ranges. [b] Results in these series are averages of independent triplicate experiments ± their standard deviations. [c] Apparent attenuation is calculated by subtracting the apparent extract from the initial wort density (21.8), dividing this number by the initial wort density (21.8) and multiply by a factor 100.

At the end of the fermentations, the attenuation limit of the worts (apparent extract after final attenuation) was determined to compare the amount of fermentable sugars remaining, expressed in delta apparent extract (Figure 8). Delta apparent extract is the difference between the apparent extract at the end of fermentation and the attenuation limit of wort (see Materials and methods). Strains GG3209 and GG3210 showed similar attenuation compared to the control strain A15 indicating that an equal amount of fermentable sugars was left in the beer at the end of fermentation.

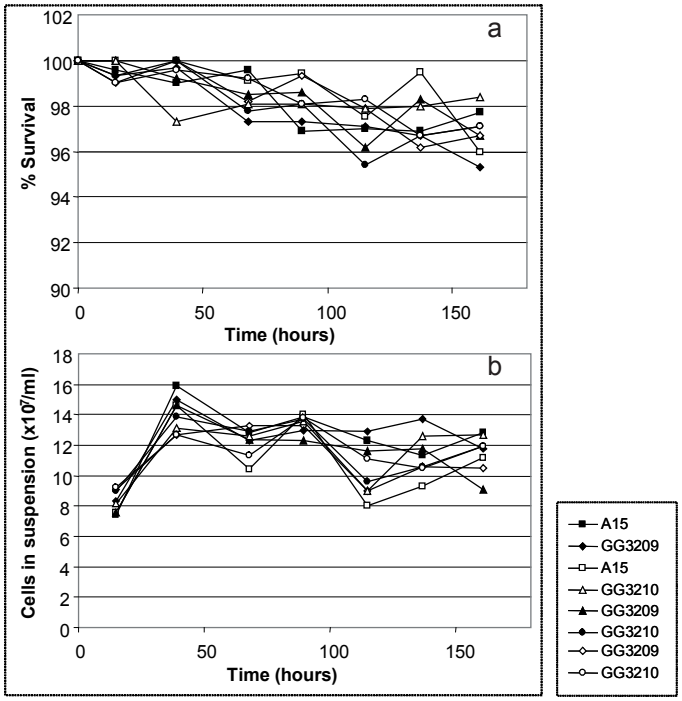

Figure 7. Viability and growth.
Viability (a) and growth (b) of the seven strains are shown during alcoholic fermentations in 2 liter tall tubes at 18 °C. Yeast was pitched in aerated wort of 21.8 °P with a pitching rate of 10⁶ cells/ ml/ °P.

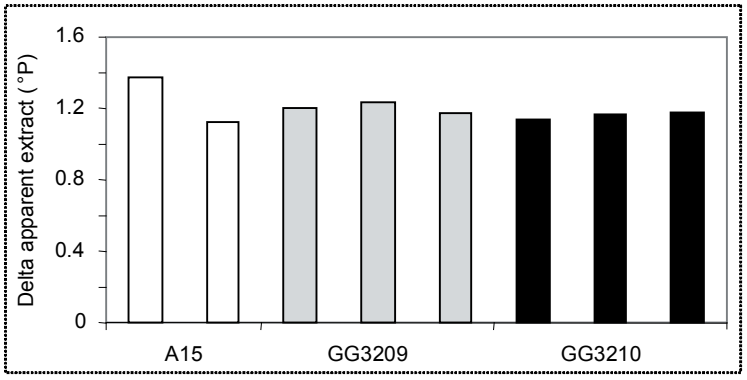

Figure 8. Delta apparent extract.
The delta apparent extract was calculated by subtracting apparent extract after final attenuation from apparent extract at the end of fermentation and shown for A15 (white bars), GG3209 (grey bars) and GG3210 (black bars) after 2 liter tall tube fermentations at 18 °C with an initial wort density of 21.8 °P.

Table 7. Average amounts of the sugars glucose, fructose, maltose and maltotriose present on different days during the 2 liter tall tube fermentations, determined by HPLC analysis

		Average amount of sugar (g/ l)				
Time (hours)	**Strain**	**Glucose**	**Fructose**	**Maltose**	**Maltotriose**	**Total (g)**
0 (start)	--	19.5	4.1	108.2	27.1	160.6 [a]
15	A15	11.1 ± 1.3	4.3 ± 0.1	99.2 ± 0.9	27.2 ± 0.7	141.8 ± 0.2
	GG3209	10.3 ± 1.2	4.1 ± 0.3	86.7 ± 6.4	26.0 ± 2.2	126.2 ± 10.5
	GG3210	11.0 ± 1.1	4.2 ± 0.2	92.4 ± 1.1	25.9 ± 1.0	133.7 ± 2.2
39	A15	0.6 ± 0.1	0.4 ± 0.1	54.3 ± 4.7	25.6 ± 0.7	80.9 ± 5.6
	GG3209	0.7 ± 0.1	0.5 ± 0.2	49.2 ± 1.3	24.3 ± 0.3	74.7 ± 1.5
	GG3210	0.9 ± 0.1	0.5 ± 0.2	49.7 ± 1.8	24.4 ± 0.2	75.6 ± 2.0
68	A15	0.5 ± 0	--	20.1 ± 3.1	18.4 ± 0.0	39.0 ± 3.1
	GG3209	0.5 ± 0	--	16.8 ± 1.9	17.2 ± 0.1	34.5 ± 1.8
	GG3210	0.5 ± 0.1	--	17.6 ± 2.2	17.6 ± 0.1	35.8 ± 2.2
90	A15	0.5 ± 0.1	--	10.5 ± 4.9	16.2 ± 1.6	27.1 ± 6.5
	GG3209	0.5 ± 0.1	--	7.6 ± 3.9	16.4 ± 0.8	24.5 ± 3.2
	GG3210	0.5 ± 0	--	7.2 ± 2.9	15.9 ± 1.0	23.6 ± 1.9
161	A15	0.4 ± 0.1	--	0.3 ± 0	13.1 ± 0.8	13.8 ± 0.9
	GG3209	0.4 ± 0.1	--	0.3 ± 0.1	11.8 ± 1.2	12.4 ± 1.4
	GG3210	0.4 ± 0	--	0.2 ± 0	11.1 ± 0.1	11.7 ± 0.1

[a] This number indicates the total amount of analyzed sugars present in wort at the start of fermentation, including 1.7 gram sucrose. Results are averages of independent triplicate experiments ± their standard deviations.

Despite the fact that the strains GG3209 and GG3210 did not clearly show a faster or more complete fermentation than the control strain, we were interested to see whether the minor differences observed in apparent attenuation in the first three days of the fermentation (Table 6), were caused by a faster consumption of maltotriose and maltose due to the extracellular expression of Mal32. The amounts of the different sugars glucose, fructose, sucrose, maltose and maltotriose present at different time-points during the fermentations were therefore determined by HPLC analysis. Results are shown in Table 7 but do not include sucrose since it disappeared by the first time point in all strains. The data confirmed that in the beginning of the fermentation, after 15 hours, the maltose concentration was lower for strains GG3209 and GG3210 compared to A15. This was also translated into a lower amount of total sugars. In addition, smaller but significant differences in amounts of maltotriose at 39 and 68 hours between the strains expressing Mal32 at their cell surface and the control strain were apparent.

In order to verify that the Mal32 fusion proteins were correctly expressed during fermentation, crude cell wall extracts were prepared from frozen cell pellets (-80 °) of seven time-points throughout the fermentation of strains A15, GG3209 and GG3210, respectively (Figure 9b). A protein of about 80 kDa, absent in the control strain A15, was detected in the crude cell wall extract from all Kre1/ HA/ Mal32/ Cwp2-expressing cells (GG3209), corresponding to the fusion protein with a predicted size of 76.1 kDa (Figure 9a). In addition, a second band of about 30 kDa was detected. In a similar approach, a protein of about 85 kDa, representing the expressed Kre1/ HA/ Mal32/ Flo1 fusion with a predicted molecular mass of 79.8 kDa, was detected in all seven crude cell wall extracts of cells expressing Kre1/ HA/ Mal32/ Flo1 (GG3210). Similar to the GG3209 experiments, a band of about 30 kDa was also detected (Figure 9a). These data indicated that Mal32 was expressed at all the different time-points during fermentation although the expression levels seemed to be lower towards the end of the fermentation (Figure 9b). The presence of the smaller band suggests that the enzyme is subjected to proteolysis.

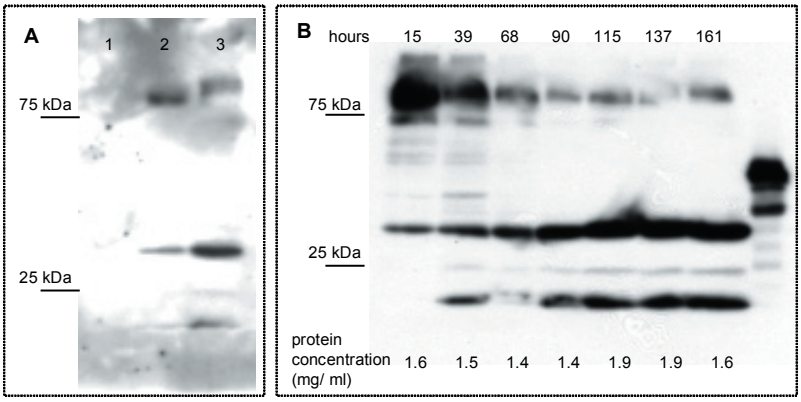

Figure 9. Western analysis of crude cell wall extracts prepared from frozen cell pellets (-80 °C) of the 2 liter tall tube fermentations.
Anti-HA-peroxidase antibody was used for the detection of the fusion proteins as described in the Materials and methods section. A. Extracts deri ved from control strain A15 (lane 1) and from yeast cells expressing Mal32 using Cwp2 (GG3209) or Flo1 (GG3210) in lanes 2 and 3, respectively, on the second day of the fermentation are shown. For the Mal32 cell wall fusion proteins bands of 76 kDa (lane 2) and 80 kDa (lane 3) respectively, were expected. B. Time course of the Mal32-Flo1 fusion protein in strain GG3210 during the fermentation. The total protein concentration in the extracts is shown in the bottom panel. The outmost right lane contains a 41 kDa control protein.

Discussion

Nowadays in brewery fermentations, the initial wort density is raised from 12 °P to 18 °P in a technology referred to as high-gravity brewing. Strains are required that are able to ferment efficiently in the presence of high sugar concentrations and high levels of ethanol and carbon dioxide. A faster or more complete fermenting strain under high-gravity conditions with high crop

viability at the end of fermentation should lead to an economical benefit for the brewer. Previous reports attributed poor fermentations to limitations in the transport of maltotriose [56,61,116,293]. This view is supported in the present work by the data obtained with yeast strains expressing maltase, encoded by the *MAL32* gene, on their cell surface and hence bypassing maltotriose transport across the plasma membrane. First, it was shown that extracellular hydrolysis of maltotriose by maltase increased the ability to grow on maltotriose of a laboratory strain lacking a functional maltose permease (CMY1050). Second, maltotriose consumption increased in the beginning of fermentations in two liter tall tubes, after 39 and 68 hours respectively, for lager yeast strain A15 expressing Mal32 at its cell surface. In this study we thus showed that extracellular hydrolysis of maltotriose is a way to bypass the rate-limiting uptake step of the trisaccharide in order to achieve more complete fermentations.

The *MAL32* gene from lager yeast was selected for expression on the cell surface since its maltase preparation showed the ability to break down maltotriose under fermentation conditions (Table 5). Under more physiological conditions, pH 7 and 30 ° C, the Mal32 preparation preferentially hydrolyzed maltose as also have been reported for the *MAL62* product of *S. carlsbergensis* [68,175]. In contrast, mimicking the conditions present at the end of fermentation, i. e. 10 °C and pH 4, revealed a much lower activity for maltotriose than at pH 7 and 30 °C whereas no activity was detected with maltose as substrate (Table 5). The switch in preference of Mal32 maltase from maltose to maltotriose hydrolysis as the pH is decreased, may be the result of a conformation change in the Mal32 protein as observed for the pH dependent inhibition of α-glucosidase by glucose analogs [287]. Regardless of the mechanism, this property might be an advantage for its application in brewing. Expression of maltase only during late fermentation would be preferred to constitutive expression as maltotriose is still only used at the later stages of fermentation. Consequently, the consistent production of flavour and aroma metabolites, an essential property of industrial brewing yeasts, might remain less or not affected.

The anchoring domains of Cwp2 or Flo1 respectively, were used for immobilizing α-glucosidase encoded by *MAL32* onto the cell surface of yeast and the leader sequence of Kre1 to ensure entry of the fusion protein into the ER lumen (Figure 3). The results in Figure 4 show that under laboratory conditions Mal32 outside the yeast cells was capable of hydrolysis of PNPG substrate by breakage of the α-1, 4-linkage. The absence of an endogenous maltase or α-glucoside permease in the laboratory strains confirmed maltase activity outside the cells. In addition, the absence of activity in the supernatants of the cells excluded that cell lysis or secretion of Mal32 were responsible for the observed activity and confirmed location of maltase at the outside of the yeast cells. We did not check the average expression rate at the single-cell level as has been done by Breinig and Schmitt for the HA epitope using the same system [26]. These authors reported that about 70 % of the yeast cells did show detectable expression of the HA peptide on the cell surface, but the expression was not homogeneously distributed among the cell population [26]. For the much larger Mal32 the number of non-expressing yeast might have been much larger, due for instance to the embedding of Mal32 within the inner cell wall mannoprotein layer. However, calculation of the expected

activity of Mal32 on the outside of the yeast cells under fermentation conditions indicated that the rate of hydrolysis of maltotriose might be in the order of $1.1*10^{-14}$ gram per minute per cell. These calculations were based on the measured activity in the lab-scale experiments, using the specific absorbance of PNPG (ε_{405} = 15 l x $mmol^{-1}$ x cm^{-1}) and the assumption that A_{600} of about 2 equals $1*10^{8}$ cells per ml. For a fermentor with $1.4*10^{11}$ cells per liter as in the 2 liter tall tube experiments (Figure 7b), this would result in a consumption of more than 10 gram maltotriose per liter in five days.

Fermentations performed at 18 °C in two liter "tall tubes" with an initial wort density of 21.8 °P, indeed showed that strains expressing Mal32 on their cell surface, used maltotriose faster than the control strain A15 at early stages of the fermentation, after 39 and 68 hours respectively (Chapter 4, Table 7). In the very beginning of the fermentations, after 15 hours, maltose was consumed faster by the genetically engineered strains. Moreover, the glucose produced from the maltotriose was apparently consumed as fast as it was produced resulting in a faster drop of total sugar. The observed differences are not due to variance in growth or viability and the results thus proved the concept of hydrolyzing the more complex sugars such as maltose and maltotriose outside the yeast cell. The high viability (>95 %) of the cells at the end of all fermentations would allow the use of the cells for repitching. If the viability of the yeast crop is below 90%, the cells can not be used to start a next round of fermentation as this will lead to a "stuck" fermentation and/or too much autolysis resulting in off-flavour compound production.

At the end of the fermentation, on day seven, averages suggest that maltotriose and total sugars are lower for strains GG3209 and GG3210 compared to the control strain but these differences are not statistically significant. This is in agreement with the measured apparent attenuation which was similar for all fermentations (Table 6). The attenuation limit of wort was also similar for all fermentations (Figure 8). However, the improved breakdown of maltose and maltotriose of the A15 strains expressing maltase outside their cells at the early stages of fermentation indicate that the use of an α-glucosidase that is more stable under conditions present at the later stages of fermentation, such as high ethanol concentrations and low pH, certainly holds promise to obtain complete fermentation.

During, and at the end of the brewing process, yeast encounters nutrient limitations, high carbon dioxide and ethanol levels, and low temperatures [2,40]. In this respect, decreased expression of Mal32 may account for its lower activity at the end of fermentation. These results are in line with previously reported changes in expression patterns resulting from the combined stress conditions exerted on the cells late in fermentation [29]. In this study, expression of the *MAL32* gene was under control of the *PGK1* promoter. This promoter has been variously reported to be induced by glucose [183,261], and to be induced under fermentation conditions after glucose depletion [224,230]. Moreover, this promoter has been used for the constitutive expression throughout fermentation of several genes [66,129,137,138,182,249]. It thus appears unlikely that changed transcription of *MAL32* is responsible for the lower maltase activity at the end of fermentation. Instability of the Mal32 protein appears to be more likely. This view is supported by Western blot analysis (Figure 9). The

analyses confirmed formation of the two fusion proteins in strains GG3209 and GG3210, Kre1/ HA/ Mal32/ Cwp2 and Kre1/ HA/ Mal32/ Flo1 respectively. Their higher abundance in crude cell wall extracts indicated that the fusion proteins were located extracellularly. The presence of a band of about 30 kDa at all days of the fermentations suggests degradation of the fusion proteins.

Moreover, at the start of fermentation the pH is just over 5 and decreases to around pH 4 at the end. As discussed before, the drop in pH caused an approximately 3-fold lower activity of a maltase preparation with maltotriose as substrate whereas no maltase activity was observed at pH 4 with maltose as substrate (Chapter 4, Table 5). In this respect, a more active α-glucosidase under conditions with low pH values would break down maltose and maltotriose at the end of the fermentation as well as at the start. The α-glucosidase from the fission yeast, *Schizosaccharomyces pombe*, (SPGase) might be a suitable candidate. SPGase is secreted into the extracellular medium and has a pH optimum of 4.5 [159,179]. Expression of the gene encoding SPGase under control of a promoter that regulates gene expression only during late fermentation, such as the *GAD1* promoter (Dietvorst, unpublished results) might induce maltotriose consumption at the final stages of fermentation. Moreover, a maltase with higher activity towards maltotriose than maltose might help to substantially decrease the amounts of residual maltotriose in beer. To this end, many α-glucosidases with diverse substrate activities have already been identified and amino acid residues involved in substrate specificity of *S. cerevisiae* α-glucosidase have been identified [286]. However, selection of the α-glucosidase most resistant to the stress conditions present in fermentation is the subject of further work.

In conclusion, the results reported indicate improved consumption of maltotriose at early stages of fermentation by yeasts expressing maltase on their cell surface and provides a potential means of conditioning yeast for more efficient fermentations in brewing. However, concerning the brewer's and consumer's resistance against the use of genetically modified organisms (GMO's) in fermentation, it remains uncertain whether this method would be applicable in the brewing industry.

Acknowledgments

We are grateful to Lies Blieck for her extensive help with the fermentation experiments and Prof. J. M. Thevelein for the opportunity to perform these experiments in his laboratory. We thank Raymond Brandt for series of Western blot analysis and Dr. G. P. H. van Heusden for helpful discussions. We thank Prof. C. A. Michels (Queens College, New York) for the supply of strains CMY1050 and CMY1001 and Dr. J. Londesborough for providing us with the A15 strain. We are grateful to Prof. F. Breinig (University of Saarland, Germany) for generously supplying us with plasmids pFB2 and pFB3. This work was financially supported by the European Union (Contract no. QLK1-CT-2001-01066).

Chapter 5

Summary and general discussion

High-gravity brewing (HGB) is a technology widely used in the brewing sector to increase productivity of the brewery and consequently competitiveness on the market [15]. In this technology, wort gravities are increased from 12 degrees Plato (° P), used in traditional brewing, to 18 ° P resulting in beers with higher strength [207]. In the last production stage, these beers are diluted to the desired alcohol content (usually 5 % as obtained in traditional brewing processes from 12 ° P wort) [23]. The use of wort gravities of more than 18 ° P in an even more advanced technology, very-high-gravity brewing, has a negative impact on yeast performance due to the many intensified stress conditions like high osmotic and ethanol concentrations, yeast encounters during such brewing processes. In this respect, loss of yeast viability, changes in flavour profiles and incomplete fermentation have been reported [36,52,191,196]. Poor yeast viability limits the brewer to re-use the yeast for another brew and consistent production of flavour and aroma metabolites is essential to maintain the desired taste and characteristics of a particular beer. In a multi-disciplinary European Union project, the various stress conditions present during high-gravity brewing have been studied (contract no. QLK1-CT-2001-01066). This study was part of the EU project and focused on the incomplete fermentation caused by the different stresses in HGB.

Incomplete fermentation is a common problem in the brewing industry since it results in high yeast fermentable extracts (carbohydrates) in the finished beer [245,247,293]. In most cases the main residual carbohydrate is maltotriose, the second most abundant fermentable sugar present in wort. The presence of residual maltotriose in beer is not due to a genetic or physiological inability of the yeast cells to utilize the sugar [56]. Due to glucose repression, the trisaccharide is only utilized by brewer's yeast after half of the less complex sugars glucose and fructose have been taken up [164]. Moreover, maltotriose transporters are also maltose transporters with higher affinity for maltose, the most abundant fermentable sugar in wort, than for maltotriose. As a consequence maltotriose is only consumed at the later stages of the fermentation when nutritional and environmental stress conditions are most prominent [56,293].

To obtain more insight in the utilization of maltotriose in alcoholic fermentation, we used a selection screen to discriminate between yeast strains that could grow on plates containing maltotriose as the sole carbon source and the respiratory inhibitor antimycin A, and strains that could not grow under these conditions [291] or showed very long lag phases on maltotriose [143]. Lager yeast strain A15 was identified to start growing particularly slow on maltotriose under conditions that prevent respiration such as at the end of large scale brewery fermentations (Chapter 3, Figure 1).

To identify genes that improved maltotriose utilization in this lager strain A15, a genetic approach was used. To this end new genetic tools for investigation of industrial yeast strains were developed (Chapter 2), since available standard genetic methods for laboratory yeast can usually not be applied for brewer's yeast strains which have a much more complex and diverse genetic constitution [125].

Lager yeasts, such as A15, are allopolyploid, containing chromosomes derived from two or more different species [20,95]. Furthermore, much variation of the copynumbers of specific genes in brewer's strains is observed due to genome rearrangements that occurred during adaptation to changing environment or any source of cellular stress [19] (Chapter 1, Figure 1). As a consequence, recessive auxotrophic markers which have been used to select yeast transformants in laboratory strains are not available [219]. The disruption and overexpression libraries developed in the framework of the EU project (Chapter 2, Figure 1) thus contain the dominant marker gene *KanMX* conferring resistance against G418 (a form of gentamycin). The presence of the *KanMX* marker makes these libraries very flexible since they can be used in laboratory strains, brewer's strains and other industrial yeast strains. Moreover, the presence of the genomes of five different yeast strains is an advantage. For other purposes this concept of using a dominant marker with a variety of DNA sources might be useful.

First a multi-copy genomic library was constructed containing chromosomal DNA fragments from one laboratory yeast strain and four lager yeast strains. A Tn5 transposon provided with the *KanMX* marker for selection in yeast as well as a bacterial selection marker and in the case of the overexpression library, with the strong phosphoglycerate kinase (*PGK1*) promoter in addition, was then inserted *in vitro* by a transposition reaction (Chapter 2, Figure 1). The resulting libraries allowed rapid identification of genes whose inactivation or overexpression respectively, enhances resistance against particular conditions i. e. the stress conditions present in HGB. In our study focusing on the maltotriose utilization in alcoholic fermentation, the libraries were used for transformation of A15 and subsequent screening of the transformants on plates containing maltotriose as the sole carbon source and the respiratory inhibitor antimycin A. In this way not only the *MTT1* gene capable of improving the ability of lager strain A15 to grow on maltotriose in the presence of antimycin A (Chapter 3, Figure 2) was identified, but also an additional feature of the disruption library. The Tn5 transposon, resided in the vector part of the genomic library, resulting in an undisrupted yeast DNA insert containing the promoter and open reading frame of gene *MTT1*. In this case the disruption library thus showed to be a tool to identify a non-functional or absent gene. The use of libraries with chromosomal DNA originating from five different strains indeed increased the changes of finding a relevant gene, as illustrated by the identification of *MTT1*. This gene is not present in the *S. cerevisiae* laboratory strain CEN.PK113-7D (Chapter 3, Figure 3).

As demonstrated, especially for the disruption library, the developed libraries provide good tools for the identification of genes in polyploid yeast strains, not only those used in the brewing industry, whose expression is relevant under specified conditions. However, a limitation is that these libraries will fail to identify two or more genes whose activation or inactivation is required at the same time. Altered expression levels of multiple genes, and not a single gene, are probably required for obtaining fully adapted cells to defined conditions as has been described for cold and freeze tolerance in baker's yeast [216]. Several genome-wide expression studies (transcriptome analyses) have proven to be a useful approach in this respect [59,114].

Another limitation is that not all *PGK1*-driven genes will be present in their full-length form on the plasmids of the overexpression library due to too small yeast DNA inserts or inappropriate location of the promoter. Therefore, direct integration of linear DNA fragments digested from the plasmid library with *Not*I into the yeast genome is required to achieve overexpression of large ORF's. However, integration via homologous recombination occurs with a much lower efficiency than transformation of intact plasmid DNA. Further improvement of the efficiency of *in vivo* transposition in yeast, as started in this study, certainly holds promise to overcome the problems concerning homologous recombination. A transposon containing a strong promoter like *PGK1* inserted at random in the yeast genome would lead to a library of overexpressed genes in an industrial strain.

The *MTT1* (*mty1*-like transporter) gene was obtained as a gene that allowed growth of lager strain A15 on maltotriose in the presence of antimycine A. The gene was initially identified to encode a transporter, later confirmed by "uptake studies", as it shared 74 % similarity with *MPH2* and *MPH3* [55], 62 % similarity with *AGT1* [94] and 91 % similarity with *MAL61* and *MAL31* from *S. cerevisiae*. Moreover, the open reading frame shared an even higher similarity (98%) with a gene from *Saccharomyces pastorianus* of which initially only the name (*mty1*) and the sequence were published (M. Salema-Oom, NCBI Accession number AJ491328). Recently also the characteristics of this gene (designated *MTY1*) have become available [223]. In that study, a strain was used lacking the *AGT1* and *MALx1* permease genes (CMY1050). *MTY1* was isolated as a gene from a genomic library of *S. pastorianus* PYCC4457 [86] that could complement the phenotype.

Using repurified [^{14}C]-maltotriose, uptake rates were studied to characterize the transport abilities of Mtt1. We found that for A15 transformants grown on maltose, the presence of *MTT1* raised maltotriose uptake by about 17 % (Chapter 3, Table 3). In addition, maltotriose uptake for A15 cells in mid-log phase containing *MTT1* was 8-fold higher after incubation for 6 hours in 2 % maltotriose with antimycin A than for control cells under the same conditions (Chapter 3, Figure 6). These uptake rates showed improved transport capacity of Mtt1 for maltotriose but are difficult to compare to published data on maltotriose transported by single transporters (Agt1, Mal31, Mal61) in strains lacking other transporters [56,223]. However, a lower affinity for maltose (K_m of 61 to 88 mM) than for maltotriose (K_m of 16 to 27 mM) has been reported for Mty1 [223] but this characteristic is not in agreement with our observation that inhibition of maltose transport by 50 mM maltotriose was low and the same for A15 cells with or without *MTT1* (Chapter 3, Table 3). These K_m values have been determinated by measuring alkalinisation of an aqueous yeast cell suspension upon sugar addition [144]. The same authors reported that Mal31 did not transport maltotriose. In contrast, previous studies using radiolabeled [^{14}C]-maltotriose showed that the maltose permease genes *MAL61* and *MAL31* facilitated high-affinity transport of maltotriose similar to that facilitated by *AGT1*.

A possible explanation for these conflicting data could be that different methods were used to study maltotriose uptake or that the purity of the labeled maltotriose was not checked in the latter study [56]. Contamination with labeled impurities might cause gross overestimation of maltotriose uptake since labeled impurities are not diluted with unlabeled carrier and therefore enter the yeast cells relatively fast. Both lots of commercial [^{14}C]-maltotriose used in our study, were found to be of low radioactive purity (84 % and 94 %). Moreover, we found that apparent maltotriose uptake rates obtained with the commercial material were 2 to 5 fold higher than rates obtained after repurification (Chapter 3). To avoid serious over-estimation of the ability of particular transporters to carry maltotriose, the purity of each batch of commercial [^{14}C]-maltotriose should therefore be checked and, if necessary, repurified.

The four lager yeast strains tested in our study, A15, CMBS-33, OG2252 and WS34/70, all contain at least one copy of the *MTT1* gene but are not characterized as strains with improved capacity to utilize maltotriose in fermentation. These findings are in agreement with those of other authors indicating the presence of *MTT1* (designated *MTY1*) in four out of the five brewer's strains examined [223]. The higher maltotriose transport capacity of the *MTT1* encoded protein in comparison to other maltose transporters and its presence in most brewer's strains suggests that it indeed is important for maltotriose utilization. Hence the PCR used in this study to quickly screen yeast strains for the presence of *MTT1* (Chapter 3, Figure 30) still might be a useful tool for the brewer for strain improvement. It should be noted though, that only the presence of the *MTT1* gene can be verified with this PCR and not the functionality of this gene. Lager strain A15 for example, may harbor a non-functional copy of *MTT1*, since introduction of the *MTT1* gene, from WS34/70 present on a single copy plasmid, in this strain improved growth on maltotriose in the presence of antimycin A (Chapter 3, Figure 5). Whether overexpression of *MTT1* during alcoholic fermentations could reduce the amount of residual maltotriose at the end of fermentation thus remains to be elucidated. However, introduction of the altered version of *MTT1* (*MTT1alt*), which was originally cloned from the mixed yeast library, might improve maltotriose utilization. The presence of this transporter encoded by *MTT1alt* in A15 cells, grown on maltose, more than doubled maltotriose uptake. The effect was even more pronounced after incubation in maltotriose under conditions that prevent respiration (such as present in our initial maltotriose selection screen). After 6 hours incubation, A15 cells carrying *MTT1alt* exhibited 2.9-fold more maltose uptake and even 22-fold more maltotriose uptake than the control cells (Chapter 3, Figure 6). The only difference between *MTT1* and *MTT1alt* is that in the product of *MTT1alt*, 21 amino acids (KEDLETSVVDEGRSTPSVVNK) from the C-terminus of the *MTT1* encoded protein have been replaced by 18 amino acids (GNVVAIAAGGELVGMEDL) encoded by vector sequences. It is not clear how these 21 amino acids influence maltotriose uptake. Further experiments are required to identify functionality of purported binding motifs like RSTPS, resembling the 14-3-3 binding motif RS*Xp*SXP [170] which might affect stability. Alternatively, the 21 amino acids might be involved in substrate affinity of the protein. Further experiments are also required to determine when the *MTT1alt* encoded transporter can help to overcome incomplete fermentation.

The consumption of maltotriose requires two steps prior to being catabolized via the glycolytic pathway, uptake and hydrolysis to glucose. This first step, active maltotriose transport across the plasma membrane appears to be the rate-limiting step for fermentation of maltotriose [290] and could be improved by introduction of an efficient maltotriose transporter, such as *MTT1alt*. Alternatively, we showed that hydrolysis of the trisaccharide maltotriose outside the yeast cell could lead to a more rapid consumption of maltotriose during fermentation (Chapter 4). To this end we focused on the anchoring of α-glucosidase protein encoded by *MAL32* within the cell wall of lager yeast strain A15. In this way maltotriose will be converted extracellularly to maltose and glucose, which are rapidly consumed. The attachment of α-glucosidase to the cell wall of the yeast instead of secreting it, is an important feature for possible applications in brewing. In the final stages of the brewing process, the beer is filtered after maturation [84] and the remaining yeast, including the extracellular enzymes attached to the cell wall, is removed. Secreted α-glucosidase on the other hand, would remain in the final product. For the attachment of Mal32 to the cell wall of A15, the N-terminal secretion signal of Kre1 was used to ensure entry of the fusion protein into the ER lumen and the C-terminal domain of either Cwp2 or Flo1 for covalently anchoring of Mal32 to cell wall β-1,3-D-glucans [26]. In fermentation performed at 18 °C in two liter "tall tubes", strains expressing Mal32 on their cell surface used maltose and to a smaller extent maltotriose, faster than the control strain A15 at early stages of the fermentation (Chapter 4, Table 7). Moreover, the subsequently produced glucose was used as fast as it was made, resulting in a faster drop of total sugar. These results proved the concept of hydrolyzing the more complex sugars such as maltose and maltotriose outside the yeast cell. However, under the conditions tested, with an initial wort density of 21.8 °P and 18 °C, the final remaining total sugar concentration was not lower than the control (Chapter 4, Figure 8). Optimization of the experimental set-up is required to actually obtain a better final attenuation of the wort and thereby leaving less sugars unfermented, especially maltotriose.

The maltase activity on the cell surface of the A15 strains constructed in this study, turned out to be insufficient to lower the sugar concentration at the end of fermentation (Chapter 4, Figure 6). This could be caused by inherent limitations of the cell surface expression system based on the leader sequence of Kre1 and the anchoring domain of Cwp2 or Flo1 respectively, used in the experiments to anchor Mal32 to the cell wall [26]. About 30 % of the yeast cells anchoring the HA peptide to the cell wall using Cwp2 and Flo1, respectively, did not show any detectable expression of the HA peptide on the cell surface [26]. The occurrence of non-expressing yeasts could have been caused by the embedding of Mal32 within the inner cell wall mannoprotein layer. As a consequence the accessibility to its sugar substrates would have been limited. In addition, it has been reported that the length of the fusion protein can decrease its accessibility on the yeast cell wall [266] and that the amount of detectable cell wall fusion proteins varies greatly within growing yeast cultures [229]. However, our experiments on lab-scale indicated that the activity of Mal32 should be sufficient for a measurable increase in the hydrolysis of maltotriose during the 2 liter tall tube experiments.

It thus seems likely that other factors, like transcription of the gene or stability of the enzyme play a role. However, the use of the *PGK1* promoter makes it unlikely that reduced transcription is the main cause for lower expression of Mal32.
Changes in expression patterns have been noted previously and may be a result of the combined stress conditions exerted on the cells late in fermentation [29]. During, and at the end of the brewing process, yeast encounters nutrient limitations, high carbon dioxide and ethanol levels, and low temperatures [2,40]. In this respect, changes in stability of Mal32 may account for its lower activity at the end of fermentation. The higher ethanol and carbon dioxide levels and the lower pH probably have a negative effect on the stability of Mal32 maltase. In addition, at the start of fermentation the pH is just over 5 and decreases to around pH 4 at the end of fermentation. The drop in pH may explain why faster sugar consumption was only observed at the start of the fermentation (Chapter 4, Table 7). With maltotriose as substrate the activity of a maltase preparation was approximately 3 fold lower at pH 4 than at pH 5, both determined at 10 °C respectively. Maltase activity was not observed at pH 4 and 10 °C with maltose as substrate (Chapter 4, Table 5).

Finally, the use of a different α-glucosidase, i. e. a maltase with higher activity under brewing conditions towards maltotriose, certainly holds promise to obtain complete fermentation. An α-glucosidase from the fission yeast, *Schizosaccharomyces pombe*, (SPGase) might be a suitable candidate. SPGase is secreted into the extracellular medium and has a pH optimum of 4.5 [159,179]. However, searching for an appropriate α-glucosidase from brewer's yeast might be a better approach. Many α-glucosidases with diverse substrate activities have already been identified and amino acid residues involved in substrate specificity of *S. cerevisiae* α-glucosidase have been identified [286]. Using a maltase from brewer's yeast will allow self-cloning, as attempted in this thesis by using α-glucosidase encoded by the *MAL32* gene from *S. cerevisiae*. Self-cloning might help to reduce the public concern about the safety of genetic engineering, as reviewed by Akada [3], and thereby enhancing the chances of future application of genetically modified strains in brewing.
In conclusion, the research described in this thesis has provided novel tools for genetic investigation of brewer's yeast strains and information regarding maltotriose utilization in alcoholic fermentation. A gene, *MTT1*, belonging to the family of α-glucoside transporters was isolated and characterized. The introduction of the altered version of this gene, *MTT1alt*, as well as expression of extracellular α-glucosidase might improve the fermentation of maltotriose in brewing to overcome incomplete fermentation.

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Nederlandse Samenvatting

Traditioneel wordt bier gebrouwen van wort met een concentratie van 12 graden Plato (°P). Een 12 °P wort bevat circa 120 gram suiker per liter en resulteert in bier met een alcoholpercentage van 5 %. Een nieuwe ontwikkeling in de brouwerssector is het gebruik van hogere wortconcentraties, zoals 18 °P, in een proces dat 'high-gravity brewing' (HGB) wordt genoemd. HGB heeft als voordeel een verhoging van de produktiviteit van de brouwerij aangezien in het laatste stadium van het HGB proces het bier verdund wordt met water om bier te verkrijgen met het gewenste alcoholpercentage. Een nadeel is echter dat in HGB en in sterkere mate in 'very-high-gravity-brewing' (VHGB), een proces waarbij nog hogere wortconcentraties worden gebruikt, de capaciteit van gist om de suikers om te zetten in alcohol en kooldioxide afneemt. Tijdens (V)HGB wordt de gist namelijk blootgesteld aan condities zoals hoge suiker- en ethanolconcentraties die 'stress' veroorzaken voor de gist. Het verlies aan 'gistviabiliteit', een afwijkend smaakprofiel en een verminderde fermentatiesnelheid kunnen de gevolgen zijn van de aanwezige condities. Door de minder levenskrachtige gist worden de mogelijkheden voor de brouwer om de gist opnieuw te gebruiken voor een volgende fermentatie beperkt. Daarnaast is een constante productie van aromacomponenten vereist om de gewenste smaak en karakteristieke eigenschappen van een bepaald bier te behouden.
De verschillende stress-situaties die zich voordoen tijdens (V)HGB processen zijn bestudeerd in een multi-diciplinair project van de Europese Unie. Het voornaamste doel van dit project was de ontwikkeling van giststammen met een verbeterde resistentie tegen de extreme condities die optreden tijdens deze alcoholische fermentaties. De studie beschreven in dit proefschrift was een onderdeel van dit EU project en was gericht op de niet complete fermentatie tijdens (V)HGB processen.

Niet complete fermentatie is een bekend probleem in de brouwerij en resulteert in de aanwezigheid van hoge concentraties fermenteerbare suikers in het bier. Het grootste gedeelte van deze achtergebleven suikers bestaat meestal uit de trisaccharide maltotriose. De aanwezigheid van een resthoeveelheid maltotriose in het bier is geen gevolg van gebrek aan capaciteit van *Saccharomyces cerevisiae* om de trisaccharide af te breken. Maltotriose is na maltose de meest voorkomende fermenteerbare suiker in wort, maar de consumptie van maltotriose vindt alleen plaats in de latere fasen van de fermentatie. In de aanwezigheid van glucose en in mindere mate fructose wordt de consumptie van minder gemakkelijk fermenteerbare suikers geremd. Door dit effect, glucoserepressie genoemd, neemt de gist maltose en maltotriose pas op nadat de helft van de sacchariden fructose en glucose zijn verdwenen. Na transport van maltose en maltotriose de cel in worden de suikers intracellulair afgebroken tot glucose. Maltose en maltotriose delen dezelfde opnamesystemen, maar de affiniteit van de transporters is groter voor maltose dan voor maltotriose.

Voor de identificatie van genen in industriële giststammen betrokken bij maltotriose consumptie tijdens de alcoholische fermentatie, is gebruikt gemaakt van een genetische aanpak. Hiervoor was de ontwikkeling van nieuwe genetische gereedschappen vereist aangezien beschikbare genetische technieken voor *S. cerevisiae* laboratoriumstammen doorgaans niet gebruikt kunnen worden voor industriële giststammen. Industriële stammen zijn vaak polyploid met een veel complexere genetische samenstelling dan haploide en diploide laboratoriumstammen. Zo bevatten brouwersstammen gebruikt voor het maken van lagerbier veelal chromosomen van twee of meer verschillende giststammen. Daarnaast is het aantal aanwezige examplaren van een specifiek gen in deze brouwersstammen vaak variabel door opgetreden genoomreorganisaties ten gevolge van aanpassing aan de verschillende condities aanwezig tijdens het brouwproces. Het gebruik van standaard genetische technieken is hierdoor veelal niet mogelijk in brouwersstammen. Als alternatief kunnen dominante selectiemarkers gebruikt worden voor de transformatie van industriële gisten. De overexpressie- en disruptiebibliotheken ontwikkeld in deze studie zijn beschreven in hoofdstuk 2. De bibliotheken bevatten het dominante markergen *KanMX* dat resistentie geeft tegen het antibioticum G418 en zijn daardoor geschikt voor gebruik in brouwersstammen.
Voor de ontwikkeling van de overexpressie- en disruptiebibliotheken, die het mogelijk maken om genen te identificeren welke door overexpressie of inaktivatie kunnen leiden tot een verhoogde weerstand tegen de condities aanwezig bij HGB, werd eerst een bibliotheek gemaakt van het genomisch DNA van vijf giststammen. Deze bibliotheek bevat een verzameling van willekeurige chromosomale DNA fragmenten afkomstig van een *S. cerevisiae* laboratoriumstam en vier brouwersstammen gekloneerd in een 'multi-copy' vektor. Een Tn5 transposon met de *KanMX* marker voor selectie in *S. cerevisiae* en een bacteriële selectiemarker werd vervolgens ingevoegd door middel van een *in vitro* transpositie reactie voor de constructie van de disruptiebibliotheek. Voor de overexpressiebibliotheek werd eenzelfde transposon met daarop ook de fosfoglyceraatkinase (*PGK1*) promoter ingevoegd. Met behulp van restrictieanalyse en de kolonie-blottechniek werd bepaald dat de verschillende genen goed vertegenwoordigd zijn in de bibliotheken.

Om meer inzicht te verkrijgen in het gebruik van maltotriose in de alcoholische fermentatie hebben we de groei van verschillende giststammen op maltotriose met antimycine A bestudeerd. Antimycine A is een ademhalingsremmer die de groei van verschillende *S. cerevisiae* laboratorium- en lagerstammen op maltotriose vertraagt. De lagerstam A15 werd hierbij geïdentificeerd als een giststam die zeer langzaam groeide op maltotriose onder condities waarbij de ademhaling geremd is, zoals aan het eind van een brouwproces. Voor verdere studie naar genen betrokken bij het maltotriosemetabolisme werden de bibliotheken vervolgens gebruikt voor de transformatie van A15. Isolatie van de transformanten die in staat waren om beter te groeien op maltotriose platen met antimycine A heeft geleid tot de isolatie van een plasmide met daarop het *MTT1*-gen zoals beschreven in hoofdstuk 3.

Het bijbehorende eiwit werd geïdentificeerd als een α-glucoside transporter aangezien DNA sequentieanalyse liet zien dat de basenvolgorde van het *MTT1*-gen grote overeenkomsten heeft met reeds gekarakteriseerde maltose-transportergenen in *S. cerevisiae*. Zo is de DNA sequentie van het *MTT1*-gen voor 74 % gelijk aan de sequenties van de genen *MPH2* en *MPH3*, voor 62 % gelijk aan het *AGT1*-gen en voor 91 % gelijk aan de genen *MAL61* en *MAL31*. Bovendien komt de sequentie van *MTT1* voor 98 % overeen met het *mty1*-gen van *Saccharomyces pastorianus*. Onlangs is de karakterisering van dit *mty1*-gen (nu *MTY1* genoemd) door de ontdekkers gepubliceerd. Uit vergelijking met het door ons geïsoleerde *MTT1*-gen blijkt dat het 'open reading frame', dat oorspronkelijk werd geïsoleerd uit de bibliotheek, 66 basen aan het 3'-einde van het *MTT1*-gen mist maar in plaats daarvan 54 basen van de vector bevat. Dit veranderde 'open reading frame' hebben wij *MTT1alt* genoemd.

Met behulp van opname-experimenten met gezuiverd [^{14}C]-maltotriose is bevestigd dat het *MTT1*-gen codeert voor een maltose/maltotriose transporter. Voor A15 transformanten gekweekt op maltose nam de opname van maltotriose toe met 17 % wanneer *MTT1* aanwezig was. Voor A15 cellen met *MTT1* in de exponentiële groeifase was de maltotriose opname zelfs 8 keer hoger na incubatie gedurende 6 uur in 2 % maltotriose met antimycine A dan voor de controlestammen. Introductie van *MTT1alt* in A15 verhoogde de maltotriose opname onder dezelfde condities zelfs 22 keer ten opzichte van de controlecellen.
De vier brouwersstammen A15, CMBS-33, OG2252 en WS34/70 die zijn gebruikt in dit promotieonderzoek bevatten allen tenminste één kopie van het *MTT1*-gen maar zijn niet gekarakteriseerd als stammen met verbeterde capaciteit om maltotriose te fermenteren. De hogere maltotriose transportcapaciteit van het eiwit gecodeerd door *MTT1* in vergelijking tot andere maltosetransporters en de aanwezigheid van *MTT1* in de geteste brouwersstammen wekt de suggestie dat de transporter toch belangrijk is voor maltotrioseconsumptie in alcoholische fermentatie. Hierdoor zou de polymerase ketting reactie (PCR), gebruikt in dit onderzoek voor het relatief snel testen van giststammen op de aanwezigheid van *MTT1,* een bruikbaar gereedschap kunnen zijn voor de brouwer ter verbetering van giststammen. Hierbij moet echter wel opgemerkt worden dat enkel de aanwezigheid van *MTT1* aangetoond kan worden met deze PCR en niet de functionaliteit van het gen. Zo lijkt het zeer waarschijnlijk dat lagerstam A15 een niet-functionele kopie bevat van het *MTT1*-gen. Introductie van het *MTT1*-gen van lagerstam WS34/70 op een 'single copy' plasmide verbeterde namelijk aanzienlijk de groei van deze stam op maltotriose met antimycine A.
Voor de consumptie van maltotriose zijn twee stappen nodig voordat de suiker afgebroken wordt in de glycolyse. De eerste stap is de aktieve opname van maltotriose. Dit transport over het plasmamembraan blijkt ook de snelheidsbeperkende stap te zijn van de fermentatie van maltotriose.

De tweede stap is de afbraak van maltotriose in de cel. Het enzym α-glucosidase katalyseert deze omzetting van maltotriose in drie glucose moleculen. In hoofdstuk 3 is aangetoond dat de introductie van een efficiënte maltotriosetransporter zoals Mtt1alt de opname van maltotriose zou kunnen verbeteren. Daarnaast is in hoofdstuk 4 nagegaan of de afbraak van maltotriose buiten de gistcel zou kunnen leiden tot een snellere consumptie van maltotriose tijdens de alcoholische fermentatie. Hiervoor hebben we het eiwit α-glucosidase gecodeerd door het *MAL32*-gen geankerd aan de celwand van de lagergist A15. De extracellulaire omzetting van maltotriose door het eiwit α-glucosidase resulteert in de suikers glucose en maltose die sneller geconsumeerd worden door *S. cerevisiae*. Aan het einde van het brouwproces wordt het bier gefiltreerd om de achtergebleven gist te verwijderen. Ook de eiwitten aanwezig op de celwand van de gist worden hierbij gescheiden van het bier. Gericht op de mogelijkheid om deze methode te gebruiken in het brouwproces, heeft de bevestiging van het enzym α-glucosidase aan de celwand hierdoor de voorkeur boven secretie van het eiwit in het medium. Voor de bevestiging van het Mal32-eiwit aan de celwand van A15 is het gefuseerd met het N-terminale secretiesignaal van het Kre1-eiwit. Dit secretiesignaal zorgt voor transport van het fusie-eiwit naar het endoplasmatisch reticulum. Verder is het fusie-eiwit gekoppeld aan het C-terminale domein van het Cwp2-eiwit of dat van het Flo1-eiwit. Deze domeinen zorgen voor verankering van het Mal32-eiwit aan de celwand.

Het fermentatieprofiel en de suikerconsumptie van de A15 stammen met en zonder het Mal32-eiwit op hun celoppervlak is vervolgens bestudeerd onder VHG-omstandigheden. De fermentaties werden uitgevoerd met een wortconcentratie van 21,8 °P in twee-liter buisfermentoren bij een temperatuur van 18 °C. In het begin van deze fermentaties (na 15 en 39 uur) verbruikte de A15 stammen met het Mal32-eiwit aan de celwand voornamelijk de suikers maltose en ook maltotriose sneller dan de controlestam A15. Hierbij werd het gevormde glucose direkt weer opgenomen door de gist. Dit resulteerde in een snellere afname van de totale hoeveelheid suiker. Met deze resultaten is dus aangetoond dat de afbraak van maltotriose buiten de gistcel zou kunnen leiden tot een snellere consumptie van maltotriose tijdens de fermentatie. Aan de andere kant is optimalisatie van de experimentele opzet nodig om ook aan het einde van de fermentatie voldoende activiteit van het Mal32-eiwit te verkrijgen. De hoeveelheid ongefermenteerde suikers die aanwezig was aan het eind van de fermentaties was namelijk gelijk voor de fermentaties met de A15 stammen met het Mal32-eiwit op het celoppervlak enerzijds als met de A15 controle stammen anderzijds.
De verminderde activiteit van het Mal32-eiwit zou veroorzaakt kunnen zijn door de veranderde omstandigheden tijdens de latere fasen van de fermentatie. Tijdens en aan het eind van de fermentatie wordt gist namelijk blootgesteld aan lage temperaturen, hoge kooldioxideconcentraties en een hoog ethanolgehalte. Deze condities zouden mogelijk een effect kunnen hebben op de de stabiliteit van het Mal32-eiwit. Gedurende een alcoholische fermentatie zakt de pH-waarde van 5 naar ongeveer 4. Deze daling van de pH-waarde zou ook een verklaring kunnen zijn voor het ontbreken van een snellere suikerconsumptie aan het eind van de fermentaties. Deze hypothese wordt ondersteund door de activiteitsbepalingen uitgevoerd op laboratoriumschaal met celextracten van het Mal32-eiwit.

De specifieke aktiviteit van het Mal32-eiwit voor zowel maltose als maltotriose bij pH 4 was lager dan die bij pH 5. Het gebruik van een α-glucosidase met een hogere affiniteit voor maltotriose onder brouwomstandigheden zou kunnen leiden tot een snellere en meer complete alcoholische fermentatie. De α-glucosidase van de gist *Schizosaccharomyces pombe* (SPGase) met een pH-optimum van 4,5 is mogelijk een geschikte kandidaat. Aan de andere kant is het wellicht beter om een α-glucosidase van brouwersgist te gebruiken. Hierdoor wordt het inbrengen van vreemd DNA in het organisme vermeden. Door het gebruik van deze methode, 'self-cloning' genoemd, zou de weerstand van de brouwerssector tegen genetische modificatie kunnen afnemen.

De resultaten van het onderzoek beschreven in dit proefschrift hebben nieuwe genetische gereedschappen opgeleverd voor het gebruik in industriële giststammen. Daarnaast heeft het onderzoek geleid tot nieuwe informatie over de consumptie van maltotriose tijdens de alcoholische fermentatie. Het *MTT1*-gen dat behoort tot de familie van α-glucoside transporters werd geïsoleerd en gekarakteriseerd. De introductie van de veranderde versie van dit gen, *MTT1alt* en de extracellulaire expressie van α-glucosidase zouden in de toekomst kunnen leiden tot verbetering van de fermentatie van maltotriose in brouwprocessen.

List of abbreviations

A_{600}	absorbance at 600 nm
AATase	alcohol acetyl transferase
AFLP	amplified fragment length polymorphism
ATP	adenosine triphosphate
bp	base pairs
cAMP	cyclic 3'5'adenosine monophosphate
CCGH	competitive comparative genome hybridization
DNA	deoxyribonucleic acid
dsRNA	double-stranded RNA
EDTA	ethylenediamino-tetraacetic acid
e.g.	for example
EMS	ethanemethyl sulphonate
G418	Geneticin
GFP	green fluorescent protein
GRAS	generally regarded as safe
HGB	high-gravity brewing
HOG	high osmolarity glycerol
HPLC	high performance liquid chromatography
Hsps	heat shock proteins
i.e.	that is
IR	illegitimate recombination
IPTG	isopropyl-beta-D-thiogalactopyranoside
kb	kilo bases
LB	Luria Bertani medium
LEU	leucine
Mb	mega bases
mRNA	messenger RNA
miRNAs	microRNAs
miRNP	miRNA containing ribonucleoprotein
mRNA	messenger ribonucleic acid
NAD^+	nicotinamide-adenine dinucleotide (oxidized)
NADH	nicotinamide-adenine dinucleotide (reduced)
NCBI	National Center for Biotechnology Information
NLS	nuclear localization sequence
nm	nano meter
O.D.	optical density
OE	outside end

ORF	open reading frame
°P	degrees Plato is a measure of the concentration of sugar in a brewer's extract of malt, such that an extract of x °P has the same specific gravity as a solution of sucrose in water containing x g of sucrose in 100 g of solution at 20 °C
PCR	polymerase chain reaction
PEG	polyethylene glycol
PFGE	pulsed field gel electrophoresis
Pi	free inorganic phosphate
PNPG	4-nitrophenyl-α-D-glucopyranoside
Ppgk1	promoter of the phosphoglycerate kinase gene
PVDF	polyvinylidene difluoride
RADP	random amplified polymorphic DNA
rasiRNAs	repeat-associated short interfering RNAs
RdRP	RNA-dependent-RNA polymerase
RI	refractive index
RISC	RNA-induced silencing complex
RNA	ribonucleic acid
RNAi	RNA interference
rpm	rotations per minute
SDS	sodium dodecyl sulfate
SR	stress response
SSC	sodium chloride-sodium citrate
STRE	stress response element
siRNAs	short-interfering RNAs
TAE	tris-acetate-EDTA
TBS	tris buffered saline
TBST	TBS with tween 20
T-DNA	transferred DNA
Tnp	transposase
UDP	uridine diphosphate
UTR	untranslated region
UV	ultraviolet
VHGB	very-high-gravity brewing
WS34/70	Weihenstephan 34/70
YP	yeast extract peptone
YPD	yeast extract peptone dextrose (glucose)

Curriculum Vitae

Judith Dietvorst, was born on the 26th of April 1977 in Eindhoven, the Netherlands. In 1996 she obtained her VWO certificate (university preparatory secondary education) at the Eckart College in Eindhoven, the Netherlands, and in 2001 her Bachelor degree in Biochemistry at the Hogeschool van Arnhem en Nijmegen in Nijmegen, the Netherlands, after an internship with Prof. dr. L. J. Kenney at the Oregon Health Science University (OHSU) in Portland, Oregon, USA on the analysis of genes regulated by OmpR in *Salmonella typhimurium*. In September 2001 she started as a graduate student at the Institute of Biology Leiden (IBL) of Leiden University, where she worked on the research described in this thesis under the supervision of Dr. ir. H. Y. Steensma in the Molecular & Developmental Genetics section of Prof. dr. P. J. J. Hooykaas. From January 2006, she is working as a post-doc in the same section at the Institute of Biology Leiden (IBL).