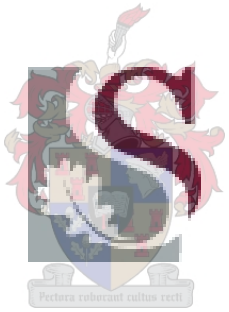
Maltotriose Transport in Yeast

by

Annél Smit



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DECLARATION

I, the undersigned, hereby declare that the work contained in this dissertation is my own original work and that I have not previously in its entirety or in part submitted it at any university for a degree.

Annél Smit **Date**

SUMMARY

The conversion of sugar into ethanol and carbon dioxide is a process that has been intertwined with human culture and long as civilized man has existed. This fermentation process has been dominated by the micro-organism *Saccharomyces cerevisiae* and from providing ancient seafaring explorers of a non perishable beverage to equipping bakers with a raising agent to turn flour into bread; this organism with its fermentative potential, has formed an essential part of most societies.

In more recent times, many industries still rely on this basic principle. The complexities and efficiencies of the conversion of sugar into its various fermentative by-products have been studied and optimised extensively to meet the specific demands of industries. Depending on the raw material used as starting point, the major beneficiaries of the useful characteristics have been alcoholic beverage producers (wine, beer, and whiskey amongst others), bakers (bread leavening) and biofuel producers.

One of the obstacles in fermentation optimisation is the sugar consumption preferences displayed by the organism used. *S. cerevisiae* can consume a wide variety of sugars. Depending on the complexities of its structures, it shows a preference for the simpler saccharides. The fermentation of certain more complex sugars is delayed and runs the risk of being left residually after fermentation. Many of the crops utilised in fermentation-based products contain large amounts of starch. During the starch degradation process many different forms of sugars are made available for fermentation. Improved fermentation of starch and its dextrin products would benefit the brewing, whiskey, and biofuel industries. Most strains of *Saccharomyces* ferment glucose and maltose, and partially ferment maltotriose, but are unable to utilise the larger dextrin products of starch. This utilisation pattern is partly attributed to the ability of yeast cells to transport the aforementioned mono-, di- and trisaccharides into the cytosol. The inefficiency of maltotriose transport has been identified as the main cause for residual maltotriose. The maltotriose transporting efficiency also varies between different *Saccharomyces* strains.

By advancing the understanding of maltotriose transport in yeast, efforts can be made to minimise incomplete fermentation. This aim can be reached by investigating the existing transporters in the yeast cell membrane that show affinity for maltotriose. This study focuses on optimising maltotriose transport through the comparison of the alpha glucoside transporter obtained from different strains of *Saccharomyces*. Through specific genetic manipulations the areas important for maltotriose transport could be identified and characterised.

This study offers prospects for the development of yeast strains with improved maltose and maltotriose uptake capabilities that, in turn, could increase the overall fermentation efficiencies in the beer, whiskey, and biofuel industries.

OPSOMMING

Die transformasie van suiker na etanol en koolstof dioksied is so oud soos die beskawing self, en dit is van die vroegste tye af onlosmaaklik met die mens se kultuur verbind. Hierdie fermentasie-proses word gedomineer deur die *Saccharomyces cerevisiae* mikro-organisme. Hierdie organisme het antieke seevaarders voorsien van ‘n nie-bederfbare drankie en van ouds af aan bakkers ‘n rysmiddel verskaf waarmee meel in brood verander kon word. As gevolg van hierdie fermenteringspotensiaal het hierdie organisme ‘n onmisbare rol in meeste beskawings gespeel.

Baie industrieë is steeds op hierdie basiese beginsel gebou. Die kompleksiteite en effektiwiteit van die transformasie van suiker na sy verskeie gefermeenteerde newe-produkte is breedvoerig bestudeer en geoptimiseer om aan die spesifieke behoeftes van verskeie industrieë te voeldoen. Afhangend van die grondstowwe wat as beginpunt gebruik is, is die primêre begunstigdes van die fermentasie proses die alkoholiese drankprodusente (onder andere die wyn-, bier- en whiskey produsente), bakkers en biobrandstofprodusente.

Die suikerverbruik-voorkeur van die organisme wat die fermentering fasiliteer is een van die struikelblokke in die optimisering van die proses. *S. cerevisiae* kan ‘n wye spektrum van suikers verbruik maar dit toon ‘n voorkeur vir die eenvoudiger suikers. Die fermentasie van sekere van die meer komplekse suikers is vertraag en loop die risiko om agtergelaat te word na fermentasie. Vele van die gewasse wat in die gefermenteerde produkte gebruik word bevat groot hoeveelhede stysel. Vele soorte suikers word gedurende die afbreek van die stysel beskikbaar gestel vir fermentasie. Die brouers-, whiskey- en biobrandstof industrieë sal almal voordeel trek uit die verbeterde fermentasie van stysel en sy gepaardgaande dekstrin produkte. Meeste *Saccharomyces* gisrasse fermenteer glucose en maltose; maltotriose word gedeeltelik gefermenteer, maar die meer komplekse dekstrien produkte gevind in stysel word nie gefermenteer nie. Hierdie verbruikerspatroon kan gedeeltelik toegeskryf word aan die vermoë van gisselle om die bogenoemde mono-, di- and trisaccharides in die sitosol op te neem. Die oneffektiwiteit van maltotriose transport is identifiseer as die hoofoorsaak van post-fermentatiewe, oortollige maltotriose. Die effektiwiteit van maltotriose transport verskil ook tussen verskillende *Saccharomyces* rasse.

Pogings om onvolledige fermentasie te veminder kan bevorder word deur die kennis rondom maltotriose transport in gis uit te bou. Hierdie oogmerk kan bereik word deur die bestaande transporters in die gissel se membraan wat ‘n affiniteit vir maltotriose toon te ondersoek. Hierdie studie fokus op die optimisering van maltotriose transport deur die vergelyking van die alpha glucoside transporter (*AGT1*) wat van verskillende *Saccharomyces* rasse afkomstig is. Die areas wat relevant is tot maltotriose transport kon deur spesifieke genetiese manipulasies identifiseer en gekarakteriseer word.

Hierdie studie bevorder die vooruitsig op die ontwikkeling van gisrasse met verbeterde maltose en maltotriose transport vermoëns wat op sy beurt weer kan aanleiding gee tot die verbeterde fermentasie effektiwiteit in die bier, whiskey en biobrandstof industrieë.

BIOGRAPHICAL SKETCH

Annél Smit was born on 17 November 1976 in Paarl, South Africa. She grew up in the Eastern Cape and matriculated in 1994 in Cape Town. She started her studies at Stellenbosch University in Parks and Recreational management and after changing her directions of study, finished her BSc Microbiology and Biochemistry in 1998. She obtained her BSc honors in Wine Biotechnology in 1999 and completed her Masters in Wine Biotechnology in April 2002 with a thesis title of “Engineering yeast for the production of optimal levels of volatile phenols in wine” all at the Stellenbosch University. She stayed at the Institute for Wine Biotechnology to continue her studies towards a PhD. She is married to André Remeires Smit.

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PREFACE

This dissertation is presented as a compilation of 6 chapters. Each chapter is introduced separately and is written according to the style of the Journal Annals of Microbiology to which Chapter 3 was submitted and accepted for publication except Chapter 4 and Chapter 5 which is written to the style of Journal of Industrial Microbiology and Biotechnology and the Journal of Microbiology respectively.

Chapter 1 **General Introduction and Project Aims**

Chapter 2 **Literature Review**
The structure and importance of α-glucoside transport in yeast

Chapter 3 **Research Results**
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Chapter 4 **Research Results**
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Chapter 1

INTRODUCTION AND PROJECT AIMS

INTRODUCTION AND PROJECT AIMS

1. INTRODUCTION

Many industries rely heavily on the unique ability of yeast cells to consume carbohydrates, and produce alcohol and carbon dioxide. The ability of yeast to consume most of the sugars present in the different substrate sources economises production processes. Various industries utilises yeast fermentation processes, including most alcoholic beverage industries, the baking industry and the biofuel industry. Productivity and cost efficiency can be greatly enhanced through reducing the time required to complete a fermentation cycle. Lower operating costs, greater flexibility, and reduction of the total fermenter volume required to reach production volume targets are achieved when fermentation rates are optimised to operate as quickly as possible (Verstrepen *et al.,* 2006). The other significant requirement is that yeast cells convert as much as possible of the raw material into a particular end product. It is therefore not surprising that the primary selection criteria applied to most strain development programmes relate to the overall objective of improving the fermentation-performance of yeast strains. For the producers of alcoholic beverages specifically, slow and incomplete yeast fermentations represent a considerable economic loss.

Three factors play a role in the rate and extent of sugar utilisation in yeast: the ability of yeast to transport sugars into the cell, the rate of the subsequent metabolism, and environmental factors present in the yeast's surroundings. For starch fermentation, *Saccharomyces* yeasts ferment glucose and maltose completely, leaving only the larger dextrins unfermented. Maltotriose is a trisaccharide that is fermented incompletely. An improvement in the ability of *Saccharomyces* to utilise and ferment maltotriose might result in more efficient fermentation processes, which would be specifically relevant for the beer and whisky industries where excess maltotriose is associated with off-flavours (Zheng *et al*., 1994).

The yeast cell is protected from the surrounding environment by a cell membrane, which provides selective permeability. Membrane proteins present in the cell membrane facilitate the influx of nutrient molecules into the cell, and many of these systems have been characterised for *S. cerevisiae*. These include transporters for carbohydrates, amino acids and phospho-organic compounds. The primary nutrient requirement in the yeast cell is a carbohydrate source for the production of energy. The sugar transporters in yeast all belong to the major facilitator superfamily (Saier, 2000). These include the glucose transporters (*HXT1-7*), the galactose transporters (*GAL2*), and the maltose transporters (*MALx1*) (Guldener *et al*., 2005). Most yeast sugar transporters have been characterised as 12-transmembrane domain proteins, and some essential residues have been identified for the individual affinities of these transporters for their specific substrates. However, no

three-dimensional model for any of these transporters has yet been characterised. The closest model for a sugar transporter was developed by the Fischbarg group of Columbia University, New York for Glut1p (human glucose transporter), comprising α-helical transmembrane segments (Salas-Burgos *et al*., 2004). This model was developed and optimised over several years and was based on the electronic density map for the oxalate transporter OxlT, glycerol 3-phosphate antiporter GlpT, and the lactose permease proton symporter LacY.

The rate-limiting factor in maltotriose fermentation is the active uptake of maltotriose by yeast cells (Zastrow *et al.,* 2001). The first approach to address this problem is to investigate the transporters in *S. cerevisiae* that are capable of transporting maltotriose. The best-known transporters are the α-glucoside transport family, expressed by the *MALx1* genes. These genes all form one of the five unlinked telomere-associated *MAL* loci. *S. cerevisiae* contains five *MAL* loci which, in turn, each contain a maltose permease (*MALx1*), a maltase (*MALx2*), and a Mal-activator (*MALx3*) gene (Needleman, 1991). The five *MAL* loci are highly homologous and the yeast cell needs at least one *MAL* loci present in its genome to utilise maltose (Charron *et al*., 1989). Mal31p is a *S. cerevisiae* maltose transporter showing affinity for maltose and turanose (Han *et al*., 1995). Speculation still exists on whether these maltose transporters show affinity for maltotriose, and many conflicting results have been obtained. However, transporters with clear affinity for maltotriose have been characterised. One such transporter was identified as the *AGT1*-encoded α-glucoside transporter I (Agt1p) for *S. cerevisiae* (Han *et al.,* 1995). *AGT1* is a mutant allele of *MAL11,* which codes for a maltose permease. *MAL11* forms part of the *MAL1* locus, which consists of *MAL11* (maltose permease), *MAL12* (maltase) and *MAL13* (activator). *MAL11* is situated in the telomeric region of chromosome VII (Needleman, 1991). Agt1p is part of the 12-transmembrane symporter family (Han *et al.,* 1995). This symporter exhibits an active transport process that requires a proton gradient across the yeast membrane. It was shown that Agt1p has a K_m value of 4±0.7 mM for maltotriose (Day *et al.,* 2002b). More recently, *MPH2* (YDL247w) and *MPH3* (YJR160c) were detected in a few industrial strains (Day *et al.,* 2002a). *MPH2* and *MPH3* are 100% identical, 75% identical to *MAL31* and *MAL61* and 55% identical to *AGT1.* The most recently identified transporter for maltotriose is the *MTT1*-encoding transporter, which is present in lager beer strains, showing 74%, 62% and 91% similarity to *MPH2*&*3, AGT1* and *MAL61*, respectively, and showing a higher affinity for maltotriose than for maltose (Salema-Oom *et al.,* 2005).

Except for the functional characteristics and regulatory influences of these transporters, not much has been characterised on the amino acid and structural level. However, in all cases glucose plays a regulatory role in repressing the expression of the maltose transporter genes, and also seems to inactivate these transporters. Most efforts have been directed at unravelling the importance of some regions of Malx1p. The secondary structure of Mal61p was characterised as two blocks of 6-transmembrane domains each, separated by a 71-residue intracellular region (Cheng and Michels, 1989).

A putative PEST sequence was identified and characterised between residues 49 and 78 in the N-terminal of Mal61p (Cheng and Michels, 1989). PEST sequences are rich in proline, aspartate, glutamate, serine and threonine. Proteins are marked for degradation through a regulated phosporylation of the PEST sequence (Rechsteiner 1988; Marchal *et al*., 1998). Brondijk *et al*. (1998) investigated the effect of Mal61p amino acid sequence modifications on inactivation through proteolysis. In order to study this, the putative protein kinase A and C phosphorylation sites were removed. Mal61p mutants (S295A, T363A, and S487A) were constructed and significantly reduced rates of glucose inactivation were observed. The inactivation rate for T363A correlated with the protein degradation rate. The reduction in protein degradation rates was much higher than the loss of activity for the S295A and S487A mutants. They concluded that some form of protein modification takes place prior to the degradation of Mal61p. Inactivation of Mal61p already takes place with this modification and proteolytic breakdown does not necessarily follow inactivation. Medintz *et al*. (2000) characterised an N-terminal PEST sequence between residues 49 and 78 of Mal61p that includes a di-leucine motif at residues 69-70 of the cytoplasmic region. They also showed that a 36-amino acid truncation at residue 581 creates a non-fermentable phenotype. Mal61p inactivation is a two-fold process. Firstly, the enzyme/transporter is inactivated due to phosphorylation, followed by degradation (Stanbrough and Magasanik, 1995; Hein *et al*., 1995). Gadura and Michels (2006) used site-directed mutagenesis to investigate the specific role of the five serine/threonine residues from the 29 to 56 N-terminal region of Mal61p. This was done with reference to glucose-induced inactivation. It was shown that the phosphorylation of the serine/threonine residues in the 29-56-residue N-terminal area is involved in delivering the internalised Mal61p permease to the vacuole for degradation. It is however not required for the induction of internalisation by glucose.

No specific characterisation of residues in Agt1p, Mph3&4p, and Mtt1p has been reported. At the time of the commencement of the present study the possibility of searching for another kind of maltotriose transporter from the whisky-isolated strains that has not been studied before was considered. Agt1p showed the higher affinity for maltotriose and, with the aim of engineering the constitutive transport of maltotriose, our focus was to start a process of characterising Agt1p. As a strategy for *AGT1* characterisation we searched for the existence of wild-type genes that carry putative mutations that could enhance maltotriose utilisation. Differences in gene sequences, when correlated with differences in maltotriose transport performances, could lead to the identification of important domains and residues in Agt1p.

Han *et al*. (1995) originally characterised Agt1p as a 12-transmembrane domain protein. *AGT1* is a mutant allele of *MAL11,* which codes for a maltose permease. *MAL11* forms part of the *MAL1* locus, which comprises *MAL11* (maltose permease), *MAL12* (maltase) and *MAL13* (activator). *MAL11* is situated in the telomeric region of chromosome VII (Needleman, 1991).

2. PROJECT AIMS

With maltotriose transport being the main problem in maltotriose utilisation, the purpose of this study was to characterise Agt1p for maltotriose transport functionality in order to engineer the existing maltotriose transporter for enhanced maltotriose transport capabilities. The specific aims of the study included the following:

(i) The assessment of possible genetic aspects that influence the transporting abilities of different strains. As a starting point, the aim was to identify *Saccharomyces* strains that are able to grow efficiently on maltotriose as sole carbon source. From these strains we wanted to map the *AGT1* loci, isolate the different *AGT1* genes and express them constitutively in the same genetic background. The maltotriose transport efficiency of the *AGT1*-encoded α-glucoside transporters isolated from different sources can thus be determined, distinguishing if the screening by growth in maltotriose is reflecting the better efficiency of transport. Sequence variations in the *AGT1* genes can be used to identify residues critical for maltotriose affinity.

(ii) The identification of specific domains for maltotriose affinity by constructing chimeric proteins showing combinations of Agt1p and Mal31p fragments from different strains. These chimeric proteins were evaluated for maltotriose transport. This can lead to the determination of the possibility of transforming Mal31p into a more efficient maltotriose transport protein, or whether the affinity for maltotriose is associated with specific domains or residues.

(iii) The characterisation of the different amino acid residues responsible for enhanced maltotriose transport efficiency. The strategy was to repair the differences in amino acid residues identified by under the first objective that are present in one of the domains identified under the second objective for the *AGT1* genes isolated from the more promising performers on maltotriose (by creating mutants). The importance of the amino acid differences between these *AGT1* genes was characterised.

3. REFERENCES

Brondijk T.H., Van Der Rest M.E., Pluim D., De Vries Y., Stingl K., Poolman B., Konings W.N. (1998). Catabolite inactivation of wild-type and mutant maltose transport proteins in *Saccharomyces cerevisiae*. J Biol Chem, 273: 15352-15357.

Charron M.J., Read E., Haut S.R., Michels C.A. (1989). Molecular evolution of the telomere-associated *MAL* loci of *Saccharomyces*. Genetics, 122: 307-316.

Day R.E., Higgins V.J., Rogers P.J., Dawes I.W. (2002). Characterization of the putative maltose transporters encoded by YDL247w and YJR160c. Yeast, 19: 1015-1027.

Day R.E., Rogers P.J., Dawes I.W., Higgins V.J. (2002). Molecular analysis of maltotriose transport and utilization by *Saccharomyces cerevisiae*. Appl Environ Microbiol, 68: 5326-5335.

Gadura N., Michels C.A. (2006). Sequences in the N-terminal cytoplasmic domain of *Saccharomyces cerevisiae* maltose permease are required for vacuolar degradation but not glucose-induced internalization. Curr Genet, 50: 101-114.

Guldener U., Munsterkotter M., Kastenmuller G., Strack N., Van Helden J., Lemer C., Richelles J., Wodak S.J., Garcia-Martinez J., Perez-Ortin J.E., Michael H., Kaps A., Talla E., Dujon B., Andre B., Souciet J.L., De Montigny J., Bon E., Gaillardin C., Mewes H.W. (2005). CYGD: the Comprehensive Yeast Genome Database. Nucleic Acids Res, 33: D364-368.

Han E.K., Cotty F., Sottas C., Jiang H., Michels C.A. (1995). Characterization of *AGT1* encoding a general α-glucoside transporter from *Saccharomyces*. Mol Microbiol, 17: 1093-1107.

Hein C., Springael J.Y., Volland C., Haguenauer-Tsapis R., Andre B. (1995). NPI1, an essential yeast gene involved in induced degradation of Gap1 and Fur4 permeases, encodes the Rsp5 ubiquitin-protein ligase. Mol Microbiol, 18: 77-87.

Marchal C., Haguenauer-Tsapis R., Urban-Grimal D. (1998). A PEST-like sequence mediates phosphorylation and efficient ubiquitination of yeast uracil permease. Mol Cell Biol, 18: 314-321.

Medintz I., Wang X., Hradek T., Michels C.A. (2000). A PEST-like sequence in the N-terminal cytoplasmic domain of *Saccharomyces* maltose permease is required for glucose-induced proteolysis and rapid inactivation of transport activity. Biochemistry, 39: 4518-4526.

Rechsteiner M. (1988). Regulation of enzyme levels by proteolysis: the role of pest regions. Adv Enzyme Regul, 27: 135-51.

Saier M.H., Jr. (2000). Families of transmembrane sugar transport proteins. Mol Microbiol, 35: 699-710.

Salas-Burgos A., Iserovich P., Zuniga F., Vera J.C., Fischbarg J. (2004). Predicting the three-dimensional structure of the human facilitative glucose transporter glut1 by a novel evolutionary homology strategy: insights on the molecular mechanism of substrate migration, and binding sites for glucose and inhibitory molecules. Biophys J, 87: 2990-2999.

Salema-Oom M., Valadao Pinto V., Goncalves P., Spencer-Martins I. (2005). Maltotriose utilization by industrial *Saccharomyces* strains: characterization of a new member of the alpha-glucoside transporter family. Appl. Environ. Microbiol. 71: 5044-5049.

Stanbrough M., Magasanik B. (1995). Transcriptional and posttranslational regulation of the general amino acid permease of *Saccharomyces cerevisiae*. J Bacteriol, 177: 94-102.

Verstrepen K.J., Chambers P., Pretorius I.S. (2006). The development of superior yeast strains for the food and beverage industries: challenges, opportunities and potential benefits. Springer-Verlag, Heidelberg, Germany.

Zastrow C.R., Hollatz C., De Araujo P.S., Stambuk B.U. (2001). Maltotriose fermentation by *Saccharomyces cerevisiae*. J Ind Microbiol Biotechnol, 27: 34-38.

Zheng X., D'Amore T., Russell I., Stewart G.G. (1994). Factors influencing maltotriose utilization during brewery wort fermentations. J Am Soc Brew, 52: 41–47.

Chapter 2

LITERATURE REVIEW

The Structure and Importance of α-Glucoside Transport in Yeast

THE STRUCTURE AND IMPORTANCE OF α-GLUCOSIDE TRANSPORT IN YEAST

1. INTRODUCTION

Saccharomyces cerevisiae is one of the most widely-used micro-organisms. It is efficient in fermenting sugars from different plant sources to produce ethanol and carbon dioxide, making it very useful in the production of beer, wine and bread. *S. cerevisiae* has been in common use for centuries and it has received GRAS (Generally Regarded As Safe) status, indicative of its acceptance as safe for use. The wide application of *S. cerevisiae* is not limited to the food and beverage industries; it is also used in the rapidly expanding biofuel production industry. The production of bio-ethanol as an alternative fuel-source forms part of the drive to replace or reduce the use of fossil fuels. Biofuels carry the significant advantage of being renewable and environmentally friendly, and the contribution of *S. cerevisiae* to the development of biofuels might be particularly important in the search for new sources of energy that will be sustainable in the global economy. A third important application of *S. cerevisiae* can be found in the field of heterologous protein production. In the research environment, *S. cerevisiae* acts as an excellent model-organism due to its susceptibility to genetic engineering and unicellular eukaryotic nature.

S. cerevisiae consumes sugars as a main carbon source. After entering the yeast cell, glycolysis is used as the pathway to convert glucose to pyruvate while producing ATP along with NADH and intermediates. The subsequent respiration of pyruvate can lead to further energy production. However, with high sugar concentrations, and despite the presence of oxygen, *S. cerevisiae* has the tendency to ferment the sugars to ethanol and carbon dioxide. The rapid and complete fermentation of all available sugars is essential when an industry is to be built on the ethanol-producing ability of *S. cerevisiae*.

α-Glucosides (maltose, maltotriose, etc.) are sugars commonly associated with all the aforementioned industries and are more complex than glucose. *S. cerevisiae* exhibits a repression system where glucose and other less complex sugars are consumed before the α-glucosides. Glucose repression occurs when the presence of glucose inhibits the systems responsible for consuming other available sugars and this often leads to the incomplete fermentation of the more complex sugars. The limiting factor in α-glucoside metabolism is widely believed to be the transport of these sugars across the plasma membrane by specific sugar carriers.

The increasing availability of biotechnological tools has lead to worldwide efforts to unravel various biological transport systems found in nature. Understandably, much attention has also been given to sugar transport systems in the hope of improving the simultaneous consumption of all sugars during fermentation. Compared to other organisms like bacteria, little is known about the structure of yeast transporters.

Transport-proteins and their three-dimensional structure characterisation remains a challenging field of study, and it profits to borrow from systems that have already been characterized in order to create a better understanding of yeast transport protein structures.

This review will focus on the importance of α-glucosides in the industries they are relevant to. A further discussion on cell membrane transport and transport proteins that have already been characterised will serve as introduction to a discussion on the yeast α-glucosides sugars transport systems. This aims to contribute to the understanding of how yeast transport structures manifest themselves.

2. THE α-GLUCOSIDES AND THEIR IMPORTANCE IN YEAST RELATED INDUSTRIES

α-Glucosides are glycosides where the sugar moiety is a glucose residue, and the anomeric carbon of the bond is in an alpha configuration (Figure 1). These sugars are available as an energy source and are usually conglomerated in the more complex form of starch. Starch and its derivatives play a role in many industries and everyday applications, including the food, beverage, paper and textile industries; in building materials, and; in some pharmaceuticals. Micro-organisms can utilize starch as a carbon source, and yeast is no exception. Yeast uses starch either in the accumulation of biomass and/or the production of by-products, like ethanol. The main application that originates from yeast utilization of α-glucosides is the production of ethanol and carbon dioxide in the fermentation process.

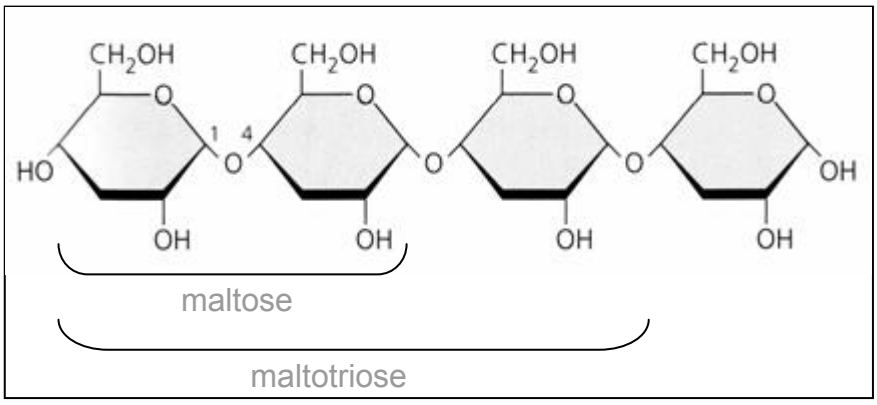

Figure 1. A schematic representation of α-Glucosides found in nature.

2.1 STARCH DEGRADATION

Starch makes up the nutritive reserves of many plants, including all major agricultural crops (Figure 2). Plants accumulate energy in the form of sugars during their growing seasons. This energy is transported to starch storing cells where sugars are converted

into starch and are stored away in intracellular organelles (amyloplasts) surrounded by a lipoprotein membrane (Raimbault 1998).

Except for serving as a food source, starch is recognised and used in many industries as a renewable energy source. There are many known applications for the utilization of starch, and in this discussion we will focus on biofuel production, beer and whiskey fermentation, and bread making.

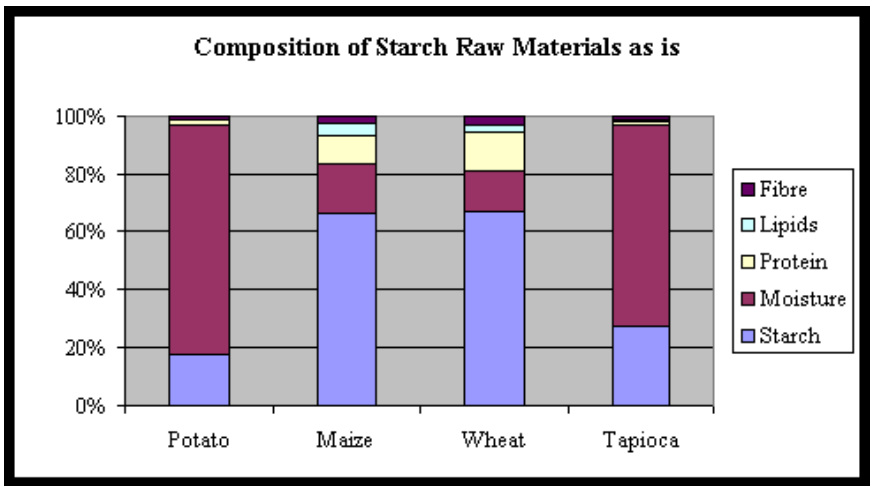

Figure 2. Histogram showing the variation in the starch composition of different crops (Copyright © 1999-2006 International Starch Institute, Science Park Aarhus, Denmark).

2.1.1 Biofuel production from starch

The availability of liquid fuel is one of the global challenges facing the industrialised world. Since 1900, fossil fuels have made up the greater part of the liquid fuel used in motorised transport modes. The problems created by fossil fuels, such as petroleum, are the finite nature of the world's deposits, and the effect of global warming created by the gases from burning fossil fuels. These problems accentuate the urgency to find alternative fuel sources.

Before World War I, ethanol produced from agricultural crops was used as a fuel source for motorised vehicles, such as automobiles. In the post-World War II era, the abundant and cheap supply of fuel extracted from petroleum oil and natural gas has caused a dramatic decrease in the importance of ethanol production as a biofuel (Bothast *et al*., 2005). In the 1970s, oil supply disruptions from the Middle East lead to renewed interest in ethanol production, and this interest was further spurned by the phasing-out of lead as an octane booster for gasoline (Hunt 1981). The worldwide trend to combat the effects of global warming has also put pressure on the use of oxygenated fuels; for example, methyl tert-butyl ether (MTBE) has been used as an oxygen source in fossil fuels (Bothast *et al*., 2005).

All factors considered, fuel ethanol remains an attractive option. When produced from plant rests, it carries the significant advantage of being renewable and

environmentally friendly while also stimulating economic development of the agricultural industry. Ethanol burns cleanly as a fuel component and increases the octane level of gasoline. According to DiPardo (2000), only half the volume of ethanol is required to produce the same oxygen level in gasoline because of its higher oxygen content as compared to MTBE. Furthermore, ethanol is biodegradable. Ethanol as a liquid fuel has re-emerged and, with its good energy density, is being used more widely.

Ethanol, as a fuel source, faces significant commercial viability obstacles when compared to petroleum (Bothast *et al*., 2005). Bio-ethanol is produced by the fermentation of plant material. In order to convert enough sugar to compete with oil use world-wide, and make this process commercially and economically feasible, it will be required to convert the total biomass of plant rests. Therefore, lignocellulose must be degraded (Figure 3). Efforts are being made around the world to create an efficient system wherein yeast can degrade lignocellulose and hence increase the commercial viability of bio-ethanol.

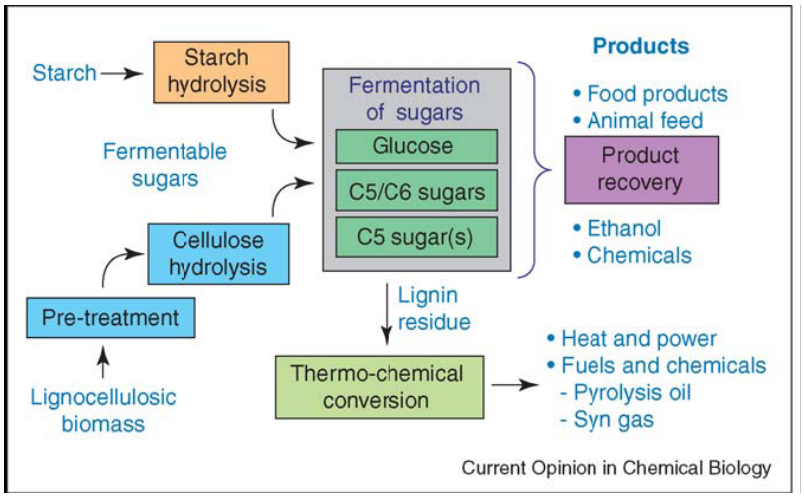

Figure 3. The three major steps in the conversion of lignocellulosic materials; thermochemical pre-treatment, enzymatic saccharification and fermentation of the released sugars by specialized organisms (Gray *et al*., 2006).

Starch fermentation is less complex than lignocellulose hydrolysis, and the optimization of the starch fermentation process has received widespread attention. Sources of starch include corn, potato, cassava, wheat and rice. Corn is the most widely used starch crop in ethanol production. Corn consists mainly of starch that is situated in the endosperm portion of the corn kernel and comprises 70-72% of the dry weight (Bothast *et al*., 2005). For the discussion of biofuel production from starch, corn will be used as an example.

2.1.1.1 Corn as a source for biofuel production

The production of ethanol from corn was first introduced in the United States in the early 20th century. In the early days of the motor car, Henry Ford had the vision of building a

vehicle that was affordable for the working class and would be powered by a fuel that would boost the economy, including the farming community. As such, the first Ford Model T carburettors had an adjustment setting which enabled it to run on either gasoline or ethanol (Kovarik 1998).

When compared to other crops containing starch, corn is the most important and most economical source. Therefore, most of the current ethanol production in the United States is sourced from field corn (Figure 4). According to the International Starch Institute (Science Park Aarhus, Denmark), the composition of corn is as follows (in averages): Protein 7.7 %; Oil 3.3 %; and Starch 61.7%. Many processes have been developed to extract as much ethanol as is possible from corn in the most economical manner.

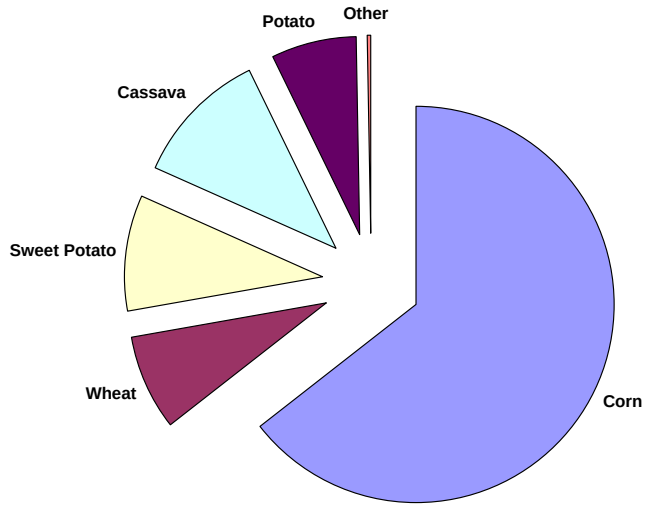

Figure 4. A pie chart indicating the distribution of 2004 world starch production, which totalled 60 million t (Copyright © 1999-2006 International Starch Institute, Science Park Aarhus, Denmark).

Corn starch, stored in the endosperm of the corn kernel, must be extracted before ethanol production commences. Two industrial processes have been used to process the corn kernel - one is known as “dry grind” and the other as “wet mill” (Bothast *et al*., 2005). Both processes vary in their outputs and by-products formed, and are the determining factors for the industry when choosing one or the other. In order to utilize starch from this energy storing form, it has to be degraded into smaller fragments. This degradation process is done by enzymes called amylases.

The bioconversion of starch into ethanol is a two-step process. The first step is saccharification, where starch is converted into sugar by the use of an amylolytic micro-organism or commercially added enzymes, such as glucoamylase and α-amylase. The second step is fermentation, where sugar is converted into ethanol using *S. cerevisiae* (Inlow *et al*., 1988; Nakamura *et al*., 1997).

When looking at the saccharification process of corn starch degradation, it is important to know the composition of the molecules that are involved in the process. Corn

starch is insoluble and partially crystalline (Figure 5) (Robertson *et al*., 2006). Starch is made up out of amyloses (20-30%) and amylopectin (70-80%). These molecules are $^4C_1$ conformation polymers of α–D-Glucose. Amylose shows α–(1,4) linked α–D-Glucose with all the ring oxygen atoms situated on the same side. Amylopectin consists of this same basic structure, except for the formation of branching points approximately every twenty residues through a α– (1,6) linkage. The ratio of amylose and amylopectin found in starch will differ depending on the particular source of starch used. In order to degrade the starch and subsequently ferment it into ethanol, the more complex molecules are degraded into simple six-carbon sugars.

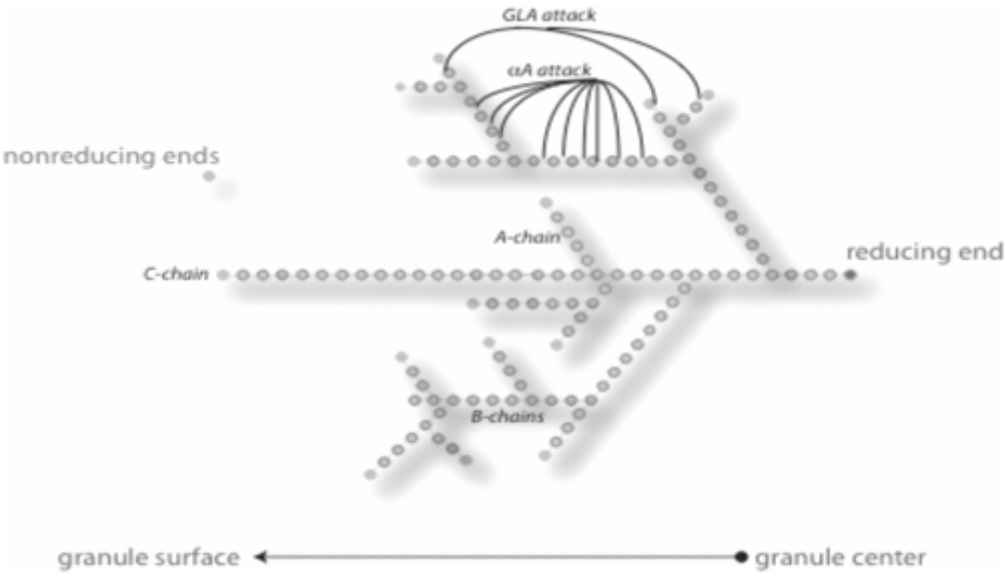

Figure 5. A simple model of starch structure, illustrating descriptive chain nomenclature and classical enzyme attack modes by R-amylase (aA) and glucoamylase (GLA) (Robertson *et al*., 2006).

The corn starch saccharification process is as follows (Bothast *et al*., 2005): the pH is kept at pH 6.0 and thermostable α-amylase enzyme is added to hydrolyze the α-1-4 bonds and create soluble dextrins, then a jet cooker is used and heated to 100°C. The high temperature and mechanical action breaks the larger starch molecules. After decreasing the temperature to between 80°C and 90°C, the α-amylases are allowed to liquefy the starch for at least 30 minutes. With the starch molecule reduced, it is cooled and the pH is lowered to pH 4.5, facilitating the addition of a glucoamylase enzyme to convert the liquefied starch into glucose. When the saccharification process is completed, the starch is complemented with a nitrogen source that is fermented by yeast at 32°C. Time needed of fermentation time typically ranges from 48 to 72 hours. The final ethanol concentration of the converted starch 'mash' is around 10–12%.

A distillation column is used to separate the ethanol from the 'mash'. Distillation extracts ethanol at 95% purity, and the excess water needs to be removed. The solid and

liquid fraction remaining after distillation is referred to as the “whole stillage”. Whole stillage includes the fibre, oil, and protein components of the grain, as well as the non-fermented starch. This co-product of ethanol manufacture is a valuable feed ingredient for livestock, poultry, and fish. Although it is possible to feed whole stillage, it is usually processed further before being sold as fodder (Bothast *et al*., 2005).

An alternative to the conventional multistage process, which offers poor economic feasibility, is the use of amylolytic yeasts for the direct fermentation of starch. These yeast cells can secrete α-amylases that hydrolyze starch into smaller oligosaccharides that are then transported into the cell where they are utilized by forming ethanol as a by-product. The advantages in combining the two processes (called simultaneous saccharification and fermentation, or SSF) are the energy efficiency, lowered microbial contamination risks and osmotic stress on the yeast in the case of high glucose concentration (Bothast *et al*., 2005).

Biofuel production from crops creates the opportunity for rural development in opening a substantial new market for corn supply. In this way, the growth of the domestic ethanol industry greatly benefits farmers in addition to the advantage of it being environmentally friendly, emphasising the importance of optimising the agricultural practises used in producing crops for fuel production. This adheres with Henry Ford’s original dream, and might be a feasible option a century after his original concept.

2.1.2 Whiskey and beer industries

The basic constituents of beer are water and a malted grass seed (barley, corn, rice, wheat, rye) or hops. Hops grow on a vine and is a member of the cannabis family. Barley is the preferred grain in beer production as it contains little gluten.

Before barley can be used in beer brewing, the grains must be ‘malted’. Malting converts the barley grain into a form of its highest starch content, where it starts to sprout in order to become a growing plant (Bamforth, 2000). At the end of malting the grain is dried. This state of chemical composition is ideal to start the process of enzymatic mashing. During mashing, the malted grains are soaked in water at temperatures favourable for naturally occurring enzymes to convert starch to smaller carbohydrate units. Amylases are abundant on the dried grain and can liquefy starches and convert them to maltose and dextrins. The soluble starch is thus converted into a sugary liquid known as 'wort' or 'beer' (Reilly *et al*., 2004)

During the beer brewing process, yeast cells convert maltose into carbon dioxide and ethanol through fermentation. The second most abundant sugar in wort is maltotriose, at 13.6% of total carbohydrate content (Hough *et al*., 1981). The brewing industry often struggles with the incomplete utilization of maltotriose, as it leads to a loss of revenue and the higher carbohydrate levels in the completed beer can result in an atypical flavour profile. It has been observed that some yeast strains used in brewing did not ferment maltotriose, but only respire it. At the initial stages, glucose repression occurs in yeast and it does not utilise any of the present alpha-glucosides. By the time the glucose repression

is elevated (at the end of glucose fermentation), no more oxygen is left for fermentation (Zastrow *et al*., 2001). Hence, during brewing, oxygen can be depleted at the beginning of fermentation.

Similar to the process of beer making, whiskey production also relies on the starch conversion to obtain the final product. Whiskey is produced in pot stills from water, barley and yeast (Russell *et al*., 2003). Malting takes place after the barley has been soaked in water for two to three days. After soaking, the germinating barley is spread out on a malting floor and regularly turned with wooden paddles. The starch takes approximately twelve days to convert to sugar, and this, combined process of germination and saccharification, is then stopped by drying in a kiln. Peat fires can also be used during drying to add a peat flavour to the malt. The dried malt is ground and mixed with water to form the wort. After the addition of yeast, the wort is fermented into weak ale referred to as 'wash'. Distillation takes place to create two consecutive stills, namely a 'wash still' and a 'spirit still'. During distillation, care is taken to collect the different stills created, for they vary in chemical composition.

In both beer and whiskey production, different styles exist and varying flavour profiles can be obtained through the adaptation of the main production processes.

2.1.3 Baking industry

The fermentation process in the baking industry utilizes the sugars found in bread dough to produce CO_2, alcohol and other volatile compounds that enables the dough to rise, whilst also contributing to the original taste of the dough. Most of these compounds, and all of the alcohol, evaporate when the dough is baked. During the fermentation of bread, dough yeast initially ferments the easily assimilated sugars, like glucose found in the flour, which usually make up 1.5% of the flour weight (Corke *et al*., 2006). Amylases are naturally occurring enzymes in the flour which can also be released by the yeast cells, but are only active after water is added to the flour; note that baking inactivates the amylases. Amylases act on starch found in the flour and releases maltose. This utilization takes place when the disaccharide is transported into the cell where it is hydrolyzed into two glucose units by the maltase enzyme. A rapid utilization of maltose is essential for CO_2 production, allowing the dough to rise sufficiently before baking. The yeast industry has studied baker's yeast strains in great detail, mainly with the view to increase the speed of the reactions, but also to adapt them for different baking applications.

The α-glucosides play an important role in all the aforementioned industries. Maltotriose, for one, is residually left after fermentation. Most α-glucosides, like maltose and maltotriose, are in competition with glucose consumption by yeast. Glucose, being the favoured sugar, will suppress the utilization of the other sugars. This leads to slow consumption and, eventually, residual sugar content. For industries relying on yeast to degrade starch, it is essential to keep the weight (in source material) : volume (ethanol produced) ratio as productive as possible. All efforts to utilize the available sugars will benefit the overall output of these industries, and it is therefore highly relevant to create yeast strains capable of utilising sugars like maltotriose.

For many sugars like maltotriose, the transport of the sugar into the yeast cell is the bottleneck in the utilization process. To enhance the ability of yeast to take up these sugars, a thorough understanding of the yeast transport systems and the specific ways in which sugars are transported is valuable. It is clear that α-glucoside transport in yeast is very important. The optimization of these transport processes can have far-reaching effects on the different industries that rely on starch degradation. Optimization can thus lead to a significant reduction in costs. The following section will introduce transport in the cell and, thereafter, proceed to a detailed discussion of α-glucoside transport in yeast.

3. TRANSPORT IN THE CELL

All living cells contain selective permeable cell membranes. These membranes consist out of structured phospholipids bilayers that contain protein molecules. Transport processes across these membranes take place continuously and can be categorised as follows: the import and export of macromolecules like proteins or peptides mediated through intracellular sorting and trafficking; the import of small molecules like nutrients (sugars, amino acids, phosphate, vitamins); and the export of small molecules like toxic compounds to prevent deleterious reactions.

Yeast cells have a protecting envelope that acts as a barrier between the internal and external areas of the cell. This area makes up about 15% of the total cell volume and consists out of the internal plasma membrane, the periplasmic space, and the external cell wall (Baron 2004). The periplasm is a thin region separating the cell membrane and the cell wall. It functions as a reservoir for mannoproteins secreted through the plasma membrane that are unable to penetrate the cell wall. These mannoproteins hydrolyse substrates that are unable to enter the cell through the plasmatic membrane (for example the conversion of sucrose into glucose and fructose by interaction of the invertase). The yeast cell wall can reach up to 200 nm in width, and consists of the polysaccharides glucan and mannan that make up 80 - 90% of the cell wall, along with a small percentage of chitin, proteins, lipids, and inorganic phosphate (Figure 6). The glucans strengthen the cell wall through the forming a microfibrillar network (Klis *et al*., 2006).

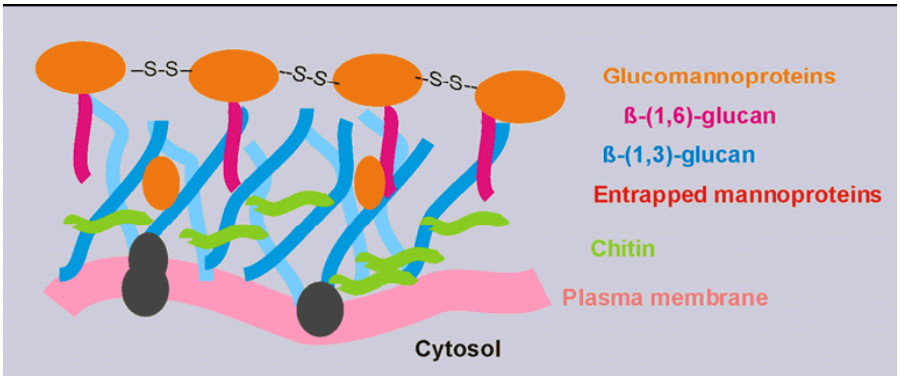

Figure 6. A simple model of the yeast cell wall (Walker 1998).

Sugar transport across the plasma membrane is one of the most important transport processes. In the case of ethanol production through fermentation, the yeast cell heavily relies on its efficiency to transport the sugars into the cell for glycolysis, and for energy production to take place. This process is facilitated by proteins situated in the plasma membrane. In order to understand the structural characteristics of a transport protein, it is necessary to understand the cell membrane.

3.1 CELL MEMBRANES

The main function of a cell membrane is to separate the interior of the cell from its surroundings and provide selective permeability. Included in this function is the maintenance of the cell's osmotic potential and the controlling of the in- and efflux of molecules, as well as regulating the cell's nutrition. In yeast, a cell wall forms the outermost boundary of the cell in addition to the function of the cell membrane. The cell membrane is 7 nm thick and a typical model of the cell membrane can be seen in Figure 7 (Lindegren *et al*., 1949). The yeast cell membrane hosts a wide variety of important proteins, including cytoskeleton anchors, enzymes for cell wall synthesis, proteins for transmembrane signal transduction, proteins for solute transport and transport facilitators.

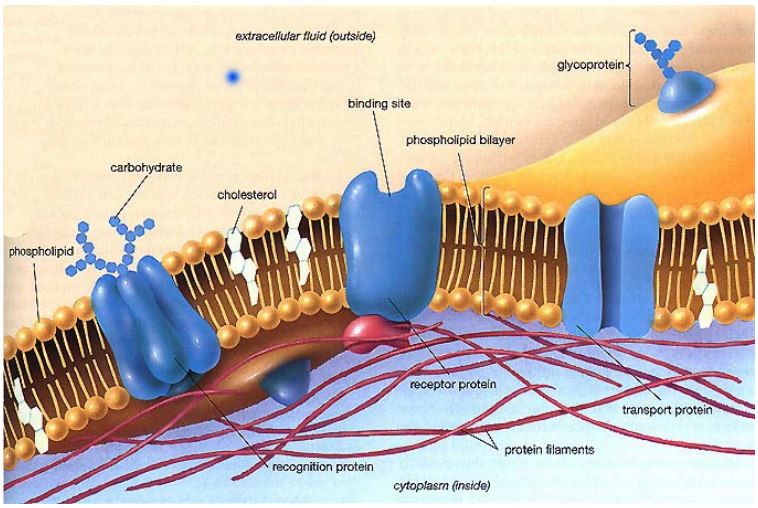

Figure 7. The plasma membrane of a cell (Audesirik 1999).

3.1.1 Phospholipid bilayers

Phospholipid bilayers have been described in the fluid mosaic model as a two-dimensional fluid of freely diffusing lipids, embedded with proteins that function as channels, transporters or receptors (Singer *et al*., 1972). Hopanoids (cholesterol in the case of human cells) assist in maintaining the fluidity. In more recent years, the lipid content of cell membranes has raised the question of how fluid the membrane truly is. Small organized groups of lipids, known as lipid rafts, have been observed. Lipid rafts can play a role in protein organization, along with the cytoskeleton, underlining the cell membrane while serving as an anchoring point for integral membrane proteins. In yeast, the lipid composition comprises mainly phosphatidylcholine and phosphatidyl-ethanolamine. Phosphaditylinositol, phosphatidylserine or phosphadityl-glycerole, as well as sterols (ergosterol and zymosterol) are also present (Walker 1998).

3.1.2 Membrane proteins

Proteins found in the cell membrane can be categorized as extrinsic proteins that simply adhere to the membrane, or as intrinsic proteins embedded in the membrane or spanning the width of the cell membrane.

Glycoproteins are membrane proteins with extracellular carbohydrates attached to them. Transport proteins fall under the intrinsic membrane proteins known as integral membrane proteins (IMP's). IMP's are processed in the cell's endoplasmic reticulum and a signal sequence determines their installation into the membrane. More than 20% of all genes sequenced up to date, code for transmembrane proteins that perform a wide range of highly critical roles in the cell (Kaback 2005). This gives a good indication of the importance of membrane proteins in cell functioning. IMP's primarily function as transport molecules, but other functions include cell-cell recognition; cell-cell or cell-media anchoring, and; cell receptors responsible for intracellular responses (for example, signal transduction).

Considering the importance of integral membrane proteins, and the role they play in the cell, one of the first questions that arise is the method of functioning. In this regard, the structure of these proteins becomes highly relevant and many efforts have been made to characterise the structures of transmembrane proteins. X-ray diffraction and nuclear magnetic resonance (NMR) are methods used to determine the 3D structures of proteins, and thousands of structures for all types of proteins have been characterised in this way (Carter *et al*., 1997). Membrane proteins denature when removed from the cell membrane. For this reason, only a small number of membrane protein structures have been characterised at atomic resolution level. Electron chrystallography has been developed to combat the problems caused by the presence of the membrane for the aforementioned methods. With electron chrystallography, protein structures can be determined from 2-dimentional helices. NMR is based on the magnetic character of the nucleus of an atom. Magnetic resonance imaging or nuclear magnetic spectroscopy

observes the alignment of the nucleus with the external magnetic field of the atom. This method can provide physical, chemical, electronic and structural information of a molecule. It is, however, not suitable for membrane proteins.

Even the strongest existing microscopes do not show atoms under normal visual light. An object can only be seen if it is at least half the wavelength of the light it is visualized by. In X-ray chrystallography, X-rays have been implemented to visualise atoms. X-rays, however, cannot be focused through a lens; this in turn rules out the microscope. Crystals can, however, diffract X-rays, and the diffraction pattern can be interpreted by a computer through mathematical calculations. Crystals of the proteins in question needs to be grown before they can be observed. Crystals are formed with edges of 0.1 - 0.3mm (Rhodes 1993). This is the principle on which X-ray chrystallography is based. The main problem with this method is determining phases in the diffraction pattern. The large 3-dimentional crystals enable this method to collect large magnitudes of diffraction patterns. In electron crystallography, an electron microscope is used to determine protein structure through electron diffraction. In comparison to X-ray chrystallography, structures are determined from 2-dimentional crystals, polyhedrons or dispersed proteins like membrane proteins. Electrons interact more strongly with proteins than X-rays; where X-rays will merely pass through a 2-dimentional crystal without diffracting, electrons will interact and diffract a clearer image. The other advantage of electron microscopes is their electron lenses that enable them to give more accurate phase information. It remains very difficult to reproduce the small-scale concentrations of crystallized proteins (Carter *et al*., 1997).

The cell membrane physiology, and the way in which proteins function inside the cell membrane, determines the ease that the in- and efflux of molecules into and out of the cell occur. Transport proteins situated in the cell membrane act as the catalysers of this highly important, permeable system.

4. TRANSPORTERS

Transporters are intrinsic membrane proteins that show a relatively high specificity and are characterized by the fact that their binding site opens alternately to the two sides of the membrane. The action of transporters can be classified as diffusion or active transport. Facilitated or mediated diffusion is driven by transporters that function without input of energy beyond the thermal movement. Primary active transport is driven by various exergonic chemical and photochemical reactions, and secondary active transport takes place over electrochemical potential gradients of H+ and Na+ and, exceptionally, of K+.

Permease is an alternative name that has been used for transporters, but is seen as a historical term applied to some secondary active transporters, and it is not to be applied to newly described transporters.

4.1 CLASSIFICATION

During the 1990s, a classification system was developed for the different transport mechanisms found in membranes of various organisms. Table 1 is a summary of these transport mechanisms as set out by a panel of the Nomenclature Committee of the IUBMB.

Table 1. Membrane transport mechanisms (Arnost Kotyk, Academy of Sciences of the Czech Republic, Prague)

A. Nonspecific permeation	**B. Specific transport**
Through hydrophobic domains of membranes (various small, medium-sized and large lipophilic molecules)	Through selective channels • Nongated, such as porins in bacterial, outer mitochondrial and chloroplast membranes, aquaporins in most cells (cations, anions, water; also nonelectrolytes) • Electrically or potential-gated, such as nerve and muscle-cell channels involved in action potential generation and propagation (Na^+, K^+, Ca^{2+}) • Chemically or ligand-gated, such as the various hormone receptor-channels (cations) • Mechanically or stress-gated, such as channels in blood capillary walls and inner ear hair cells (cations, especially K^+)
Through water-filled pores; some permanently opened, such as the complement complex, bacteriocins, defensins, polyene antibiotics; some opening only after a stimulus, such as connexons between adjacent cells (virtually all solutes up to a certain size)	On specific carriers • Through mediated (or facilitated) diffusion (monosaccharides in animal, noninsulin-dependent tissues, some yeast cells) • By primary active transport 1. Driven by ATP or diphosphate hydrolysis by P-type, F-type and ABC-type ATPases in all cells (cations, anions, amino acids, sugars, xenobiotics) 2. Driven by oxidation reactions, in bacterial, inner mitochondrial and chloroplast membranes (H^+, Na^+) 3. Driven by light absorption, in halobacteria (H^+, Cl^-) 4. Driven by decarboxylation, in bacteria (Na^+) 5. Driven by methyl transfer, some methanobacteria (Na^+) • **by secondary active transport** **1. Of symport type, using H^+, Na^+ and, exceptionally, K^+ as driving ion, in bacterial, fungal, plant and animal cells, net charge transporting (various non--electrolytes, mainly nutrients)** 2. Of antiport type, electrically silent, in a variety of cells (anions, cations, often in combination) 3. Of antiport type, net charge transporting, in outer mitochondrial membranes (ADP/ATP)
Through true pores in the lipid bilayer, as are transiently formed at higher temperature and with applied transmembrane electric potential (all solutes, including macromolecules ("electroporation"))	By group translocation, mainly in Gram-positive and Gram-negative bacteria, in brain tissue (mono- and disaccharides; amino acids)
Via non-receptor endocytosis in membrane vesicles (all solutes present in extracellular aqueous medium)	By receptor-mediated pinocytosis • By endocytosis, mainly in animal cells (ferritransferrin) • By exocytosis, mainly in fungal and animal cells (hormones)

During the last decade, many transporter proteins have been sequenced and, along with the amino acid sequence information generated, phylogenetic relationships pertaining

to the evolutionary history of a particular group of organisms could be classified. This led to a more detailed classification system, where specificity toward a substrate or molecule being transported was no longer the only criteria used. Enzymes are classified according to the system developed by the Enzyme Commission (EC). Until recently, no comparable system was developed for transport proteins catalyzing transmembrane reactions. Saier (1998) developed the Transport Commission (TC) system, which is an analogy to the EC system. According to the TC system, transport protein families and subfamilies are grouped into classes and subclasses (Table 2 and Figure 7), and an abbreviation system was developed to organize these classes. The TC system has made it possible to classify vast amounts of transport proteins without having information on their specificity. As research continues, this information can be added, but in the interim, separate tables of information on transport specificity is available Transport Protein Classification on World Wide Web (Saier 2000).

Table 2. Classes and subclasses of transporters in the TC system[a] (Saier 2000).

1.	Channels and pores
1.A	α-Type channels
1.B	β-Barrel porins
1.C	Pore-forming toxins (proteins and peptides)
1.D	Non-ribosomally synthesized channels
2.	Electrochemical potential-driven transporters
2.A	Porters (uniporters, symporters, and antiporters)
2.B	Nonribosomally synthesized porters
2.C	Ion gradient-driven energizers
3.	Primary active transporters
3.A	Diphosphate bond hydrolysis-driven transporters
3.B	Decarboxylation-driven transporters
3.C	Methyl transfer-driven transporters
3.D	Oxidoreduction-driven transporters
3.E	Light absorption-driven transporters
4.	Group translocators
4.A	Phosphotransfer-driven group translocators
8.	Accessory factors involved in transport
8.A	Auxiliary transport proteins
9.	Incompletely characterized transport systems
9.A	Recognized transporters of unknown biochemical mechanism
9.B	Putative but uncharacterized transport proteins
9.C	Functionally characterized transporters lacking identified sequences

[a]This system of classification was approved by the transporter nomenclature panel of the International Union of Biochemistry and Molecular Biology in Geneva, 28–30 November 1999. No assignment has been made for categories 5 to 7. These will be reserved for novel types of transporters, yet to be discovered, that do not fall within categories 1 to 4.

The current system makes use of a five-digit code which is assigned as follows: the first number (class of transport protein) and second a letter (subclass of protein) refer to the mechanism of translocation and/or the source of energy used; the third number (family of transporter) and the fourth number (subfamily of transporter) refer to the basis of their primary structure; the fifth number refers to the specific transport protein.

In Figure 8, a flow diagram indicates the basic division of primary types of transport. They are firstly divided into channels and carriers, and the further division of transporters into classes link to their structure and mode of action.

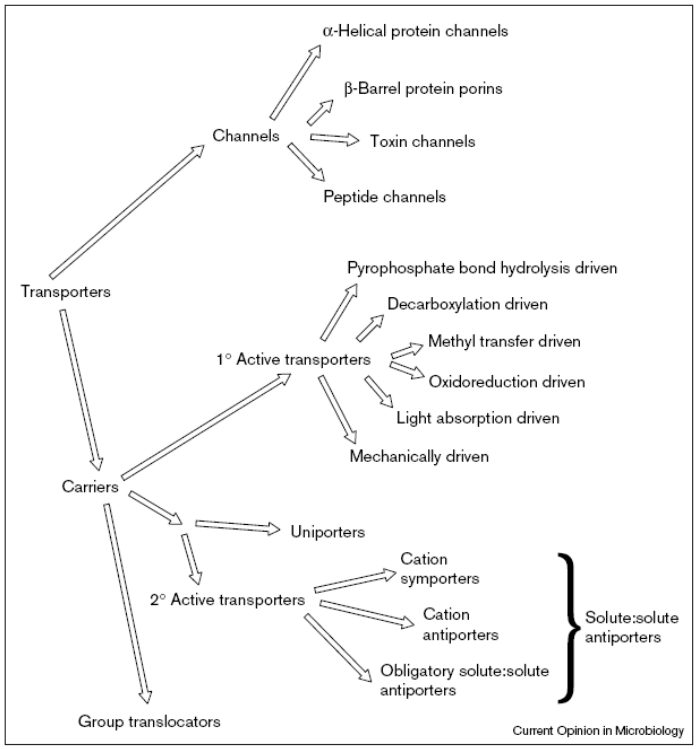

Figure 8. Scheme illustrating the currently recognized primary types of transporters found in nature (Saier 2000).

4.2 MODE OF ACTION

Solutes can cross the cell membrane either through simple diffusion (non-specific permeation), or protein mediated transport (through channels). In the case of polar, charged or macro molecules, proteins (carriers) are essential in mediating transport. Sugar molecules are polar and rely on transport proteins to cross the cell membrane and enter the cytosol (Johnson *et al*., 2002). In yeast, sugars are transported mainly through active transport.

The first type of transport process is called passive diffusion (Figure 9). During passive diffusion, water and water-soluble substances (as well as small lipids) are transported through the lipid bilayers by a concentration gradient.

Facilitated diffusion can be carried out by channel-type transporters (Figures 8 and 9). The channel is lined with appropriately hydrophilic, hydrophobic, or amphipathic amino acid residues, depending on the type of substrate being transported (Saier 2000). Most channels are oligomeric complexes consisting out of more than one subunit.

Channel functioning differs from carrier transport functioning. During facilitated diffusion, the transport is mediated by the channel creating a water filled pore. It takes place over a concentration gradient in order to reach equilibrium. Solutes will diffuse from a higher concentration to a lower concentration through the membrane, making use of transporters until the intracellular and extracellular concentrations are equal. Facilitated diffusion does not require any energy. Instead, it increases entropy through decreasing free energy.

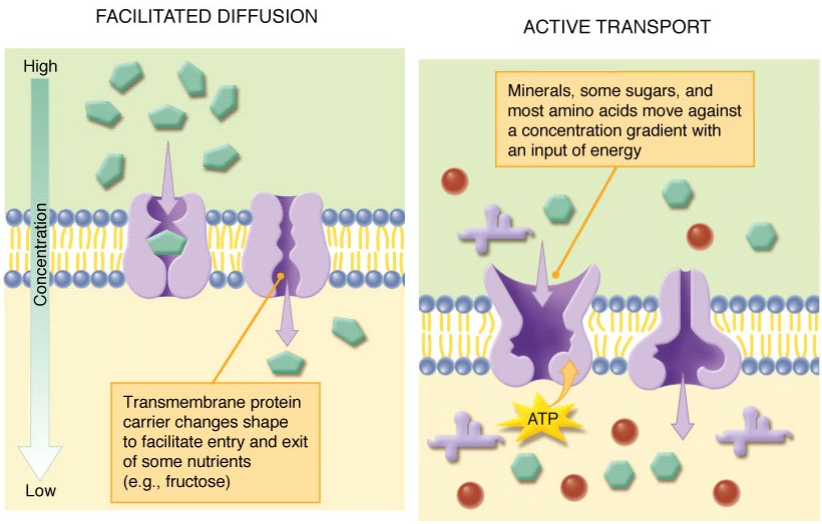

Figure 9. A schematic model of the processes of facilitated diffusion and active transport taking place in the cell wall (Insel *et al*., 2005).

Active transport takes place against a concentration gradient. Transmembrane proteins (porters) force ions and small molecules through the membrane. Chemical energy is needed for this process to take place. Solutes are transported into the cell, and a difference in extracellular and intracellular concentration is created. These carriers create unfavourable entropy by moving molecules against their electrochemical gradient. This is remedied by the hydrolysis of ATP. Carriers usually exhibit rates of transport that are several orders of magnitude lower than those of channels. They also exhibit saturation kinetics and stereo-specific substrate specificity, and can function as monomeric proteins (Saier 2000). Active transporters can be subdivided into primary and secondary transporters.

Primary transporters can be classified as transmembrane ATPases. ATPases are large groups of proteins known for their ability to obtain energy through the hydrolysis of the phosphate of ATP (ATP $\rightarrow$ ADP + Pi). Primary transporters directly bind the ATP to obtain energy for its transport action and are independent of any other action.

Secondary transporters are cation gradient dependent. They carry two substrates at the same time and create energy through this cation gradient (Van der Rest *et al.,* 1995). There is thus no direct binding of ATP involved. The diffusion of the cation over the membrane drives the transport of another. This takes place in either the same direction (symport) or in the opposite direction (antiport). Protons are the most common cations used in secondary transport and function as the driving force of these reactions.

Sugar transporters in yeast all make use of active transport and can also be classified as high-affinity or low-affinity transporters. These terms are indicative of the transporters' affinity for the specific substrate they transport and is usually expressed by the Michaelis constant (*K*m-value). High-affinity transporters have low *K*m values and the genes expressing these proteins are usually induced by low concentrations of the substrate sugar. Low-affinity transporters have higher *K*m values and the genes expressing these proteins are usually constitutively expressed (Lagunas 1993). Yeast sugar transporters can be seen as biological catalysts. While transporting substrates they undergo conformational changes and they only bind specific substrates.

4.3 STRUCTURAL FEATURES

Genes in the genome of organisms carry a code (transcription) for the production of proteins. This code acts as a signal for the consecutive attachment of amino acids to each other (translation) on order to form the specific protein. The primary (1°) structure of a protein is simply covalently linked amino acids (Figure 10). All amino acids exhibit certain characteristics like being charged, polar, hydrophobic, and hydrophilic and - depending on where they are positioned relative to all the other amino acids - are responsible for conformational tendencies in a protein (Figure 10) (Campbell and Farrell 2006). In examining the amino acid sequence of a certain protein, a lot can be estimated about the final structural characteristics of the protein. In the case of the transmembrane proteins, the areas that are situated in the membrane are commonly associated with a high concentration of hydrophobic residues. Thus on amino acid level it can already be predicted where the putative transmembrane domains (TMD) could be situated. The charged amino acids situated inside the transport protein have been known to act as a binding site and assist the sugar molecule in moving through the membrane. Other important amino acids identified in transport are the phenolic amino acids (phenylalanine and tyrosine) that can act as recognition sites on the exterior of the cell membrane recognising the sugar-substrate.

While forming what is known as the secondary (2°) structure of a protein, hydrogen bonding interactions between adjacent amino acids take place. The polypeptide arranges itself into characteristic patterns known as helical or pleated segments (Figure 11). This occurs as result of carbonyl oxygens and amide protons participating in numerous H-bonds, linking one peptide group to another. The α-helix has been characterized as a coil-shaped structure where one turn contains 3.6 amino acid residues (Pauling *et al*., 1951,

Perutz *et al*., 1969). When viewing the extension of one amino acid residue along the helical axis, it amounts to 1.5 Å (0.15nm). The core of a helix is 6 Å in diameter. The α-helix is the most common helix shape found in proteins, even though some others do exist. The other typical pattern found in a protein structure is the β-pleated sheet. These sheets can be visualized as a peptide backbone running straight with a zigzag conformation. These sheets also interact with each other to form parallel or anti-parallel sheets. Although these sheets are present in some bacterial porins (Johnson *et al*., 2002) for the uptake of sugars, they are not associated with the family of transporters with 12 transmembrane domains. The interaction of these basic helices with each other and the type of conformations they form are highly relevant for the folding of transport proteins. The transfer of a transport protein, characterized by polar and charged molecules into the lipid bilayers of the membrane, creates a high thermodynamic cost. This can be remedied by high amounts of non polar amino acids in the transmembrane domains. Another way of reducing this cost is for the highly polar polypeptide backbone of transmembrane domains to form H-bonds. This H-bond structure is characteristic of the α-helices and β-sheets. In all proteins that have been three-dimensionally characterised, these structures have been confirmed (White 1999).

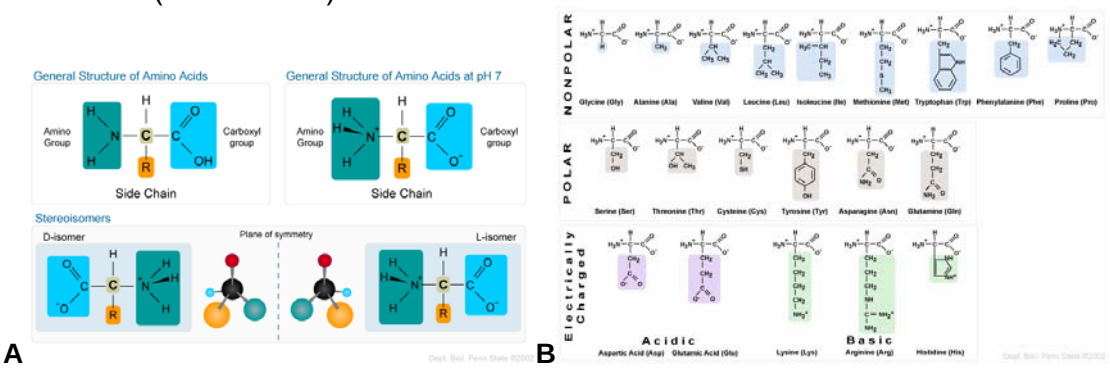

Figure 10 A The general structure of amino acids. **B** Amino acids are grouped as nonpolar, polar and electrically charged, based on their R-groups shaded in purple blue or green (© Penn State Biology Department 208 Mueller Lab, University Park). The amino group (dark blue), the carboxyl group (light blue), the alpha carbon (red), and the R-group (orange) are indicated.

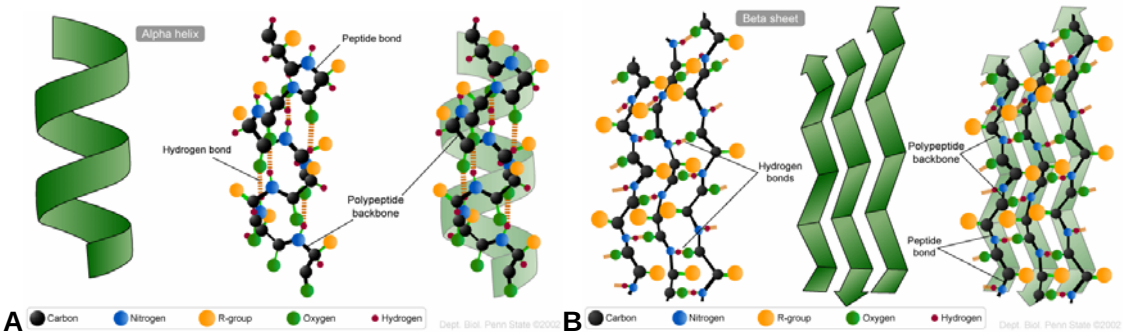

Figure 11 The alpha helix (A) and beta sheet (B) (© Penn State Biology Department 208 Mueller Lab, University Park).

Protein folding takes place in order to obtain a final protein conformation from the original primary (1°) structure of the protein. Engelman *et al*., (1986) proposed a model for the folding methods of membrane proteins. His model can be divided into two stages, the first of which is the stage of independent helix formation. Each helix is immersed in a lipid as they become hydrophobic. The second stage of folding takes place when the helices interact with each other to form the next level of structure. Popot *et al*., (1990) proposed a next stage, where the protein helix is incorporated into a lipid vesicle, and when the vesicles fuse, the fragments are placed in the same lipid bilayers. Engelman *et al*., (2003) proposed a model for the successive steps following the first stages of membrane protein folding (that include the effects of the polar backbone, partitioning of space away from the lipid and the creation of binding surfaces).

The tertiary (3°) level of structure is generated when the polypeptide chains of a protein bend and fold to create a three-dimensional shape with greater complexity. In general, proteins fold to create the most stable structure possible. Many bacterial transport proteins consist out of two or more subunits, each being a separate polypeptide chain folded into a tertiary conformation. All the higher protein structures exits through noncovalent forces, such as hydrogen bonds, ionic bonds, van der Waals and hydrophobic interactions (Campbell and Farrell 2006). The three-dimensional structure of a protein is known as the protein conformation.

Many of the bacterial transport proteins have been well characterised on structural level and it is worth while to look into what is known about them to better understand yeast transport proteins.

4.3.1 The structure of porins and channels

Lipid bilayers show low permeability for hydrophilic solutes. Channel forming proteins are known to allow the influx of nutrients. Examples of these transmembrane proteins, of which many have been structurally characterised, are α-type channels, specific channels and β-barrel porins.

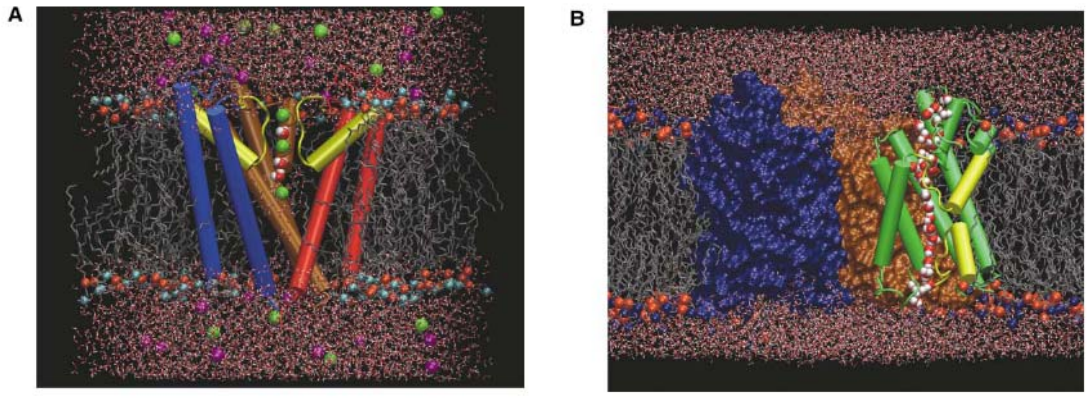

Figure 12 Atomic Models of Membrane Channels KcsA K+ channel (A) (Berne`che *et al*., 2001) and aquaporin (B) (Tajkhorshid *et al*., 2002). The extracellular side is at the top and the intracellular side at the bottom. The α-helices (yellow), K+ (green) (Roux *et al*., 2004).

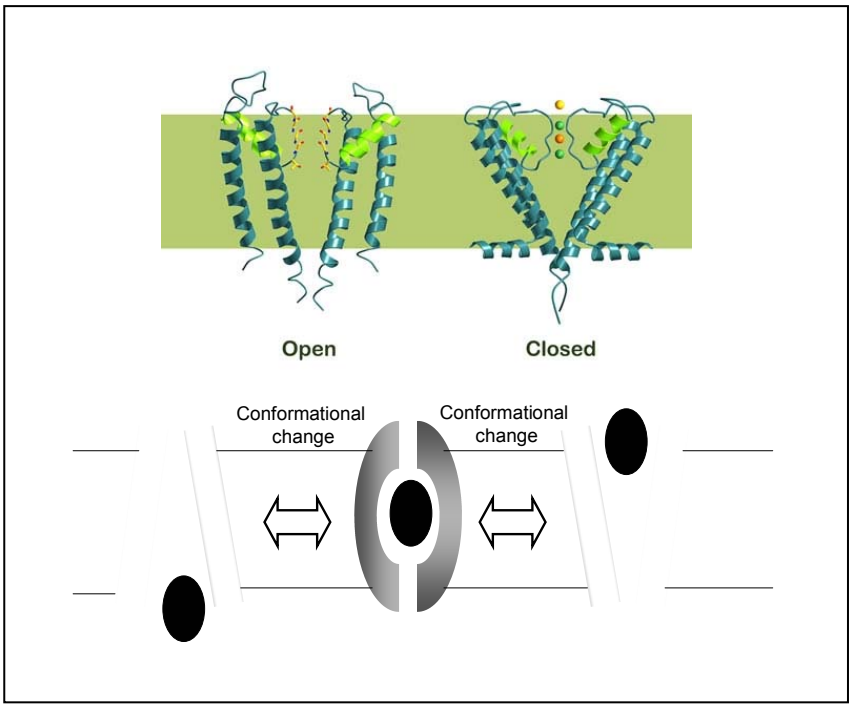

Figure 13 The 'gating' action of the potassium channel and a simplified schematic representation of the conformational change that takes place during this process.

The α-type channels (that are associated with all organisms) have been classified as transporters moving solutes with an energy independent process through an aqueous pore. This channel-type primarily comprises of α-helices, although β-sheets can be present in parts of the final structure. Some of the channels that have been structurally characterised are the potassium channels and the water channels. The basic structures of these channels consist out of α-helices (Figure 12). The potassium channel is known to undergo conformational changes in response to signals leading to the opening and closing of the pore. This process is referred to as 'gating' and a simplified representation of this action is shown in Figure 13 (Roux *et al*., 2004).

More specific channels also exist, like the LamB channel responsible for maltose transport in *Escherichia coli* (Figure 14) (Nikaido 2003). For specific diffusion, the binding of the substrate is essential. Channels responsible for specific sugar transport contain a 'sugar binding site' to catalyze the transport process. LamB is characterized by a monomeric β-barrel structure.

The β-barrel porins are transmembrane pores allowing energy independent flow of solutes across the membrane. These porins are exclusively made up out of β-sheets, and are commonly found in the outer membrane of bacterial cells and eukaryotic organelles (peroxisomal membranes and mitochondrial membranes which are voltage dependent anion channels).

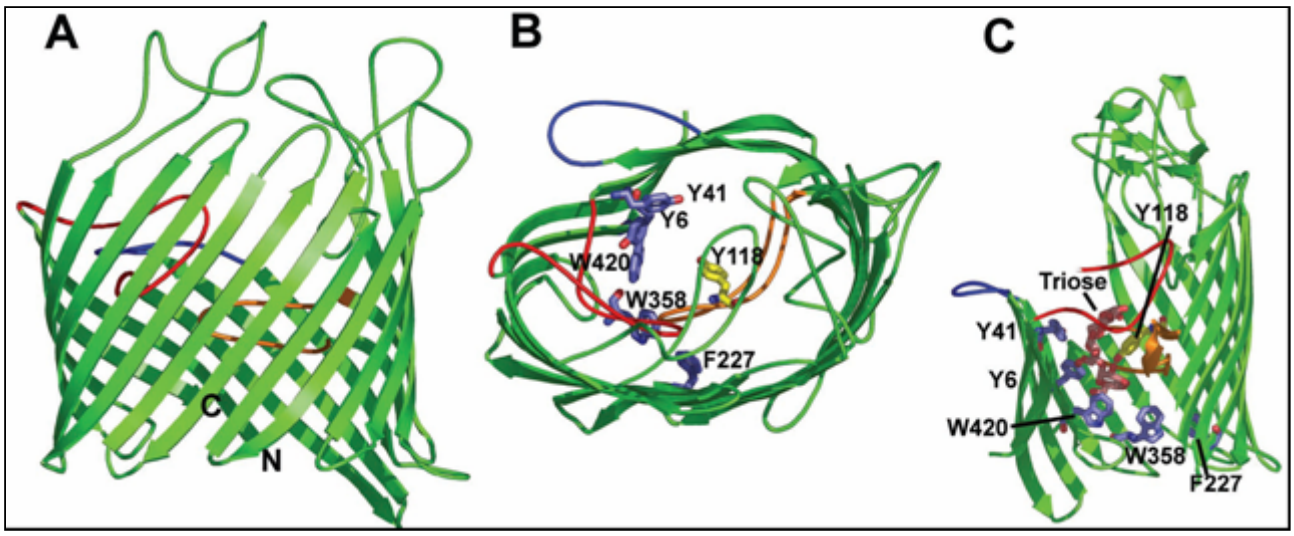

Figure 14 LamB X-ray crystallographic structure with the side view of the monomeric unit (A); top view of the monomeric unit including the greasy slides with (Tyr41, Tyr6, Trp420, Trp358, and Phe227) in blue (B); side view of the greasy slide and its interaction with maltotriose in orange (C) (Nikaido 2003).

In gram-negative bacteria, the β-sheets porins are located in the protective outer membrane (Hofnung 1995). These β-sheets are structured in such a way that they form large channels (β-barrel) across the membranes that allow free diffusion of molecules with a molecular weight of 10 000 Da or less. An example can be seen in Figure 15 that shows the OmpF porin of *Escherichia coli*. The amino acid R-groups point above and below this sheet structure. The structure of a porin contains alternating polar and non-polar amino acids to distinguish between the area facing the aqueous lumen and the phospholipids membrane. The channel remains inert during the transport process. Intra- and extracellular loops connect the β-sheets to each other and the extracellular loops are exposed to the exterior of the cell (Schirmer *et al*., 1995).

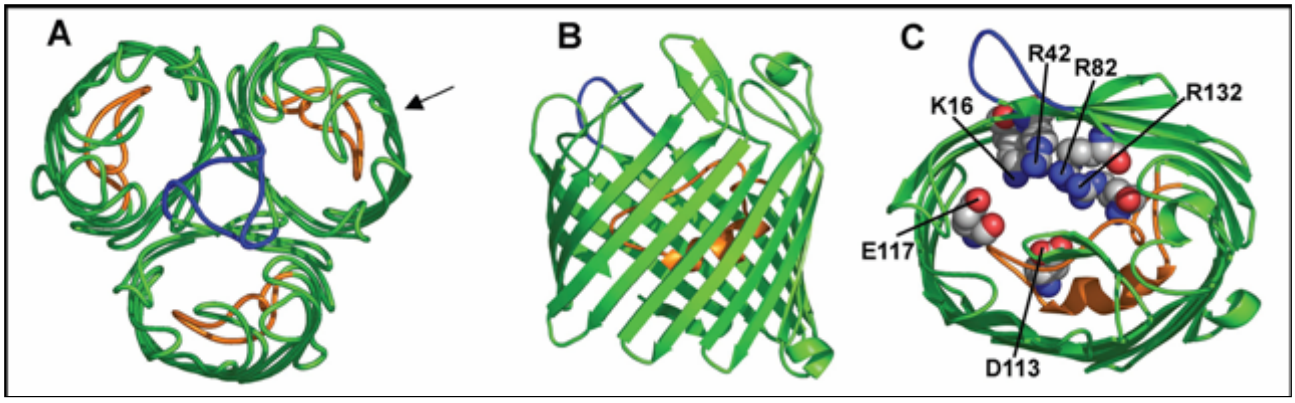

Figure 15 The *E. coli* OmpF porin structure with the trimer perpendicular to the plane of the membrane (A); The monomeric unit from the side (B); The monomeric unit from the top (C) (Nikaido 2003).

What is clear from the structures of these channels and porins is that the centrally positioned amino acids in the pore play an essential role in the movement of substances through the pore. Furthermore, there are many functional and structural features that are highly dependent on the exact way in which the different transmembrane domains and intra- and extracellular loops interact with each other. All these characteristics are also present in the yeast sugar transporters and can be interpreted to understand their structure and functioning.

4.3.2 The structure of eukaryotic sugar transporters

The structure for the transporters that make use of active transport is mainly associated with an α-helix structure that characterises the transmembrane domain (TMD) segments of the proteins (Figure 11A) (Campbell and Farrell 2006). The number of residues found in one α-helix varies from protein to protein and, in the case of transmembrane domains can, for example, range between 18 and 23 for Mal31p. A typical structure for a transmembrane transporter is a collection of transmembrane domains in the α-helix form crossing the membrane and linking with each other through extra- and intracellular loops. The transmembrane regions are known to be highly hydrophobic and are arranged in a circular form. The extracellular loops might contain amino acids involved in substrate recognition, and charged residues inside the circular formation could be involved in the process of moving the sugar through the membrane. Transporters can undergo conformational changes while moving a molecule through the membrane. The intracellular loop contain ATP binding sites in the case of active transporters and can also contain domains that play a role in signalling the protein degradation process.

4.3.3 *GLUT1* as an example for the three-dimensional structure of a eukaryotic sugar transporter

GLUT 1 (human facilitative glucose transporter) forms part of the major facilitator superfamily (MFS) grouped as transmembrane proteins that are characterised by their ability to transport a wide range of solutes across the cell membrane. It is also an example of a eukaryotic sugar transporter. Collection from human erythrocytes has made it possible to obtain large amounts of purified Glut1p. Through mutagenesis studies on the accessibility of cysteins, the effect of inhibitors, labelling reactions with metabolites and antibodies, and the evaluation of the effect of protein degradation, much was revealed about the structural and functional attributes of Glut1p (Hruz *et al*., 2001). This information was incorporated into a prediction according to the hydrophobicity patterns found in Glut1p to create a model for a protein. The model consists of 12 transmembrane domains separated by a large intracellular loop between the sixth and seventh helix into two transmembrane domain segment of six TMD's each (Figure 16). The N- and C-termini of Glut1p are intracellular (Baldwin 1993; Hresko *et al*., 1994). The similarity in the primary

sequences of the MFS family members suggests that they display a conserved architecture.

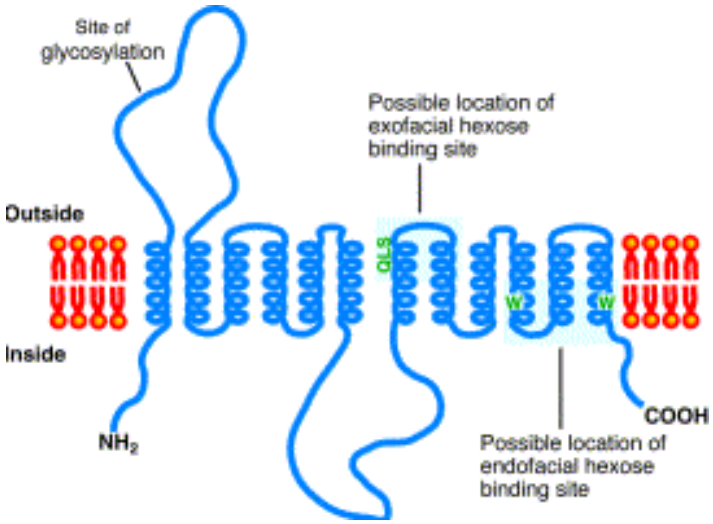

Figure 16 Structural model of major-facilitator transporters showing the 12 membrane-spanning helices (I–XII; numbered from N- to C-terminus). The regions thought to make up the exofacial and endofacial substrate-binding sites, together with important amino acid residues discussed in the text – notably the tryptophan residues involved in CB binding – and the glutamine residues thought to be important for substrate selection, are shown. (Wamsley *et al*., 1998)

For Glut1p, up to 22 essential residues have been characterised which are essential for the transport of glucose take place (Garcia *et al*., 1992, Mueckler *et al*., 1994, Mueckler *et al*., 1997, Kasahara *et al*., 1998, Seatter *et al*., 1998, Hurz *et al*., 1999, Klepper *et al*., 1999, Olsowski *et al*., 2000, Wang *et al*., 2000, Brockmann *et al*., 2001, Klepper *et al*., 2001, Mueckler *et al*., 2002, Pascual *et al*., 2002). As well, Asano *et al*., (1991) characterised the glycosylation site as N45.

Advancements in the structural characterization of the MFS proteins have led to the production of crystallographical data. Hirai *et al*., (2002) produced an electronic density map for the *Oxalobacter formigens* oxalate transporter OxlTp. It was determined that OxlTp possesses a 6.5 Å resolution. Additionally, two other constructed maps were elucidated for *E. coli* glycerol 3-phophate antiport (GlpTp) at a resolution of 3.3 Å, and the lactose permease proton Symporter (LacYp) at 3.5 Å (Huang *et al*., 2003, Abramson *et al*., 2003).

Through homology studies, models have been proposed for the structure of Glut1p, the most recent one being the model by Salas-Burgos *et al*., (2004) (PDB No. 1SUK). Using the three proteins already crystallized in the MFS family, conserved areas in the helical packaging were observed and thus gave confidence in the credibility of modelling other members of the family to these structures. Modelling needs to undergo extensive refinement and validation, and bioinformatics applications plays a large role herein. In

figures 18 and 19, graphical views are given on the model for Glut1p created by Salas-Burgos *et al*., (2004).

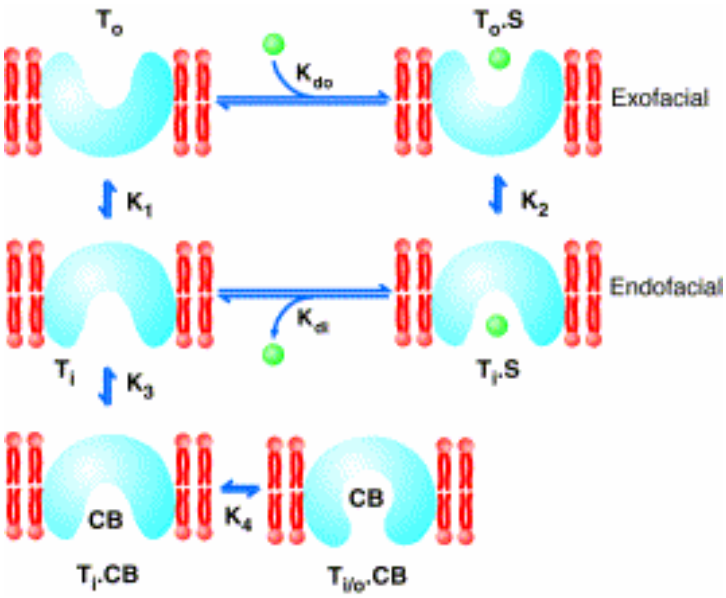

Figure 17 The model for the translocation cycle of a sugar molecule through Gult1p (blue). Reorientation takes place between exofacial (To) and endofacial (Ti) conformations in the presence and absence of the sugar molecule (green) (Wamsley *et al*., 1998).

Glut1p contains endofacial (inward facing) and exofacial (outward facing) sugar-binding sites (Wamsley *et al*., 1998). This forms the basis for the kinetic mechanism whereby the sugar molecule is moved through Glut1p. One model proposed the translocation of the sugar molecule between the endo- and exofacial sites while both sites are exposed simultaneously (Helgerson *et al*., 1987, Carruthers 1990). The more generally accepted model is that by Baldwin (1993), based on the principle that the transporter exists in two conformations where either the endo- or exofacial binding site is exposed. Movement of the sugar through the membrane occurs when the transporter oscillates between these two conformations (Figure 17). Residues crucial for transport of glucose are located in a channel segment between T310 and the Q161 (Salas-Burgos *et al*., 2004).

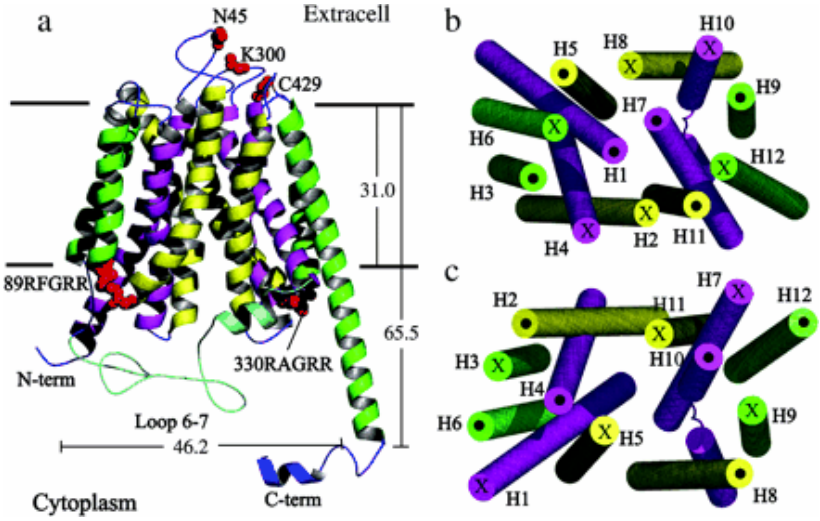

Figure 18 Side-view of Glut1p showing relative positions of the helices (A). Top view from the extracellular side showing the tilt of the 12 transmembrane helices (B). Cytoplasmic view; X, loops entering; dots, loops exiting (C) (Salas-Burgos *et al*., 2004).

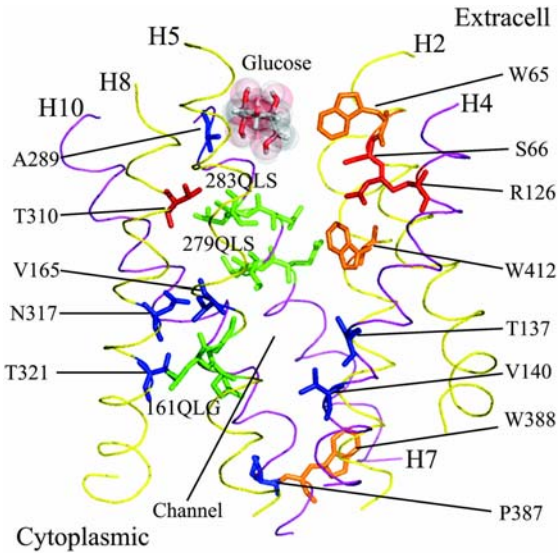

Figure 19 Glut1 side view showing the helical ribbons surrounding the putative channel. Residue shown as follows: crucial for transport (blue); tryptophans lining the channel (orange) with W388 and W412 crucial for transport; QLS and QLG motifs (green) (Salas-Burgos *et al*., 2004).

Hirai *et al*., (2002) developed a template for the symmetry of the helix arrangement of OxlTp. Glut1 was modelled according to this template (Figure 18C). This symmetry template gives an indication of the positions of the transmembrane helixes, but does not assign specific helix's numbers to those positions. Hirai *et al*., (2002) used molecular modelling to unravel the symmetry and in comparing it to the VIC family of transporters (Hvorup *et al*., 2003) showed that OxlTp might exibit four-fold symmetry. LacY and GlpT from *E. coli* show two-fold symmetry (Huang *et al*., 2003; Abramson *et al*., 2003). Glut1p

gives a very good putative view on how the yeast sugar transporters might look when seen three-dimensionally. The twelve transmembrane domain elements correlate well, and the intra- and extracellular loops can be related to the hydrophobicity plots of the yeast sugar transporters. Hopefully, future advances in chrystallography or the development of new methods to view membrane proteins can lead to a speedier solution for the unravelling of the structures of the yeast α-glucoside transporters.

5. YEAST SUGAR TRANSPORTERS

Yeast possesses a variety of membrane transporters that serve the (energy independent) uniport, symport, or antiport of ions or small (charged) metabolites, the (energy independent) uptake of nutrients, the energy-dependent (ATP-dependent) uptake or exchange of ions, or the efflux of toxic compounds. Several of these are bound to specific intracellular membranous compartments, such as mitochondria. For nutrient molecules, the yeast plasma membrane contains a large variety of transporters. This includes transporters for carbohydrates, amino acids and phospho-organic compounds. The primary nutrient requirement in the yeast cell is a carbohydrate source for the production of energy. A catalogue of yeast sugar transporters was compiled, and Table 3 contains the genes expressing these transporters (Guldener *et al*., 2005).

Table 3. Yeast sugar transporters (Guldener *et al*., 2005).

Gene name	ORF name	Brief description
HXT1	YHR094C	Low-affinity hexose facilitator
HXT2	YMR011W	Hexose facilitator of moderately low affinity for glucose
HXT3	YDR345C	Low-affinity hexose facilitator
HXT4	YHR092C	Hexose facilitator of moderately low affinity
HXT5	YHR096C	Hxt family protein with intrinsic hexose transport activity
HXT6	YDR343C	High-affinity hexose facilitator
HXT7	YDR342C	High-affinity hexose facilitator
HXT14	YNL318C	Protein of the sugar transporter superfamily – able to sustain slow growth on galactose
GAL2	YLR081W	Galactose permease
SNF3	YDL194W	Transporter-like sensor of low glucose concentrations
RGT2	YDL138W	Transporter-like sensor of high glucose concentrations
MAL61	YBR298C	Maltose permease
AGT1	YGR289C	Alpha-glucoside and trehalose permease
YFL040W	YFL040W	Protein of the sugar transporter superfamily – unknown biological function
ITR1	YDR497C	Inositol permease
ITR2	YOL103W	Inositol permease
GIT1	YCR098C	Glycerophosphoinositol transporter

Yeast sugar transporters belong to the major facilitator superfamily and to the sugar transporter family (TC 2.A.1.1). The MFS includes over a thousand sequenced members. They are characterized by having 12, 14 or 24 putative transmembrane α-helical domains. MFS proteins show affinity for a wide range of molecules, and they are found ubiquitously in all three kingdoms of living organisms. The MFS transport reactions can be categorised as shown in Table 4.

Table 4. MFS transport reactions (Nelissin *et al*.,1997).

MFS transport reaction	Action
Uniport	substrate (out) or substrate (in)
Symport	substrate (out) & (H^+ or Na^+) (out) or substrate (in) & (H^+ or Na^+) (in)
Antiport	substrate 1 (out) & substrate 2 (in) or substrate 1 (in) & substrate 2 (out) (substrate 1 being H^+ or a solute)

Yeast utilizes the monosaccharide glucose as a main carbon source. Glucose enters the cell and is incorporated into glycolysis to create energy for the cell. Another monosaccharide transported by the yeast cell is galactose. The transport system for glucose and galactose in yeast has been well documented. Collectively, they are known as the hexose transporters and make use of facilitated transport. These hexose transporters consist out of two structural units each having six transmembrane α-helixes connected by a cytoplasmic loop (Nelissin *et al*., 1997). Glucose transporters (*HXT1-7*) are also responsible for the transport of D-fructose, D-xylose and D-mannose. The galactose transporter (*GAL2*) also transports D-fucose and L-arabinose. (Lagunas 1993). Chimeric studies of Gal2p and Hxt2p showed that Tyr 446 is essential, and Trp 455 is important for galactose recognition by Gal2p (Nishizawa *et al*., 1995, Kasahara *et al*., 1996, Kasahara *et al*., 1997). Both of these residues are located in putative transmembrane domain 10 of Gal2p. The Phe 431 of Hxt2p corresponds to Tyr 446 of Gal2p and is critical for glucose transport (Kasahara and Maeda 1998). The Glut1p family demonstrated the importance of the aromatic amino acids. The residues important for glucose transport in the rat Glut1p is Trp 388 corresponding to Trp 455 of Gal2p, and Phe 379 corresponding to Tyr 446 of Gal2p (Kasahara and Kasahara 1998). In addition to Tyr 446, the aromatic sites Tyr 352 and Phe 504 of Gal2p are also critical for galactose transport (Kasahara and Kasahara 2000).

Kasahara *et al*., 2004 also investigated the structural differences between high and low affinity glucose transport in the Hxtp glucose transporters. The approach was to create chimeric transporters out of a combination of Hxt1p and Hxt2p transmembrane domains. It was found that at least TMDs 1, 5, 7, and 8 of Hxt2p are required for high affinity glucose transport. They also showed that Leu 201 in TMD 5 of Hxt2p is most important for high affinity transport, and that either Cys 195 or Phe 198 is required for maximal activity. Figure 20 indicates the characterized transmembrane domain segments where the relevant residues are highlighted.

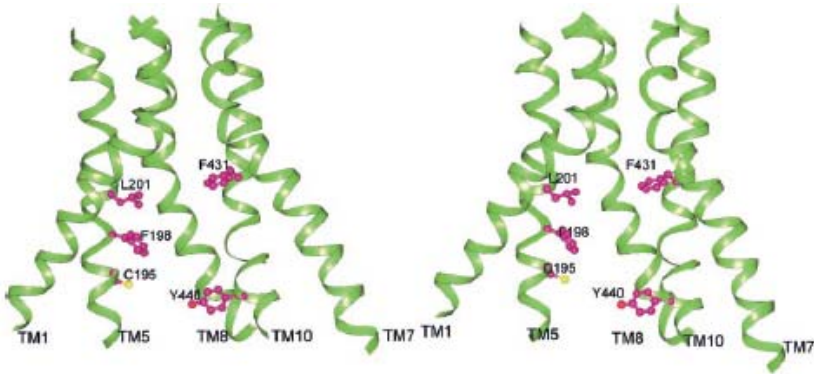

Figure 20 Putative locations of amino acid residues in TMDs 5 and 10 of Hxt2p important for high affinity, high capacity glucose transport activity. TMD 1 (residues 63–93), TMD 5 (residues 190–215), TMD 7 (residues 310–342), TMD 8 (residues 348–375), and TMD 10 (residues 417–444) are shown by ribbon depiction. Cys 195, Phe 198, and Leu 201 in TMD 5, as well as Phe 431 and Tyr 440 in TMD 10, are shown by ball-and-stick representation (Kasahara *et al*., 2004).

However, if we focus on the di- and trisaccharides, the ATP dependent system of symporters is used to transport these sugars. A proton gradient is essential and H^+ is used as cation in this mode of action. A symporter is a secondary active transporter binding two molecules at a time using the one's concentration gradient to force the other molecule against its own gradient. The α-glucoside transporters are classified as 12 transmembrane domain symporters that form part of the MFS. The α-glucosides are transported into the yeast by proteins that can collectively be named maltose transporters. All these transporters show mayor affinity for maltose and vary in their affinity for maltotriose and other α-alpha glucosides.

5.1 MALTOSE TRANSPORTERS

All α-glucosides are actively transported into yeast cells by a H^+-symport mechanism. Maltose transporters are the most studied of all the α-glucosides transporter systems in yeast. The first step in maltose utilization by yeast is driven by the maltose transporter, which catalyzes the transport of maltose into the cell. After entering the cell, the maltose is hydrolysed into two glucose molecules by a 1,4-α-glucosidase known as maltase. The main system for maltose utilization is the *MAL* locus. The *MAL* locus consists of three genes. They can vary in their arrangement, but in essence consist out of *MALx1* (maltose permease), *MALx2* (maltase) and *MALx3* (*MAL*-activator). The "x" denotes for the 5 unlinked *MAL* loci already described in yeast (*MAL1, MAL2, MAL3, MAL4*, and *MAL6*). The *MAL* loci are telomere associated and highly homologous, and are situated on Chromosome VII, III, II, XI, and VIII for *MAL1*, *MAL2*, *MAL3*, *MAL4*, and *MAL6* respectively (Table 5). One *MAL* locus is sufficient for the yeast to utilize maltose. The maltose permease and the maltase genes share a combined promoter region. The *MAL*-activator is, in turn, responsible to activate the expression of the other two genes. Maltose

utilization is inhibited and repressed by glucose and induced by maltose. The glucose repression is mediated by one of the main glucose repression pathways via the transcription factor Mig1p, which is binding to the promoter region of the maltose transport and maltase genes. The second way in which glucose influences the maltose system is through catabolite inactivation. The presence of glucose inactivates the maltose transporter in targeting it for inactivation, and eventual proteolysis in the vacuole (Lucero *et al*., 1993, Hicke *et al*., 1997, Brondijk *et al*., 1998, Medintz 2000). Attempts to determine the α-glucoside substrate range for the Malx1ps were inconclusive, often conflicting with reference to maltotriose uptake. However, it can be said that the Malx1p transporters show affinity for maltose and turanose. The affinity of Malx1p for maltose ranges at a K_m of 2 to 4 mM (Day *et al*., 2002). Other maltose transporters have also been characterised. The *AGT1*-encoded α-glucoside transporter I (Agt1p) of *S. cerevisiae* is a mutant allele of *MAL11,* which codes for a maltose permease (Han *et al*., 1995). *MAL11* is situated in the telomeric region of chromosome VII (Needleman 1991). Agt1p is part of the 12 transmembrane symporter families. This Symporter exhibits an active transport process that requires a proton gradient over the yeast membrane. It was shown that Agt1p has a K_m value (mM) of 4±0.7 for maltotriose and 5.1±6 for maltose (Day *et al*., 2002). Agt1p show affinity for maltose, turanose, isomaltose, alpha-methylglucoside, maltotriose, palatinose, trehalose and melezitose (Han *et al.,* 1995). Two other yeast maltose transporters that have been characterised are *MPH2* (YDL247w) and *MPH3* (YJR160c) (Day *et al*., 2002b). Mph2p and Mph3p have K_m values (mM) of 7.2±1.0 for maltotriose and 4.4±0.5 for maltose. *MTY1* of *Saccharomyces pastorianus* encodes a maltose transporter, which shows a higher affinity for maltotriose than for maltose (Salema-Oom *et al*., 2005). This unique characteristic was also reported for the *MTT1* gene (Dietvorst *et al*., 2005).

Table 5. Location on the chromosomes of the different MAL loci (Needleman 1991, Han *et al*., 1995, Day *et al*., 2002).

Band	Chromosome	*MAL* loci	*AGT1* loci	*MPH2*and *MPH3* loci
12	IV			*MPH2*
11A	XV			
11B	VII	*MAL1*	*AGT1*	
10A	XIII			
10B	XVI			
9	II	*MAL3*		
8	XIV			
7	X			*MPH3*
6	XI	*MAL4*		
5A	VIII	*MAL6*		
5B	V			
4	IX			
3	III	*MAL2*		
2	VI			
1	I			

Amongst all the α-glucoside transporters, Malx1p has been studied more extensively than the others, and is used as a reference in discussing the structure and functioning of this group of transporters.

5.1.1 The structural features of maltose transporters

Membrane proteins, like all other proteins, can be divided into a primary, secondary, and tertiary structure. As mentioned before, the first key to the structure of a protein is the amino acid sequence coded from the gene of origin. Many deductions can be made from this primary structure and it can serve as a tool to manipulate specific areas of a protein in order to unravel some clues to the roles these domains play in the final structure of the protein.

The maltose transporter is inactivated in the presence of glucose by proteolysis (Lucero *et al*.,1993). Proteolysis is an ongoing event and is used by the cell to keep the intracellular functioning intact by degrading the bulk of abnormal and unnecessary proteins. Throughout the cell, proteins destined for proteolysis firstly gets phosphorylated. This phosphorylated state acts as a signal for ubiquitination to take place. During ubiquitination, an ubiquitin molecule binds to the protein which gives the targeting signals for internalization into the endocytic pathway for subsequent degradation to take place (Hicke *et al*., 1997). Membrane proteins differ from cytosol proteins in that they are phosphorylated on serine residues to signal for ubiquitination, compared to the usual Lys-linked ubiquitination.

Brondijk *et al*., (1998) did a study to determine whether modifications in the Mal61p amino acid sequence influence the inactivation through proteolysis. This was done by removing putative protein kinase A and C phosphorylation sites. They constructed the three Mal61p mutants S295A, T363A, and S487A and observed significantly reduced rates of glucose inactivation. For T363A, the inactivation rate correlated with the protein degradation rate. For the S295A and S487A mutants, the reduction in protein degradation rates was much higher than the loss of activity. Their findings showed that some form of protein modification does take place prior to degradation of Mal61p. This modification already inactivates Mal61p, and that proteolytic breakdown does not necessarily follow inactivation. Another structural component found to be involved in membrane proteins are the ubiquitination signals known as PEST. A PEST sequence is a region rich in proline, glutamate, aspartate, serine, and threonine. Phosphorylation of the serine and threonine residues in a PEST sequence is associated with degradation signals. Medintz *et al.* (2000) further investigated Mal61p on amino acid level and showed that a 36 amino acid truncation creates a non fermentable phenotype. They also characterized an N-terminal PEST sequence at the 49-78 residue sequence, including a di-leucine motive at residues 69-70 of the cytoplasmic region (Figure 22). With reference to glucose induced inactivation, Gadura *et al*., (2006) made use of site-directed mutagenesis to investigate the specific role of residues, in particular the serine/theonine residues in the N-terminal region of Mal61p. The data showed that the phosphorylation of the serine/threonine residues in the 29 to 56 residue N-terminal area is involved in the delivering of the internalized Mal61p permease to the vacuole for degradation, but it is not required for glucose induced internalization.

No data of the other maltose transporters have been shown specifically on amino acid level and little is known about their inactivation signals. A basic method used for unravelling the first clue to transmembrane domain structure is making use of a hydrophobicity plot. Various bioinformatics tools exist to identify the highly hydrophobic regions in an amino acid sequence. In the case of the transmembrane proteins, these sequences are indicative of the domains where the protein spans the membrane. Figure 21 shows a hydrophobicity plot for Agt1p.

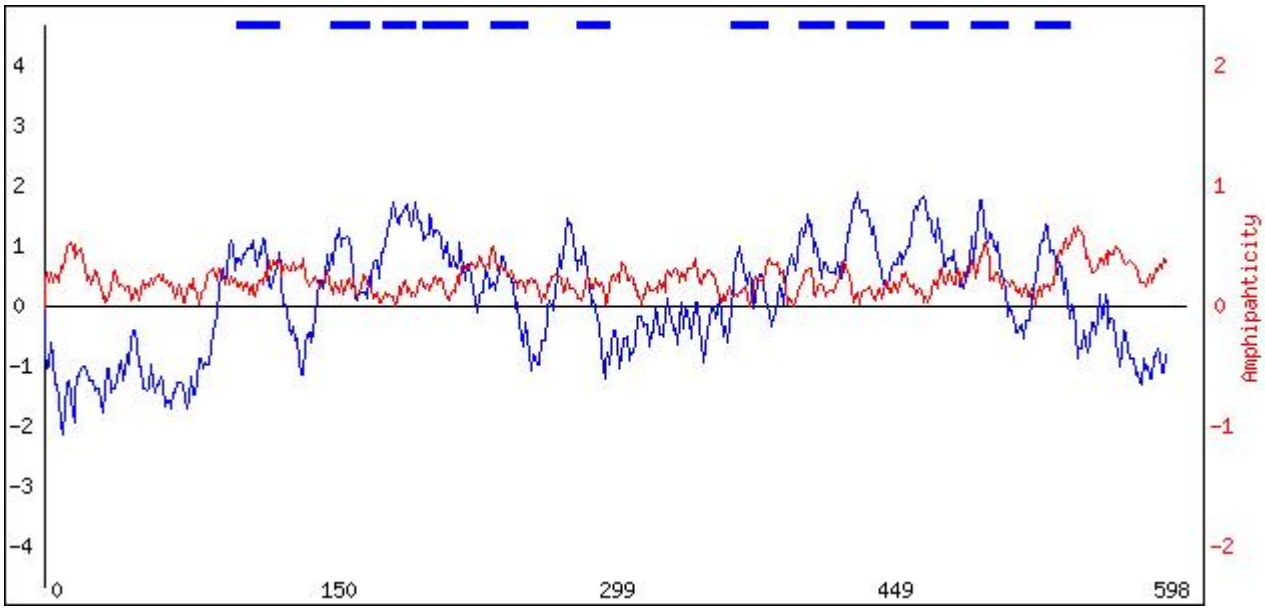

Figure 21 A hydrophobicity plot for Agt1p showing the hydropathy (blue), hydrophobic moment or amphipathicity (red) and the putative transmembrane segments (blue bars) (Saier 2007).

The transmembrane domain regions can also be determined through bioinformatical tools available on the worldwide web. The predictions of the different tools vary to some extent. According to the Saier (2000) classification system, the maltose transporters are seen as 12 transmembrane domain spanners. Han *et al*., (1995) also originally characterised Agt1p as a 12 transmembrane domain protein. In Figure 22, an alignment of the main maltose transporters is given with the putative transmembrane domains as 12 spanners. The residue previously identified for the hexose transporter in yeast can be correlated with the maltose transport sequences. It is well documented that phenolic residues are essential in sugar recognition. In observing the transmembrane domain segments of the maltose transporters, many Tyr, Phe and Tyr residues can be observed. The charged residues Asp, Glu, Lys, Arg, and His, are also present in the channel regions, and can assist in the movement of the sugar molecule through the channel. It should always be taken into consideration that all residues inside the channel with relative proximity to each other can interact and contribute to the transport process. Taking this interaction into account, it is not simple to identify possible residues, and the

combined effect of these interactions might be difficult to predict. Single amino acids can, however, be critical and essential for transport. Further structural evaluation on the exact position of the transmembrane domains of the maltose transporters will assist in determining which amino acid residue could play a role in substrate recognition. Mutational studies on the effect of these phenolic residues are therefore necessary before any conclusions can be made. Further structural analysis is required to fully characterize this group, and this can lead to determining their precise functioning. Information pertaining to this can lead to the successful manipulation of these proteins and enhance their energy efficiency, accurately determining the sugars required, and optimize the entirety of the transport system that contains many obstacles for the industries relying on this information.

```
Agt1p    MKNIISLVSKKKAASKNEDKNISESSRDIVNQQEVFNTEDFEEGKKDSAFELDHLEFTTNSAQLGDSDEDNENVINEMNATDDANEANSEEKSMTLKQAL
Mal31p   MKGLSSLINRKKDRNDSHLDEIENGVN-----ATEFNSIEMEEQGKKSDFDLSHLEYGPGSLIPNDNNE--EVPDLLDEAMQDAKEADESERGMPLMTAL
Mty1p    MKGLSSLINRKKDRNDSHLDEIENGVN-----ATEFNSIEMEEQGKKSDFDLSHHEYGPGSLTPNDNNE--EVPDLLDEAMQDAKEADESERGMPLMTAL
Mph23p   MKNLSFLINRRKE-NTSDSNVYPGKAK-----SHEPSWIEMDDQTKKDGLDIVHVEFSPDTRAPSDSNK---VITEIFDATEDAKEADESERGMPLATAL
         **.:  *:.::*  . .. .      .          .  ::::  *.. ::: * *: ..:   .*.::         :* :**:**:..*:.*.*  **
Agt1p    LKYPKAALWSILVSTTLVMEGYDTALLSALYALPVFQRKFGTLNG-EGSYEITSQWQIGLNMCVLCGEMIGLQITTYMVEFMGNRYTMITALGLLTAYIF
Malx1p   KTYPKAAAWSLLVSTTLIQEGYDTAILGAFYALPVFQKKYGSLNSNTGDYEISVSWQIGLCLCYMAGEIVGLQMTGPSVDYMGNRYTLIMALFFLAAFIF
Mty1p    KTYPKAAAWSLLVSTTLIQEGYDTAILGSFYALPVFQKKYGSLNSNTGDYEISASWQIGLSLCVTAGEIVGLQMTGPFVDYMGNRYTLILALILLAAFTF
Mph23p   NTYPKAAAWSLLVSTTLIMEGYDTAILGAFYALPIFQRKFGSQNDKTGEWEISASWQIGLTLCYMAGEIVGLQLTGPSVDLVGNRYTLIIALFFLAAFTF
          .***** **:******: ******:*.::****:**:*:*: *.  *.:**: .***** :*  .**::***:*   *: :*****:* ** :*:*: *
Agt1p    ILYYCKSLAMIAVGQILSAIPWGCFQSLAVTYASEVCPLALRYYMTSYSNICWLFGQIFASGIMKNSQENLGNSDLGYKLPFALQWIWPAPLMIGIFFAP
Mal31p   ILYFCKSLGMIAVGQALCGMPWGCFQCLTVSYASEICPLALRYYLTTYSNLCWAFGQLFAAGIMKNSQNKYPNSDLGYKLPFALQWIWPLPLAVGIFFAP
Mty1p    ILYFCKGLGMIAVGQVLCGMPWGCFQCLTVSYASEICPMALRYYLTTYSNLCWTFGQLFAAGIMKNSQNKYPNSELGYKLPFALQWIWPAPLAIGIFFAP
Mph23p   ILYFCNSLGMIAVGQALCGMPWGCFQCLTVSYASEICPLALRYYLTTYSNLCWLFGQLFAAGIMKNSQKKYADSELGYKLPFALQWILPVPLALGIFFAP
         ***:*:.*.****** *..:******.*:*:****:**:*****:*:***:** ***:**:*******::  :*:************ * ** :******
Agt1p    ESPWWLVRKDRVAEARKSLSRILSGKGAEKDIQVDLTLKQIELTIEKERLLASKSGSFFNCFKG-VNGRRTRLACLTWVAQNSSGAVLLGYSTYFFERAG
Mal31p   ESPWWLVKKGRIDQARRSLERTLSGKGPEKELLVSMELDKIKTTIEKEQKMSD-EGTYWDCVKDGINRRRTRIACLCWIGQCSCGASLIGYSTYFYEKAG
Mty1p    ESPWWLVKKGRIDQARRSLERTLSGKGPEKELLVSMELDNIKVTIEKEKKLSDSEGSYWDCLKDSVNRRRTRIACLCWVGQTTCGTSLIGNSTYFYEKAG
Mph23p   ESPWWLVKKGRFDEARRSLRRTLSGKGPEKEILVTLEVDKIKVTIDKEKRLTSKEGSYSDCFEDKINRRRTRITCLCWAGQATCGSILIGYSTYFYEKAG
         *******:*.*. :**:** * *****.**:: * : :.:*: **:**: ::. .*:: :*.:. :* ****::** * .* :.*: *:* ****:*:**
Agt1p    MATDKAFTFSLIQYCLGLAGTLCSWVISGRVGRWTILTYGLAFQMVCLFIIGGMGFGSGSSASNGAGGLLLALSFFYNAGIGAVVYCIVAEIPSAELRTK
Mal31p   VSTDTAFTFSIIQYCLGIAATFVSWWASKYCGRFDLYAFGLAFQAIMFFIIGGLGCSDTHGAKMGSGALLMVVAFFYNLGIAPVVFCLVSEIPSSRLRTK
Mty1p    VGTDTAFTFSIIQYCLGIAATFLSWWASKYFGRFDLYAFGLAIQTVSLFIIGGLGCSDSHGAEMGSGSLLMVLSFFYNLGIAPVVFCLVSEIPSSRLRTK
Mph23p   VSTEMSFTFSIIQYCLGICATFLSWWASKYFGRYDLYAFGLAFQTIVFFIIGGLGCSSTHGSKMGSGSLLMAVAFFYNLGIAPVVFCLVSEMPSSRLRTK
         :.*: :****:******:..*: **  *   **: : ::***:* : :*****:* ..  .:. *:*.**:.::**** **..**:*:*:*:**:.****
Agt1p    TIVLARICYNLMAVINAILTPYMLNVSDWNWGAKTGLYWGGFTAVTLAWVIIDLPETTGRTFSEINELFNQGVPARKFASTVVDPFGKGKTQHDSLADES
Mal31p   TIILARNAYNVIQVVVTVLIMYQLNSEKWNWGAKSGFFWGGFCLATLAWAVVDLPETAGRTFIEINELFRLGVPARKFKSTKVDPFAAAKAAAAEINVKD
Mty1p    SIILARNAYNMASIVTTVLIMYQLNSEKWNWGAKSGFFWGGLCFATLVWAVIDLPETAGRTFIEINELFRLGVPARKFKSTKVDPFAAAKAAAAEINVKD
Mph23p   TIILARNTYNVVSIICSVLILYQLNSKKWNWGAKSGFFWGVLCFCTLIWAVVDLPETAGKTFVEINELFKLGVSARKFKSTKVDPFVVKTPPKDVSHN-D
         :*:***  **:  :: ::*  * ** ..******:*::** :   ** *.::*****:*:** ******. **.**** ** ****   ..       .
Agt1p    ISQSSSIKQRELNAADKC----
Mal31p   PKEDLETSVVDEGRSTPSVVNK
Mty1p    PKEDLETSVVDEGRSTPSVVNK
Mph23p   PKGDIEASIAEE----------
          . . . .  :
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Figure 22 Alignment of the amino acid sequences of Agt1p, Mal31p, Mty1p and Mph23p. Mutants observed in literature are indicated in blue and underlined with a dotted line. Putative transmembrane domains are underlined as predicted by the Transport classification website (Saier 2007) and are shown in blue. The sequence data was also retrieved from the Transport classification website. The alignment was performed through ClustalW application.

6. SUMMARY

The α-glucosides are groups of sugar molecules that impact on the very important beverage, baking and the rapidly growing biofuel production industries. They are released during starch degradation can be utilized by yeast cells in order to produce ethanol and carbon dioxide during fermentation. This basic reaction of sugar conversion to ethanol is very efficiently performed by *S. cerevisiae*; it is, however, not without the scope for further optimisation, as quality enhancement and cost-effectiveness are critical factors considered in the aforementioned industries. Ways to enhance these production systems include the simultaneous fermentation of all sugars present in the different source materials; the total consumption of all available sugars; the enhancement of the speed at which fermentation occurs, and; the simultaneous fermentation and saccharification (degradation) of starch. These goals can all be achieve through the adaptation of *S. cerevisiae*.

Along with the age-old method of breeding, biotechnological techniques have been developed to improve yeast strains and to customize them to satisfy specific requirements. In the process of starch degradation, a great deal of attention has been given in developing amylolytic yeast strains that can degrade starch through the release of α-amylases and glucoamylases, and the simultaneous fermentation of sugars into ethanol. The motivation behind this drive is the reduction in degradation time and the use of one fermentation tank for the whole production process, resulting in a dramatic reduction in the cost of production. Whilst no single strain meets all of the requirements, great strides have been made in the development of micro-organisms for industrial ethanol production. Except for the successful secretion of effective saccharification enzymes, it is very important for ethanol production to efficiently transport the smaller sugar molecules into the yeast for utilization purposes. It is therefore critical to optimise the effectiveness of the transport proteins situated in the yeast cell membrane. Sugar transport is an acknowledged bottleneck in many sugar utilization processes, as incomplete fermentation of sugars leads to a reduced ethanol yield and increased residual carbohydrate contamination in the final product. While effective transporters exist for the favoured *S. cerevisiae* sugar glucose, a great need exists to improve the specificity of sugar transporters for the α-glucosides and to eliminate the effects of glucose repression on them. In this regard, efforts to unravel the functioning and structure of the transport proteins remain critical and highly topical. Through identifying PEST sequences (protein degradation sites) and sugar recognition sites, proteins can be engineered to overcome the effects of glucose inactivation and the inability to transport all α-glucosides simultaneously. All these modifications potentially lead to the improvement of maltotriose utilization and the solving of the post fermentation dilemma of residual sugars. Through mutagenesis studies on specific amino acid residues, the effect of inhibitors, and the evaluation of the effect of protein degradation, much can be revealed about the structural and functional attributes of the α-glucoside transporters.

Since genetically engineered strains (GMO's) might not be accepted in certain markets and industries for differing reasons, it is an incredible tool to 'program' DNA and direct cellular machinery in order to obtain products that were unimaginable a few years ago. The challenge is to redirect genetic and cellular machinery to manufacture economically critical molecules for application in environments that exhibit specific biotechnological challenges. One such application is the production of an amylolytic *S. cerevisiae* strain containing specific non repressible α-glucosides transporters that are not sensitive to inactivation. A better understanding of the structural components of these transporters is critical in unravelling the modifications necessary for adapting these transporters. Sequence and domain characterization of the amino acid residues are crucial steps in reaching this goal. Recently Betzig *et al*., (2006) reported on a method to optically image intracellular proteins at nanometer-spatial resolution. The development of these and similar new methods might lead to valuable insight into transport proteins which, in the future, can lead to a more direct evaluation of α-glucoside transporters and their three-dimensional functioning.

7. REFERENCES

Abramson J., Smirnova I., Kasho V., Verner G., Kaback H.R., Iwata S. (2003). Structure and mechanism of the lactose permease of *Escherichia coli.* Science. 301: 610-615.

Asano T., Katagiri H., Takata K., Lin J.L., Ishihara H., Inukai K., Tsukuda K., Kikuchi M., Hirano H., Yazaki Y., et al. (1991). The role of N-glycosylation of *GLUT1* for glucose transport activity. J. Biol. Chem. 266: 24632-24636.

Audesirik T, Audesirik G. (1999) Biology, Life on Earth. Prentice Hall, New Jersey.

Baldwin S.A. (1993). Mammalian passive glucose transporters: members of an ubiquitous family of active and passive transport proteins. Biochem. Biophys. Acta. 1154: 17-49.

Bamforth C.W. (2000). Review Brewing and brewing research: past, present and future. Journal of the Scienceof Food and Agriculture. 80:1371-1378.

Baron S. (2004). Medical Microbiology. 4th edition. The University of Texas Medical Branch.

Berneche S., Roux B. (2001). Energetics of ion conduction through the K+ channel. Nature. 414: 73-77.

Betzig E., Patterson G.H., Sougrat R., Lindwasser O.W., Olenych S., Bonifacino J.S., Davidson M.W., Lippincott-Schwartz J., Hess H.F. (2006). Imaging intracellular fluorescent proteins at nanometer resolution. Science. 313: 1642-1645

Bothast R.J., Schlicher M.A. (2005). Biotechnological processes for conversion of corn into ethanol. Appl. Microbiol. Biotechnol. 67: 19-25.

Brockmann K., Wang D., Korenke C.G., Moers A.V., Ho Y.Y., Pascual J.M., Kuang K., Yang H., Ma L., Kranz-Eble P., Fischbarg J., Hanefeld F., Vivo D.C.D. (2001). Autosomal dominant Glut-1deficiency syndrome and familial epilepsy. Ann. Neurol. 50: 476-485.

Brondijk T.H., Van Der Rest M.E., Pluim D., De Vries Y., Stingl K., Poolman B., Konings W.N. (1998). Catabolite inactivation of wild-type and mutant maltose transport proteins in *Saccharomyces cerevisiae*. J. Biol. Chem. 273: 15352-15357.

Campbell M.K., Farrell S.O. (2006). Biochemistry. Thomson Brooks/Cole.

Carruthers A. (1990). Facilitated diffusion of glucose. Physiol. Rev. 70: 1135-1176.

Carter C., Sweet R. (1997). Methods in Enzymology. Volume 276. Academic Press. New York.

Corke H., Cross N., Nip W., Hui Y.H. (2006). Bakery Products: Science and Technology. 1st edition. Blackwell Publishing Professional.

Day R.E., Higgins V.J., Rogers P.J., Dawes I.W. (2002). Characterization of the putative maltose transporters encoded by YDL247w and YJR160c. Yeast. 19: 1015-1027.

Day R.E., Rogers P.J., Dawes I.W., Higgins V.J. (2002). Molecular analysis of maltotriose transport and utilization by *Saccharomyces cerevisiae*. Appl. Environ. Microbiol. 68: 5326-5335.

Dietvorst J., Londesborough J., Steensma H.Y. (2005). Maltotriose utilization in lager yeast strains: *MTT1* encodes a maltotriose transporter. Yeast. 22: 775-788.

DiPardo J. (2000). Outlook for biomass ethanol production and demand. Energy Information Administration, US Department of Energy. Washington D.C.

Engelman D.M., Steize T.A. Goldman A. (1986) Identifying nonpolar transbilayer helices in amino acid sequences of membrane proteins, Annu. Rev. Biophys. Biophys. Chem. 15: 321–353.

Engelman D.M., Chen Y., Chin C.N., Curran A.R., Dixon A.M., Dupuy A.D., Lee A.S., Lehnert U., Matthews E.E., Reshetnyak Y.K., Senes A., Popot J.L. (2003). Membrane protein folding: beyond the two stage model. FEBS Lett. 555: 122-125.

Gadura N., Michels C.A. (2006). Sequences in the N-terminal cytoplasmic domain of *Saccharomyces cerevisiae* maltose permease are required for vacuolar degradation but not glucose-induced internalization. Curr. Genet. 50: 101-114.

Garcia J.C., Strube M., Leingang K., Keller K., Mueckler M.M. (1992). Amino acid substitutions at tryptophan 388 and tryptophan 412 of the HepG2 (Glut1) glucose transporter inhibit transport activity and targeting to the plasma membrane in Xenopus oocytes. J. Biol. Chem. 11: 7770-7776

Gray K.A., Zhao L., Emptage M. (2006). Bioethanol. Curr. Op. Chem. Biol. 10: 141-146.

Guldener U., Munsterkotter M., Kastenmuller G., Strack N., Van Helden J., Lemer C., Richelles J., Wodak S.J., Garcia-Martinez J., Perez-Ortin J.E., Michael H., Kaps A., Talla E., Dujon B., Andre B., Souciet J.L., De Montigny J., Bon E., Gaillardin C., Mewes H.W. (2005). CYGD: the Comprehensive Yeast Genome Database. Nucleic Acids Res. 33: D364-368.

Han E.K., Cotty F., Sottas C., Jiang H., Michels C.A. (1995). Characterization of *AGT1* encoding a general alpha-glucoside transporter from *Saccharomyces*. Mol. Microbiol. 17: 1093-1107.

Helgerson A.L., Carruthers A. (1987). Equilibrium ligand binding to the human erythrocyte sugar transporter. Evidence for two sugar-binding sites per carrier. J. Biol. Chem. 262: 5464-5475.

Hicke L., Zanolari B., Pypaert M., Rohrer J., Riezman H. (1997). Transport through the yeast endocytic pathway occurs through morphologically distinct compartments and requires an active secretory pathway and Sec18p/N-ethylmaleimide-sensitive fusion protein. Mol. Biol. Cell. 8: 13-31.

Hirai T., Heymann J.A., Shi D., Sarker R., Maloney P.C., Subramaniam S. (2002). Three-dimensional structure of a bacterial oxalate transporter. Nat. Struct. Biol. 9: 597-600.

Hough J.S., Briggs D.E., Stevens R., Young I.W. (1981). Malting and brewing sciences. Cambridge: Cambridge University Press.

Hresko R.C., Kruse M., Strube M., Mueckler M. (1994). Topology of the Glut 1 glucose transporter deduced from glycosylation scanning mutagenesis. J. Biol. Chem. 269: 20482-20488.

Hruz P.W., Mueckler M.M. (1999). Cysteine-scanning mutagenesis of transmembrane segment 7 of the *GLUT1* glucose transporter. J. Biol. Chem. 274: 36176-36180.

Hruz P.W., Mueckler M.M. (2001). Structural analysis of the *GLUT1* facilitative glucose transporter (review). Mol. Membr. Biol. 18: 183-193.

Huang Y., Lemieux M.J., Song J., Auer M., Wang D.N. (2003). Structure and mechanism of the glycerol-3-phosphate transporter from *Escherichia coli*. Science. 301: 616-620.

Hunt D. (1981). The gasohol handbook. New York: Industrial New York.

Hvorup R., Chang A.B., Saier M.H., Jr. (2003). Bioinformatic analyses of the bacterial L-ascorbate phosphotransferase system permease family. J. Mol. Microbiol. Biotechnol. 6: 191-205.

Inlow D., Mcrae J., Ben-Bassat A. (1988). Fermentation of corn starch to ethanol with genetically engineered yeast. Biotechnol. Bioeng. 32: 227-234.

Insel P., Turner E., Ross D. (2005). Discovering nutrition. 2nd edition. The Human Body: From food to Fuel. American dietetic association. Jones and Bartlett publishers. Boston.

Johnson G.B., Raven P.H. (2002). Biology. Sixth edition. New York. USA. McGraw-Hill.

Kaback H.R. (2005). Structure and mechanism of the lactose permease. C. R. Biol. 328: 557-567.

Kasahara M., Maeda M. (1998). Contribution to substrate recognition of two aromatic amino acid residues in putative transmembrane segment 10 of the yeast sugar transporters Gal2 and Hxt2. J. Biol. Chem. 273: 29106-29112.

Kasahara M., Shimoda E., Maeda M. (1996). Transmembrane segment 10 is important for substrate recognition in Ga12 and Hxt2 sugar transporters in the yeast *Saccharomyces cerevisiae*. FEBS Lett. 389: 174-178.

Kasahara M., Shimoda E., Maeda M. (1997). Amino acid residues responsible for galactose recognition in yeast Gal2 transporter. J. Biol. Chem. 272: 16721-16724.

Kasahara T., Ishiguro M., Kasahara M. (2004). Comprehensive Chimeric Analysis of Amino Acid Residues Critical for High Affinity Glucose Transport by Hxt2 of *Saccharomyces cerevisiae*. J. Biol. Chem. 279: 30274–30278.

Kasahara T., Kasahara M. (1998). Tryptophan 388 in putative transmembrane segment 10 of the rat glucose transporter Glut1 is essential for glucose transport. J. Biol. Chem. 273: 29113-29117.

Kasahara T., Kasahara M. (2000). Three aromatic amino acid residues critical for galactose transport in yeast Gal2 transporter. J. Biol. Chem. 275: 4422-4428.

Klepper J., Fischbarg J., Vera J.C., Wang D., De Vivo D.C. (1999). *GLUT1*-deficiency: barbiturates potentiate haploinsufficiency in vitro. Pediatr. Res. 46: 677-683.

Klepper J., Monden I., Guertsen E., Voit T., Willemsen M., Keller K. (2001). Functional consequences of the autosomal dominant G272A mutation in the human *GLUT1* gene. FEBS Lett. 498: 104-109.

Klis F.M., Boorsma A., Groot P.W.J.D. (2006). Cell wall construction in *Saccharomyces cerevisiae*. Yeast. 23: 185–202.

Kovarik B. (1998). Henry Ford, Charles F. Kettering and the fuel of the future. Automot. Hist. Rev. 32: 7-27.

Lagunas R. (1993). Sugar transport in *Saccharomyces cerevisiae*. FEMS Microbiol. Rev. 10: 229-242.

Lindegren, C.C. (1949). The yeast cell, its genetics and cytology. Educational Publishers. St. Louis.

Lucero P., Herweijer M., Lagunas R. (1993). Catabolite inactivation of the yeast maltose transporter is due to proteolysis. FEBS Lett. 333: 165-168.

Medintz I., Wang X., Hradek T., Michels C.A. (2000). A PEST-like sequence in the N-terminal cytoplasmic domain of *Saccharomyces* maltose permease is required for glucose-induced proteolysis and rapid inactivation of transport activity. Biochemistry. 39: 4518-4526.

Mueckler M., Kruse M., Strube M., Riggs A.C., Chiu K.C., Permutt M.A. (1994). A mutation in the Glut2 glucose transporter gene of a diabetic patient abolishes transport activity. J. Biol. Chem. 269: 17765-17767.

Mueckler M., Makepeace C. (1997). Identification of an amino acid residue that lies between the exofacial vestibule and exofacial substrate-binding site of the Glut1 sugar permeation pathway. J. Biol. Chem. 272: 30141-30146.

Mueckler M., Makepeace C. (2002). Analysis of transmembrane segment 10 of the Glut1 glucose transporter by cysteine-scanning mutagenesis and substituted cysteine accessibility. J. Biol. Chem. 277: 3498-3503.

Nakamura Y., Kobayashi F., Ohnaga M., Sawada T. (1997). Alcohol fermentation of starch by a genetic recombinant yeast having glucoamylase activity. Biotechnol. Bioeng. 53: 21-25.

Needleman R. (1991). Control of maltase synthesis in yeast. Mol. Microbiol. 5: 2079-2084.

Nelissen B., De Wachter R., Goffeau A. (1997). Classification of all putative permeases and other membrane plurispanners of the major facilitator superfamily encoded by the complete genome of *Saccharomyces cerevisiae*. FEMS Microbiol. Rev. 21: 113-134.

Nikaido H. (2003). Molecular basis of bacterial outer membrane permeability revisited. Microbiol. Mol. Biol. Rev. 67: 593-656.

Nishizawa K., Shimoda E., Kasahara M. (1995). Substrate recognition domain of the Gal2 galactose transporter in yeast *Saccharomyces cerevisiae* as revealed by chimeric galactose-glucose transporters. J. Biol. Chem. 270: 2423-2426.

Olsowski A., Monden I., Krause G., Keller K. (2000). Cysteine scanning mutagenesis of helices 2 and 7 in GLUT1 identifies an exofacial cleft in both transmembrane segments. Biochemistry. 39: 2469-2474.

Pascual J.M., Van Heertum R.L., Wang D., Engelstad K., De Vivo D.C. (2002). Imaging the metabolic footprint of Glut1 deficiency on the brain. Ann. Neurol. 52: 458-464.

Pauling L., Corey R., Branson H. (1951). The structure of proteins: two hydrogen-bonded helical configurations of the polypeptide chain. PNAS, 37: 205-211.

Perutz M.F., Randall J.T., Thomson L., Wilkins M.H., Watson J.D. (1969). DNA helix. Science. 164: 1537-1539.

Popot J.L., Engelman D.M., Zaccai G., Vitry C.D. (1990). The "microassembly" of integral membrane proteins: applications & implications. Prog. Clin. Biol. Res. 343: 237-262.

Raimbault M. (1998). General and microbiological aspects of solid substrate fermentation. EJB Electr. J. Biotechnol. 1: 174-188.

Reilly D.I., O'Cleirigh C., Walsh P.K. (2004). Laboratory-scale production of High-gravity wort suitable for a broad variety of research applications. J. Am. Soc. Brew. Chem. 62:23-28.

Rhodes G. (1993). Crystallography Made Crystal Clear. San Diego. Academic Press.

Robertson G.H., Wong D.W., Lee C.C., Wagschal K., Smith M.R., Orts W.J. (2006). Native or raw starch digestion: a key step in energy efficient biorefining of grain. J. Agric. Food Chem. 54: 353-365.

Roux B., Schulten K. (2004). Computational studies of membrane channels. Structure. 12: 1343-1351.

Russell I., Bamforth C., Stewart G. (2003). Whisky: Technology, Production and Marketing. 1st edition. Academic press.

Saier M.H., Jr. (1998). Molecular phylogeny as a basis for the classification of transport proteins from bacteria, archaea and eukarya. Adv. Microb. Physiol. 40: 81-136.

Saier M.H., Jr. (2000). Families of proteins forming transmembrane channels. J. Membr. Biol. 175: 165-180.

Saier M.H., Jr. (2000). Families of transmembrane sugar transport proteins. Mol. Microbiol. 35: 699-710.

Saier M.H., Jr. (2000). A functional-phylogenetic classification system for transmembrane solute transporters. Microbiol. Mol. Biol. Rev. 64: 354-411.

Saier M.H., Jr. (2007). Transport classification website. http://www.tcdb.org/tcdb. Division of Biological Sciences, University of California San Diego, La Jolla.

Salas-Burgos A., Iserovich P., Zuniga F., Vera J.C., Fischbarg J. (2004). Predicting the three-dimensional structure of the human facilitative glucose transporter glut1 by a novel evolutionary homology strategy: insights on the molecular mechanism of substrate migration, and binding sites for glucose and inhibitory molecules. Biophys. J. 87: 2990-2999.

Salema-Oom M., Valadao Pinto V., Goncalves P., Spencer-Martins I. (2005). Maltotriose utilization by industrial *Saccharomyces* strains: characterization of a new member of the alpha-glucoside transporter family. Appl. Environ. Microbiol. 71: 5044-5049.

Schirmer T., Keller T.A., Wang Y.F., Rosenbusch J.P. (1995). Structural basis for sugar translocation through maltoporin channels at 3.1 A resolution. Science. 267: 512-514.

Seatter M.J., De La Rue S.A., Porter L.M., Gould G.W. (1998). QLS motif in transmembrane helix VII of the glucose transporter family interacts with the C-1 position of D-glucose and is involved in substrate selection at the exofacial binding site. Biochemistry. 37: 1322-1326.

Singer S.J., Nicolson G.L. (1972). The fluid mosaic model of the structure of cell membranes. Science. 175: 720-731.

Tajkhorshid E., Nollert P., Jensen M.O., Miercke L.J., O'connell J., Stroud R.M., Schulten K. (2002). Control of the selectivity of the aquaporin water channel family by global orientational tuning. Science. 296: 525-530.

Van Der Rest M.E., De Vries Y., Poolman B., Konings W.N. (1995). Overexpression of Mal61p in *Saccharomyces cerevisiae* and characterization of maltose transport in artificial membranes. J. Bacteriol. 177: 5440-5446.

Walker G. (1998). Yeast Physiology and Biotechnology. Chichester. John Wiley & Sons.

Walmsley A.R., Barrett M.P., Bringaud F., Gould G.W. (1998). Sugar transporters from bacteria, parasites and mammals: structure-activity relationships. Trends. Biochem. Sci. 23: 476-481.

Wang D., Kranz-Eble P., De Vivo D.C. (2000). Mutational analysis of *GLUT1* (SLC2A1) in Glut-1 deficiency syndrome. Hum. Mutat. 16: 224-231.

White S.H. (1999). Membrane Protein Folding and Stability: Physical Principles. Ann. Rev. Biophy. Biomol. Struc. 28: 319-365

Zastrow C.R., Hollatz C., De Araujo P.S., Stambuk B.U. (2001). Maltotriose fermentation by *Saccharomyces cerevisiae*. J. Ind. Microbiol. Biotechnol. 27: 34-38.

Chapter 3

RESEARCH RESULTS

Differences among *AGT1*-encoded α-glucoside transporters and their ability to transport maltotriose in *Saccharomyces yeasts*

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DIFFERENCES AMONG *AGT1*-ENCODED α-GLUCOSIDE TRANSPORTERS AND THEIR ABILITY TO TRANSPORT MALTOTRIOSE IN *SACCHAROMYCES YEASTS*

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1. ABSTRACT

Improved fermentation of starch and its dextrin products would benefit the brewing and whiskey industries. Most strains of *Saccharomyces* ferment glucose and maltose and partially ferment maltotriose, but are unable to utilise the larger dextrin products of starch. This utilisation pattern is partly attributed to the ability of yeast cells to transport the aforementioned mono-, di- and trisaccharides into the cytosol. The maltotriose transporting efficiency varies between different *Saccharomyces* strains. In this study, several *Saccharomyces* strains, including whiskey strains, were screened for growth on maltotriose. The *AGT1* genes, which encode a maltose transporter that show affinity for maltotriose uptake, were isolated from the strains that grew strongest in media with maltotriose as sole carbon source. The isolated *AGT1* alleles were sequenced and their chromosomal locations determined in the strains from which they were cloned. Nucleotide and deduced amino acid sequences of the isolated genes shared 95% and 98% identity, respectively. The efficiency of maltotriose transport was determined by expressing the *AGT1* variants in an identical genetic background. The *K*m values obtained for all the permeases were very similar (≈ 3), but the permease with improved performance for maltotriose transport showed an approximately 30% higher *V*max value than for the others. The data obtained suggest that the genetic variation among the *AGT1*-encoded transporters is reason for the variation in maltotriose transport efficiency among different *Saccharomyces* strains. This study offers prospects for the development of yeast strains with improved maltose and maltotriose uptake capabilities that, in turn, could increase the overall fermentation efficiencies in the beer and whiskey industries.

2. INTRODUCTION

Slow and incomplete yeast fermentations represent a considerable economic loss for the producers of alcoholic beverages. The time needed to complete a fermentation cycle is a major determinant of productivity and cost efficiency; a faster fermentation rate results in lower running costs, greater flexibility, and the total fermenter volume needed to obtain a

targeted production volume is dramatically reduced (Verstrepen *et al.,* 2006). Equally important is the extent and completeness with which the yeast cells convert the raw material into the end product; the more carbohydrates that remain unfermented, the higher the residual sugars and the lower the cost-efficiency of the fermentation process. It is therefore not surprising that the primary selection criteria applied to most strain development programs relate to the overall objective of improving the fermentation performance of yeast strains.

The rate and extent of sugar utilisation in yeast are controlled by the ability of yeast to transport sugars, the rate of the subsequent metabolism, and environmental factors. In starch fermentation, *Saccharomyces* yeasts ferment glucose and maltose, leaving the larger dextrins unfermented. In addition, maltotriose is fermented incompletely. An improvement in the ability of *Saccharomyces* to utilise and ferment maltotriose might result in more efficient fermentation processes in the beer and whiskey industries.

It was shown that the rate-limiting step in maltotriose fermentation is the active uptake of maltotriose by yeast cells (Zastrow *et al.,* 2001). Many studies have been conducted in an attempt to better understand the transport of maltotriose by yeast in order to improve this process. A departure point in such studies was to identify proteins responsible for maltotriose uptake, and an affinity for maltotriose was exhibited by certain maltose transporters. One such transporter was identified as the *AGT1*-encoded α-glucoside transporter I (Agt1p) of *S. cerevisiae* (Han *et al.,* 1995). *AGT1* is a mutant allele of *MAL11,* which codes for a maltose permease. *MAL11* forms part of the *MAL1* locus, which comprise *MAL11* (maltose permease), *MAL12* (maltase) and *MAL13* (activator). *MAL11* is situated in the telomeric region of chromosome VII Needleman, 1991). Agt1p is part of the 12 transmembrane symporter families (Han *et al.,* 1995). This symporter exhibits an active transport process that requires a proton gradient over the yeast membrane. It was shown that Agt1p has a *K*m value (mM) of 4 ± 0.7 for maltotriose (Day *et al.,* 2002b).

Two other *S. cerevisiae* maltose transporters were also characterised, *i.e. MPH2* (YDL247w) and *MPH3* (YJR160c), and were only detected in few industrial strains (Day *et al.,* 2002a). *MPH2* and *MPH3* exhibit 100% identity to each other, 75% identity to *MAL31* and *MAL61* and 55% identity to *AGT1* and have *K*m values (mM) of 7.2 ± 1.0 for maltotriose (Day *et al*., 2002a). The strain used to sequence the yeast genome, S288C must, however, have contained the two genes (YDL247w and YJR160c). More recently, a fourth transporter gene was described; *MTY1*/*MTT1* encodes a maltotriose transporter present in *Saccharomyces pastorianus* and lager beer strains respectively (Dietvorst *et al.,* 2005; Salema-Oom *et al.,* 2005). This gene shows 74, 62 and 91% similarity to *MPH2* and *MPH3, AGT1* and *MAL61,* respectively and shows a higher affinity for maltotriose than for maltose (Salema-Oom *et al.,* 2005).

The question arises as to why there are at least five variations of a maltose/maltotriose transporter in *Saccharomyces*. Industrial yeast strains are selected for the robust characteristics relevant under fermentation conditions (Querol *et al.,* 2003).

Compared to laboratory strains, industrial yeast strains are not genetically uniform. Evolution might have taken place for adaptive purposes under the specialised environmental conditions of industrial strains origins (Pretorius, 2000). Another common characteristic of industrial strains is the presence of highly polymorphic chromosomes (Codon *et al.,* 1998). One of *S. cerevisiae'*s well-known characteristics, which is well exploited during fermentation, is its ability to withstand the osmotic stress imposed by high sugar concentrations (Bauer and Pretorius, 2000). Another characteristic of *S. cerevisiae* is its high ethanol tolerance (Fleet and Heard, 1992). *Saccharomyces* strains have also developed the ability to ferment and utilise different carbon sources. Some of these spontaneous mutations in the *Saccharomyces* genome include gene translocations and chromosome-length polymorphisms (Codon *et al*., 1998). It has been suggested that *AGT1* was formed through two recombination events: the first was the translocation of *AGT1* to chromosome VII, and the second the retention of the regulating sequence upstream of *AGT1* and *MAL12* (maltase) (Han *et al.,* 1995).

The objective of this study was to investigate genetic aspects that might influence the ability of different *Saccharomyces* yeasts to transport maltotriose. The first aim was to identify *Saccharomyces* strains that are able to grow efficiently on maltotriose as sole carbon source. The second aim was to use these strains to map the *AGT1* loci, isolate the different *AGT1* genes and express them constitutively in the same genetic background. This enabled us to observe a direct correlation between the degree of cell growth on maltotriose as the sole carbon source and the efficiency of maltotriose transport in strains transformed with the various *AGT1*-encoded α-glucoside transporters. The results indicated that the differences found in the sequences of the *AGT1*-encoded transporters might be responsible for varying abilities to transport maltotriose. Thus, these sequence variations in the *AGT1* genes illustrate the tendency of industrial strains to create genomic variation in order to utilise available carbon sources in their immediate surroundings.

3. MATERIALS AND METHODS

3.1 MICROBIAL STRAINS AND PLASMIDS.

The sources and relevant genotypes of bacterial and yeast strains, together with the plasmids used in this study, are listed in Table 1.

3.2 GROWTH MEDIA AND CULTURE CONDITIONS.

Escherichia coli transformants were cultured in a Luria-Bertani (LB) medium (Sambrook *et al.,* 1989) containing ampicillin (100 μg/ml). The yeast strains were grown in the following media: YPD (1% yeast extract, 2% bacto-peptone and 2% glucose), YPM (1% yeast extract, 2% bacto-peptone and 2% maltose) and YPMt (1% yeast extract, 2% bacto-peptone and 2% maltotriose). The *S. cerevisiae* transformants were cultured and selected

on synthetic complete (SC) medium containing 2% maltose and 0.67% yeast nitrogen base (YNB; Difco, Sparks, MD, USA) without amino acids but supplemented with uracil, leucine, histidine and/or tryptophan as required by the various strains. All solid media contained 2% agar (Difco) and bacteria and yeasts were routinely cultured by shaking at 150 rpm at 37°C and 30°C, respectively. Growth quantification was performed on the AlphaImager v. 5.5 and the integrated density values over four days at 30 °C were calculated with the AlphaEase v. 5.5 software.

3.3 CHROMOSOME RESOLUTION OF YEAST STRAINS.

Yeast cells were grown in 100 ml of YPD medium overnight and prepared according to the embedded-agarose procedure (Carle and Olson, 1985). Separation of the chromosomes was performed according to the technique described by Van der Westhuizen and Pretorius (1991). The samples were run on a 2% agarose gel in the pulsed-field gel electrophoresis (PFGE) system CHEF-DR11 (Bio-Rad laboratories, Richmond, VA, USA) for 42 h; first for 14 h with a switching interval of 60 s, then for 28 h with a switching interval of 90 s. The temperature of the 0.5 x TBE electrophoresis buffer (Sambrook *et al.,* 1989) was maintained at 14 °C; the pump speedpulse time was set at 55 s and the voltage at 200 V.

3.4 SOUTHERN BLOT ANALYSIS.

DNA probes *MALx1*, *AGT1*, *LEU1* and *SUP61* were amplified by the polymerase chain reaction (PCR) method. Genomic DNA from strain CEN.PK2-1D was used as template DNA for the PCR reactions and the PCR primers used in these reactions are listed in Table 2. All DNA probes were labelled with a non-radioactive DIG Labelling System (Roche Biochemical Products, Mannheim, Germany), and Southern hybridisation was performed according to the recommendations of the DIG Application Manual.

3.5 DNA CLONING, AMPLIFICATION, AND MICROORGANISM TRANSFORMATIONS.

Different chromosomes were isolated from the CHEF gel using the Qiagen gel purification kit (Qiagen, West Sussex, UK), and used as template to amplify the *AGT1* genes for CEN.PK2-1D, WH310 and WH314, by PCR, using the primers AGT1-F and AGT1-R (Table 2). These genes were then cloned into the pGEM-Teasy (Promega, Madison, WI, USA) vector, according to the manufacturer's instructions and sequenced using the standard Promega primers, AGT1nt685-F and AGT1nt1298-R (Table 32). The sequenced *AGT1* genes were isolated from the pGEM-T constructs with *Eco*RI and *Xho*I and ligated independently into pSTA1, creating pSTACENPK, pSTAWH310, pSTAWH314 and pSTAWH314III (Table 1). The five pSTA1 vectors were separately linearised at the *Stu*I restriction site of the *URA3* marker, transformed into *S. cerevisiae* EBY.VW4000 using the method described by Gietz *et al*. (1995) and selected on SCM-Ura plates. Genomic DNA was digested with *Nco*I restriction enzyme, which cuts once in the *AGT1* gene and once in

URA3. An *AGT1*-probe was used to confirm integration using the Southern blot method (data not shown). The ΔMALx1DLEU2-F and ΔMALx1DLEU2-R primers (Table 2) were used to PCR-amplify a disruption cassette containing the gene *LEU2* flanked by the *MAL31* promoter and terminator sequences, using YCplac111 as template. This cassette was transformed into the strains EBYCENPK, EBY310 and EBY314, resulting in EBYΔMAL31CENPK, EBYΔMAL31WH310 and EBYΔMAL61WH314, respectively (Table 1). These transformants were confirmed by Southern blot evaluation using a *LEU2MALx1*-probe amplified from YCplac181, using LEU2MALx1probe-F and LEU2MALx1probe-R as primers. The 100-μl PCR reactions contained: standard PCR buffer, 1.25 mM dNTPs, 1.5 mM MgCl2, 0.3 μM of each primer, 1 ng template DNA per μl and DNA polymerase 5 U (Roche). The following PCR program was used to amplify all probes and *AGT1* genes: denaturation, 5 min at 95 °C; primer annealing, 30 s at 55 °C; and primer extension, 30 s at 72 °C.

3.6 SUGAR TRANSPORT ASSAY.

Two methods were used to assay the maltotriose transport activity of the *S. cerevisiae* strains. The uptake of 14C-maltotriose (American Radiolabeled Chemicals Inc., St. Louis, MO, USA) was assayed as previously described in Zheng *et al*. (1994) and the method using *p*-nitrophenyl-α-D-maltoside (*p*NPM) (Sigma-Aldrich, St. Louis, MO, USA) as a substrate, according to Stambuk *et al.* (1999) was performed. The amount of maltotriose transported is expressed as the quantity of intracellular *p*-nitrophenyl measured at absorbance 400 nm (A400) when the substrate is *p*NPM. After entering the cell, the substrate is hydrolysed by the native maltase, releasing *p*-nitrophenyl, which, after boiling, can be quantified. The maltotriose transport activity of the different strains was determined and results are given in nmol/mg dry weight at 1-min intervals. All assays were done in duplicate with at least two different transformants for each strain, with a maximum deviation of less than 5%. The specificity for maltotriose was determined by using *p*NPM over a range of 1- 120 mM. The transport of maltotriose was linear over the period measured (300 s) hence computer-assisted kinetic analysis Eadee Hoffstee plot was used to determine the characteristics of different *AGT1*-encoded transporters (ChemSW Enzyme Kinetics!Pro http://www.chemsw.com/16029.htm).

4. RESULTS AND DISCUSSION

4.1 PERFORMANCE OF DIFFERENT *SACCHAROMYCES* STRAINS ON MALTOTRIOSE

Considering the fact that transport is the rate-limiting step in maltotriose utilisation (Zastrow *et al.,* 2001), it is important to assess the various aspects that influence the

transporting abilities of different strains. In evaluating the growth of the different S*accharomyce*s strains on 2% maltotriose plates, differences in their ability to utilise maltotriose were detected (Fig. 1A). It was clear that the industrial strains WH310, WH313 and WH314 showed the strongest growth on maltotriose, followed by the haploid strain CEN.PK2-1D. The strains were then cultivated in 2% maltotriose liquid medium and their growth curves were developed (Fig. 1B). The faster maltotriose-user strains were found to be WH310 and WH313, followed by CEN.PK2-1D. It is generally accepted that whiskey strains, such as WH310 and WH313, are polyploidy, and this factor can partly explain their better growth performance on solid media than the CEN.PK2-1D strain.

To determine whether there is a relationship between growth performance and maltotriose uptake, the ability of the yeast strains to transport maltotriose was investigated. This was done by performing the *p*NPM transport assay (Fig. 2). The performance of the strains in biomass production, using maltotriose as sole carbon source, has a direct correlation with the efficiency of maltotriose transport. Although other unknown mechanisms for maltotriose utilisation may exist, the relation between utilisation and transport is direct in this instance. This correlates with the findings of Zastrow *et al.* (2001) where they show that transport is the rate-limiting factor for maltotriose utilisation.

These findings lead to the question of the presence and functionality of the *AGT1* genes in these strains, and whether the *AGT1*-encoded proteins are responsible for the transport efficiency of these strains. It also prompts discussion of the industrial strains selected for their varying abilities to utilise certain carbon sources, and whether *AGT1* has been part of a genome evolution process in order to achieve this.

4.2 CHROMOSOMAL DISTRIBUTION OF THE α-GLUCOSIDE TRANSPORTER GENES

To carry out a more detailed evaluation of the different abilities of the *Saccharomyces* strains to utilise maltotriose, we examined their genetic constitution. The CHEF pulsed-field gel electrophoresis system was used for the resolution of chromosomes from each of the *Saccharomyces* strains. After blotting onto nylon membranes the chromosomes were hybridised with specific *AGT1* and *MALx1* DNA probes. The *MAL* locus, consisting of three genes (the first encodes a maltose transporter, the second a maltase, and the third a transcriptional activator), necessary for maltose utilisation by yeast, was characterised in the late 1980s and early 1990s (Needleman, 1991). Although one such locus is sufficient for growth on maltose, many strains are found to contain more than one. Multiple *MAL* loci were detected for the industrial strains evaluated. *MAL1* (chromosome VII), *MAL4* (chromosome XI) and *MAL6* (chromosome VIII) loci were detected in all the industrial strains: *MAL2* (chromosome III) in WH314 and *MAL3* (chromosome II) in WH313. The CEN.PK2-1D strain carries *MAL4,* and *MAL2* loci. Furthermore, all the studied yeast strains contain a copy of the *AGT1* locus on chromosome VII, except WH314 that has a second copy of *AGT1* on chromosome III.

The chromoblot results for WH310, WH314 and CEN.PK2-1D are shown in Fig. 3. The chromosomal locations of the *AGT1* and *MAL* genes were confirmed by hybridising with specific probes for *LEU1*, and *SUP61* mapping to chromosome VII and chromosome III, respectively. The results correlate with the previous reports of *AGT1* locus being situated on the telomere of chromosome VII (Han *et al*., 1995), and being a partially functional allele of *MAL1* (Chow *et al*., 1989). The CEN.PK2-1D data also correlates with the findings of Vidgren *et al*. (2005). Interestingly, a second *AGT1* sequence was found for WH314 on chromosome III. In their analysis of *AGT1*, Han *et al.* (1995) commented that *AGT1* is a telomeric associated gene, which appeared not to be part of a family of highly repetitive gene sequences. They suggested that this *MAL1/AGT1* allele might be the result of some telomere translocation, similar to the formation of *MAL* and *SUC* loci, and contribute to the highly polymorphic chromosomes typical of the industrial yeast strains (Codon *et al*., 1998).

Additionally, the industrial *Saccharomyces* strains' adaptive response to the variable environmental conditions during fermentation should induce aneuploidy and polyploidy, thus generating multiple copies of certain genes essential for fermentation (Bakalinsky and Snow, 1990; Salmon, 1997). Moreover, some industrial strains show chromosome length polymorphism, which can be generated by subtelomeric repeated sequences (Rachidi *et al*., 1999). *Saccharomyces cerevisiae* contains numerous non-coding open reading frames (ORFs). It has been proposed that they originated from gene duplications followed by mutations, throwing the duplicated copy out of frame (Mackiewicz *et al*., 1999). Independently duplicated genes usually map to the telomeric regions and are often associated with genes involved in sugar uptake and metabolism (e.g., sucrose and maltose assimilation) (Grieg and Travisano, 2004). The sequence analysis shows that WH314's *AGT1* from chromosome III contains a frame shift mutation. Taking into consideration all that is mentioned above, it is possible that this non-coding WH314 *AGT1* sequence might have some mapping proximity to the *MAL2* locus situated on the same chromosome (Fig. 3). The question that remains is whether the *AGT1* genes from the *Saccharomyces* industrial strains have undergone some mutational changes for the improvement of maltotriose uptake.

4.3 STRUCTURE-FUNCTION ASSESSMENT OF *AGT1* GENES

For many industries relying on starch fermentation technology, certain *Saccharomyces* strains have been selected for a better fermentation performance of α-maltosides, maltose and maltotriose. The question arises as to whether these different strains show variations in their Agt1p sequences, and whether these sequence differences account for the varying abilities of these strains to transport maltotriose. Therefore, the variations in both the copy number of genes and the levels of expression of the *AGT1* genes could influence the capability of yeast cells to uptake maltotriose. To evaluate the maltotriose transport efficiency of various Agt1p, we expressed separately, the mapped *AGT1* genes in the

same genetic background. The *AGT1* genes of CEN.PK2-1D, WH310 and WH314 were independently amplified by PCR using CHEF-resolved chromosomal DNA as template from each of these strains, thereby ensuring specific linkage between the cloned *AGT1* sequence and the specific chromosome on which it is located. The amplified sequences *AGT1CENPK* from CEN.PK2-1D strain, *AGT1WH310* from WH310, *AGT1WH314* from WH314, and *AGT1WH314III* from WH314 (chromosome III) were cloned separately into pSTA1 under the control of the *PGK1* promoter and terminator sequences, generating the yeast integrating plasmids pSTACENPK, pSTAWH310, pSTAWH314 and pSTAWH314III, respectively (Table 1). Expression with *PGK1* control eliminates possible differences in regulation found in native promoters and ensures the equal expression of the different *AGT1* genes.

The *S. cerevisiae* EBY.VW4000 strain, deleted for all the maltotriose transporters (*AGT1*, *MPH2* or *MPH3*), showed low maltotriose activity. This might be due to the presence of *MALx1*. Conflicting results have been shown for the ability of the Malx1p's in transporting maltotriose (Day *et al*., 2002b). The ability of EBY.VW4000 to grow on maltotriose can, however, be due to other reasons and not necessarily transport by the Malx1p's, but when maltotriose transport was evaluated for this strain, it showed no transport over a 5-min period of time. That phenotype has promoted this strain as an adequate genetic background with which to test the different Agt1p transporter proteins.

The plasmids containing the four constructs were transformed separately and integrated into the genome of the laboratory strain *S. cerevisiae* EBY.VW4000, resulting in EBYCENPK, EBYWH310, EBYWH314III and EBYWH314 (Table 1). Southern-blot hybridisation was performed to confirm the single-copy integrations into the yeast genome (data not shown). Once integrations had been confirmed, the maltotriose transporting ability of the different Agt1p's was evaluated by the method developed by Hollatz and Stambuk (2001), using the chromogenic substrate *p*NPM to analyse the uptake of maltotriose by yeast (Fig. 4).

The EBY310 strain performed better than all the other strains over a 5-min period. The EBYCENPK and EBY314 strains showed relatively similar transport rates, and no transport was detected for EBY314III Agt1p. As previously described by Stambuk and De Araujo (2001), the rate of transport is expressed as the slope of the linear increase of nmol/mg over a 5-min period (Fig. 4B). The EBY310 strain shows a 3.38- and 7.52-fold higher rate of transport than the EBYCENPK and EBY314 strains, respectively. These results indicate that there could be some variations in the sequences of the *AGT1* genes isolated from the different strains, as different maltotriose transport efficiencies were detected.

In order to determine the kinetic data for the Agt1p transporter proteins, as a precaution, *MAL31* was disrupted from EBYCENPK, EBY310 and EBY314, creating EBY∆MAL31CENPK, EBY∆MAL31310 and EBY∆MAL31314, respectively. The gene disruptions were confirmed by Southern blot (data not shown). EBY.VW4000 was derived from CEN.PK21-D. CEN.PK2-1D is known to contain *MAL2*. Our reports also confirm

previous results showing the presence of *MAL3* (Vidgren *et al*., 2005). The possible false detection caused by the low-affinity maltose transporter in *S. cerevisiae*, reported by Benito and Lagunas (1992), raised some more questions on the methods used for maltotriose transport evaluation. This prompted us to determine the kinetic values of maltotriose transport in the different developed yeast strains by two different methods, which use *p*NPM and C14maltotriose as substrate (Zheng *et al*., 1994; Hollatz and Stambuk, 2001). C14maltotriose have been shown to be highly contaminated leading to an overestimation of transport efficiency (Dietvorst *et al*., 2005). Our *p*NPM data correlated well with previous reports and with the tendencies seen in our C14maltotriose (data not shown). We continued to use the *p*NPM assay method because of its cost effectiveness and the high rate of repeatability. The *K*m values obtained for all the strains were very similar. The maltotriose transporter *AGT1WH310* yielded an approximately 30% higher *V*max value than for the *AGT1CENPK* and *AGT1WH314* (Table 3).

Growth evaluation of the EBY.VW4000, EBYCENPK, EBYWH310, and EBYWH314 on maltotriose was facilitated through growth dilutions blotted onto maltotriose minimal media (Fig. 5). The growth intensity was quantified by AlphaEase v. 5.5 and expressed as integrated density values (IDV) of 17, 7 and 4 for EBYΔMAL31WH310, EBYΔMAL31CENPK, and EBYΔMAL31WH314, respectively. A correlation was observed between maltotriose growth efficiency of the reference strains, (donors of the *AGT1* genes), and maltotriose growth efficiency and the efficiency of maltotriose transport of the transformed strains. These efficiencies were consistently found to be best for strain WH310, followed by CEN.PK2-1D and then by WH314. The uniform tendencies of the above mentioned strains' performance on maltotriose can be attributed to the differences in their *AGT1* sequences.

We also initiated a structure-function study in order to identify amino acid residues that could play a role in maltotriose or proton binding. The sequences were evaluated by sequence alignment and an overall identity of above 95% was obtained for all four *AGT1* nucleotide sequences. When translated into amino acid sequences, it was clear that WH314's *AGT1* (located on chromosome III) coded for a non-functional protein due deletion of the 1772 A-residue creating frame shift mutation and a premature TGA stop codon. When comparing the amino acid sequences of WH310's Agt1p and WH314's (chromosome VII) Agt1p to the database sequence of the Han *et al*. (1995) 617 amino acid characterised sequence, the following was found (amino acid data presented as: database/position on sequence /relevant *AGT1* gene and absent amino acids indicated with a Δ): Agt1p from WH314 has a point mutation V549A and a 23 amino acid truncation at the C-terminal consisting of H591L, D592I, S593R, and the absence of residues 594-616. Similar truncations were reported for Agt1p found in brewing strains. Vidgren *et al*. (2005) observed an insertion of an extra T in position 1183 of the *AGT1* sequence leading to a frame-shift mutation that creates an early stop codon. This mutation leads to a 394 amino acid protein and was observed in several of the strains they evaluated.

WH310's Agt1p contains the following point mutations: I505T and T557S. WH314 and WH310 both contain the following point mutations: D40N, M78T, A80T, K102I, S128N, L163Q, T175P, I198V, I215V, I219M, S226G, A228T, V333I, N359D, S381T, V385C, K396R, K397Δ, Q398A, V399G, L410V, S460G, A489T, and L510I. The mutations observed for the Agt1p sequences of these whiskey strains correlate with similar variations observed in brewing strains except for I505T (Vidgren *et al*., 2005). The CEN.PK2-1D Agt1p is 100% identical to the database sequence characterised by Han *et al*. (1995) except for K396R, K397Δ, Q398A, and V399G. It is, however, identical to the polymorphism observed by Volckaert *et al*. (1997) for the 616 amino acid Agt1p of the sequenced yeast strain (ORF YGR289c). The differences found in the sequences of the *AGT1* genes isolated could indicate that some genomic changes had taken place in order to adapt the ability of the *AGT1*-encoded transporters to transport maltotriose over the yeast membrane. These differences might be very important for maltotriose specificity, as there is a relevant difference in their maltotriose transporting ability. Sequence differences might also influence the rate of catabolite inactivation, *i.e.* the process in which proteins are degraded from the plasma membrane (Busturia and Lagunas, 1986). In order to characterise specific domains in Agt1p that are important for maltotriose transport, these sequence differences will be further investigated.

5. CONCLUSION

Maltotriose transport differences can be directly correlated to the same differences observed in growth. Thus the differences found in the Agt1p sequences of the evaluated strains, when expressed in an identical genetic background account for the variations in the rate of maltotriose transport. Selective pressure present in the environment that these strains originated from must have been the cause of these variations to occur, and reflects on the diversity of sugar transporters. The three permeases that were tested in this study transported maltotriose. The improved performance of the *AGT1*310 transporter, compared to the *AGT1CENPK* and *AGT1WH314* transporters, was caused by a higher *V*max and not by a lower *K*m value. The sequence discrepancies of Agt1p should therefore unravel some of the answers needed for improved maltotriose utilisation in yeast. Small variations in protein sequences could lead to varying functional characteristics. Therefore, it is important to note these differences and determine their exact influence on protein function and specificity. In results to be reported elsewhere, the relevance of amino acid sequence differences among the various *AGT1*-encoded maltotriose transporters that were identified in this study will be characterised. Further, this should facilitate the development of engineered transporters adapted to starch-efficient fermentation systems.

6. ACKNOWLEDGEMENTS

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TABLE 1 - Strains and plasmids used in this study

Strain and Plasmid	Relevant features	Source
Plasmids:		
YCplac181	Ap^R *LEU2*	Gietz and Sugino, 1988
pSTA1	Ap^R $PGK1_{PT}$ *URA3*	Gundllapalli *et al.*, 2001
pSTACENPK	Ap^R $PGK1_P$ $AGT1_{CENPK}$ $PGK1_T$ *URA3*	This study
pSTAWH310	Ap^R $PGK1_P$ $AGT1_{WH310}$ $PGK1_T$ *URA3*	This study
pSTAWH314	Ap^R $PGK1_P$ $AGT1_{WH314}$ $PGK1_T$ *URA3*	This study
pSTAWH314III	Ap^R $PGK1_P$ $AGT1_{WH314III}$ $PGK1_T$ *URA3*	This study
Eschericchia coli strains:		
DH5α	*sup*E44 ρ*lac*U169 (ϕ*80lac*ZρM15) *hsd*R17 *rec*A1 *gyr*A96 *thi*-1 *rel*A1	GIBCO/Bethesda Research Laboratories
Saccharomyces cerevisiae strains:		
WH301	Wild-type	DCL Scotland
WH310	Wild-type	DY10; ex Anchor
WH313	Wild-type	NRRL Y-987
WH314	Wild-type	NRRL Y-567
CEN.PK2-1D	*MATα leu2-3, 112 ura3-52 trp1-289 his3-Δ1*	Entian and Kötter, 1998
∑1278b	*MATα ura3*	Liu *et al.*, 1993
EBY.VW4000	*MATα leu2-3,112 ura3-52 trp1-289 his3-Δ1 MAL2-8 SUC2 hxt8-17Δ hxt514Δ hxt2Δ hxt367Δ gal2Δ stl1Δ agt1Δ ydl247wΔ yjr160cΔ*	Wieczorke *et al.*, 1999
EBYCENPK	EBY.VW4000, *URA3*::pSTACENPK	This study
EBYWH310	EBY.VW4000, *URA3*::pSTAWH310	This study
EBYWH314	EBY.VW4000, *URA3*::pSTAWH314	This study
EBYWH314III	EBY.VW4000, *URA3*::pSTAWH314III	This study
EBYΔMAL31CENPK	EBYCENPK, *LEU2*::*mal2-8Δ*	This study
EBYΔMAL31WH310	EBYWH310, *LEU2*::*mal2-8Δ*	This study
EBYΔMAL31WH314	EBYWH314, *LEU2*::*mal2-8Δ*	This study

CBS: Centraalbureau voor Schimmelcultures, The Netherlands; NRRL-ARS Culture Collection, USA.

TABLE 2 - Primers used in this study

Primer	Oligonucleotide sequence[a]
AGT1-F	5'-**GAATTC**ATGAAAAATATCATTTCATT-3'
AGT1-R	5'-**CTCGAG**TTAACATTTATCAGCTG-3'
AGT1p-F	5'-GCCCAGTTAGGAGATTCTG-3'
AGT1p-R	5'-TTTTTTCCTCGCTGTTAGCT-3'
AGT1-684F	5'-GTTACTTATGCTTCGGAAG-3'
AGT1-1298R	5'-CCAAGACCATAGGTCAGT-3'
MALx1p-F	5'-CAGCAGCTGCAGAAATTAAT-3'
MALx1p-R	5'-CATTTGTTCACAACAGATGG-3'
SUP61p-F	5'-GGCACTATGGCCG-3'
SUP61p-R	5'- GCTGTGGTCGTCCT-3'
LEU1p-F	5'-GTTGATTGTACTCTAGCTACTG-3'
LEU1p-R	5'-CTCTAAAGTTTTGACTTGTAAAC-3'
LEU2MALx1p-F	5' CAGTATAACAATAAGAATTACATCCAAG 3'
LEU2MALx1p-R	5' GGAAGTGGAACACCTGTAGC 3'
ΔMALx1LEU2-F	5'-CAGTATAACAATAAGAATTACATCCAAGACTATTAATTAACGGCGTATCACGAGGCCCTTT-3'
ΔMALx1LEU2-R	5'-AAAAAAAAGTCATAATGTCGAGTAAAAATAAAATCCCATTCCATGCGGGGTA-3'

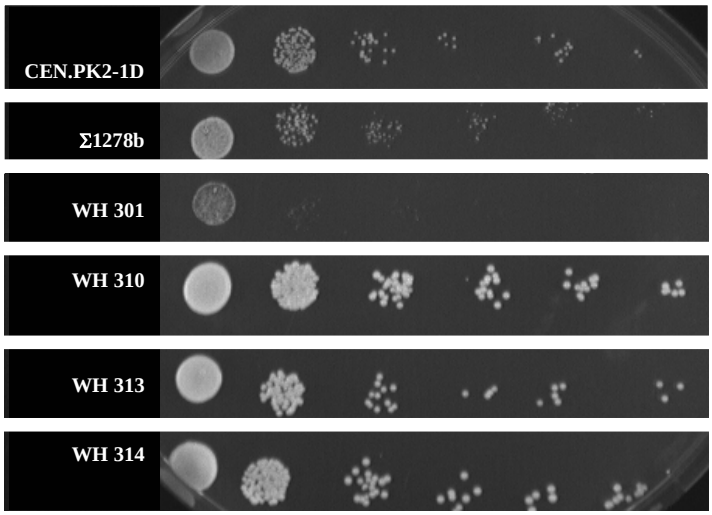
[a] The restrictions sites are indicated in bold

TABLE 3 - Kinetic constants for maltotriose transport in the generated strains

Strain	Permease	Maltotriose	
		K_m [a]	V_{max} [b]
EBYΔMAL31CENPK	*AGT1*$_{CENPK}$	3.1 ± 0.5	66
EBYΔMAL31310	*AGT1*$_{WH310}$	3.3 ± 0.7	83
EBYΔMAL31314	*AGT1*$_{WH314}$	3.0 ± 0.3	60

[a]mM
[b]nmol/min mg of dry wt

A



B

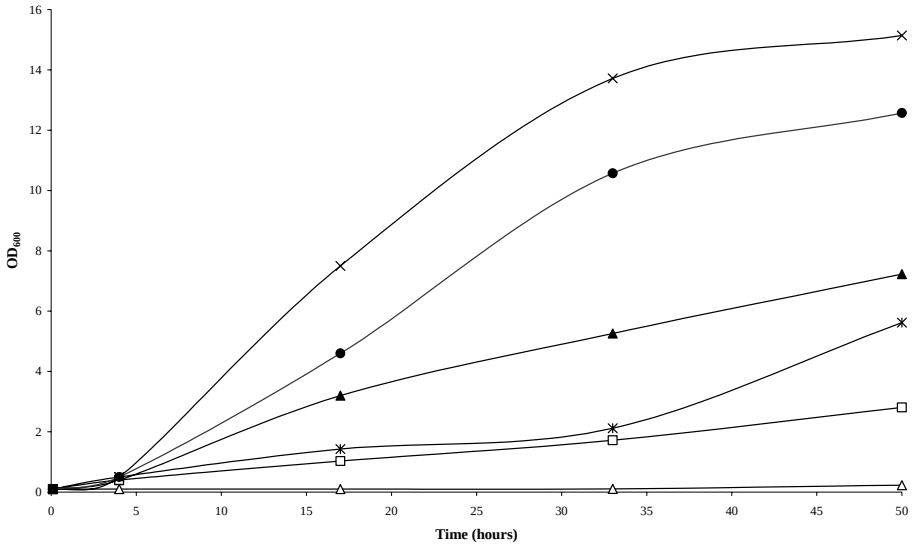


FIG. 1 – (A) A dilution series of growth on 2% maltotriose for different *Saccharomyces* strains was made starting at 1X10^6 cells. (B) Growth in 2% maltotriose broth was measured for CEN.PK2-1D, ▲; Σ1275b, □; WH310, ×; WH314, ✳; WH313, •; and WH301, +. Cells were precultured on YPM.

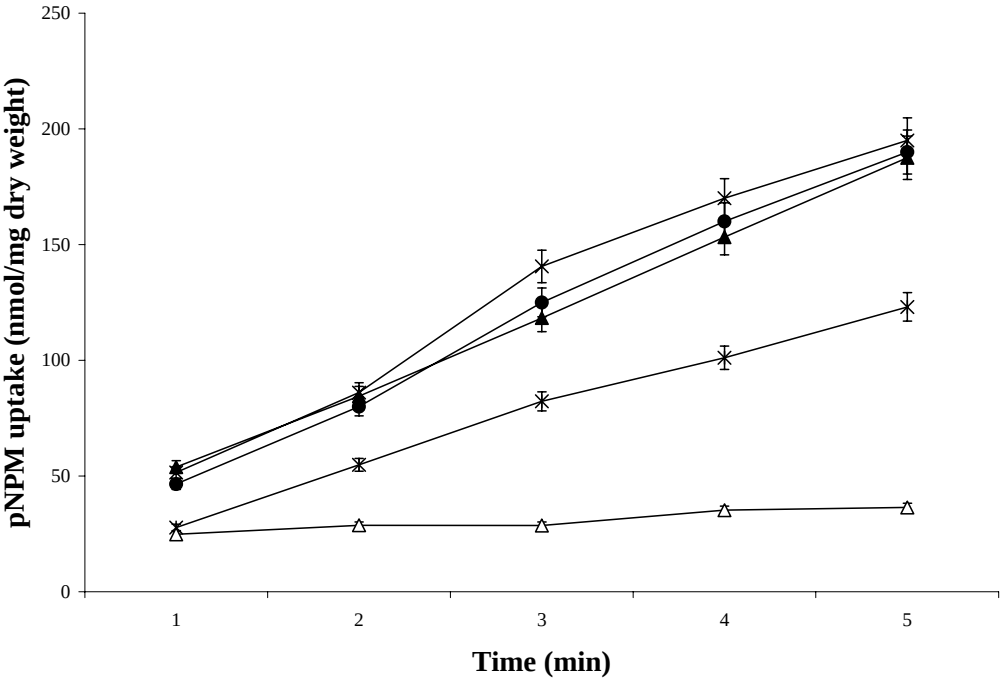


FIG. 2 - *p*-Nitrophenyl-α-D-maltoside uptake of the *Saccharomyces* strains CEN.PK2-1D, ▲; WH310, ×; WH314, ✳; WH313, •; and WH301, +; expressed in nmol/min dry weight for time intervals of 1 min.

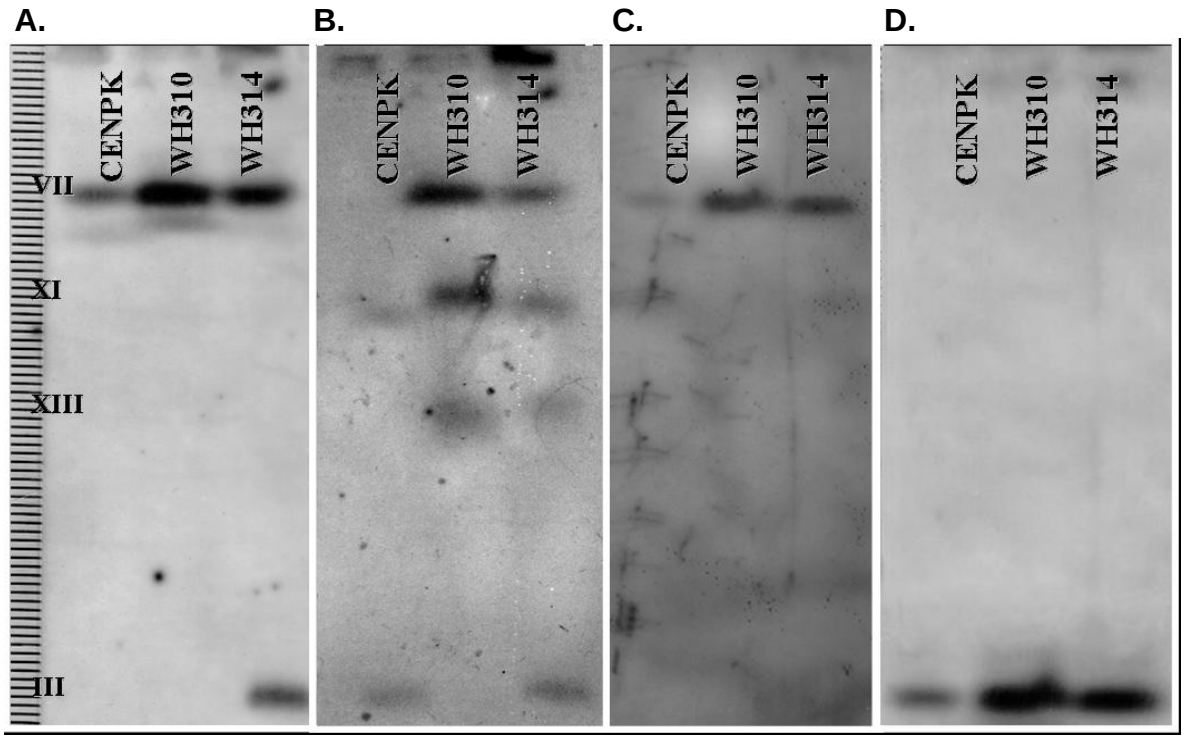
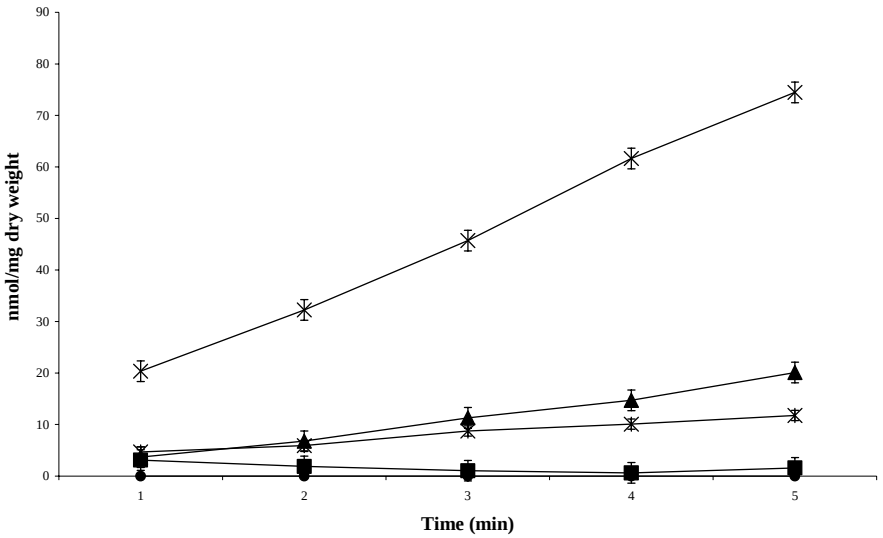


FIG. 3 - Chromosomal mapping of the *AGT1* and *MALx1* genes in different *Saccharomyces* strains. A CHEF pulsed-field gel was blotted onto a nitrocellulose membrane, and hybridised with a probe annealing to *AGT1* in A, *MALx1* in B, *LEU2* in C, and *SUP61* in D. The lanes are represented by CEN.PK2-1D, WH310, and WH314 respectively, and rows are assigned with the different chromosomes present in the far left lane. The *LEU2* and *SUP61* represent Chromosome VII (*MAL1*) and Chromosome II (*MAL2*), respectively.

A.



B.

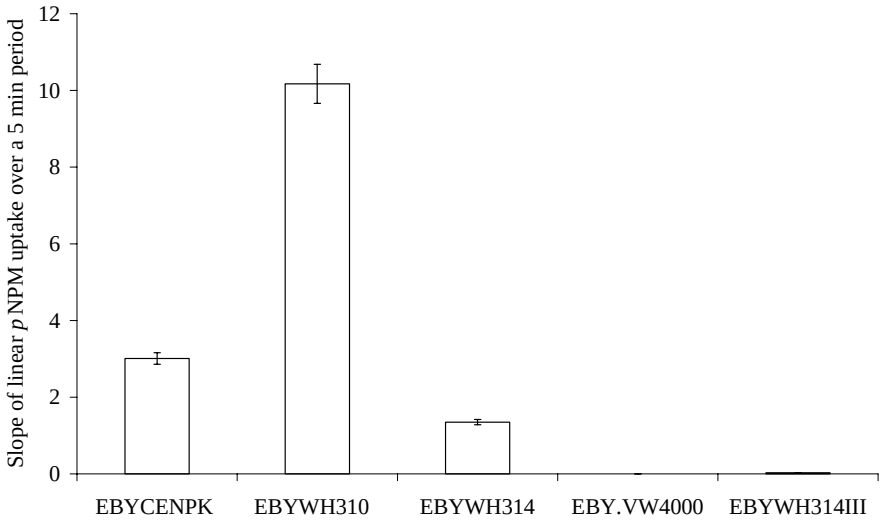


Fig. 4 – (A) *p*-Nitrophenyl-α-D-maltoside uptake of the *Saccharomyces cerevisiae* strains EBYWH310, ×; EBYCENPK, ▲; EBYWH314, ✳;and EBYWH314III, ■. (B) The transport rate for *p*NPM determined from the slope of the linear uptake of *p*NPM over a 5-min time period, as seen in Fig. 4A. EBY representing the wild-type EBY.VW4000.

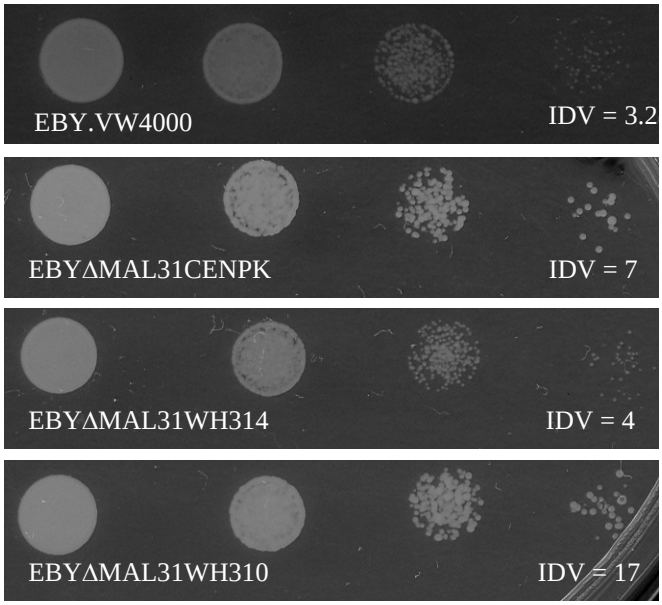


Fig. 5 - Growth of EBYΔMAL31CENPK, EBYΔMAL31WH310, and EBYΔMAL31WH314 on 2% maltotriose minimal media with a 10-times dilution range starting at an OD_{600} of 0.5. The integrated density value was calculated as the average value over four days at 24-h intervals.

7. REFERENCES

Bakalinsky A.T., Snow R. (1990). The chromosomal constitution of wine strains of *Saccharomyces cerevisiae*. Yeast, 6: 367-82.

Bauer F.F., Pretorius I.S. (2000). Yeast stress response and fermentation efficiency: How to survive the making of wine - a review. S. Afr. J. Enol. Vitic., 21: 27-51.

Benito B., Lagunas R. (1992). The low-affinity component of *Saccharomyces cerevisiae* maltose transport is an artifact. J. Bacteriol., 174: 3065-3069.

Busturia A., Lagunas R. (1986). Catabolite inactivation of the glucose transport system in *Saccharomyces cerevisiae*. J. Gen. Microbiol., 132: 379-385.

Carle G.F., Olson M.V. (1985). An electrophoretic karyotype for yeast. Proc. Natl. Acad. Sci., 82: 3756-3760.

Chow T.H., Sollitti P., Marmur J. (1989). Structure of the multigene family of MAL loci in *Saccharomyces*. Mol. Gen. Genet., 217: 60-69.

Codon A.C., Benitez T., Korhola M. (1998). Chromosomal polymorphism and adaptation to specific industrial environments of *Saccharomyces* strains. Appl. Microbiol. Biotechnol., 49: 154-163.

Day R.E., Higgins V.J., Rogers P.J., Dawes I.W. (2002a). Characterization of the putative maltose transporters encoded by YDL247w and YJR160c. Yeast, 19: 1015-1027.

Day R.E., Rogers P.J., Dawes I.W., Higgins V.J. (2002b). Molecular analysis of maltotriose transport and utilization by *Saccharomyces cerevisiae*. Appl. Environ. Microbiol., 68: 5326-5335.

Dietvorst J., Londesborough J., Steensma H.Y. (2005). Maltotriose utilization in lager yeast strains: *MTT1* encodes a maltotriose transporter. Yeast, 22: 775-788.

Entian K-D., Kötter P. (1998). Yeast mutant and plasmid collections. In: Methods in Microbiology, Vol. 26, Brown A.J.P., Tuite M., Eds., Academic Press, San Diego.

Fleet H., Heard G.M. (1992). Yeast growth during fermentation. In Wine Microbiology and Biotechnology, Fleet G., edn., Harwood Academic Publishers, Chur, Switzerland.

Gietz R.D., Sugino A. (1988) New yeast Escherichia coli shuttle vectors constructed with in vitro mutagenized yeast genes lacking six-base pair restriction sites. Gene, 74: 527-534.

Gietz R.D., Schiestl R.H., Willems A.R., Woods R.A. (1995). Studies on the transformation of intact yeast cells by the LiAc/SS-DNA/PEG procedure. Yeast, 11: 355-360.

Greig D., Travisano M. (2004). The Prisoner's Dilemma and polymorphism in yeast *SUC* genes. Proc. Biol. Sci., 271: S25-26.

Gundllapalli Moses S., Cordero Otero R.R., La Grange D.C., Pretorius I.S., Van Rensburg P. (2001). Different genetic backgrounds influence the expression, secretion and substrate utilization of recombinant *Saccharomyces cerevisiae* strains. Biotechnol. Lett., 24: 651-656.

Han E.K., Cotty F., Sottas C., Jiang H., Michels C.A. (1995). Characterization of *AGT1* encoding a general alpha-glucoside transporter from *Saccharomyces*. Mol. Microbiol., 17: 1093-1107.

Hollatz C., Stambuk B.U. (2001). Colorimetric determination of active alpha-glucoside transport in *Saccharomyces cerevisiae*. J. Microbiol. Methods., 46: 253-259.

Jiang H., Medintz I., Michels C.A. (1997). Two glucose sensing/signaling pathways stimulate glucose-induced inactivation of maltose permease in *Saccharomyces*. Mol. Biol. Cell., 8: 1293-1304.

Liu H., Styles C.A., Fink G.R. (1993). Elements of the yeast pheromone response pathway required for filamentous growth of diploids. Science, 262: 1741-1744.

Mackiewicz P., Kowalczuk M., Gierlik A., Dudek M.R., Cebrat S. (1999). Origin and properties of non-coding ORFs in the yeast genome. Nucleic. Acids. Res., 27: 3503-3509.

Needleman R. (1991). Control of maltase synthesis in yeast. Mol. Microbiol., 5: 2079-2084.

Pretorius I.S. (2000). Tailoring wine yeast for the new millennium: novel approaches to the ancient art of winemaking. Yeast, 16: 675-729.

Querol A., Fernandez-Espinar M.T., Del Olmo M., Barrio E. (2003). Adaptive evolution of wine yeast. Int. J. Food. Microbiol., 86: 3-10.

Rachidi N., Barre P., Blondin B. (1999). Multiple Ty-mediated chromosomal translocations lead to karyotype changes in a wine strain of *Saccharomyces cerevisiae*. Mol. Gen. Genet., 261: 841-850.

Salema-Oom M., Valadao Pinto V., Goncalves P., Spencer-Martins I. (2005). Maltotriose utilization by industrial *Saccharomyces* strains: characterization of a new member of the alpha-glucoside transporter family. Appl. Environ. Microbiol., 71: 5044-5049.

Salmon J.M. (1997). Enological fermentation kinetics of an isogenic ploidy series derived form an industrial *Saccharomyces cerevisiae* strain. J. Ferment. Bioeng., 83: 253–260.

Sambrook J., Fritsch E.F., Manitis T. (1989). Molecular Cloning: A Laboratory Manual, 2nd ed., Cold Spring Harbor Laboratory, Cold Spring Harbor, New York.

Stambuk B.U., Da Silva M.A., Panek A.D., De Araujo P.S. (1999). Active alpha-glucoside transport in *Saccharomyces cerevisiae*. FEMS Microbiol. Lett., 170: 105-110.

Stambuk B.U., De Araujo P.S. (2001). Kinetics of active alpha-glucoside transport in *Saccharomyces cerevisiae*. FEMS Yeast Res., 1: 73-78.

Van der Westhuizen T.J., Pretorius I.S. (1991). The value of electrophoretic fingerprinting and karyotyping in wine yeast breeding programmes. Antonie van Leeuwenhoek, 61: 249–257.

Verstrepen K.J., Chambers P., Pretorius I.S. (2006). The development of superior yeast strains for the food and beverage industries: challenges, opportunities and potential benefits. Springer-Verlag. Heidelberg.

Vidgren V., Ruohonen L., Londesborough J. (2005). Characterization and Functional Analysis of the *MAL* and *MPH* Loci for Maltose Utilization in Some Ale and Lager Yeast Strains. Appl. Environ. Microbiol., 71: 7846-7857.

Volckaert G., Voet M., Robben J. (1997). Sequence analysis of a near-subtelomeric 35.4 kb DNA segment on the right arm of chromosome VII from *Saccharomyces cerevisiae* carrying the *MAL1* locus reveals 15 complete open reading frames, including *ZUO1*, *BGL2* and *BIO2* genes and an ABC transporter gene. Yeast, 13: 251-259.

Wieczorke R., Krampe S., Weierstall T., Freidel K., Hollenberg C.P., Boles E. (1999). Concurrent knock-out of at least 20 transporter genes is required to block uptake of hexoses in *Saccharomyces cerevisiae*. FEBS Lett., 464: 123-128.

Zastrow C.R., Hollatz C., De Araujo P.S., Stambuk B.U. (2001). Maltotriose fermentation by *Saccharomyces cerevisiae*. J. Ind. Microbiol. Biotechnol., 27: 34-38.

Zheng X., D'Amore T., Russell I., Stewart G.G. (1994). Factors influencing maltotriose utilization during brewery wort fermentations. J. Am. Soc. Brew., 52: 41–47.

Chapter 4

RESEARCH RESULTS

Maltotriose-specific domain characterization of chimeric *AGT1* and *MAL31* encoded α-glucosidases in *Saccharomyces cerevisiae*

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MALTOTRIOSE-SPECIFIC DOMAIN CHARACTERIZATION OF CHIMERIC *AGT1* AND *MAL31* ENCODED α-GLUCOSIDASES IN *SACCHAROMYCES CEREVISIAE*

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1. ABSTRACT

Efficient uptake of maltose, maltotriose and other starch breakdown products is an essential criterion in the development of improved yeast strains for the baking, brewing, whiskey and biofuel industries. This study focuses on genetic aspects that might influence the efficiency of maltotriose transport by *Saccharomyces* yeasts. The *AGT1*-encoded α-glucosidase transporter (Agt1p) forms part of a group of maltose transporters and shows affinity for maltotriose. The *MAL3*-encoded permease (Mal31p), one of the five *MAL* loci-associated maltose transporters of *Saccharomyces cerevisiae*, does not exhibit affinity for maltotriose. The specific aim of this study was to identify definite protein domains responsible for maltotriose affinity by constructing a range of eight chimeric proteins combining areas of Mal31p and Agt1p. These chimeric proteins were evaluated for maltotriose transport. It was found that the first 56 residues of Agt1p are not essential for maltotriose transport, whereas the region from 56 to 617 of contains critical sites for maltotriose affinity. However, Mal31p could not be converted into a maltotriose transporter by the addition of this single domain, leading to the conclusion that more than one specific domain is necessary for maltotriose recognition.

2. INTRODUCTION

Efficient transport of maltose and maltotriose across the cell membrane of yeast cells is of key importance for starch and malt-based fermentation processes in industries such as the baking, brewing, whiskey and biofuel industries. Strain development programs in these industries are therefore aimed, amongst others, to improve the maltose and maltotriose uptake capabilities of yeast that, in turn, would increase the overall fermentation efficiencies.

The *MAL31*-encoded permease (Mal31p) is a *Saccharomyces cerevisiae* maltose transporter showing affinity for maltose and turanose [8]. *S. cerevisiae* contains five unlinked *MAL* loci (*MAL1-4* and *MAL6*), each containing a maltose permease (*MALx1*), a maltase (*MALx2*), and an activator (*MALx3*) gene [10]. The five *MAL* loci are highly homologous and the yeast cell needs at least one functional *MAL* locus present in its genome to utilise maltose [3]. The *AGT1*-encoded α-glucosidase (Agt1p) is a Mal11p-derived protein that shows affinity for maltose, turanose, isomaltose, maltotriose, palatinose and α-methylglucoside [8]. There is 57% identity and 75% similarity between Agt1p and Mal61p, and this homology is seen throughout the length of these proteins [8]. Both transporters are proton gradient-dependent symporters and form part of the 12-transmembrane domain sugar transport family, exhibiting 12 hydrophobic domains spanning the membrane of the cell and forming a channel-like structure for the transport of sugars [11].

Site-directed mutagenesis has commonly been used to identify important amino acids in the *MALx1*-encoded transporters [1, 9, 5]. Such a specific and targeted approach usually followed a broad, robust evaluation of the functionality of these permeases. For example, unlike the *MALx1*-encoded transporters, Agt1p was found to have affinity for maltotriose [8]. Based on that, our group investigated further which specific amino acids of various *S. cerevisiae* strains' *AGT1*-encoded proteins are essential for maltotriose transport. In a previous study, we identified two critical amino acids for maltotriose affinity in the *AGT1*-encoded transporter of a whiskey strain (WH310) that allow its permease ($Agt1p_{WH310}$) to transport maltotriose at a much higher rate than the *AGT1*-encoded α-glucosidases of other *Saccharomyces* strains [14]. However, the specific protein domain of Agt1p responsible for maltotriose when compared to Malx1p, which shows no affinity for maltotriose, has not yet been localised.

The purpose of this study was to identify a specific Agt1p domain for maltotriose affinity. The approach taken was to construct chimeric proteins that combined specific peptides from both Agt1p and Mal31p. These chimeric proteins were evaluated for their differential capacity to transport maltotriose, leading to the characterisation of putative Agt1p domains, which play a role in maltotriose transport.

3. MATERIALS AND METHODS

3.1 MICROBIAL STRAINS AND PLASMIDS

The sources and relevant genotypes of the bacterial and yeast strains, together with the plasmids used in this study, are listed in Table 1.

3.2 GROWTH MEDIA AND CULTURE CONDITIONS

Escherichia coli transformants were cultured in a Luria-Bertani (LB) medium [13] containing ampicillin (100 μg/ml). The yeast strains were grown in the following media: YPD (1% yeast extract, 2% bacto-peptone and 2% glucose), YPM (1% yeast extract, 2% bacto-peptone and 2% maltose) and YPMt (1% yeast extract, 2% bacto-peptone and 2% maltotriose). The *S. cerevisiae* transformants were cultured and selected on synthetic complete (SC) medium [6.7 g l^{-1} yeast nitrogen base without amino acids (Difco Laboratories, MI, USA), 20 g l^{-1} glucose (SCD), 20 g l^{-1} maltose (SCMaltose), or 20 g l^{-1} (SCMaltotriose)] supplemented with amino acids essential for selection (50 mg/l tryptophan, 240 mg/l leucine, 50 mg/l histidine, and/or 40 mg/l uracil). Solid media contained 2% agar (Difco). Bacteria and yeasts were routinely cultured by shaking at 150 rpm at 37°C and 30°C, respectively.

3.3 RECOMBINANT DNA METHODS AND TRANSFORMATION

Standard procedures for isolation and manipulation of DNA were used throughout this study [13]. Restriction enzymes (Roche, Mannheim, Germany), T4 DNA-ligase (Promega, Madison, WI, USA) and Takara *Ex-Taq* DNA polymerase (TakaRa Bio, Shiga, Japan) were used according to the specifications of the supplier. Standard procedures were used for the transformation of bacteria [13] and yeast [6]. Nucleotide sequences of the yeast genes were obtained from the *Saccharomyces* genome database (http://genome-www2.stanford.edu/cgi-bin/SGD), and protein sequences were retrieved from the yeast proteome database (http://www.proteome.com/databases/YPD). Sequence analysis and the alignment of sequences were conducted using Vector NTI 9.

The *AGT1* and *MAL31* genes were amplified from the genomic DNA of *S. cerevisiae* CEN.PK2-1D by the polymerase chain reaction (PCR) technique, using the primers AGT1-F and AGT1-R, and MAL31-F and MAL31-R (Table 2). These PCR products were cloned into the pGEM-T-Easy vector (Promega, Madison, WI, USA), according to the manufacturer's instructions, and sequenced using the standard Promega primers. The sequenced *AGT1* and *MAL31* genes were isolated as *Eco*RI-*Xho*I DNA fragments from the pGEM-T constructs, and ligated separately into the *Eco*RI and *Xho*I sites of pSTAH, creating pSTAH pSTAHAGT1 and pSTAHMAL31 (Table 1). The gene fragments depicted in Fig. 1 were all amplified using pGEMMAL31 and pGEMAGT1, respectively, as templates, and the correspondingly named primers listed in Table 2: AA349, MB268, AA56, MB561, AA458, MB159, MA349, AB268, MA56, AB561, MA458 and AB159. All fragments were cloned into pGEM-T. Plasmids pGEMAA349, pGEMAA56 and pGEMAA458 were cut with *Eco*RI and *Bam*HI and the fragments were gel-isolated [13]. Plasmids pGEMMB268, pGEMMB561, pGEMMB159, pGEMMA349, pGEMAB268, pGEMMA56, pGEMAB561, pGEMMA458 and pGEMAB159 were cut with *Bam*HI and *Xho*I and gel-isolated. The following gene fragments were ligated in pairs into pSTAH to

create the consecutive plasmids: *Eco*RI-*Bam*HI AA349 *Xho*I-*Bam*HI MB268 fragments for pSTAHA349M; *Eco*RI-*Bam*HI AA56 and *Xho*I-*Bam*HI MB458 fragments for pSTAHA56M; *Eco*RI-*Bam*HI AA458 and *Xho*I-*Bam*HI MB56 fragments for pSTAHA458M; *Xho*I-*Bam*HI MA349 and *Xho*I-*Bam*HI AB268 fragments for pSTAHM349A; *Xho*I-*Bam*HI MA56 and *Xho*I-*Bam*HI AB458 fragments for pSTAHM56A; and *Xho*I-*Bam*HI MA458 and *Xho*I-*Bam*HI AB56 fragments for pSTAHM458A.

Sequencing was performed for all positive transformants to confirm the correct nucleotide sequences. The eight aforementioned pSTAH plasmids were separately linearised at the *Kpn*I restriction site of the *HIS3* marker, transformed into *S. cerevisiae* EBY.VW4000 and selected on SCMaltose^{-His} plates, and the transformants EBYAGT1, EBYMAL31, EBYA349M, EBYA56M, EBYA458M, EBYM349A, EBYM56A, EBYM458A and EBY∆MAL31 were obtained.

The ∆MALx1DLEU2-F and ∆MALx1DLEU2-R primers (Table 2) were used to PCR-amplify a gene disruption cassette containing the gene *LEU2* flanked by the *MAL31* promoter and terminator sequences, using YCplac181 as template. To knock out the *MALx1* gene of EBY.VW4000, this gene disruption cassette was transformed into the strains EBYAGT1, EBYMAL31, EBYA349M, EBYA56M, EBYA458M, EBYM349A, EBYM56A, EBYM458A and EBY∆MAL31, respectively (Table 1). The following *S. cerevisiae* disruptants were obtained: EBY∆MAL31AGT1, EBY∆MAL31MAL31, EBY∆MAL31A349M, EBY∆MAL31A56M, EBY∆MAL31A458M, EBY∆MAL31M349A, EBY∆MAL31M56A and EBY∆MAL31M458A. The *MALx1* gene disruptions were confirmed by PCR characterisation using the LEU2MALx1p-F and LEU2MALx1p-R (Table 2). The 100-µl PCR reactions contained standard PCR buffer, 1.25 mM dNTPs, 1.5 mM $MgCl_2$, 0.3 µM of each primer, 1 ng template DNA per µl and DNA polymerase 5 U (Roche). The following PCR programme was used to amplify all genes: denaturation, 5 min at 95 °C; primer annealing, 30 sec at 55°C; and primer extension, 30 sec at 72°C.

3.4 SOUTHERN BLOT ANALYSIS

To confirm integration of the gene cassettes into the *HIS3* locus, EBY∆MAL31AGT1 genomic DNA was digested with *Cla*I, EBY∆MAL31A349M, EBY∆MAL31A56M and EBY∆MAL31A458M with *Pst*I, and EBY∆MAL31MAL31, EBY∆MAL31M349A, EBY∆MAL31M56A and EBY∆MAL31M458A with *Bgl*I. Southern blotting was performed and EBY∆MAL31AGT1, EBY∆MAL31A349M, EBY∆MAL31A56M and EBY∆MAL31A458M were probed with AA56 DNA. EBY∆MAL31M349A, EBY∆MAL31M56A, EBY∆MAL31M458A were probed with AB56 DNA, and EBY∆MAL31MAL31 was probed with MA56 DNA. Probes MA56, AA56 and AB159 were amplified by PCR using primers MA56-R and MAL31-F, AA56-R and AGT1-F, and AB56-F and AGT1-R, respectively (Table 2). All DNA probes were labelled with a non-radioactive DIG Labelling System (Roche), and Southern hybridisation was performed according to the recommendations of the DIG Application Manual.

3.5 SUGAR TRANSPORT ASSAY

p-Nitrophenyl-α-D-maltoside (*p*NPM) (Sigma-Aldrich, St. Louis, MO, USA) was used as a substrate [15] to assess the maltotriose-transporting ability of the transformed strains. The amount of maltotriose transported is expressed as the quantity of intracellular *p*-nitrophenyl measured at an absorbance of 400 nm (A_{400}) when the substrate is *p*NPM. After entering the cell, the substrate is hydrolysed by the native maltase, releasing *p*-nitrophenyl, which can be quantified after boiling. The maltotriose transport activity of the different strains was determined and the results are given in nmol/mg dry weight at one-minute intervals. All assays were done in duplicate with at least two different transformants for each strain, with a maximum deviation of less than 5%. The specificity for maltotriose was determined by using *p*NPM over a range of 1-120 mM. The transport of maltotriose was linear over the period measured (300 sec), hence a computer-assisted kinetic analysis Eadee Hoffstee plot was used to determine the characteristics of the different chimeric transporters (ChemSW Enzyme Kinetics!Pro http://www.chemsw.com/16029.htm).

3.6 GROWTH CURVES

The growth performance of the transformed strains for maltose and maltotriose was determined in SCMaltose- and SCMaltotriose minimal medium, supplemented with the necessary amino acids, in 50 ml flasks shaken at 30°C. All sugars were filter-sterilised and added to SC media after autoclaving. The growth curves were performed in triplicate with a standard deviation of less than 5%.

4. RESULTS AND DISCUSSION

4.1 CHIMERIC PROTEIN CONSTRUCTION

A strategy was developed to express eight chimeric protein combinations between Agt1p and Mal31p in order to identify specific domains responsible for maltotriose specificity. Primers were used to introduce a *Bam*HI site at differently-sized fragments in order to obtain different combinations of *AGT1* and *MAL31* ligated together, as shown in Fig. 1. Agt1p and Mal31p are both 12-transmembrane domain proteins and all transmembrane segments play a structural role in creating a channel through which the sugar molecule is transported into the yeast cell [8, 12]. Using *AGT1*-encoded α-glucosidase's ability to transport maltotriose when compared to Mal31p, the rationale was to determine whether certain domains in Agt1p can convert Mal31p into a protein capable of maltotriose uptake, and therefore to indirectly identify domains responsible for the transport of the maltotriose of Agt1p.

The following chimeric proteins were expressed under *PGK1*$_P$ control in the same genetic background (EBY.VW4000): Agt1p, Mal31p, A349Mp, A56Mp, A458Mp, M349Ap, M56Ap and M458Ap (Fig. 1). Southern blots were performed to confirm the integration of these respective genes into *HIS3* (data not shown). EBY.VW4000 also contains copies of the *MALx1* genes and, even though these proteins have not been shown to transport maltotriose [14], a *MAL*-disruption cassette was transformed into the eight strains to knock out one of the *MALx1* copies. These knockouts were confirmed with specific PCR amplifications and characterisations using the LEU2MALx1p-F and LEU2MALx1p-R primers. Along with the EBYDMAL strain, (the reference strain EBY.VW4000 with *MALx1* knocked out) nine strains were developed to evaluate on maltotriose.

4.2 GROWTH ON MALTOTRIOSE AND FUNCTIONALITY OF PROTEINS

Growth of the eight chimeric proteins on maltotriose was determined, and Agt1p showed much faster growth on maltotriose than the rest of the chimeric transporters, reaching an OD_{600} of approximately 6.5 (Fig. 3). The only other *S. cerevisiae* transformant showing growth on maltotriose was EBYΔMAL31M56A, but it grew slower than Agt1p and only reached an OD_{600} of 1.99. All the other chimeric proteins did not show any significant growth on maltotriose.

It has been shown that chimeric proteins are not always expressed in cells [2, 7]. The question arose whether the chimeric proteins constructed here are functional and, if not, whether that accounts for their loss in maltotriose transport ability. To answer this question, the chimeric proteins were assessed for their ability to support growth on maltose. It was hypothesised that if they show a similar growth on maltose to the reference strain, the chimeric proteins must still be functional. All the strains containing the chimeric proteins showed similar growth rates for maltose as the reference strain EBYΔMAL31MAL31, with a final optical density measure at 600 nm (OD_{600}) ranging between 6.3 and 6.9. When these growth performances were compared to that of reference strain EBYΔMAL, containing a single copy of Malx1p, the reference strain was found to grow markedly slower on maltose and only reached a final OD_{600} of 5.8. These results indirectly support the conclusion that the chimeric proteins expressed by the transformants are functional transporters, as they transport maltose.

4.3 MALTOTRIOSE TRANSPORT

The ability of the nine strains to transport maltotriose was determined through a pNPM assay, where the amount of pNP released provides an indication of the amount of maltotriose molecules transported into the cell. In Fig. 2A it is shown that, except for Agt1p and M56Ap, none of the other chimeric proteins showed affinity for maltotriose transport. EBYDMAL was used as the reference strain. From Fig. 2B it is clear that Agt1p shows a

2.44-fold higher transport rate than M56Ap. Agt1p has a V_{max} of 66 nmol/min mg of dry weight for maltotriose [14]. We determined the V_{max} for M56Ap as being 40 nmol/min mg of dry wt for maltotriose. The loss in maltotriose transport efficiency of M56Ap, when compared to Agt1p, has to do with a drop in V_{max}.

Mal31p, A349Mp, A56Mp, A458Mp, M349Ap and M458Ap showed no affinity for maltotriose. This provides an indication that the residues of Agt1p 56 to 617 contain critical sites for maltotriose recognition.

4.4 ALIGNMENT COMPARISON

From the evaluation of the chimeric proteins, the only chimeric permease that still transported maltotriose was the chimeric protein with the first 56 residues of Agt1p exchanged for the first 56 residues of Mal31p. According to VectorNTI alignment, AA56p and MA56p show a 33.9% identity and 42.9 % positivity. For the Agt1p and Mal31p full-length proteins, 54.2% identity and 64.8% positivity were found after a VectorNTI evaluation. It is interesting to note that the first 56 residues of these two proteins showed considerable differences. For Mal61p, it is known that residues 49 to 78 contain a PEST sequence involved in the glucose inactivation of this protein [9], and that the five serine/theonine residues in the 29 to 56 N-terminal region of Mal61p are involved in delivering the internalised Mal61p permease to the vacuole for degradation, although it is not required for glucose-induced internalisation [5]. Glucose was not present under the conditions in which the transformants were grown and the chimeric transporters were evaluated, so no conclusions can be drawn about the effect of these residues in Agt1p. However, it is interesting to note that in the same area that Mal31p also contains five serine/threonine residues, Agt1p contains only two.

5. CONCLUSION

The yeast α-glucoside transporter, Agt1p, shows high transport levels for maltotriose in comparison with the yeast maltose transporter Mal31p, although they share high levels of homology. Functional chimeras of Agt1p and Mal31p were constructed and expressed in the same genetic background. This led to an in-depth evaluation of the ability of these proteins to transport maltotriose in an attempt to identify the domains in Agt1p responsible for maltotriose affinity.

The growth of the eight chimeric proteins on maltotriose showed similar results to the maltotriose transport assays (Fig. 3). The chimeric proteins Mal31p, A349Mp, A56Mp, A458Mp, M349Ap and M458Ap did not transport maltotriose. Agt1p and M56Ap showed transport activity toward maltotriose, with Agt1p showing the highest transport efficiency. The improved performance of the Agt1p transporter when compared to the M56Ap transporter, was caused by a higher V_{max}. The sequence discrepancies of Agt1p compared

to M56Ap indicate that the first 56 residues of Agt1p are not critical for maltotriose transport, although the exchange of the 56 residues of Agt1p for the first 56 residues of Mal31p did cause a dramatic decrease in maltotriose transport. Except for the above, no specific domain for maltotriose specificity was identified.

In light of these results it seems reasonable to assume that more than one specific domain is required for maltotriose recognition. The only chimerc Mal31p able to transport maltotriose is the chimeric M56Ap, indicating that some part of Agt1p from the position 56 to position 617 is instrumental in maltotriose transport. The fragment AB561 in the chimeric A458Mp has a C-terminal deletion of 159 residues creating a maltotriose transport deficient Agt1p. We can thus hypothesise that AB159 (D), is relevant for maltotriose transport. Conversely, the presence of the MA56 fragment in the chimeric M56Ap does not knock out maltotriose transport, but the fragment M349 does that in the chimeric M349Ap. It is therefore tempting to speculate that the fragment of Agt1p between the insertion of the fragment MA56 and M349 (B) is required for maltotriose transport. In Fig. 4, a hypothetical chimeric is created with regions A, B, C, and D where we can suggest that the Agt1p fragments B and D of the chimeras are required for maltotriose transport. When either B or D, or both regions are absent, maltotriose transport does not occur.

The differences between these two proteins are very important in maltotriose specificity, and transforming Mal31p into a protein with affinity for maltotriose is probably not only domain specific.

6. ACKNOWLEDGEMENTS

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Table 1 Strains and plasmids used in this study

Strain and plasmid	Relevant features	Source
Plasmids:		
YCplac181	Ap^R *LEU2*	[6]
pSTAH	Ap^R $PGK1_{PT}$ *HIS3*	[16]
pSTAHAGT1	Ap^R $PGK1_P$ $AGT1_{AGT1}$ $PGK1_T$ *HIS3*	This study
pSTAHMAL31	Ap^R $PGK1_P$ $AGT1_{MAL31}$ $PGK1_T$ *HIS3*	This study
pSTAHA349M	Ap^R $PGK1_P$ $AGT1_{A349M}$ $PGK1_T$ *HIS3*	This study
pSTAHA56M	Ap^R $PGK1_P$ $AGT1_{A56M}$ $PGK1_T$ *HIS3*	This study
pSTAHA458M	Ap^R $PGK1_P$ $AGT1_{A458M}$ $PGK1_T$ *HIS3*	This study
pSTAHM349A	Ap^R $PGK1_P$ $AGT1_{M349A}$ $PGK1_T$ *HIS3*	This study
pSTAHM56A	Ap^R $PGK1_P$ $AGT1_{M56A}$ $PGK1_T$ *HIS3*	This study
pSTAHM458A	Ap^R $PGK1_P$ $AGT1_{M458A}$ $PGK1_T$ *HIS3*	This study
Escherichia coli:		
DH5α	*sup*E44 ρ*lac*U169 (ϕ*80lac*ZρM15) *hsd*R17 *rec*A1 *gyr*A96 *thi*-1 *rel*A1	GIBCO/Bethesda Research Laboratories
Saccharomyces cerevisiae:		
CEN.PK2-1D	*MATα leu2-3, 112 ura3-52 trp1-289 his3-Δ1*	[4]
EBY.VW4000	*MATα leu2-3,112 ura3-52 trp1-289 his3-Δ1 MAL2-8 SUC2 hxt8-17Δ hxt514Δ hxt2Δ hxt367Δ gal2Δ stl1Δ agt1Δ ydl247wΔ yjr160cΔ*	[17]
EBYAGT1	EBY.VW4000, *HIS3*::pSTAHAGT1	This study
EBYMAL31	EBY.VW4000, *HIS3*::pSTAHMAL31	This study
EBYA349M	EBY.VW4000, *HIS3*::pSTAHA349M	This study
EBYA56M	EBY.VW4000, *HIS3*::pSTAHAA56M	This study
EBYA458M	EBY.VW4000, *HIS3*::pSTAHA458M	This study
EBYM349A	EBY.VW4000, *HIS3*::pSTAHM349A	This study
EBYM56A	EBY.VW4000, *HIS3*::pSTAHM56A	This study
EBYM458A	EBY.VW4000, *HIS3*::pSTAHM458A	This study
EBYΔMAL31	EBY.VW4000, *LEU2*::*mal2-8Δ*	This study
EBYΔMAL31AGT1	EBYAGT1, *LEU2*::*mal2-8Δ*	This study
EBYΔMAL31MAL31	EBYMAL31, *LEU2*::*mal2-8Δ*	This study
EBYΔMAL31A349M	EBYA349M, *LEU2*::*mal2-8Δ*	This study
EBYΔMAL31A56M	EBYA56M, *LEU2*::*mal2-8Δ*	This study
EBYΔMAL31A458M	EBYA458M, *LEU2*::*mal2-8Δ*	This study
EBYΔMAL31M349A	EBYM349A, *LEU2*::*mal2-8Δ*	This study
EBYΔMAL31M56A	EBYM56A, *LEU2*::*mal2-8Δ*	This study
EBYΔMAL31M458A	EBYM458A, *LEU2*::*mal2-8Δ*	This study

CBS: Centraalbureau voor Schimmelcultures, The Netherlands; NRRL-ARS Culture Collection, USA.

Table 2 Primers used in this study

Primer	Oligonucleotide sequence[a]
AGT1-F	5'-**GAATTC**ATGAAAAATATCATTTCATT-3'
AGT1-R	5'-**CTCGAG**TTAACATTTATCAGCTG-3'
AGT349-F	5'-CA**GGATCC**TTCTTTAATTGTTTCAAGG-3'
AGT349-R	5'-GAA**GGATCC**TGATTTAGATGCTAAAAG-3'
AB56-F	5'-GC**GGATCC**TTCACCACCAATTCA-3'
AA458-R	5'-CG**GGATCC**AGAACCAAAACCCATTC-3'
AB458-F	5'-CG**GGATCC**AGCGCTAGTAATG-3'
AA56-R	5'-GC**GGATCC**GTGGTCTAGCTCAAA-3'
AGT1p-F	5'-GCCCAGTTAGGAGATTCTG-3'
AGT1p-R	5'-TTTTTTCCTCGCTGTTAGCT-3'
MAL31-F	5'-CC**CTCGAG**ATGAAGGGATTATC-3'
MAL31-R	5'-CC**CTCGAG**TCATTTGTTCACAAC-3'
MAL349-F	5'-GAA**GGATCC**TACTGGGATTGTG-3'
MAL349-R	5'-GTA**GGATCC**TTCATCAGACATTTTC-3'
MB56-F	5'-GC**GGATCC**CTAATACCAAACGATAAT-3'
MA458-R	5'-CG**GGATCC**CATTTTAGCGCCATG-3'
MB458-F	5'-GC**GGATCC**GGTGCTCTTCTAATG-3'
MA56-R	5'-GC**GGATCC**TGGACCGTACTCAA-3'
LEU2MALx1p-F	5' CAGTATAACAATAAGAATTACATCCAAG 3'
LEU2MALx1p-R	5' GGAAGTGGAACACCTGTAGC 3'
ΔMALx1LEU2-F	5'-CAGTATAACAATAAGAATTACATCCAAGACTATTAATTAACGGCGTATCACGAGGCCCTTT-3'
ΔMALx1LEU2-R	5'-AAAAAAAAGTCATAATGTCGAGTAAAAATAAAATCCCATTCCATGCGGGGTA-3'

[a] The restrictions sites are indicated in bold

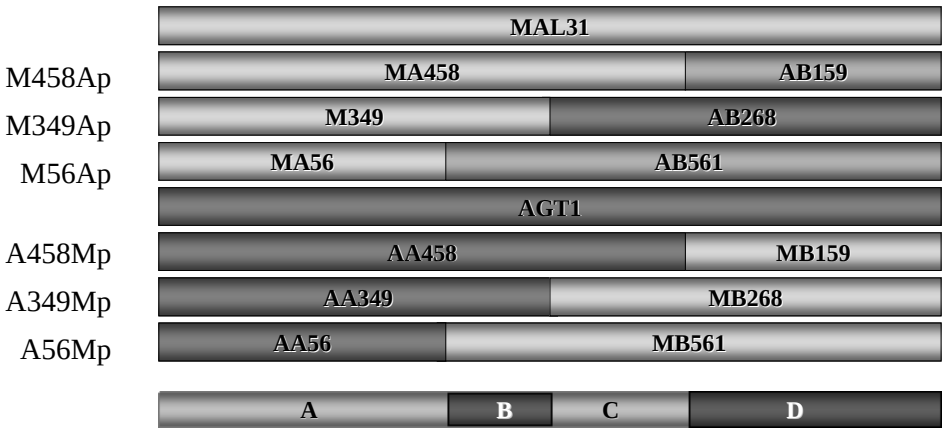
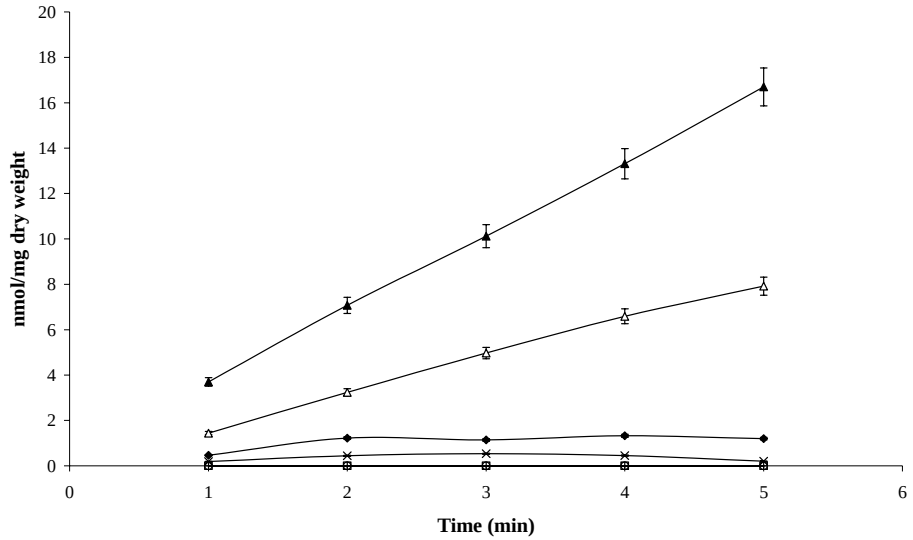


Fig. 1 Chimeric Agt1p and Mal31p proteins that were constructed. The colours correspond to the origin of the peptides.

A.



B.

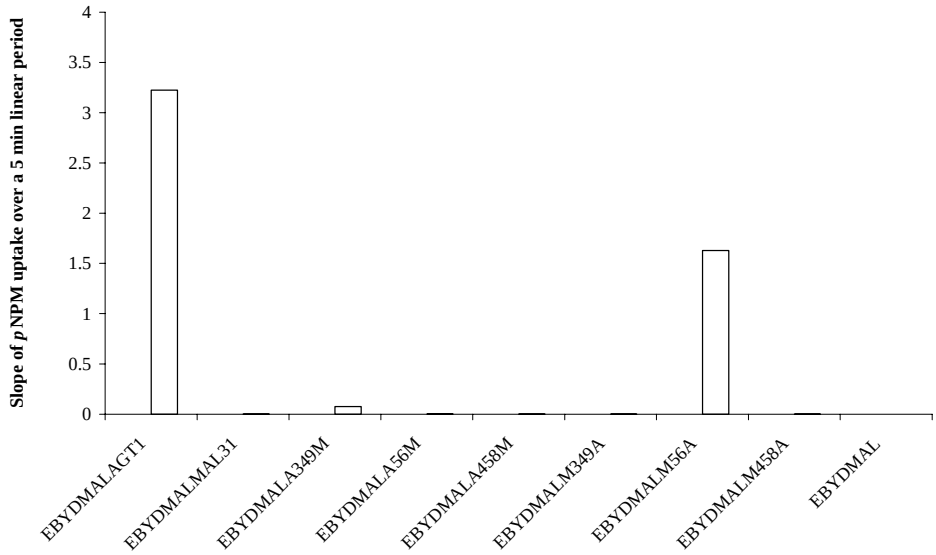


Fig. 2 (**a**) *p*-Nitrophenyl-α-D-maltoside uptake of the *Saccharomyces cerevisiae* strains EBYDMALAGT1, ▲; EBYDMALMAL31, ■; EBYDMALA349M, ♦; EBYDMALA56M, ×; EBYDMALA458M, ○; EBYDMALM349A, +; EBYDMALM56A, ∆; EBYDMALM458A, □; EBYDMAL, ●. (**b**). The transport rate for the ρNPM transport of the EBY strains. The transport rate is determined from the slope of the linear uptake of *p*NPM over a 5 min time period.

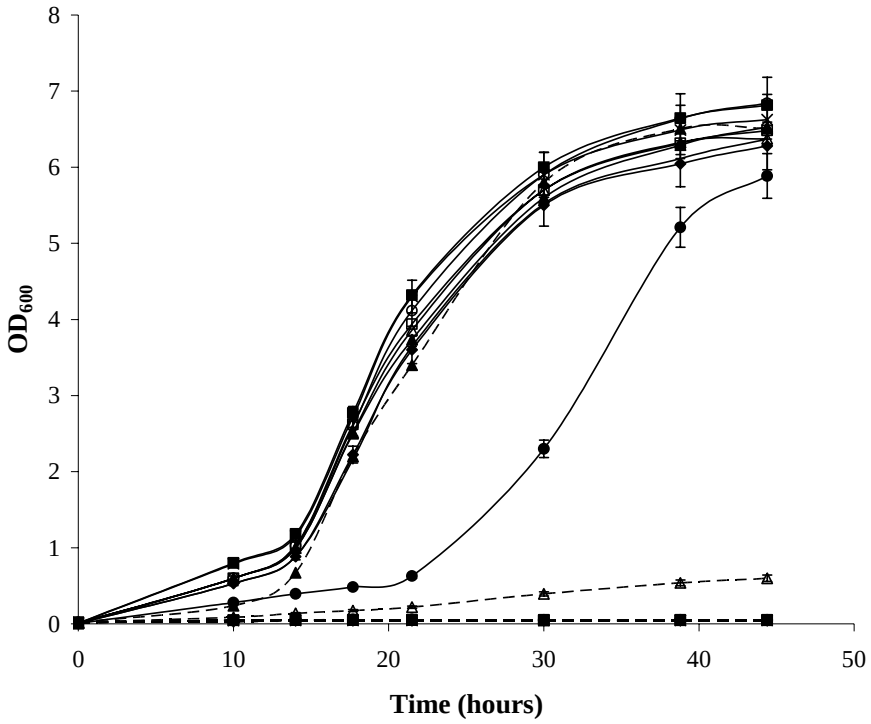


Fig. 3 Growth in 2% maltose (solid lines) and 2% maltotriose (dashed lines) minimal broth was measured for EBYDMALAGT1, ▲; EBYDMALMAL31, ■; EBYDMALA349M, ♦; EBYDMALA56M, ×; EBYDMALA458M, ○; EBYDMALM349A, +; EBYDMALM56A, △; EBYDMALM458A, □; EBYΔMAL, •

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Agt1p      MKNIISLVSKKKAASKNEDKNISESSRDIVNQQEVFNTEDFEEGKKDSAFELDHLEFTTNSAQLGDSDEDNENVINEMNATDDANEANSEEKSMTLKQAL
Mal31p     MKGLSSLINRKKDRNDSHLDEIENGVN-----ATEFNSIEMEEQGKKSDFDLSHLEYGPGSLIPNDNNE--EVPDLLDEAMQDAKEADESERGMPLMTAL

Agt1p      LKYPKAALWSILVSTTLVMEGYDTALLSALYALPVFQRKFGTLNG-EGSYEITSQWQIGLNMCVLCGEMIGLQITTYMVEFMGNRYTMITALGLLTAYIF
Malx1p     KTYPKAAAWSLLVSTTLIQEGYDTAILGAFYALPVFQKKYGSLNSNTGDYEISVSWQIGLCLCYMAGEIVGLQMTGPSVDYMGNRYTLIMALFFLAAFIF

Agt1p      ILYYCKSLAMIAVGQILSAIPWGCFQSLAVTYASEVCPLALRYYMTSYSNICWLFGQIFASGIMKNSQENLGNSDLGYKLPFALQWIWPAPLMIGIFFAP
Mal31p     ILYFCKSLGMIAVGQALCGMPWGCFQCLTVSYASEICPLALRYYLTTYSNLCWAFGQLFAAGIMKNSQNKYPNSDLGYKLPFALQWIWPLPLAVGIFFAP

Agt1p      ESPWWLVRKDRVAEARKSLSRILSGKGAEKDIQVDLTLKQIELTIEKERLLASKSGSFFNCFKG-VNGRRTRLACLTWVAQNSSGAVLLGYSTYFFERAG
Mal31p     ESPWWLVKKGRIDQARRSLERTLSGKGPEKELLVSMELDKIKTTIEKEQKMSD-EGTYWDCVKDGINRRRTRIACLCWIGQCSCGASLIGYSTYFYEKAG

Agt1p      MATDKAFTFSLIQYCLGLAGTLCSWVISGRVGRWTILTYGLAFQMVCLFIIGGMGFGSGSSASNGAGGLLLALSFFYNAGIGAVVYCIVAEIPSAELRTK
Mal31p     VSTDTAFTFSIIQYCLGIAATFVSWWASKYCGRFDLYAFGLAFQAIMFFIIGGLGCSDTHGAKMGSGALLMVVAFFYNLGIAPVVFCLVSEIPSSRLRTK

Agt1p      TIVLARICYNLMAVINAILTPYMLNVSDWNWGAKTGLYWGGFTAVTLAWVIIDLPETTGRTFSEINELFNQGVPARKFASTVVDPFGKGKTQHDSLADES
Mal31p     TIILARNAYNVIQVVVTVLIMYQLNSEKWNWGAKSGFFWGGFCLATLAWAVVDLPETAGRTFIEINELFRLGVPARKFKSTKVDPFAAAKAAAAEINVKD

Agt1p      ISQSSSIKQRELNAADKC----
Mal31p     PKEDLETSVVDEGRSTPSVVNK
```

Fig. 4 Vector NTI alignment of Agt1p and Mal31p. The first 56 residues are shown in bold and the transmembrane domains are underlined.

7. REFERENCES

1. Brondijk TH, Van Der Rest ME, Pluim D, De Vries Y, Stingl K, Poolman B, Konings WN (1998) Catabolite inactivation of wild-type and mutant maltose transport proteins in *Saccharomyces cerevisiae*. J Biol Chem 273:15352-15357
2. Caron MG (1993) The chimaeras speak again. Nature 366:409 – 410
3. Charron MJ, Read E, Haut SR, Michels CA (1989) Molecular evolution of the telomere-associated *MAL* loci of *Saccharomyces*. Genetics 122:30716
4. Entian K-D, Kötter P (1998) Yeast mutant and plasmid collections. In: Methods in Microbiology. Academic Press, San Diego
5. Gadura N, Michels CA (2006) Sequences in the N-terminal cytoplasmic domain of *Saccharomyces cerevisiae* maltose permease are required for vacuolar degradation but not glucose-induced internalization. Curr Genet 50:101-114
6. Gietz RD, Schiestl RH, Willems AR, Woods RA (1995) Studies on the transformation of intact yeast cells by the LiAc/SS-DNA/PEG procedure. Yeast 11:355-360
7. Giros B, Wang YM, Suter S, McLeskey SB, Pifl C, Caron MG (1994) Delineation of discrete domains for substrate, cocaine, and tricyclic antidepressant interactions using chimeric dopamine-norepinephrine transporters. J Biol Chem 269:15985-15988
8. Han EK, Cotty F, Sottas C, Jiang H, Michels CA (1995) Characterization of *AGT1* encoding a general alpha-glucoside transporter from *Saccharomyces*. Mol Microbiol 17:1093-1107
9. Medintz I, Wang X, Hradek T, Michels CA (2000) A PEST-like sequence in the N-terminal cytoplasmic domain of *Saccharomyces* maltose permease is required for glucose-induced proteolysis and rapid inactivation of transport activity. Biochemistry 39:4518-4526
10. Needleman R (1991) Control of maltase synthesis in yeast. Mol Microbiol 5:2079-2084
11. Saier MH Jr (2000) A functional-phylogenetic classification system for transmembrane solute transporters. Microbiol Mol Biol Rev 64:354-411
12. Salas-Burgos A, Iserovich P, Zuniga F, Vera JC, Fischbarg J (2004) Predicting the three-dimensional structure of the human facilitative glucose transporter glut1 by a novel evolutionary homology strategy: insights on the molecular mechanism of substrate migration, and binding sites for glucose and inhibitory molecules. Biophys J, 87:2990-2999
13. Sambrook J, Fritsch EF, Manitis T (1989) Molecular Cloning: A Laboratory Manual. 2nd ed. Cold Spring Harbor, Laboratory Cold Spring Harbor, New York

14. Smit A, Cordero Otero RR, Pretorius IS (2007) Differences among *AGT1*-encoded alpha-glucoside transporters and their ability to transport maltotriose in *Saccharomyces* yeasts. Annals of Microbiology 57:
15. Stambuk BU, Da Silva MA, Panek AD, De Araujo PS (1999) Active alpha-glucoside transport in *Saccharomyces cerevisiae*. FEMS Microbiol Lett 170:105-110
16. Thanvanthri Gururajan V, Gorwa-Grauslund M-F, Hahn-Hägerdal B, Pretorius IS, Cordero Otero RR (2007) A constitutive catabolite repression mutant of a recombinant *Saccharomyces cerevisiae* strain improves xylose consumption during fermentation. Ann Microbiol 57:85-92
17. Wieczorke R, Krampe S, Weierstall T, Freidel K, Hollenberg CP, Boles E (1999) Concurrent knock-out of at least 20 transporter genes is required to block uptake of hexoses in *Saccharomyces cerevisiae*. FEBS Lett 464:123-128

Chapter 5

RESEARCH RESULTS

Thr^{505} and Ser^{557} of Agt1p are Critical for Maltotriose Transport in *Saccharomyces cerevisiae*

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THR^{505} AND SER^{557} OF AGT1P ARE CRITICAL FOR MALTOTRIOSE TRANSPORT IN *SACCHAROMYCES CEREVISIAE*

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1. ABSTRACT

1.1 AIMS

The main objective of this study was to identify amino acid residues in the *AGT1*-encoded α-glucoside transporter (Agt1p) that is critical for efficient transport of maltotriose in the yeast *Saccharomyces cerevisiae.*

1.2 METHODS AND RESULTS

The sequences of two *AGT1*-encoded α-glucoside transporters with different efficiencies of maltotriose transport in two *Saccharomyces* strains (WH310 and WH314) were compared. The sequence variations and discrepancies between these two proteins ($Agt1p_{WH310}$ and $Agt1p_{WH314}$) were investigated for potential effects on the functionality and maltotriose transport efficiency of these two *AGT1*-encoded α-glucoside transporters. A 23 amino acid C-terminal truncation proved not to be critical for maltotriose affinity. The identification of three amino acid differences, which potentially could have been instrumental in the transportation of maltotriose, were further investigated. Single mutations were created to restore the point mutations I505T, V549A and T557S one by one. The single site mutant V549A showed a decrease in maltotriose transport ability, and the I505T and T557S mutants showed complete reduction in maltotriose transport.

1.3 CONCLUSIONS

The amino acids Thr^{505} and Ser^{557}, which are respectively located in the transmembrane (TM) segment TM^{11} and on the intracellular segment after TM^{12} of the *AGT1*-encoded α-glucoside transporters, are critical for efficient transport of maltotriose in *S. cerevisiae.*

1.4 SIGNIFICANCE AND IMPACT OF THE STUDY

Improved fermentation of starch and its dextrin products, such as maltotriose and maltose, would benefit the brewing and whisky industries. This study could facilitate the development of engineered maltotriose transporters adapted to starch-efficient fermentation systems, and offers prospects for the development of yeast strains with improved maltose and maltotriose uptake capabilities that, in turn, could increase the overall fermentation efficiencies in the beer and whisky industries.

2. INTRODUCTION

Complete starch degradation and rapid assimilation of fermentable sugars by specialised *Saccharomyces* strains is an important contributor to the efficiency of fermentation and the overall cost-effectiveness in the brewing, whisky, baking and bioethanol industries. The efficiency of the uptake and utilisation of breakdown products of starch, such as maltotriose and maltose, is therefore a key attribute of yeast strains used for the production of beer, whisky, dough and bioethanol. For example, at 13.6% of total carbohydrate content, maltotriose is the second most abundant sugar in beer wort (Hough *et al*. 1981). Inefficient transport of maltotriose across the yeast cell membrane during the brewing process does not only compromise the efficiency with which the raw material is converted into beer, but a high concentration of maltotriose residues in the final product can also lead to atypical flavour profiles, reduced product quality and potential loss of revenue.

The rate-limiting step in maltotriose fermentation is the active uptake of maltotriose by yeast cells (Zastrow *et al.* 2001). Although effective transporters exist for the favoured *S. cerevisiae* sugar, glucose, a significant requirement exists in the aforementioned industries for the improvement of the efficiency of maltose and maltotriose transport in their respective starter yeast strains. Several *S. cerevisiae* maltose transport proteins show an affinity for maltotriose, including the *AGT1*-encoded α-glucoside symporter (Agt1p), which facilitates an active transport process that requires a proton gradient over the yeast membrane. It has been shown that Agt1p has a K_m value (mM) of 4±0.7 for maltotriose (Day *et al*. 2000). Other related transporters, such as Mph2p and Mph3p, have K_m values (mM) of 7.2±1.0 for maltotriose (Day *et al*. 2000) while the *MTT1*-encoded maltose transporter (with 91% homology to the maltose transporter, *MALx1*) facilitates maltotriose transport slightly more efficiently when compared with other *S. cerevisiae* maltose transporters (Dietvorst *et al*. 2005; Salema-Oom *et al.,* 2005). This indicates that there is a wide variation amongst the various transporters of maltotriose in terms of their efficiency as regards their ability to facilitate uptake of maltotriose by yeast cells.

One of the main factors influencing the efficiency with which a carrier can transport maltotriose across the yeast cell membrane is catabolite repression by glucose. The presence of glucose represses the uptake of maltose and maltotriose in yeast by

repressing the expression of the transporter-encoding genes, as well as by inactivating the maltose/maltotriose transporters. When studying the physical structure of a transporter as the main contributing factor to transport efficiency, the effects of protein specificity for the substrates in question and the rate of protein degradation should be considered. Both of these aspects are also highly relevant in the studies of maltotriose transport.

Through hydropathy analysis, the secondary structure of Mal61p was characterised as two blocks of six-transmembrane domains each, separated by a 71-residue intracellular region (Cheng and Michels 1989). Mal61p and Agt1p are 57% identical and Han *et al*. (1995) originally characterised Agt1p as a 12-transmembrane domain protein. According to the Saier (2000) classification system, maltose transporters are classified as 12-transmembrane domain spanners. However, predictions by different software and computer programmes vary and some contradict the 12-spanner analysis. A putative PEST sequence [rich in proline (P), glutamic acid (E), serine (S) and threonine (T)] was identified between residues 49 and 78 in the N-terminal sequence of Mal61p (Cheng and Michels 1989). Proteins are marked for degradation through a regulated phosphorylation of the PEST sequence (Rechsteiner 1988; Marchal *et al*. 1998).

Brondijk *et al*. (1998) did a study to determine whether modifications to the Mal61p amino acid sequence influence the inactivation through proteolysis. The study was conducted by removing the putative protein kinase A and C phosphorylation sites. The Mal61p mutants (S295A, T363A and S487A) were constructed and significantly reduced levels of glucose inactivation were observed. For T363A, the inactivation rate correlated with the protein degradation rate. For the S295A and S487A mutants, the reductions in protein degradation rates were much higher than the loss of activity. Their findings showed that some form of protein modification took place prior to the degradation of Mal61p. This modification already inactivated Mal61p and proteolytic breakdown did not necessarily follow inactivation. Medintz *et al*. (2000) characterised an N-terminal PEST sequence between residues 49 and 78 of Mal61p which includes a di-leucine motif at residues 69-70 of the cytoplasmic region. They also showed that a 36-amino acid truncation at residue 581 created a non-fermentable phenotype. Mal61p inactivation is a two-step process, i.e. the enzyme/transporter is first inactivated by phosphorylation and then degradation follows (Hein *et al*. 1995; Stanbrough and Magasanik 1995). No specific characterisation of Agt1p residues has been reported and no PEST sequences have been characterised to date.

Previously, our group identified various point mutations on the Agt1p proteins of *Saccharomyces* $AGT1_{WH310}$ (EF628289) and $AGT1_{WH314}$ (EF628288) and compared the results with the database sequence of Agt1p (Smit *et al*. 2007). Notable differences were observed in the ability of these proteins to transport maltotriose when expressed in a homogenous genetic background (in the absence of glucose). It was concluded that the improved performance of the $AGT1_{310}$ transporter, compared to the $AGT1_{CENPK}$ and $AGT1_{WH314}$ transporters, was caused by a higher V_{max} and not by a lower K_m value.

The objective of this study was to characterise the different amino acid residues responsible for enhanced maltotriose transport efficiency in the $AGT1_{WH310}$ transporter.

The strategy was to eliminate the sequence differences in amino acid residues identified by Smit *et al*. (2007) for both *AGT1*$_{WH310}$ and *AGT1*$_{WH314}$ through the development of newly mutated genes. The importance of the 23-amino acid truncation found in the C-terminal of *AGT1*$_{WH314}$, and the three amino acid differences between *AGT1*$_{WH310}$ and *AGT1*$_{WH314}$ were investigated. Site-directed mutagenesis was used to demonstrate that the Thr^{505} and Ser^{557} are critical for maltotriose specificity, and V549A and the 23-C-terminal amino acids might be responsible for decreased maltotriose transport efficiency.

3. MATERIALS AND METHODS

3.1 MICROBIAL STRAINS, PLASMIDS, GROWTH MEDIA AND CULTURE CONDITIONS

The sources and relevant genotypes of bacterial and yeast strains, together with the plasmids used in this study, are listed in Table 1.

Ampicillin-resistance (Ap^{R}) transformants of *Escherichia coli* were grown in Luria Bertani (LB) broth (Ausubel *et al*. 1989) supplemented with ampicillin at a concentration of 100 μg ml^{-1}. Yeast strains were cultivated in the following media: YPD (1% yeast extract, 2% bacto-peptone and 2% glucose), YPM (1% yeast extract, 2% bacto-peptone and 2% maltose) and YPMt (1% yeast extract, 2% bacto-peptone and 2% maltotriose). *S. cerevisiae* transformants were cultivated on synthetic complete (SC) medium containing 2% glucose and 0.67% yeast nitrogen base (YNB) without amino acids (Difco, Sparks, MD, USA). No growth factors were added and uracil was used as the selectable marker. SCM^{-Ura} (containing 2% maltotriose, 0.67% YNB lacking uracil) was used to select *S. cerevisiae* transformants. Solid media contained 2% agar (Difco). Bacteria and yeasts were routinely cultured at 37°C and 30°C, respectively.

3.2 RECOMBINANT DNA TECHNIQUES AND METHODS

Standard methods for DNA amplification by the polymerase chain reaction (PCR) technique, manipulating and subcloning of DNA fragments, plasmid DNA isolations, *E. coli* transformation and Southern blot hybridisation were used (Ausubel *et al*. 1996). *S. cerevisiae* was transformed by using the Gietz and Schiestl method (1995).

All primers used for PCR amplifications are shown in Table 2. *AGT1*$_{WH310\Delta}$was amplified using AGT1-F and AGT1Δ-R with pSTAWH310 as the template. *AGT1*$_{WH310\Delta1}$, *AGT1*$_{WH310\Delta2}$ and *AGT1*$_{WH310\Delta3}$ were amplified through the use of AGT1-F and AGT1-R, and 310Δ1fix-F, 310Δ2fix-F, and 310Δ3fix-F, respectively, with AGT1-F pSTA1WH310 as the template. The 50-μl PCR reaction mixtures contained standard PCR buffer, 1.25 mM dNTPs (for *AGT1*$_{WH310\Delta}$) and 0.625 mM dNTPs (for the rest of the reactions), 1.5 mM $MgCl_2$, 0.3 μM of each primer, 1 ng template μl^{-1} and 5 U DNA polymerase (Roche, Mannheim, Germany). The following PCR programme was used to amplify *AGT1*$_{WH310\Delta}$:

denaturation, 5 min at 95°C; primer annealing, 30 s at 55°C; and primer extension, 30 s at 72°C. The following PCR programme was used to amplify *AGT1*$_{WH310\Delta1}$, *AGT1*$_{WH310\Delta2}$, and *AGT1*$_{WH310\Delta3}$: denaturation, 5 min at 95°C; primer annealing, 45 s at 48 °C and primer extension, 1 min at 72°C for 10 cycles. The amplification was followed by another cycle of denaturation, 30 s at 94°C; primer annealing, 45 s at 48°C with a 0.25 degree/cycle increment; and primer extension, 30 s at 72°C. All PCR products were thereafter cloned into the pGEM-Teasy (Promega, Madison, WI, USA) vector, according to the manufacturer's instructions and sequenced using the standard Promega primers.

The sequenced *AGT1* genes were isolated from the pGEM-T constructs with *Eco*RI and *Xho*I and ligated independently into plasmid pSTA1 (linearised with *Eco*RI and *Xho*I), generating plasmids pSTAWH310Δ, pSTAWH310Δ1, pSTAWH310Δ2 and pSTAWH310Δ3. These plasmids were separately linearised at the *Stu*I restriction site of the *URA3* marker gene, transformed into *S. cerevisiae* EBY.VW4000, plated separately on SCM^{-Ura}, and transformants EBYWH310Δ, EBYWH310Δ1, EBYWH310Δ2, and EBYWH310Δ3 were selected for further study.

Genomic DNA of the control and transformed yeast strains was digested with *Nco*I, which cuts once in the *AGT1* gene and once in the *URA3* gene. An *AGT1*-probe was used to confirm integration of the reconstructed *AGT1* genes into the genomes of the transformants through the Southern blot method (data not shown). The ΔMALx1DLEU2-F and ΔMALx1DLEU2-R primers (Table 2) were used to PCR-amplify a disruption cassette containing the *LEU2* gene flanked by the *MAL31* promoter and terminator sequences, using YCplac111 as template. These transformants were confirmed by Southern blot evaluation using a *LEU2*-probe amplified from ΔMALx1DLEU2 disruption cassette, using LEU2MALx1p-F and LEU2MALx1p-R as primers. All DNA probes were generated by PCR and the primers used to develop the 0.1-kb *AGT1* and *LEU2* probes are listed in Table 2. All DNA probes were labelled with a non-radioactive DIG labelling system (Roche), and Southern hybridisation was performed according to the recommendations of the DIG Application Manual.

3.3 SEQUENCE ANALYSES

Nucleotide sequences of yeast genes were obtained from the *Saccharomyces* genome database (http://genome-www2.stanford.edu/cgi-bin/SGD), and protein sequences were retrieved from the yeast proteome database (http://www.proteome.com/databases/YPD). Sequence analysis and alignment of sequences were conducted by using ClustalW (http://www.ebi.ac.uk/clustalw/). The transmembrane domain regions were determined through Transport Classification Database (http://www.tcdb.org/tcdb). Amino acids data presented as: database/position on sequence/Agt1p$_{WH310}$.

3.4 SUGAR TRANSPORT ASSAY

Maltotriose transport activity of the *S. cerevisiae* strains was assayed using *p*-nitrophenyl-α-D-maltoside (*p*NPM) (Sigma-Aldrich, St. Louis, MO, USA) as a substrate (Stambuk *et al.,* 1999). The amount of maltotriose transported is expressed as the quantity of intracellular *p*-nitrophenyl measured at a wavelength of 400 nm (absorbance at 400 nm; A_{400}) when the substrate is *p*NPM. The maltotriose transport activities of the different strains were determined and results are given in nmol mg^{-1} dry weight at 1-min intervals. All assays were done in duplicate with at least two different transformants for each strain, with a maximum deviation of less than 5%. The specificity for maltotriose was determined by using *p*NPM over a range of 1-80 mM. The transport of maltotriose was linear over the period measured (300 s); hence a computer-assisted kinetic analysis Eadee Hoffstee plot was used to determine the characteristics of different *AGT1*-encoded transporters (http://www.chemsw.com).

4. RESULTS

4.1 SEQUENCE ANALYSIS OF THE *AGT1* GENE

According to the transmembrane domain prediction by Han *et al*. (1995), the differences in the sequences of $Agt1p_{WH310}$ and $Agt1p_{WH314}$ can be located at the transmembrane domain 11 (TMD11) changing Ile^{505} to Thr (I505T), at TMD12 Val^{549} to Ala (V549A) and at the intracellular C-terminal region of TMD12 Thr^{557} to Ser (T557S) (Fig. 1). The other significant difference is found in the C-terminal region where a truncation of 23 amino acids has occurred (Smit *et al*. 2007). The Thr^{505} residue of $Agt1p_{WH310}$ is a polar residue that exhibits a hydrophilic character. In comparison, Ile^{505} of $Agt1p_{WH314}$ exhibits a hydrophobic character. These residues are found in the highly hydrophobic, intra-membrane region of the membrane protein, and the change in affinity for water can account for the transport of sugar through the membrane. Ala^{549} (CH_3-) and Val^{549} (CH3-CH(CH2)-) are both non-polar, but differ in side chain length. The differing side chain length might have functional consequences. Thr^{557} (CH_3-CH(OH)-) and Ser^{557} (HO-CH_2-) are both polar and exhibit hydrophilic characters. Once again, the side chain length variations might be significant in transport. This residue forms part of the long intracellular loop on the C-terminal and might be involved in various intracellular interactions.

4.2 ASSESSMENT OF STRUCTURE-FUNCTION RELATIONSHIPS OF THE α-GLUCOSIDE TRANSPORTERS

$Agt1p_{WH310}$ shows a 7.5 fold increase in rate of transport maltotriose over $Agt1p_{WH314}$ (Smit *et al*. 2007). The first question to answer when unravelling the relevance of the amino acid differences between $Agt1p_{WH310}$ and $Agt1p_{WH314}$ for maltotriose transport is how relevant the 23-amino acid truncation is. To determine its significance, the full length $AGT1_{WH310}$

was truncated to obtain *AGT1*$_{WH310\Delta}$. The *AGT1*$_{WH310\Delta}$ sequence was placed under control of the yeast phosphoglycerate kinase I gene (*PGK1*) promoter (*PGK1*$_P$) and terminator (*PGK1*$_T$) sequences in plasmid pSTAWH310Δ. This plasmid was linearised and integrated into the *URA3* locus of *S. cerevisiae* EBY VW4000. It was found that the resulting strain, EBYWH310Δ continued to show constitutive expression of the *AGT1* gene with the same genetic background, with all other maltotriose transporters knocked out. In Fig. 2 the level of maltotriose transport in strains EBYWH310, EBY314, and EBYWH310Δ is expressed as the amount of *p*NPM transported through the cell membrane in nmol/mg dry weight. The transport rate of EBYWH310Δ was found to be lower than that of EBYWH310, but not as low as that of EBYWH314. Thus, EBYWH310Δ is capable of more efficient transport of maltotriose than EBYWH314. This led to the conclusion that the 23 amino acids on the C-terminal of EBY310 are not essential for maltotriose transport, and to the assumption that the three point mutation differences between Agt1p$_{WH314}$ and Agt1p$_{WH310}$ are highly relevant for maltotriose specificity of Agt1p$_{WH310}$.

The putative implication of the individual point mutations present in the C-terminal of maltotriose transport was determined by replacing the Agt1p$_{WH310}$ amino acids Thr^{505} with Ile (Agt1p$_{WH310\Delta1}$), and Ser^{557} with Thr (Agt1p$_{WH310\Delta3}$) as compared to the database sequence. The Agt1p$_{WH310}$ V^{549} (Agt1p$_{WH310\Delta2}$) was replaced by an Ala residue as compared to the Agt1p$_{WH314}$ sequence. This was done by performing a three primer PCR reaction that changed T 1517 to C, T 1646 to C, and G 1670 to C, respectively for *AGT1*$_{WH310\Delta1}$, *AGT1*$_{WH310\Delta2}$ and *AGT1*$_{WH310\Delta3}$. These mutated *AGT1* genes were expressed under *PGK1* control and in the genetically uniform background of EBY VW4000. The growth of the strains containing the *AGT1*$_{WH310}$-expressed transporters was evaluated for maltotriose and maltose uptake. In Fig. 3A, growth on 2% maltotriose is shown for EBYΔMAL31WH310, EBYΔMAL31Δ1, EBYΔMAL31Δ2 and EBYΔMAL31Δ3. From the photograph presented in Fig. 3A, it is clear that EBYΔMAL31WH310 and EBYΔMAL31Δ2 grew more efficiently on the maltotriose agar plates than EBYΔMAL31Δ1 and EBYΔMAL31Δ3. The improved growth was also reflected in the integrated density values calculated for the growth density (Fig. 3B). EBYΔMAL31WH310 and EBYΔMAL31Δ2 exhibited an integrated density value of 17 and 15 respectively, with EBYΔMAL31Δ1 and EBYΔMAL31Δ3 having integrated density values of 3. Aerobic growth of these cultures was also observed on 2% maltose and 2% maltotriose. All strains showed growth on maltose, including the EBYVW4000 wild-type strain containing the *MAL31* gene. The transformed strains outperformed the wild-type strain by reaching the stationary phase with an optical density value at 600 nm (OD_{600}) of 8. By comparison, the wild-type strain reached the stationary phase at an OD_{600} of 5.85. This can be attributed to the overexpression of the transformed *AGT1* genes that led to multiple copies of the transporters present in the medium. The wild-type *MAL31* gene is expressed by its native promoter. Aerobic growth of the strains on maltotriose compared favourably with their growth on agar plates containing maltotriose. Once again, EBYΔMAL31WH310 grew fastest on maltotriose, reaching stationary phase at an OD_{600} value of 10. The growth

EBYΔMAL31Δ2 was less efficient, reaching the stationary phase at an OD_{600} value of 4.5. The EBYVW4000 wild-type strain did not grow well on maltotriose, reaching a maximum OD_{600} value of less than 1. The growth of EBYΔMAL31Δ1 and EBYΔMAL31Δ3 was less efficient than the wild-type strain, reaching an OD_{600} value of only 0.5.

Fig. 4 shows the maltotriose transport efficiencies of EBYWH310Δ1, EBYWH310Δ2, and EBYWH310Δ3. The mutated peptide $Agt1p_{WH310\Delta2}$ showed a 3.3–fold decrease in maltotriose transport efficiency, but it was still able to transport maltotriose. $Agt1p_{WH310\Delta1}$ and $Agt1p_{WH310\Delta3}$ showed a total absence of activity as regards maltotriose. $Agt1p_{WH310}$ is thus not capable of transporting maltotriose without Thr^{505} and Ser^{557}.

No V_{max} and K_m values could be calculated for EBYΔMAL31Δ1 and EBYΔMAL31Δ3. The results obtained for these strains over a concentration range of substrate were comparable with the background exhibited by the blank. Transformant EBYΔMAL31Δ2 showed a decrease in V_{max} with a value of approximately 57 nmol min^{-1} mg of dry wt^{-1} as compared to EBYΔMAL31WH310 at approximately 80 nmol min^{-1} mg of dry wt^{-1} (Smit *et al.* 2007).

5. DISCUSSION

In our previous study, we evaluated three different *AGT1*-encoded permeases that were obtained from three different *S. cerevisiae* strains ($AGT1_{310}$, $AGT1_{CENPK}$, and $AGT1_{WH314}$) with identical genetic backgrounds. Variations in the rate of maltotriose transport were also shown. The improved maltotriose uptake efficiency by the $AGT1_{310}$ transporter, compared to the $AGT1_{CENPK}$ and $AGT1_{WH314}$ transporters, was caused by a higher V_{max} and not by a lower K_m value (Smit *et al*. 2007). In the present study, a site-specific mutagenesis approach was implemented to characterise the importance of a 23-amino acid truncation on $Agt1p_{WH314}$, and the three amino acid differences of Thr^{505}, Val^{549} and Ser^{557} between $Agt1p_{WH310}$ and $Agt1p_{WH314}$. The 23-amino acid truncation was shown not to be essential for maltotriose transport, paving the way for the characterisation of the three individual point mutations Thr^{505}, Val^{549} and Ser^{557}. The Ser^{557} or Thr^{557} residues are known to be involved in protein phosphorylation that leads to catabolite inactivation of maltose transporters (Brondijk *et al*. 1998). Phosphorylation of the serine and threonine residues in a PEST sequence is associated with degradation signals (Medintz *et al*. 2000). Although no PEST sequence has been characterised for Agt1p, the T557S mutation on the intracellular loop of Agt1p might play a role in decreasing the rate of protein degradation.

Side-chain lengths and hydrophobicity in Agt1p play a significant role in maltotriose transport ability. This has been shown for Thr^{505}, Val^{549} and Ser^{557}. Although low sequence conservation for Thr^{505} and Ser^{557} is observed when compared to the other maltose transporters that show affinity for maltotriose, they are essential in the transport of maltotriose by $Agt1p_{WH310.}$ Val^{549} contributes to maltotriose transport, but does not appear to be as critical as Thr^{505} and Ser^{557}. It is interesting to note that Mal31p, thought it does not aid in the transport of maltotriose, also contains Ala^{549} in stead of Val^{549}.

Although no three-dimensional model is available for the maltose transporters, it is interesting to compare the maltose transporters to the model available for the human glucose transporter Glut1p. TMD11 for Glut1p is positioned inside the channel with TMD12 not forming part of the inner layer of the channel. If the three-dimensional model of Glut1p can act as a possible reference for Agt1p, Thr^{505} might play an essential role in interacting with maltotriose when moving through the channel. Val^{549} might be more relevant to protein folding and stability than direct interaction with maltotriose, which can explain the fact that Val^{549} has proven not to be essential for maltotriose transport. Ser^{557} may have an essential intracellular role, which might have to do with ATP activity, interaction with inhibitors, activators or any other related intracellular reaction.

The loss of the ability of $Agt1p_{WH310\Delta1}$ and $Agt1p_{WH310\Delta3}$ to transport maltotriose raises the question of whether these mutations led to non-functional proteins. The fact that EBYΔMAL31Δ1 and EBYΔMAL31Δ3 grew more efficiently on maltose than the reference strain led to the conclusion that $AGT1_{WH310\Delta1}$ and $AGT1_{WH310\Delta3}$ code for functional proteins still capable of transporting sugar – in the present instance, maltose.

The decrease in V_{max} of EBYΔMAL31Δ2 – when compared to EBYΔMAL31WH310 – indicated a decrease in the rate with which the molecule moved through the membrane. However, the K_m value remained unchanged, indicating that Val^{549} might not have any relation with substrate specificity. The 7.5-fold difference present in maltotriose transport efficiency of $Agt1p_{WH310}$ when compared to $Agt1p_{WH314}$ can be directly linked to Thr^{505}, Val^{549} and Ser^{557}.

Thr^{505}, located in transmembrane segment (TM) 11 and Ser^{557}, located on the intracellular segment after TM 12 of $Agt1p_{WH310}$, are essential for efficient maltotriose transport by this protein. $Agt1p_{CENPK}$ and $Agt1p_{WH310}$ show 26 amino acid point mutation differences, including Thr^{505} and Ser^{557} (Smit *et al*. 2007). It would be interesting to see whether Thr^{505} and Ser^{557}, when mutated in $Agt1p_{CENPK}$, can lead to elevated maltotriose transport efficiency in $Agt1p_{CENPK}$, or whether the other 24 amino acids contribute to the difference in maltotriose transport efficiency when compared to $Agt1p_{WH310}$.

In conclusion, the Thr505 and Ser557 residues, which are respectively located in the transmembrane (TM) segment TM11 and on the intracellular segment after TM12 of the *AGT1*-encoded α-glucoside transporters, are critical for efficient transport of maltotriose in *S. cerevisiae*. This study paves the way for the development of engineered maltotriose transporters adapted to starch-efficient fermentation systems, and offers prospects for the development of yeast strains with improved maltose and maltotriose uptake capabilities that, in turn, could increase the overall fermentation efficiencies in the beer, whisky, baking and bioethanol industries.

6. ACKNOWLEDGEMENTS

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Table 1 Strains and plasmids used in this study

Plasmids and strains	Relevant features	Sources/References
Plasmids:		
YCplac111	Ap^R *LEU2*	Gietz and Sugino, 1988
pSTA1	Ap^R $PGK1_{PT}$ *URA3*	Gundllapalli *et al*., 2001
pSTACENPK	Ap^R $PGK1_P$ $AGT1_{CENPK}$ $PGK1_T$ *URA3*	Smit *et al*., 2007
pSTAWH310	Ap^R $PGK1_P$ $AGT1_{WH310}$ $PGK1_T$ *URA3*	Smit *et al*., 2007
pSTAWH314	Ap^R $PGK1_P$ $AGT1_{WH314}$ $PGK1_T$ *URA3*	Smit *et al*., 2007
pSTAWH310Δ	Ap^R $PGK1_P$ $AGT1_{WH310\Delta}$ $PGK1_T$ *URA3*	This study
pSTAWH310Δ1	Ap^R $PGK1_P$ $AGT1_{WH310\Delta1}$ $PGK1_T$ *URA3*	This study
pSTAWH310Δ2	Ap^R $PGK1_P$ $AGT1_{WH310\Delta2}$ $PGK1_T$ *URA3*	This study
pSTAWH310Δ3	Ap^R $PGK1_P$ $AGT1_{WH310\Delta3}$ $PGK1_T$ *URA3*	This study
Eschericchia coli strains:		
DH5α	*sup*E44 ρ*lac*U169 (ϕ*80lac*ZρM15) *hsd*R17 *rec*A1 *gyr*A96 *thi*-1 *rel*A1	GIBCO/Bethesda Research Laboratories
Saccharomyces strains:		
WH301	Industrial strain	DCL Scotland
WH310	Industrial strain	DY10; ex Anchor
WH313	Industrial strain	NRRL Y-987
WH314	Industrial strain	NRRL Y-567
S. cerevisiae CEN.PK2-1D	*MATα leu2-3, 112 ura3-52 trp1-289 his3-Δ1*	Entian and Kötter, 1998
S. cerevisiae EBY VW4000	*MATα leu2-3,112 ura3-52 trp1-289 his3-Δ1 MAL2-8 SUC2 hxt8-17Δ hxt514Δ hxt2Δ hxt367Δ gal2Δ stl1Δ agt1Δ ydl247wΔ yjr160cΔ*	Wieczorke *et al.,* 1999
EBYWH310	EBY VW4000 *URA3*::pSTAWH310	Smit *et al*., 2007
EBYWH314	EBY VW4000 *URA3*::pSTAWH314	Smit *et al*., 2007
EBYWH310Δ	EBY VW4000 *URA3*::pSTAWH310Δ	This study
EBYWH310Δ1	EBY VW4000 *URA3*::pSTAWH310Δ1	This study
EBYWH310Δ2	EBY VW4000 *URA3*::pSTAWH310Δ2	This study
EBYWH310Δ3	EBY VW4000 *URA3*::pSTAWH310Δ3	This study
EBYΔMAL31WH310	EBYWH310, *LEU2*::*mal2-8Δ*	Smit et al., 2007
EBYΔMAL31WH310Δ1	EBYWH310Δ1, *LEU2*::*mal2-8Δ*	This study
EBYΔMAL31WH310Δ2	EBYWH310Δ2, *LEU2*::*mal2-8Δ*	This study
EBYΔMAL31WH310Δ3	EBYWH310Δ3, *LEU2*::*mal2-8Δ*	This study

CBS: Centraalbureau voor Schimmelcultures, The Netherlands; NRRL-ARS Culture Collection, USA.

Table 2 Primers used in this study

Primer	Oligonucleotide sequence*
AGT1-F	5'-**GAATTC**ATGAAAAATATCATTTCATT-3'
AGT1-R	5'-**CTCGAG**TTAACATTTATCAGCTG-3'
AGT1p-F	5'-GCCCAGTTAGGAGATTCTG-3'
AGT1p-R	5'-TTTTTTCCTCGCTGTTAGCT-3'
AGT1Δ-R	5'-**CTCGAG**TTATTGAGTTTTTCCC-3'
310Δ1fix-F	5'-GCTGGCCCGTATTTGCTAC-3'
310Δ2fix-F	5'-TAGCTTGGGCCATCATCG-3'
310Δ3fix-F	5'-GCCTGAGACAACTGGTAGAACC-3'
MALx1p-F	5'-CAGCAGCTGCAGAAATTAAT-3'
MALx1p-R	5'-CATTTGTTCACAACAGATGG-3'
ΔMALx1LEU2-F	5'-CAGTATAACAATAAGAATTACATCCAAGACTATTAATTAACGGCGTATCACGAGGCCCTTT-3'
ΔMALx1LEU2-R	5'-AAAAAAAAGTCATAATGTCGAGTAAAAATAAAATCCCATTCCATGCGGGGTA-3'
LEU2MALx1p-F	5' CAGTATAACAATAAGAATTACATCCAAG 3'
LEU2MALx1p-R	5' GGAAGTGGAACACCTGTAGC 3'

* The restrictions sites are indicated in bold

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Agt1p           MKNIISLVSKKKAASKNEDKNISESSRDIVNQQEVFNTEDFEEGKKDSAFELDHLEFTTNSAQLGDSDEDNENVINEMNATDDANEANSEEKSMTLKQAL
Agt1310p        MKNIISLVSKKKAASKNEDKNISESSRDIVNQQEVFNTENFEEGKKDSAFELDHLEFTTNSAQLGDSDEDNENVINETNTTDDANEANSEEKSMTLKQAL
Agt1314p        MKNIISLVSKKKAASKNEDKNISESSRDIVNQQEVFNTENFEEGKKDSAFELDHLEFTTNSAQLGDSDEDNENVINETNTTDDANEANSEEKSMTLKQAL
Mal31p          MKGLSSLINRKKDRNDSHLDEIENGVN-----ATEFNSIEMEEQGKKSDFDLSHLEYGPGSLIPNDNNE--EVPDLLDEAMQDAKEADESERGMPLMTAL

Agt1p           LKYPKAALWSILVSTTLVMEGYDTALLSALYALPVFQRKFGTLNG-EGSYEITSQWQIGLNMCVLCGEMIGLQITTYMVEFMGNRYTMITALGLLTAYIF
Agt1310p        LIYPKAALWSILVSTTLVMEGYDTALLNALYALPVFQRKFGTLNG-EGSYEITSQWQIGLNMCVQCGEMIGLQITPYMVEFMGNRYTMITALGLLTAYVF
Agt1314p        LIYPKAALWSILVSTTLVMEGYDTALLNALYALPVFQRKFGTLNGEGSYEITSQWQIGLNMCVQCGEMIGLQITPYMVEF MGNRYTMITALGLLTAYVF
Malx1p          KTYPKAAAWSLLVSTTLIQEGYDTAILGAFYALPVFQKKYGSLNSNTGDYEISVSWQIGLCLCYMAGEIVGLQMTGPSVDYMGNRYTLIMALFFLAAFIF

Agt1p           ILYYCKSLAMIAVGQILSAIPWGCFQSLAVTYASEVCPLALRYYMTSYSNICWLFGQIFASGIMKNSQENLGNSDLGYKLPFALQWIWPAPLMIGIFFAP
Agt1310p        ILYYCKSLAMIAVGQVLSAMPWGCFQGLTVTYASEVCPLALRYYMTSYSNICWLFGQIFASGIMKNSQENLGNSDLGYKLPFALQWIWPAPLMIGIFFAP
Agt1314p        ILYYCKSLAMIAVGQVLSAMPWGCFQGLTVTYASEVCPLALRYYMTSYSNICWLFGQIFASGIMKNSQENLGNSDLGYKLPFALQWIWPAPLMIGIFFAP
Mal31p          ILYFCKSLGMIAVGQALCGMPWGCFQCLTVSYASEICPLALRYYLTTYSNLCWAFGQLFAAGIMKNSQNKYPNSDLGYKLPFALQWIWPLPLAVGIFFAP

Agt1p           ESPWWLVRKDRVAEARKSLSRILSGKGAEKDIQVDLTLKQIELTIEKERLLASKSGSFFNCFKG-VNGRRTRLACLTWVAQNSSGAVLLGYSTYFFERAG
Agt1310p        ESPWWLVRKDRVAEARKSLSRILSGKGAEKDIQIDLTLKQIELTIEKERLLASKSGSFFDCFKG-VNGRRTRLACLTWVAQNTSGACLLGYSTYFFERAG
Agt1314p        ESPWWLVRKDRVAEARKSLSRILSGKGAEKDIQIDLTLKQIELTIEKERLLASKSGSFFDCFKG-VNGRRTRLACLTWVAQNTSGACLLGYSTYFFERAG
Mal31p          ESPWWLVKKGRIDQARRSLERTLSGKGPEKELLVSMELDKIKTTIEKEQKMSD-EGTYWDCVKDGINRRRTRIACLCWIGQCSCGASLIGYSTYFYEKAG

Agt1p           MATDKAFTFSLIQYCLGLAGTLCSWVISGRVGRWTILTYGLAFQMVCLFIIGGMGFGSGSSASNGAGGLLLALSFFYNAGIGAVVYCIVAEIPSAELRTK
Agt1310p        MATDKAFTFSVIQYCLGLAGTLCSWVISGRVGRWTILTYGLAFQMVCLFIIGGMGFGSGSGASNGAGGLLLALSFFYNAGIGAVVYCIVTEIPSAELRTK
Agt1314p        MATDKAFTFSVIQYCLGLAGTLCSWVISGRVGRWTILTYGLAFQMVCLFIIGGMGFGSGSGASNGAGGLLLALSFFYNAGIGAVVYCIVTEIPSAELRTK
Mal31p          VSTDTAFTFSIIQYCLGIAATFVSWWASKYCGRFDLYAFGLAFQAIMFFIIGGLGCSDTHGAKMGSGALLMVVAFFYNLGIAPVVFCLVSEIPSSRLRTK

Agt1p           TIVLARICYNLMAVINAILTPYMLNVSDWNWGAKTGLYWGGFTAVTLAWVIIDLPETTGRTFSEINELFNQGVPARKFASTVVDPFGKGKTQHDSLADES
Agt1310p        TIVLARTCYNIMAVINAILTPYMLNVSDWNWGAKTGLYWGGFTAVTLAWVIIDLPETSGRTFSEINELFNQGVPARKFASTVVDPFGKGKTQHDSLADES
Agt1314p        TIVLARICYNIMAVINAILTPYMLNVSDWNWGAKTGLYWGGFTAVTLAWAIIDLPETTGRTFSEINELFNQGVPARKFASTVVDPFGKGKTQLIR-----
Mal31p          TIILARNAYNVIQVVVTVLIMYQLNSEKWNWGAKSGFFWGGFCLATLAWAVVDLPETAGRTFIEINELFRLGVPARKFKSTKVDPFAAAKAAAAEINVKD

Agt1p           ISQSSSIKQRELNAADKC----
Agt1310p        ISQSSSIKQRELNAADKC----
Agt1314p        ----------------------
Mal31p          PKEDLETSVVDEGRSTPSVVNK
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Fig. 1 Amino acid alignment of Mal31p, Agt1p$_{WH310}$, Agt1p$_{WH314}$, and Agt1p$_{DB}$. Mutations observed in literature are underlined with a dotted line. Putative transmembrane domains are underlined as predicted by the Transport classification website http://www.tcdb.org/tcdb. The mutations addressed in this study is underlined and shown in bold. The alignment was performed through ClustalW application

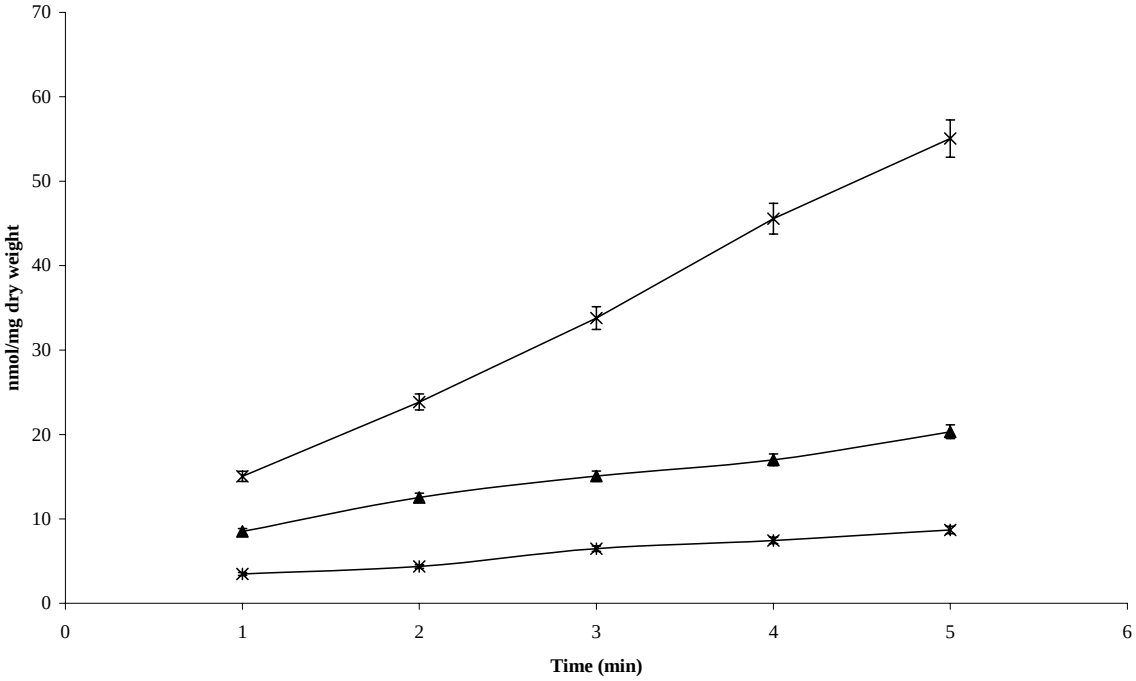
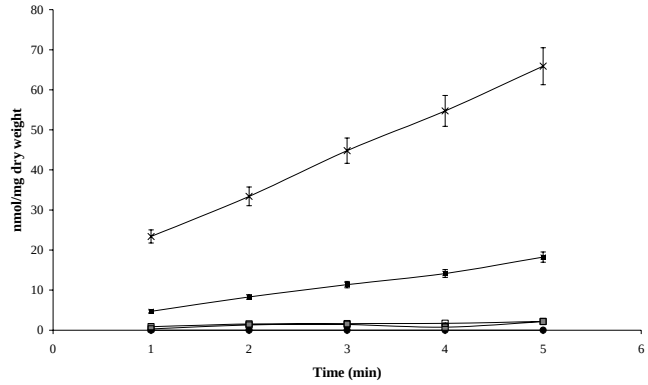


Fig. 2 *p*-Nitrophenyl-α-D-maltoside uptake of the *Saccharomyces cerevisiae* strains EBYWH310(×), EBYWH314(✳), and EBY310Δ (▲), expressed in nmol.min^{-1} dry weight for time intervals of 1 min

A



B.

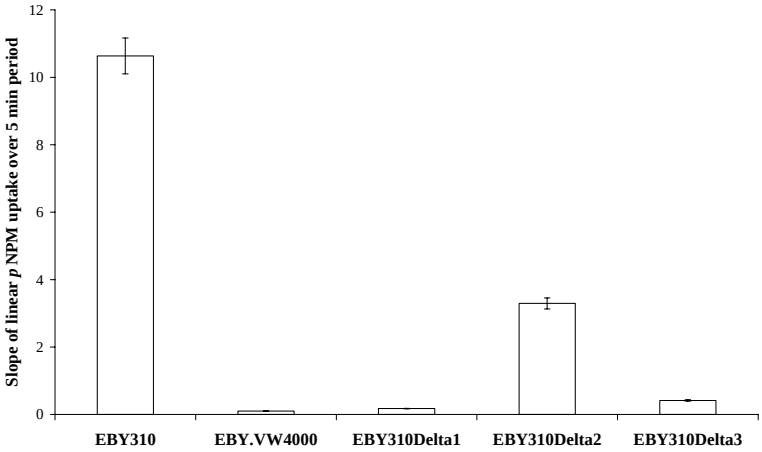


Fig. 3 The rate of *p*-Nitrophenyl-α-D-maltoside (*p*NPM) uptake in various *Saccharomyces cerevisiae* strains. (**A**) *p*NPM uptake of *S. cerevisiae* strains EBYΔMAL31WH310(×), EBYVW4000(•), EBYΔMAL31Δ1(□), EBYΔMAL31Δ2(■) and EBYΔMAL31Δ3(□). (**B**) The transport rate for *p*NPM transport of the EBY strains. The transport rate is determined from the slope of the linear uptake of *p*NPM over a 5 min time period

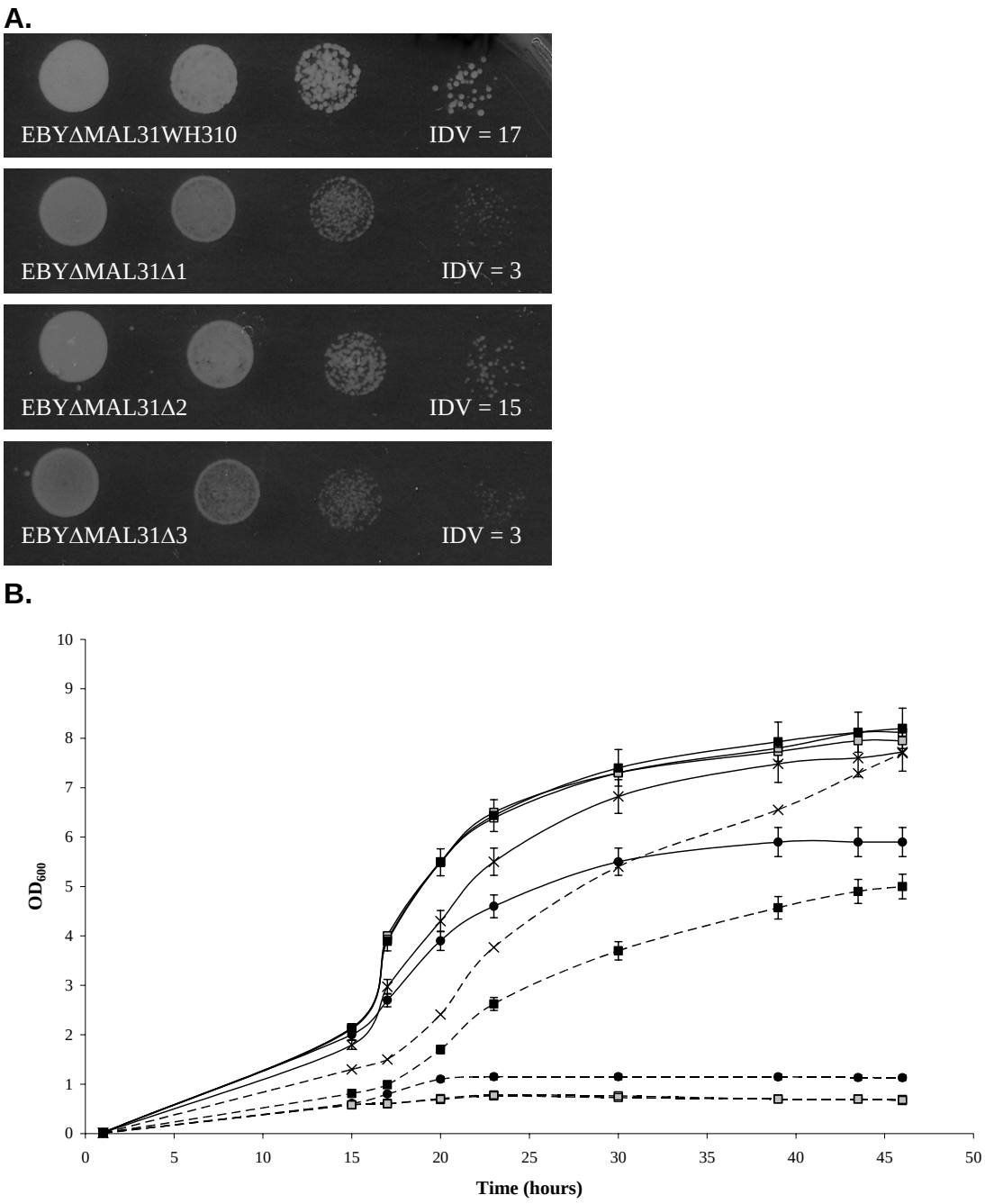


Fig. 4 Time-course growth curves of various *Saccharomyces cerevisiae* strains in different culture media. (**A**) Growth of the *S. cerevisiae* strains EBYΔMAL31WH310, EBYΔMAL31Δ1, EBYΔMAL31Δ2 and EBYΔMAL31Δ3 on 2% maltotriose minimal media with a 10 X dilution range starting at OD_{600} = 0.5. The integrated density value was calculated as the average value over four days at 24 hour intervals. (**B**) Growth in 2% maltose (solid lines) and 2% maltotriose (dashed lines) minimal broth was measured for EBYΔMAL31WH310(×), EBYVW4000(•), EBYΔMAL31Δ1(□), EBYΔMAL31Δ2(■) and EBYΔMAL31Δ3(□). Cells were pre-cultured on YPM

7. REFERENCES

Abramson, J., Smirnova, I., Kasho, V., Verner, G., Kaback, H.R. and Iwata, S. (2003) Structure and mechanism of the lactose permease of *Escherichia coli*. Science 301, 610-5.

Ausubel, F.M., Brent, R., Kingston, R.E., Moore, D.D., Seidman, J.G., Smith, J.A. and Strul, K. (1996). Current Protocols in Molecular Biology, Wiley, New York.

Brondijk, T.H., Van Der Rest, M.E., Pluim, D., De Vries, Y., Stingl, K., Poolman, B. and Konings, W.N. (1998) Catabolite inactivation of wild-type and mutant maltose transport proteins in *Saccharomyces cerevisiae*. Journal of Biological Chemistry 273, 15352-15357.

Chang, Y.S., Dubin, R.A., Perkins, E., Michels, C.A. and Needleman, R.B. (1989) Identification and characterization of the maltose permease in genetically defined *Saccharomyces* strain. Journal of Bacteriology 171, 6148-6154.

Day, R.E., Higgins, V.J., Rogers, P.J. and Dawes, I.W. (2002) Characterization of the putative maltose transporters encoded by YDL247w and YJR160c. Yeast 19, 1015-1027.

Dietvorst, J., Londesborough, J. and Steensma, H.Y. (2005) Maltotriose utilization in lager yeast strains: *MTT1* encodes a maltotriose transporter. Yeast 22, 775-788.

Gadura, N. and Michels, C.A. (2006) Sequences in the N-terminal cytoplasmic domain of *Saccharomyces cerevisiae* maltose permease are required for vacuolar degradation but not glucose-induced internalization. Current Genetics 50, 101-114.

Goswitz, V.C. and Brooker, R.J. (1995) Structural features of the uniporter/symporter/antiporter superfamily. Protein Science 4, 534-537.

Entian, K-D. and Kötter, P. (1998) Yeast mutant and plasmid collections. In: Methods in Microbiology ed. Brown A.J.P. and Tuite M. San Diego: Academic Press.

Gietz, R.D., Schiestl, R.H., Willems, A.R. and Woods, R.A. (1995) Studies on the transformation of intact yeast cells by the LiAc/SS-DNA/PEG procedure. Yeast 11, 355-360.

Gundllapalli Moses, S., Cordero Otero, R.R., La Grange, D.C., Pretorius, I.S. and Van Rensburg, P. (2001) Different genetic backgrounds influence the expression, secretion and substrate utilization of recombinant *Saccharomyces cerevisiae* strains. Biotechnology Letters 24, 651-656.

Han, E.K., Cotty, F., Sottas, C., Jiang, H. and Michels, C.A. (1995) Characterization of *AGT1* encoding a general α-glucoside transporter from *Saccharomyces*. Molecular Microbiology 17, 1093-1107.

Hein, C., Springael, J.Y., Volland, C., Haguenauer-Tsapis, R. and Andre, B. (1995) *NPI1*, an essential yeast gene involved in induced degradation of Gap1 and Fur4 permeases, encodes the Rsp5 ubiquitin-protein ligase. Molecular Microbiology 18, 77-87.

Hirai, T., Heymann, J.A., Shi, D., Sarker, R., Maloney, P.C. and Subramaniam, S. (2002) Three-dimensional structure of a bacterial oxalate transporter. Nature Structural Biology 9, 597-600.

Hough, J.S., Briggs, D.E., Stevens, R. and Young, I.W. (1981) Malting and brewing sciences. Cambridge University Press. Cambridge.

Huang, Y., Lemieux, M.J., Song, J., Auer, M. and Wang, D.N. (2003) Structure and mechanism of the glycerol-3-phosphate transporter from *Escherichia coli*. Science 301, 616-620.

Kasahara, T. and Kasahara, M. (1998) Tryptophan 388 in putative transmembrane segment 10 of the rat glucose transporter Glut1 is essential for glucose transport. Journal Biological Chemistry 273, 29113-29117.

Marchal, C., Haguenauer-Tsapis, R. and Urban-Grimal, D. (1998) A PEST-like sequence mediates phosphorylation and efficient ubiquitination of yeast uracil permease. Molecular and Cellular Biology 18, 314-321.

Medintz, I., Wang, X., Hradek, T. and Michels, C.A. (2000) A PEST-like sequence in the N-terminal cytoplasmic domain of *Saccharomyces* maltose permease is required for glucose-induced proteolysis and rapid inactivation of transport activity. Biochemistry 39, 4518-4526.

Rechsteiner, M. (1988) Regulation of enzyme levels by proteolysis: the role of pest regions. Advances in Enzyme Regulation 27, 135-151.

Saier, M.H.Jr. (2000) Families of transmembrane sugar transport proteins. Molecular Microbiology 35, 699-710.

Salas-Burgos, A., Iserovich, P., Zuniga, F., Vera, J.C. and Fischbarg, J. (2004) Predicting the three-dimensional structure of the human facilitative glucose transporter glut1 by a novel evolutionary homology strategy: insights on the molecular mechanism of substrate migration, and binding sites for glucose and inhibitory molecules. Biophysical Journal 87, 2990-2999.

Salema-Oom M., Valadao Pinto V., Goncalves P., Spencer-Martins I. (2005). Maltotriose utilization by industrial *Saccharomyces* strains: characterization of a new member of the α-glucoside transporter family. Appl Environ Microbiol, 71: 5044-5049.

Smit, A., Cordero Otero, R.R. and Pretorius, I.S. (2007) Differences among *AGT1*-encoded alpha-glucoside transporters and their ability to transport maltotriose in *Saccharomyces* yeasts. Annals of Microbiology 57,.

Stambuk, B.U., Da Silva, M.A., Panek, A.D. and De Araujo, P.S. (1999) Active alpha-glucoside transport in *Saccharomyces cerevisiae*. FEMS Microbiology Letters 170, 105-110.

Stanbrough, M. and Magasanik, B. (1995) Transcriptional and posttranslational regulation of the general amino acid permease of *Saccharomyces cerevisiae*. Journal of Bacteriology 177, 94-102.

Wieczorke, R., Krampe, S., Weierstall, T., Freidel, K., Hollenberg, C.P. and Boles, E. (1999) Concurrent knock-out of at least 20 transporter genes is required to block uptake of hexoses in *Saccharomyces cerevisiae*. FEBS Letters 464, 123-128.

Zastrow, C.R., Hollatz, C., De Araujo, P.S., Stambuk, B.U. (2001). Maltotriose fermentation by *Saccharomyces cerevisiae*. Journal of Industrial Microbiology and Biotechnology 27, 34-38.

Chapter 6

GENERAL DISCUSSION AND CONCLUSIONS

GENERAL DISCUSSION AND CONCLUSIONS

Starch degradation comes into play in beer brewing, bread leavening, whisky production and the fermentation of starchy crops to produce biofuel. With starch degradation, sucrose and glucose are fermented before maltose and maltotriose. Maltose and maltotriose are widely believed to share the same transport system; however, the rate of maltotriose uptake is lower than that of maltose (Day *et al*., 2002; Zheng *et al*., 1994). These findings all support the conclusion that maltotriose is only utilised in the later stages of fermentation.

A common problem in the brewing industry is the incomplete fermentation of the available carbohydrates and, in many instances the main residual sugar is maltotriose (Stewart *et al*., 1997). Glucose repression is largely responsible for the late utilisation of maltotriose after the less complex sugars - glucose and fructose - have been taken up (Meneses *et al*., 2002). Another contributing factor is that the yeast maltotriose transporters are also maltose transporters, with high affinities for maltose (the most abundant sugar in wort). This leads to belated maltotriose consumption. Many studies have been conducted in an attempt to improve maltotriose utilisation and fermentation in yeast, mainly focusing on identifying new transporters for maltotriose.

Several maltose transporters have been characterised for yeast: the Malx1p maltose permeases, Agt1p, Mtt1p, Mph2p and Mph3p. The substrate ranges for these permeases have been evaluated with conflicting results. The Malx1p have mainly been inconclusive for maltotriose transport. Tests using radio-labelled maltotriose showed that Mal31p and Mal61p were capable of maltotriose transport (Day *et al*., 2002). Han *et al*. (1995), however, reported that a yeast strain containing only Mal61p could not grow on maltotriose, with Mal21p being incapable of H^+ symport activity (Stambuk and De Araujo, 2001; Stambuk *et al*., 1999). Day *et al*. (2002) showed that only Agt1p, Mph2p and Mph3p are capable of transporting maltotriose. Recently, Mty1p/Mtt1p has been identified as another maltose transporter capable of transporting maltotriose (Dietvorst *et al*., 2005; Salema-Oom *et al*. 2005). The conflicting data could possibly be explained by the variations in maltotriose transport determination. The most significant reason is probably the high levels of contamination observed in commercial [^{14}C]-maltotriose. Contamination with impure labelled sugars might cause gross overestimation of maltotriose uptake (Dietvorst *et al.,* 2005).

Various approaches can be followed to address the problems occurring during fermentation and to combat the high levels of residual maltotriose. Firstly, the transport efficiency of the available maltotriose transporters can be evaluated, characterised and possibly improved. As more information becomes available on which domains of the transporters are responsible for maltotriose recognition and movement through the membrane, one could get closer to characterising and improving the efficiency of these

transporters in maltotriose uptake. Another important focus area is the characterisation of the glucose repression and inactivation system. For example, if the recognition areas responsible for protein degradation due to the presence of glucose can be identified, efforts to nullify their effects can lead to the availability of maltotriose transporters in the membrane, despite the presence of glucose. This might lead to simultaneous consumption of the simple and complex sugars available in the fermentation mix. The third aspect is the competition between maltose and maltotriose uptake at the same time by the same transporter. The Mtt1p maltose transporter seems to show a higher affinity for maltotriose than for maltose and, when characterised, could lead to interesting conclusions on the uptake competition between maltose and maltotriose. The effect this competition has on fermentation has also not been fully characterised. Agt1p (showing higher affinity for maltose than maltotriose), shows an increase in maltotriose transport and maltotriose fermentation when overexpressed in media containing a maltose and maltotriose mixture (Stambuk *et al*., 2006).

When this study was initiated, Agt1p was reported as the maltose transporter showing the highest affinity for maltotriose of all the maltose transporters then identified. Our approach was to focus on Agt1p characterisation in order to unravel its maltotriose transport functionality.

As part of our first aim, namely to assess the possible genetic aspects that influence the transporting abilities of different strains, *Saccharomyces* strains that are able to grow efficiently on maltotriose as sole carbon source were identified. The *AGT1* loci were mapped and isolated and expressed constitutively in the same genetic background. Maltotriose transport differences can be directly correlated with the same differences observed in growth. Thus, the differences found in the Agt1p sequences of the evaluated strains, when expressed in an identical genetic background, account for the variations in the rate of maltotriose transport. The three permeases that were tested in this study transported maltotriose. The improved performance of the $AGT1_{310}$ transporter, compared to the $AGT1_{CENPK}$ and $AGT1_{WH314}$ transporters, was caused by a higher $V_{max,}$ and not by a lower K_m value.

Functional chimerics of Agt1p and Mal31p were constructed and expressed in the same genetic background. This led to an in-depth evaluation of the ability of these proteins to transport maltotriose in an attempt to identify the domains in Agt1p responsible for maltotriose affinity. Hypothetical domains were identified in Agt1p, which can be essential for maltotriose transport.

Mal31p, A349Mp, A56Mp, A458Mp, M349Ap and M458Ap show no affinity for maltotriose. The specificity for maltotriose could not be localised to one area of Agt1p. The differences between these two proteins, Mal31p and Agt1p, are very important in maltotriose specificity, and transforming Mal31p into a protein with affinity for maltotriose seem not to be solely domain driven, but rather linked to specific essential residues in Agt1p.

A site-specific mutagenesis approach was implemented to characterise the importance of a 23-amino acid C-terminal truncation on Agt1p$_{WH314}$ and the three amino acid differences of Thr^{505}, Val^{549} and Ser^{557} between Agt1p$_{WH310}$ and Agt1p$_{WH314}$. The 23-amino acid truncation was shown to not be essential for maltotriose transport, and this paved the way for the characterisation of the three individual point mutations.

Side chain lengths and hydrophobicity in Agt1p play a big role in maltotriose transport ability. This is shown for Thr^{505}, Val^{549} and Ser^{557}. Although low conservation for Thr^{505} and Ser^{557} is observed when compared to the other maltose transporters that show affinity for maltotriose, they are essential for the transport of maltotriose by Agt1p$_{WH310.}$ Val^{549} contributes to maltotriose transport, but does not appear to be as critical as Thr^{505} and Ser^{557}.

The decrease in V_{max} showed by EBYΔMAL31Δ2 when compared to EBYΔMAL31WH310 indicates a decrease in the speed with which the molecule moves through the membrane. The K_m, however, remained unchanged, indicating that Val^{549} might not have any relation with substrate affinity. The 7.5-fold difference in maltotriose transport efficiency between Agt1p$_{WH310}$ and Agt1p$_{WH314}$ can be directly linked to Thr^{505}, Val^{549} and Ser^{557}**.**

In conclusion, the research described in this thesis has provided interesting insights into the importance of specific amino acid residues in Agt1p for maltotriose transport. Thr^{505} (located in transmembrane segment (TM) 11) and Ser^{557} (located on the intracellular segment after TM 12 of Agt1p$_{WH310}$) are essential for efficient maltotriose transport by this protein. Agt1p$_{CENPK}$ and Agt1p$_{WH310}$ show 26 amino acid point mutation differences, including Thr^{505} and Ser^{557} (Smit *et al*., 2007). It would be interesting to determine whether Thr^{505} and Ser^{557}, when mutated in Agt1p$_{CENPK}$, can lead to elevated maltotriose transport efficiency in Agt1p$_{CENPK}$, or whether the other 24 amino acids differences also shown for Agt1p$_{CENPK}$ and Agt1p$_{WH310}$ contribute to the difference in maltotriose transport efficiency when compared to Agt1p$_{WH310}$. Stambuk *et al*., 2006 showed that an increase in maltotriose transport directly correlated with an increased fermentation capacity. Seeing that Agt1p$_{WH310}$ outperformed all other proteins tested for maltotriose transport in this study and might be used to improve the fermentation of maltotriose to overcome incomplete fermentation. It might also be feasible to test the chimeric proteins on other sugars for which Agt1p shows affinity, coupled with test of glucose inactivation. The information gathered can facilitate the development of engineered transporters adapted to efficient starch fermentation systems.

1. REFERENCES

Day R.E., Rogers P.J., Dawes I.W., Higgins V.J. (2002). Molecular analysis of maltotriose transport and utilization by *Saccharomyces cerevisiae*. Appl Environ Microbiol, 68: 5326-5335.

Dietvorst J., Londesborough J., Steensma H.Y. (2005). Maltotriose utilization in lager yeast strains: *MTT1* encodes a maltotriose transporter. Yeast, 22: 775-788.

Han E.K., Cotty F., Sottas C., Jiang H., Michels C.A. (1995). Characterization of *AGT1* encoding a general α-glucoside transporter from *Saccharomyces*. Mol Microbiol, 17: 1093-1107.

Meneses F.J., Henschke P.A., Jiranek V. (2002). A survey of industrial strains of *Saccharomyces cerevisiae* reveals numerous altered patterns of maltose and sucrose utilisation. J Inst Brew, 108: 310-321.

Salema-Oom M., Valadao Pinto V., Goncalves P., Spencer-Martins I. (2005). Maltotriose utilization by industrial *Saccharomyces* strains: characterization of a new member of the α-glucoside transporter family. Appl Environ Microbiol, 71: 5044-5049.

Smit A., Otero R.R.C., Pretorius I.S. (2007). Differences among *AGT1*-encoded α-glucoside transporters and their ability to transport maltotriose in *Saccharomyces* yeasts. Annals of Microbiology, 57: 77-84.

Stambuk B.U., Da Silva M.A., Panek A.D., De Araujo P.S. (1999). Active α-glucoside transport in *Saccharomyces cerevisiae*. FEMS Microbiol Lett, 170: 105-110.

Stambuk B.U., De Araujo P.S. (2001). Kinetics of active alpha-glucoside transport in *Saccharomyces cerevisiae*. FEMS Yeast Res, 1: 73-78.

Stambuk B.U., Alves S.L.Jr., Hollatz C., Zastrow C.R. (2006). Improvement of maltotriose fermentation by *Saccharomyces cerevisiae*. Letters in Applied Microbiology, 43: 370–376.

Stewart G.G., Bothwick R., Bryce J., Cooper D., Cunningham S., Hart C., Rees E. (1997). Recent developments in High Gravity Brewing. Tech Q Master Brew Assoc Am, 34: 264-270.

Zheng X., D'Amore T., Russell I., Stewart G.G. (1994). Factors influencing maltotriose utilization during brewery wort fermentations. J Am Soc Brew, 52: 41–47.