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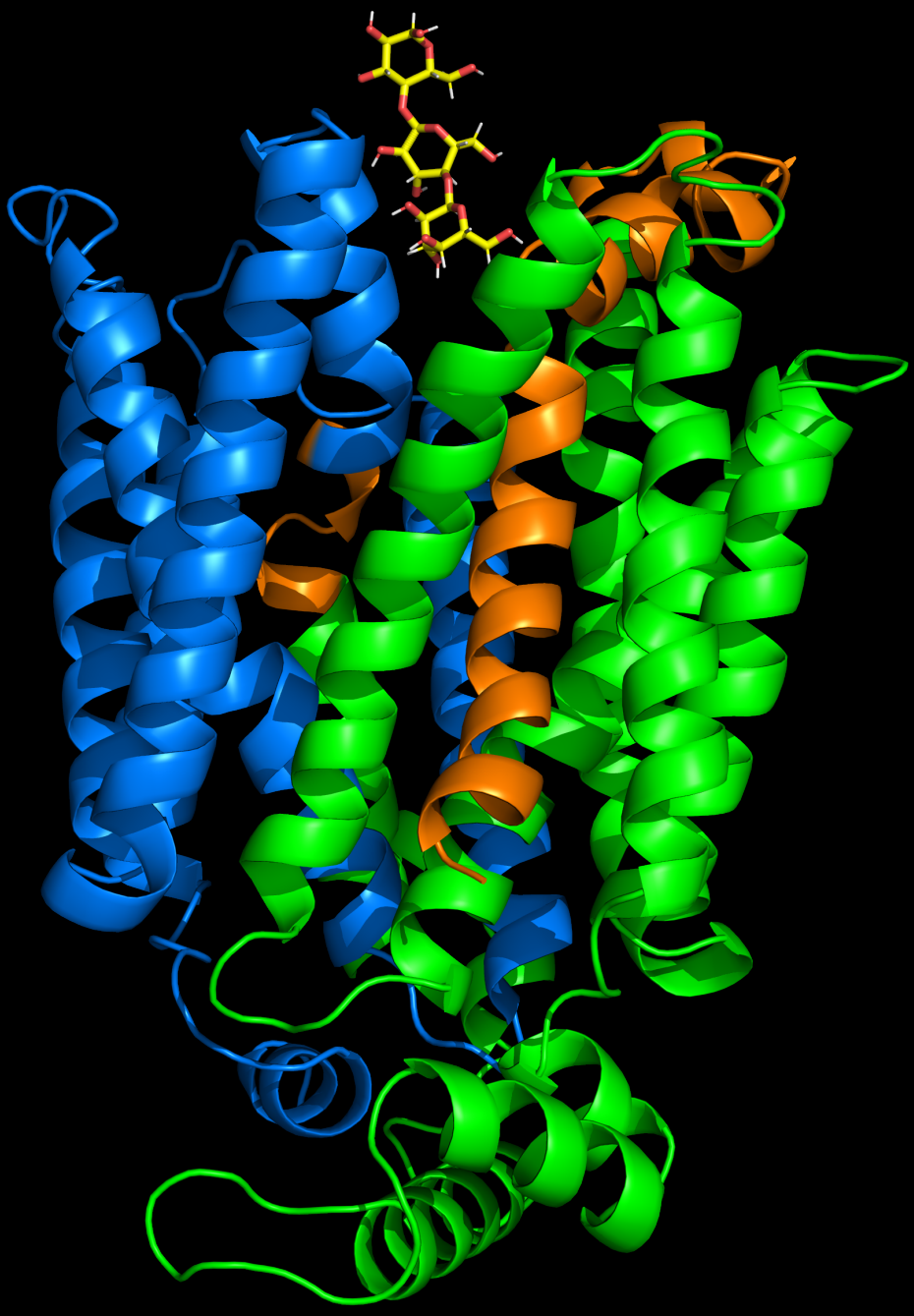
Nick Brouwers



Genetics of maltose and maltotriose metabolism in *Saccharomyces eubayanus*

Dissertation

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Samenvatting

Summary

Samenvatting

De eerste productie van alcoholische dranken uit plantaardige materialen vond duizenden jaren geleden plaats. In 15e eeuw leidde productie van lagerbier met lichte (pils-)mout, zacht water en Saaz nobele hop tot pils, een helder gouden, fris smakend bier. Dit lagerbier is nu 's werelds meest geproduceerde alcoholische drank. De gist die verantwoordelijk is voor de omzetting van wortsuikers in alcohol, CO_2 en aromatische stoffen is *S. pastorianus*, een hybride van *S. cerevisiae* en de recent ontdekte koude-tolerante gistsoort *S. eubayanus*. Eigenschappen van beide ouders maken *S. pastorianus* een superieure vergister van brouwerswort bij temperaturen beneden 15 °C. Omdat *S. cerevisiae* geen koude-tolerante gist is, worden de prestaties van S. pastorianus-hybriden toegeschreven aan het *S. eubayanus* sub-genoom. De geografische oorsprong van het *S. eubayanus* sub-genoom van *S. pastorianus*-hybriden, alsmede de bijdrage van dit sub-genoom aan de omzetting van belangrijke oligosachariden in brouwerswort, worden echter niet volledig begrepen. Het in dit proefschrift beschreven onderzoek richt zich daarom op de genetica van de stofwisseling van maltose en maltotriose, twee belangrijke suikers in brouwerswort, in *S. eubayanus*.

Hoofdstuk 2 beschrijft het bepalen van de volledige DNA-volgorde van de type-stam van *S. eubayanus*, CBS 12357^T. Door gebruik te maken van 'long-read' technologie (Oxford Nanopore), konden volledige 'telomeer-tot-telomeer' DNA-volgorden van chromosomen worden geassembleerd en geannoteerd. Deze aanpak maakte het mogelijk om de *MAL*-loci van de *S. eubayanus* CBS 12357^T volledig te reconstrueren. Van vier complete maltosetransportergenen, *SeMALT1, SeMALT2, SeMALT3* en *SeMALT4*, bevonden alleen *SeMALT2* en *SeMALT4* zich in een volledig *MAL*-locus. De DNA-volgorden van *SeMALT2* en *SeMALT4* waren 99.7% identiek en codeerden voor volledig identieke eiwitten. Voor een functionele analyse werden de unieke *SeMALT* genen apart tot overexpressie gebracht in een maltosetransport-negatieve *S. cerevisiae* stam. Aan de hand van groei van de resulterende stammen op synthetisch medium met alleen maltose of maltotriose als koolstofbron werd geconcludeerd dat alle drie *Se*Malt-transporters (*Se*Malt1, *Se*Malt2/4 en *Se*Malt3) wel maltose, maar geen maltotriose kunnen transporteren. Parallel aan deze heterologe complementatie-studie werden deleties in *SeMALT*-genen geïntroduceerd en bestudeerd in *S. eubayanus* CBS 12357^T. Hierbij werd voor het eerst gebruik gemaakt van CRISPR-Cas9 voor 'genome editing' in *S. eubayanus*. Een dubbele deletie van *SeMALT2* en *SeMALT4* leidde tot drastisch verminderde groei op maltose in synthetisch medium, daarmee aantonend dat deze genen een belangrijke rol spelen in maltosetransport. Deze conclusie werd bevestigd door analyse van de mRNA-niveaus van de *SeMALT*- genen, waarbij werd vastgesteld dat *SeMALT2* en *SeMALT4* de enige maltosetransportgenen zijn die hoog tot expressie komen bij groei op maltose.

Hoofdstuk 3 beschrijft hoe een combinatie van niet-gerichte mutagenese en laboratorium-evolutie werd ingezet om de genetische basis van maltotriose-transport nader te onderzoeken. Hierbij werd de *S. eubayanus*-stam CBS 12357^T, die niet op maltotriose kan groeien, eerst met ultraviolet licht bestraald. Deze behandeling leverde mutanten die in synthetisch medium op maltotriose konden groeien, maar geen maltotriose consumeerden bij groei op brouwerswort. Laboratoriumevolutie met deze mutanten in chemostaten, gekweekt op met maltotriose verrijkte brouwerswort, leverde mutanten die maltotriose konden consumeren in aanwezigheid van glucose, fructose en maltose en bovendien ook maltotriose omzetten in wort. DNA-analyse met 'short-read'- en 'long-read'-technologieën toonde aan dat 'in-frame'-recombinatie van de *SeMALT4*-,

SeMALT1- en *SeMALT3*-genen had geleid tot vorming van een nieuw, chimeer *SeMALT413*-gen. Expressiestudies bewezen dat dit chimere gen voor een functionele maltotriosetransporter codeerde. Deze resultaten vormden een unieke 'real-time' registratie van neofunctionalisatie als evolutionair mechanisme en boden tegelijkertijd een mogelijke verklaring voor de oorsprong van maltotriosetransporters in brouwgisten.
Hoofdstuk 4 beschrijft onderzoek aan een *S. eubayanus*-stam die afkomstig is van de Tibetaanse Hoogvlakte en waarvan het genoom, op grond van beschikbare informatie, de hoogste verwantschap vertoont met het *S. eubayanus*-subgenoom van *S. pastorianus*-stammen. De volledige DNA-volgorde van deze stam, *S. eubayanus* CDFM21L.1, werd geanalyseerd met een combinatie van 'long-read'- en 'short-read'-technologieën, geassembleerd en geannoteerd. In het genoom van *S. eubayanus* CDFM21L.1 werden drie kopieën aangetroffen van *SeAGT1*, een belangrijk maltotriose-transportergen in de biergiststam *S. pastorianus* CBS 1483. Kweekexperimenten met CDFM21L.1 en met de nauw verwante Tibetaanse *S. eubayanus*-stam ABFM5L.1 toonden echter verrassenderwijs aan dat deze stammen noch maltose, noch maltotriose konden omzetten.
Expressie van de *SeMALT1-, SeMALT2-, SeMALT3-* en *SeAGT1*-genen uit *S. eubayanus* CDFM21L.1 in een maltose-transport-negatieve *S. cerevisiae*-stam liet zien dat alleen *SeMALT1* en *SeAGT1* codeerden voor functionele maltosetransporters. *Se*Agt1 kon bovendien maltotriose transporteren terwijl *Se*Malt1 dit niet kon. Expressie in een maltase-negatieve *S. cerevisiae*-stam bewees de functionaliteit van de *SeMALS1*- en *SeMALS2*-maltasegenen uit *S. eubayanus* CDFM21L.1. Deze resultaten suggereerden dat het onvermogen van de Tibetaanse *S. eubayanus*-stammen om op maltose en maltotriose te groeien, voortkwam uit een probleem in de regulatie van genexpressie. Expressie van het *S. cerevisiae* regulatorgen *ScMAL13* in *S. eubayanus* CDFM21L.1 maakte inderdaad groei op maltose of maltotriose als enige koolstofbron mogelijk. Nadat in de resulterende *S. eubayanus*-stam, deletiemutanten van *SeMALT1* en *SeAGT1* waren gemaakt, bleek alleen de deletie van *SeAGT1* een negatieve invloed te hebben op gebruik van maltose en maltotriose. In overeenstemming met deze resultaten bleek uit analyse van mRNA-niveaus dat *SeMALT1* slechts zeer laag tot expressie kwam.
Hoewel de Tibetaanse *S. eubayanus*-stammen geen maltose en maltotriose kunnen omzetten, boden de in Hoofdstuk 4 beschreven waarnemingen nieuwe mogelijkheden voor het maken van hybride biergiststammen. Kruising van de Tibetaanse *S. eubayanus*-stam ABFM5L.1 met de maltotriose-negatieve 'ale'-gist *S. cerevisiae* CBC-1 leverde een kunstmatige *S. pastorianus* hybride. Door de wisselwerking tussen de twee sub-genomen, waarbij de *ScMALx3*-genen expressie van *SeAGT1* induceerden, kon deze hybride, in tegenstelling tot beide ouderstammen, maltotriose transporteren. Deze wisselwerking treedt mogelijk ook op in de biergiststam *S. pastorianus* CBS 1483, waarin *SeAGT1* het enige gen is dat codeert voor een maltotriosetransporter.

Summary

The first production of alcoholic beverages by fermentation of plant-derived materials occurred many thousands of years ago. In the 15th century, production of lager beer with pale barley malt, soft water and Saaz noble hops, first resulted in pilsner, a clear golden, crispy tasting beer. Today, this type of lager beer is the most produced alcoholic beverage in the world. The yeast responsible for conversion of wort sugars into ethanol, CO_2 and aromatic compounds is *S. pastorianus*, a hybrid of *S. cerevisiae* and the recently discovered cryotolerant species *S. eubayanus*. Properties from both parents make *S. pastorianus* a superior fermenter of brewer's wort at temperatures below 15 °C. Since *S. cerevisiae* is not a cryotolerant yeast, the low-temperature performance of *S. pastorianus* hybrids has been attributed to the *S. eubayanus* sub-genome. However, the geographical origin of the *S. eubayanus* sub-genome of *S. pastorianus* hybrids, as well as its contribution to metabolism of key oligosaccharides in brewer's wort, is not fully understood. The research described in this thesis therefore focused on the genetics of the metabolism of maltose and maltotriose, two key sugars in brewer's wort, in *S. eubayanus*.

Chapter 2 describes whole-genome sequencing of the *S. eubayanus* type strain CBS 12357T. Use of 'long-read' technology (Oxford Nanopore) enabled complete telomere-to-telomere assembly and annotation of chromosomes. This approach also allowed for full reconstruction of *MAL* loci of *S. eubayanus* type CBS 12357T. Of four complete maltose transporter genes, *SeMALT1, SeMALT2, SeMALT3* and *SeMALT4*, only *SeMALT2* and *SeMALT4* were located in a complete *MAL* locus. The DNA sequences of *SeMALT2* and *SeMALT4* were 99.7% and encoded identical proteins. For a functional analysis, the unique *SeMALT* genes were expressed separately in a maltose-transport-negative *S. cerevisiae* strain. Based on growth of the resulting strains on synthetic medium with only maltose or maltotriose as carbon source, it was concluded that all three *Se*Malt transporters (*Se*Malt1, *Se*Malt2/4 and *Se*Malt3) were able to transport maltose, but not maltotriose. In parallel with this heterologous-complementation study, deletions in the *SeMALT* genes were introduced and studied in *S. eubayanus* CBS 12357T. These experiments encompassed the first use of CRISPR-Cas9-mediated genome editing in *S. eubayanus*. A double deletion of *SeMALT2* and *SeMALT4* led to drastically reduced growth on maltose in synthetic medium, indicating that these two genes play an important role in maltose transport. Consistent with this conclusion, analysis of mRNAs of the *SeMALT* genes indicated that *SeMALT2* and *SeMALT4* were the only maltose-transport genes that were highly expressed during growth on maltose.

Chapter 3 outlines how a combination of non-targeted mutagenesis and laboratory evolution was used to further investigate the genetic basis of maltotriose transport. In this study, the *S. eubayanus* strain CBS 12357T, which cannot grow on maltotriose, was first irradiated with ultraviolet light. This treatment yielded mutants that were able to grow on maltotriose in synthetic medium, but did not consume maltotriose during growth on brewer's wort. Laboratory evolution experiments with these mutants, performed in chemostat cultures grown on maltotriose-enriched brewer's wort, yielded mutants that could consume maltotriose in the presence of glucose, fructose and maltose and, moreover, converted maltotriose in wort. DNA-analysis with short-read and long-read technologies showed that in-frame recombination of the *SeMALT4, SeMALT1* and *SeMALT3* genes had led to the formation of a novel, *SeMALT413* gene. Expression studies proved that this chimeric gene encoded a maltotriose transporter. These results provided a unique, real-time registration of neofunctionalization as evolutionary mechanism and,

at the same time, offered a possible explanation for the origin of maltotriose transporter genes in brewing yeasts.

Chapter 4 describes research on an *S. eubayanus* strain that originates from the Tibetan Plateau and whose genome, based on available information, shows the strongest relatedness with the *S. eubayanus* subgenome of *S. pastorianus* strains. The complete DNA sequence of this strain, *S. eubayanus* CDFM21L.1, was analyzed by combination of long-read and short-read technologies, assembled and annotated. In the genome of *S. eubayanus* CDFM21L.1, three copies of *SeAGT1* were found, an important maltotriose-transporter gene in the brewing yeast strain *S. pastorianus* CBS 1483. However, growth experiments with CDFM21L.1 and with the closely related Tibetan *S. eubayanus*-strain ABFM5L.1 surprisingly showed that these strains converted neither maltose nor maltotriose.

Expression of the *SeMALT1, SeMALT2, SeMALT3* and *SeAGT1* genes of *S. eubayanus* CDFM21L.1 in a maltose-transport-negative *S. cerevisiae* strain indicated that only *SeMALT1* and *SeAGT1* encoded functional maltose transporters. *Se*Agt1 could, in addition, transport maltotriose, while *Se*Malt1 could not. Expression in a maltase-negative *S. cerevisiae* strain proved the functionality of the *SeMALS1* and *SeMALS2* maltase genes from *S. eubayanus* CDFM21L.1. These results suggested that the inability of the Tibetan *S. eubayanus* strains to grow on maltose and maltotriose reflected a problem in regulation of gene expression. Indeed, expression of the *S. cerevisiae* regulator gene *ScMAL13* in *S. eubayanus* CDFM21L.1 enabled growth on maltose or maltotriose as sole carbon source. When deletion mutants of *SeMALT1* and *SeAGT1* were subsequently constructed in the resulting *S. eubayanus* strain, only the deletion of *SeAGT1* negatively affected consumption of maltose and maltotriose. Consistent with this observation, analysis of mRNA levels confirmed that *SeMALT1* was expressed at a very low level.

Although the Tibetan *S. eubayanus* strains did not convert maltose or maltotriose, the results presented in Chapter 4 did offer new options for the construction of hybrid brewing yeast strains. Mating of the Tibetan *S. eubayanus* strain ABFM5L.1 with the maltotriose-negative ale strain *S. cerevisiae* CBC-1 generated an *S. pastorianus*-like laboratory hybrid. The interaction of its two sub-genomes, in which *ScMALx3*-genes induced expression of *SeAGT1*, enabled this hybrid, in contrast to its parental strains, to transport maltotriose. This interaction may also be involved in maltotriose utilization by the brewing strain *S. pastorianus* CBS 1483, in which *SeAGT1* is the only gene encoding a maltotriose transporter.

Chapter 1 Introduction

1.1 History of lager beer and brewing yeast

Alcoholic beverages have been associated with human culture for thousands of years. Since the early Neolithic plant domestication led to farming and more permanent human settlements. The earliest archaeological evidence for the use alcoholic fermentation from this period, which started over 12,000 years ago, were found in caves located in Mesopotamia [1, 2]. Further evidence for the use alcoholic fermentation during the Neolithic was found on sites around the world [3, 4]. In these early processes, storage and processing of domesticated crops led to microbial conversion of plant sugars into alcohol (ethanol) and carbon dioxide. The currently best-known ethanol-producing microorganism is the yeast *Saccharomyces cerevisiae*, a unicellular eukaryote. The capacity to not only produce but also tolerate high concentrations of ethanol provided *Saccharomyces cerevisiae* with an advantage in-sugar rich environments by inhibiting growth of competing microorganisms [5-7]. Examples of such sugar-rich substrates include cereals, fruits and honey, whose alcoholic fermentation yields beer, wine and mead, respectively.

Fermentation was essentially used to preserve and flavor processed food and beverages [6, 8]. The first documented example of fermentation and beer dates to around 4000 B.C. and was written by the Sumerians, a literate agrarian society. Stories about brewing and beer consumption were written in hieroglyphs on stone tablets [1]. The stone tablets mentioned that beer was made by fermenting pieces of bread and herbs (Figure 1) [1].

Figure 1 Depiction of beer consumption by Sumerians around 4000 B.C. based on tablets found in Mesopotamia (Wooley 1934). Fermentation took place in large vessels and beer was consumed with long straws. From Ancient-origins.

Although it is unclear when malted cereals were first used for brewing, it is speculated that this innovation occurred soon after the introduction of baking and brewing. Archaeological evidence from ancient Egypt indicates that fermented malt beverages were drunk from large jars, using straws to filter out the residues. [6, 9-11].

Fermented, alcohol-containing products played an important role in social and cultural activities, festivities and medicine. Besides preservation and flavoring, alcohol also acted as a mind-altering, analgesic and disinfecting substance, which made alcoholic drinks important medicinal products [3]. Spontaneously fermented, cereal-based beverages from this era were the precursors of the beer that is consumed today.

Until the late Roman period, corresponding to the end of the 5th century, brewing in Central Europe was mainly a women's job as the men ploughed the fields and harvested the cereals [1]. Beer was among the most consumed drinks in Central Europe and brewing was generally done with malted wheat, barley and water [12]. While preparing the resulting mash, also known as wort, different herbs, also called "gruit", were added to the brew to improve the beer flavor, but also to counter-balance off-flavors caused by bacterial contamination. The low alcohol concentrations of these early fermented beverages resulted in a limited shelf life [1].

As Christianity spread through Central Europe in the 6th century, beer brewing became increasingly associated with monasteries. Some monks and nuns specialized in brewing, which contributed to the transformation of household brewing into a well-established profession. Around this time, brewing practices improved and became increasingly standardized. Several critical steps were implemented such as direct heating above fire and addition of hops [1, 13, 14]. Monks discovered that hops gave the beer a nice bitter character but also led to longer shelf lifes, which we now know are due to their antibacterial properties [15]. The oldest written reference to hops used in brewing is a document found in Benedictine Abbey of Weihenstephan and dates back to 768 [14]. During the fermentation process, a foam layer with yeast was formed on top of the brew. These medieval brewing processes are therefore referred to as top fermenting. The monks discovered that inoculation of new fermentation by adding an aliquot of the previous batch of fermented wort reduced the fermentation time, introducing the technique referred to as re-pitching [1, 13]. The use of re-pitching became an important factor in yeast domestication, since successful brews were more likely used for propagation[16], which increased selective pressure for successful natural strains and mutants. Several performance indicators may have been used to guide this process, including residual sweetness, ethanol concentration and flavor formation.

Around the Middle Ages, beer quality in Bavaria still varied a lot and was season dependent. During summer, beers often tasted sour but winter beers had a much better taste. In an attempt to increase beer quality, several regulations were implemented. In 1156, a regulation came into force that bad beer had to be destroyed or given for free to poor people [1]. In 1516. the Reinheitsgebot was issued by the Bavarian Duke Wilhelm IV. This still famous rule stated that only water, barley and hops could be used for beer brewing. Despite these regulations, summer beer could often still taste sour or worse and in 1553 a decree came into force that forbade brewing between the Feast of Saint George (April 23) and Michaelmas (September 29). As a result, most brewing took place in the cold season, thereby subjecting brewing yeast cultures to low temperatures. Beer was then stored underground or in caves, a method that started around the 15th century. Beer storage spaces filled with river ice facilitated beer supply during the warm summer months. Further yeast domestication and cold storage of beer led to a novel beer style named lager, which comes from "lagern" meaning "to store" in German.

The cause for the poor taste of summer beer were still poorly understood, since microbes were only discovered when Antonie van Leeuwenhoek invented the microscope in 1673 and observed microorganisms for the first time. He observed yeast for the first time in 1680 although he did not yet consider it a living organism. Only in the 1850s did Louis

Pasteur show that yeast is a living organism and responsible for alcoholic fermentation [17].
During the lager process at low temperature a natural selection for new cold tolerant yeasts occurred. A hybrid of two different *Saccharomyces* yeast species, *Saccharomyces cerevisiae* and *Saccharomyces eubayanus* apparently thrived under these conditions [18, 19]. At the end of this slow and cold fermentation, the yeast would flocculate (form flocks), and sink to the bottom. This new type of yeast was classified as *Saccharomyces pastorianus* by Max Reess in 1870, named after Louis Pasteur. *Saccharomyces pastorianus* became the preferred yeast for brewing and its type of fermentation was described as "bottom-fermenting" which became the Bavarian style. This style was adopted by the Bohemian Citizens' Brewery in Pilsen (Měšťanský pivovar Plzeň) in 1839, which was built after, in the preceding year, dissatisfied consumers had dumped beer vessels with top-fermented (predominantly *Saccharomyces cerevisiae* yeast) beer. Josef Groll, who worked at the Citizens' Brewery used Bavarian brewing techniques, Pilsen's soft water, Saaz noble hops, lager yeast and pale malt to brew the first pale lager beer in 1842.
In 1883, a mycologist who worked at the Carlsberg brewery, Emil Christian Hansen, developed microbial techniques to isolate and grow the first pure microbial culture originating from a single cell [20-22]. Monoculture brewing led to enhanced, consistent product quality and spread through breweries in Europe. Simultaneously, characterization of pure cultures emerged and resulted in even more selective pressure [21]. Due to the invention of refrigeration, it became possible to brew lager beers in places that were not near rivers or lakes that froze during winter. This development led to an enormous increase of the number of lager breweries. Around 1870 ,already 97% of the Bohemian breweries were lager breweries [23]. Lager beer currently has a global market, contributing 83.8% of the total beer volume produced in 2013. In 2013, a total of 1.7 billion hectoliter beer was sold, worth €387.4 billion in revenue [24].

1.2 Domestication of brewing yeast

During the transition from ale to lager brewing, the selective pressure on brewing yeast changed. As the lager-brewing environment was much colder than the ale-brewing environment, yeast strains had to adapt to ferment at temperatures below 10 °C [8, 25]. Process intensification involved the use of higher-gravity wort, so more standardized beer could be brewed using smaller fermentation volumes. As a consequence, selective pressure on ethanol tolerance also increased. One genetic adaptation to cope with these new environments was to change ploidy, leading to polyploid and aneuploid (unequal amount of chromosome copies) strains. By (partial) genome duplication, gene copy number and gene dosage change, thereby possibly conferring favorable traits such flocculation [26, 27]. Hybridization is a common mechanism that results in altered ploidy. Combining positive traits from different parental strains in a hybrid can lead to outperformance of both parents, a phenomenon called heterosis. This phenomenon explains the common occurrence of hybrids among domesticated organisms [28]. However, hybrid genomes also confer genetic redundancy and initial instability. The latter issues have been proposed to contribute to the aneuploidy of lager brewing yeasts. However, a recent study demonstrated that the genome of an artificial yeast hybrid was relatively stable [29].
By applying monoculture techniques, yeasts with desired traits were selected and thus evolved further. Many industrial yeast strains have an aneuploid genome, including strains used in bioethanol fermentation [30, 31]. Genome duplication and hybrids are often linked to domestication. For example, many food crops, including domesticated

wheat and maize varieties, have polyploid genomes [32, 33]. Additional gene copies enable mutational freedom yielding further options for functional improvement, even extending to gene neofunctionalization [34, 35].
In wort, the majority of fermentable sugars consist of maltose and maltotriose, which are a di- and tri-mer of glucose, respectively. Many sugar transporters in yeast, including the maltose transporters, have likely evolved from an ancestral transporter with 12 α-helical transmembrane domains. Transporters from this family evolved to specialize in certain substrates. Use of maltotriose likely evolved during domestication and enabled more substrate consumption and ethanol production. The resulting yeast strains were likely selected as near complete sugar metabolism is important for a fresh taste, a key-characteristic for pilsner.

1.3 Modern process of brewing lager beer

The lager-beer brewing process can be dated back to the 14th century. The original lager beers, like the Bavarian Dunkel, were not the pilsner type that is predominantly consumed today [1]. Malts were much darker and pure culture techniques did not yet exist. A major step towards standardization was accomplished by the Bohemian Citizens' Brewery in Pilsen by introducing pale malt and soft water [1]. The general outline of the process is still in use today but was gradually improved to be more efficient and better controlled. Beer brewing manufacturing including pilsner brewing, starts with **malting**.
Harvested barley is moisturized to start germination of the barley grain, which is accompanied by the production of a variety of barley enzymes [36]. Some of these enzymes help in protein and cell wall degradation, thereby releasing amino acids, glucans and fatty acids. Others, including feruloyl esterase, release flavor precursors such as ferulic acid [37, 38]. Amylases hydrolyze starch, a homopolymer of α-1,4-linked glucose residues [39]. After a few days, germination is stopped by drying and/or roasting the germinated barley, the product of which is called malt. The roasting temperature has a strong impact on the color and flavor of the malt. Pale malt used for brewing lager beer is dried at a relative low temperature to prevent a burnt flavor and darkening color. Malt can be stored until further processing. Since the starch in the kernel is still compact and poorly available to amylases, malted barley is **milled** into a fine powder. This milling exposes the starch and, in the subsequent steps of the process, makes it more easily accessible to enzymes that digest it into fermentable sugars.
The milled malt is mixed with water to dissolve and convert nutrients, a process called **mashing**. The mash consists of soluble and insoluble parts and is heated to different specific temperatures to activate enzymes and optimize their activity. In the resulting solution, also called wort, different enzymes convert polymeric starch and proteins into simple sugars and free amino nitrogen (FAN). Proteinases hydrolyze polypeptide chains into individual amino acids and short peptide chains while α-amylases (1,4-α-D-glucan glucanohydrolases) digest the α-1,4 glycosidic bonds of starch, thereby generating oligosaccharides (predominantly the glucose trimer maltotriose and the glucose dimer maltose) and glucose [39]. β-amylase (1,4-α-D-glucan maltohydrolase) is only able to hydrolyze starch at the non-reducing end, thereby releasing one maltose molecule at the time [40]. Wort is separated from the mash (spent grains) via a process called **lautering** and then transferred to the boiling vessel.
After addition of hops, the wort is **boiled** to inactivate all enzymes and kill all microorganisms. During the boiling step, hop α-acids are isomerized, which yields a characteristic bitter flavor. The β-acids and essential oil compounds in hops, such as humulene, contribute to the characteristic hop aroma. The anti-bacterial activity of hop

components furthermore contributes to expansion the shelf life of hopped beers. For brewing specialty beers, other ingredients, such as spices and herbs, may also be added. Wort boiling takes at least one hour before the wort is again separated from the hops and other residual insoluble particles by whirlpool separation.

Prior to fermentation, the wort needs to be **cooled**. To this end, hot wort is led through a heat exchanger until it reaches a desired temperature for **fermentation**. For lager brewing the fermentation temperature is generally between 8 and 15 °C [41].

Typically, the bottom-fermenting lager yeast *S. pastorianus* used to inoculate the cooled wort is derived from a previous fermentation (re-pitching). Between fermentations, the yeast biomass is stored at a temperature of approximately 4 °C. Prior to fermentation the wort is aerated to provide the oxygen necessary for the synthesis of anaerobic growth factors such as ergosterol and unsaturated fatty acids. These compounds, which are essential for cell membrane integrity, cannot be synthesized by yeast cells under the anaerobic conditions to which the yeast cells are exposed during most of the fermentation process [42].

During the low-temperature fermentation of lager beer, the yeast cells divide in suspension and convert the glucose, maltose and maltotriose into alcohol, CO_2 and flavor compounds. Alcoholic fermentation already occurs during the initial aeration phase. This aerobic fermentation by *Saccharomyces* yeasts in the presence of excess sugars is referred to as the Crabtree effect.

When glucose, fructose and nearly all maltose have been consumed, most yeast cells flocculate and sediment to the bottom of the fermentation vessel. Fermentation continues slowly while the remaining sugars, mostly consisting of maltotriose, are further metabolized [43]. During this first stage of fermentation, the yeast cells not only produces CO_2, alcohol and desirable flavor compounds. In particular, diacetyl is a well-known off-flavor compound in lager beer that confers a buttery taste [44]. Diacetyl is only taken up by the yeast cells and further reduced to acetoin and 2,3-butanediol after the primary fermentation has been completed. Before the viability drops too much, the majority of the cells are recovered and stored for re-pitching [42]. Yeast cells cannot be perpetually re-pitched. To prevent contamination, genetic deterioration and reduced viability caused by replicative ageing, the number of repitching cycles is usually limited to five to fifteen [45]. Most of the flocculation yeasts are separated from the beer to prevent excessive autolysis, which can cause off-flavors and increased haze formation [46]. The brew is now called young beer or green beer and is transferred to a lager tank for the second stage of fermentation, which is also called **lagering** or beer maturation.

In the lager tank, the young beer is cooled to a few degrees above 0 °C. In the lager process, off-flavors dissipate and the remaining yeast cells slowly consume maltotriose further [44]. Depending on the beer and brewery, lagering can take up to several weeks. After lagering, the beer is filtered to completely remove yeast cells, resulting in a clear beer. Carbon dioxide, which is almost completely removed during this process, is re-introduced during **bottling**.

Bottles are filled with an adequate dilution of the fermentation broth so that the alcohol percentage and flavor intensity is standardized. Then, the beer is mechanically carbonated right before capping the bottles. Beer inevitably contains some residual carbohydrates that were not consumed by the yeast [47]. To prevent microbial growth of wild yeast and bacteria on these residual carbohydrates, industrial lager beers are pasteurized. Finally, the beer is labeled and ready for distribution. The entire brewing process is schematically represented in Figure 2.

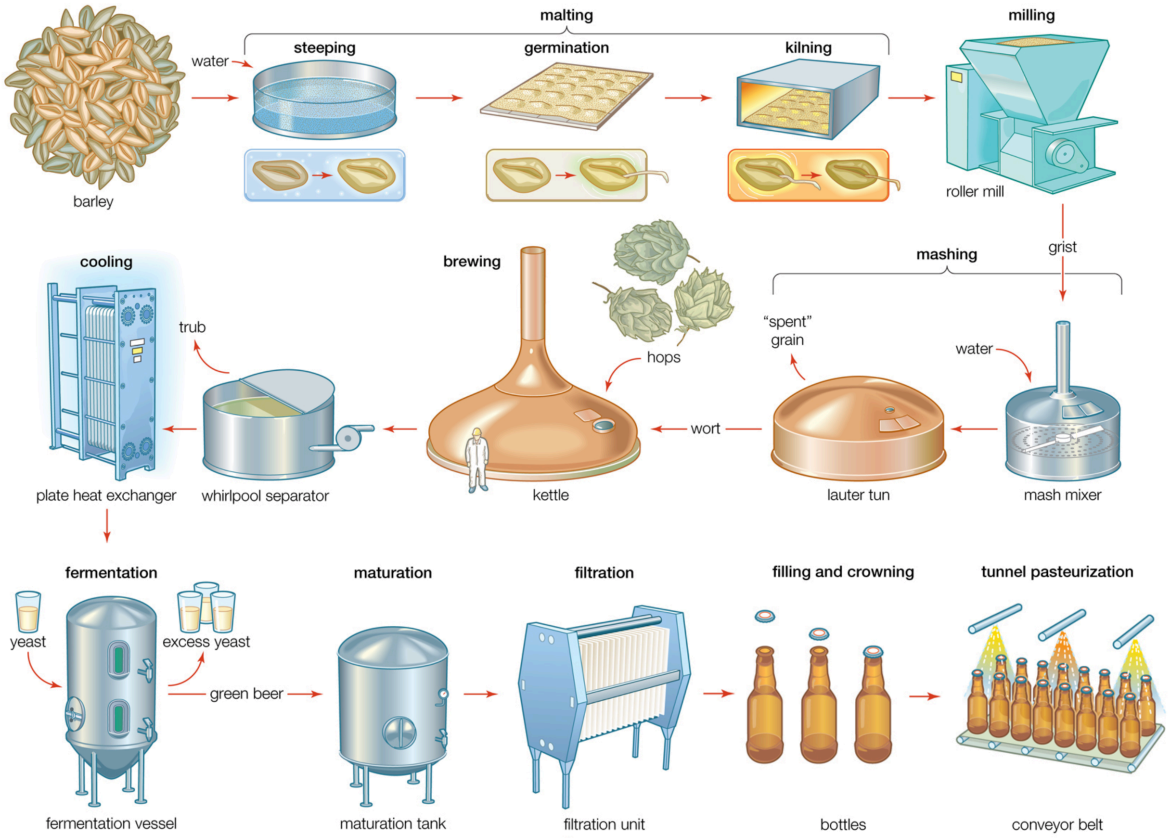


Figure 2 Schematic representation of the brewing process, from raw material to final product. Barely is malted, milled, mixed with water and heated to make wort. Hops are added and wort is cooked. Cooled wort is fermented and beer is stored for maturation. Finally, beer is filtered, bottled, and pasteurized. Adapted from Encyclopedia Britannica.

1.4 *Saccharomyces* yeasts used in the brewing industry

In the beer brewing industry, different *Saccharomyces* species and their hybrids are used. Characterization of pure cultures led to a distinction between different classes of brewing yeast. Top-fermenting ale yeasts were generally classified as *S. cerevisiae*, whereas the bottom- fermenting brewing yeasts were taxonomically classified into three different groups: *S. carlsbergensis* (Unterhefe Nr. I), *S. pastorianus* and *S. monacensis* (Unterhefe Nr. II), each having different physiological properties [22]. Later, the bottom-fermenting yeasts turned out to be hybrids and were all renamed *S. pastorianus* [48, 49]. Based on brewing properties, *S. pastorianus* strains were divided into two main groups: Saaz (Group I) and Frohberg (Group II). The main difference between these groups relates to growth performance at low temperature and ability to consume maltotriose [43]. Yeast strains belonging to the Saaz group grow better at low temperature but typically exhibit

a lower fermentation performance than yeast strains of the Frohberg group, a difference that has been attributed to the lower ability of Saaz strains to assimilate maltotriose [43]. However, maltotriose utilization can vary significantly among Frohberg brewing strains and even some Saaz strains have been demonstrated to ferment maltotriose [50].

Genetically, the Saaz and Frohberg groups can be clearly distinguished based on chromosome content. In strains belonging to the Saaz group, the number of *S. cerevisiae* chromosomes makes up less than half of the total number of chromosomes, which varies between 45 and 55. In the Saaz group, some *S. cerevisiae* chromosomes are often completely absent. Strains belonging to the Frohberg group typically contain more *S. cerevisiae* chromosomes than *S. eubayanus* chromosomes. The total chromosome copy number of currently analyzed Frohberg-type strains varies between 70 and 79 [51]. The difference in *S. cerevisiae* content between the two groups may explain their different brewing properties, with a lower *S. cerevisiae* chromosome content leading to a better growth at low temperature but a poorer fermentation capacity [50].

In the late 1990's, high gene copy numbers led to a growing suspicion that *S. pastorianus* was a hybrid of *S. cerevisiae* and another *Saccharomyces* species [48, 52-54]. Support for this hypothesis was strengthened by the application of multiple techniques, such as DNA sequence analysis, Southern blotting and fluorescent amplified fragment length polymorphism (AFLP) analysis based on genomic DNA [54, 55]. Analysis of parts of the hybrid genomes of different *S. pastorianus* strains showed substantial variation of their sub-genomic contents [56].

The complete genome of an *S. pastorianus* strain was first sequenced in 2009 [18] and confirmed its hybrid nature, with *S. cerevisiae* and an *S. bayanus*-type as parental species. Sub-genome analysis showed that sequence identity of the *S. cerevisiae*-type ORFs with their *S. cerevisiae* S288C counterparts was 99.2%. The identity of the *S. bayanus*-type ORF sequences compared to those of *S. bayanus* var. *uvarum* CBS7001 was 92.7%.

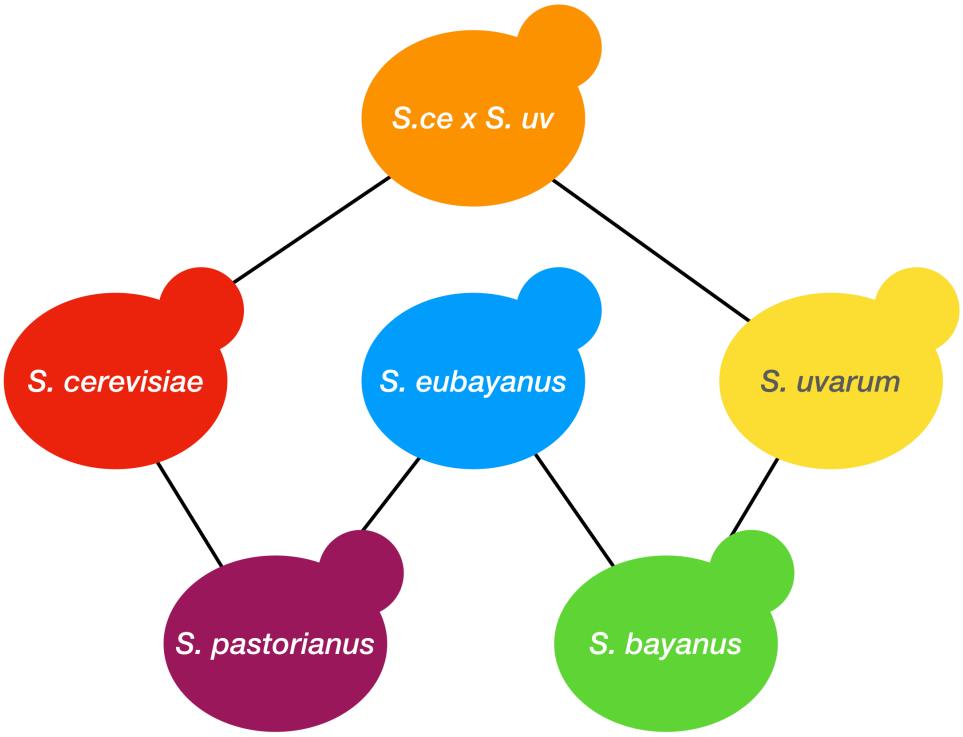


Figure 3 Brewing-related Saccharomyces species (*S. cerevisiae, S. eubayanus* and *S. uvarum*) and their hybrids (*S. pastorianus, S. bayanus* and *S. cerevisiae x S. uvarum*).

These observations demonstrated that, while *S. bayanus* was the most closely related known species to the donor of the non-*S. cerevisiae* sub-genome of *S. pastorianus*, the real parent had not yet been identified.
In 2011, a new cryo-tolerant *Saccharomyces* species, named *S. eubayanus*, was isolated from the cold Patagonian plateau in Argentina [19]. This research showed that *S. eubayanus* and *S. uvarum* were separate species and that *S. bayanus* was a hybrid of both. Moreover, sequence analysis of *S. eubayanus* unveiled a sequence identity of 99.56% with the non-*S. cerevisiae* sub-genome of *S. pastorianus*, thus indicating that *S. pastorianus* strains are hybrids of *S. cerevisiae* and *S. eubayanus* [19]. Based on these results, it could be unequivocally concluded that *S. cerevisiae*, *S. eubayanus* and *S. uvarum* are separate species, whereas *S. pastorianus* and *S. bayanus* are hybrids (Figure 3).

1.5 *Saccharomyces eubayanus*: the missing parent in the brewing yeast family

S. eubayanus was first discovered in Patagonia. This cold South-American region is characterized a maximum average of 15 °C in summer and a -9 °C average in winter [19]. *S. eubayanus* was isolated from fruiting bodies of the fungus *Cyttaria hariotti*, which grow on *Nothophagus* trees native to the southern hemisphere [19]. This tree species is a close relative of *Quercus* (oak) species, from which *Saccharomyces* strains have frequently been isolated in Europe [57]. Following its discovery in Patagonia, ecologists and biologists sampled worldwide to find more strains of *S. eubayanus*. Its cryo-tolerant properties indicated that *S. eubayanus* might be isolated from regions with a low average temperature, for instance at high altitudes. This hypothesis was initially strengthened by the subsequent isolation of *S. eubayanus* on the Tibetan plateau [58]. Since these discoveries, *S. eubayanus* has been isolated from many more locations distributed worldwide (Figure 4) [58-61].

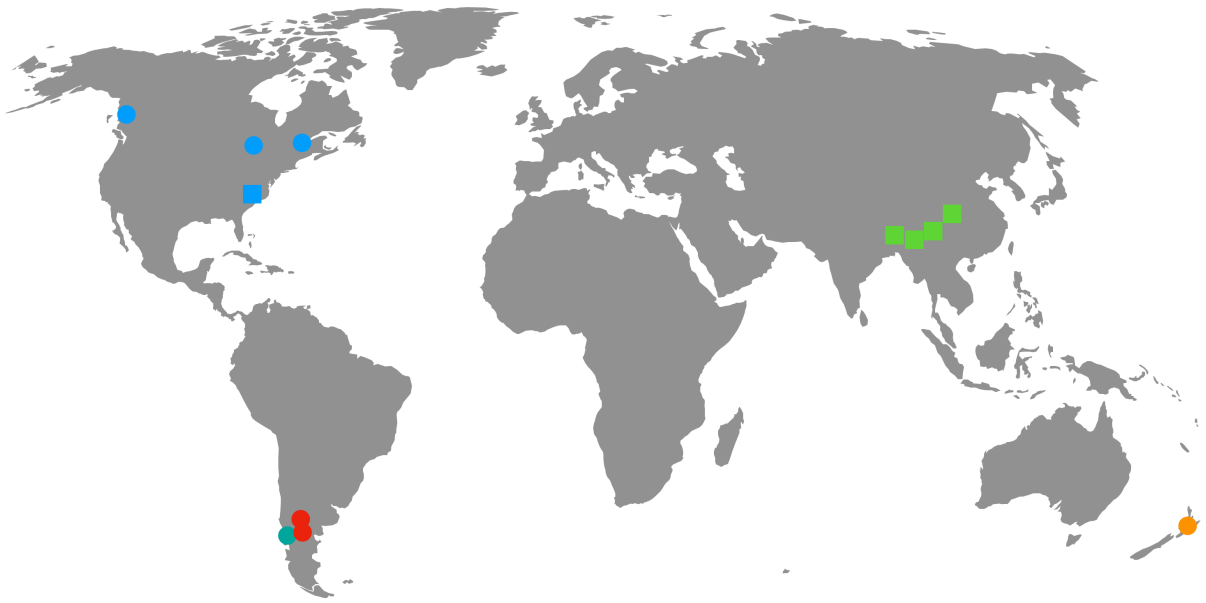


Figure 4 Approximate sample locations from which *S. eubayanus* has been isolated. Argentina (red), Chile (turquoise), North-America (blue), China (green) and New-Zealand (orange). *S. eubayanus* strains isolated from locations indicated by squares are the most closely related to European *S. pastorianus*. [58, 60-62]

Phylogenetic studies on wild *S. eubayanus* isolates divided its known worldwide population in three distinct groups; Patagonia A, Patagonia B and an admixed population [59, 61]. Patagonia A and B strains were both found in South-America. Some isolates from North-America clustered to Patagonia B, but most isolates clustered to Patagonia A and B. Multi-locus sequence analysis showed that these isolates had an admixed genome [59]. Comparison of *S. eubayanus* isolates worldwide showed that the populations could be subdivided into sub-populations Patagonia A-1, A-2, B-1, B-2 and B-Holarctic [61]. The *S. eubayanus* sub-genome of *S. pastorianus* is mostly related to the Patagonia B-Holarctic population. Surprisingly, *S. pastorianus* seems to cluster strongly with a few specific *S. eubayanus* strains, isolated from Tibet and North-Carolina, USA. The *S. eubayanus* isolate from Tibet, CDFM21L.1, shows the highest sequence similarity (99.82%) to the *S. eubayanus* sub-genome of *S. pastorianus* [58, 59, 63].
Lager brewing originated in Europe and *S. pastorianus* has so far only been isolated from man-made environments. The current hypothesis suggests that hybridization between *S. cerevisiae* and *S. eubayanus* could have occurred in Europe since this is where lager brewing originated. However, *S. eubayanus* has not been isolated in Europe yet. Isolation of *S. eubayanus* could be more difficult since most of the primeval oak forests, with which *Saccharomyces* is associated [57], disappeared after the 15th century. This disappearance could have led to a possible extinction of *S. eubayanus* if the species was originally present in Europe. Alternatively, more thorough sampling is required in case *S. eubayanus* isolation is rare like in North-America [59].

Recent studies show that the *S. eubayanus* sub-genome in lager yeast relates partly to the Tibetan and partly to the North-Carolina *S. eubayanus* isolates [22, 61] which suggests that a possible European *S. eubayanus* is a combination of the two isolates.
Since its discovery, *S. eubayanus* has not been intensively characterized, despite its important contributions to *S. pastorianus* and its brewing properties. In the meantime, *S. eubayanus* itself has also been commercially applied for brewing. Heineken, as well as craft breweries in South America, brewed beer with *S. eubayanus*, advertising *S. eubayanus* as the wild mother yeast of lager yeast (Figure 5) [64].

Figure 5 Commercial products brewed with *S. eubayanus* at different breweries. Top left: Berlina-Argentina, top right Bachmann-Argentina, bottom left: Heineken, bottom right: Blest-Argentina.

1.6 Advances in DNA sequencing reveal unique properties and complex genomes of lager yeast

Performance of *S. pastorianus* in the lager brewing process is related to a unique set of properties, which include flocculation, cryotolerance, fermentative capacity and flavor formation [65]. Progress in genome sequencing and molecular genetics eventually helped to study the genotypes underlying these brewing relevant phenotypes. A better understanding of the molecular mechanisms behind these traits has contributed to further improve and develop strains for the brewing industry.

The first whole-genome sequence of *S. pastorianus* WS34/70 was determined with first-generation Sanger sequencing, a labor-intensive and low-throughput method [18]. First-generation ABI Sanger sequencing requires single sequence reactions. The sequence run has an output of ~2 mega base of sequence data resulting in fragmented genomes with a low coverage [66]. Because of limitations in chemical reactions, up to 1,000 base pairs (bp) can be sequenced per sequence run, making it quite challenging for whole-genome sequencing [67]. The final, low- coverage (7.8) and fragmented assembly of strain WS34/70 encompassed 3184 contigs, scaffolded into 796 scaffolds.

In 2015, another *S. pastorianus* strain (CBS1483) was sequenced with second-generation Illumina technology. In contrast to Sanger sequencing, second-generation sequencing

platforms such as Illumina enable sequencing of several thousands of fragments in a single reaction, generating up to 1500 Gb of sequence in a single run [66]. While still based on *in situ* DNA amplification, these small-scale techniques sequence up to 6 billion reads [66, 68]. After fragmentation into 100-300 bp fragments, DNA molecules are sequenced with high accuracy (99.9%). Second-generation sequencing generates up to 1.5 Tb of sequence data, almost a million times more than first-generation sequencing [66]. Sequencing of *S. pastorianus* CBS1483 resulted in ~7Gb of sequence data with a coverage of around 270-fold [51]. The genome was assembled in 908 contigs that were organized into 59 scaffolds. The high coverage made ploidy estimation possible, unveiling the complex aneuploid genome of *S. pastorianus*. Chromosome copy number variation was shown to have a direct impact on brewing related properties such as flocculation [51]. Short-read sequence information is, however, rarely sufficient to assemble a complete yeast genome including sub-telomeres, especially because they contain Ty elements and (long) repetitive regions that complicates genome assembly (Figure 6B)[69]. These regions are mostly found in the sub-telomeres, making it harder to study gene loci located there [70]. Many important brewing-related gene families are located in the sub-telomeric regions, involving those encoding sugar transporters (*HXT* and *MAL* families) and lectin-like proteins involved in flocculation (*FLO* family) [71]. For proper analysis of gene families in subtelomeric regions, a complete genome assembly is required. In an ideal situation, a full chromosome sequence should represent a single contig. In contrast, short-read sequence information typically requires many contigs, representing a partial chromosome, as illustrated by the first published genome sequence of *S. pastorianus* CBS1483.

The third and latest generation sequencing tries to reduce the problem of gaps in scaffolds caused by short reads by an approach called single molecule sequencing (Figure 6B)[67]. One of the third-generation sequence methods is marketed by Oxford Nanopore Technologies. It uses a flow cell containing a membrane with biological nanopore proteins, over which a voltage is applied. Complete DNA strands with an adapter are loaded into the flow cell and guided through the nanopore. As the DNA molecule passes through the nanopore, the electrical current over the membrane changes. depending on the bases present in the nanopore. The change in electrical current can be measured in real time and is converted into a DNA sequence (Figure 6A). The only limitations of this technique are the length of the DNA fragment and the lifetime of the pores. Nanopore sequencing, which makes it possible to sequence complete chromosomes, is still under development and. because the sequence accuracy of the original Nanopore platforms was ~90 %, this technique was not ideally suited for generating high-quality genomes. However, combining Nanopore sequencing with second-generation Illumina sequencing enables correction of false base pairs, resulting in good quality genomes. Oxford Nanopore is continually developing the chemistry and nanopores and enormous improvement has been made to the scalability, processing time and data output. Recent developments show that a new generation of pores (R10) is even able to reach a 99.999% accuracy [72]. Recently, the genome of *S. pastorianus* CBS1483 was re-sequenced with a combination of Nanopore and Illumina platforms, resulting in 29 annotated single-chromosome contigs that included sub-telomeric regions and 2 annotated chromosome scaffolds, making this the highest quality reference genome for a *S. pastorianus* strain [63]. Having a reference genome is very useful when multiple strains are studied, since it would be expensive and time consuming to assemble high-quality genomes for every strain. Reference-based comparison is an effective approach to obtain valuable sequence information without requiring multiple independent genome assemblies. In reference-based comparisons,

sequencing reads are aligned to a reference genome. Based on mapping, it can be seen which sequence is present or absent, and which variations occur. For proper analysis, it is essential that a reference genome covers as much of the genome as possible and is properly annotated. The ability to assemble complete loci in the sub-telomeres, enables genetic studies on brewing-related genes such as the *MAL* genes.

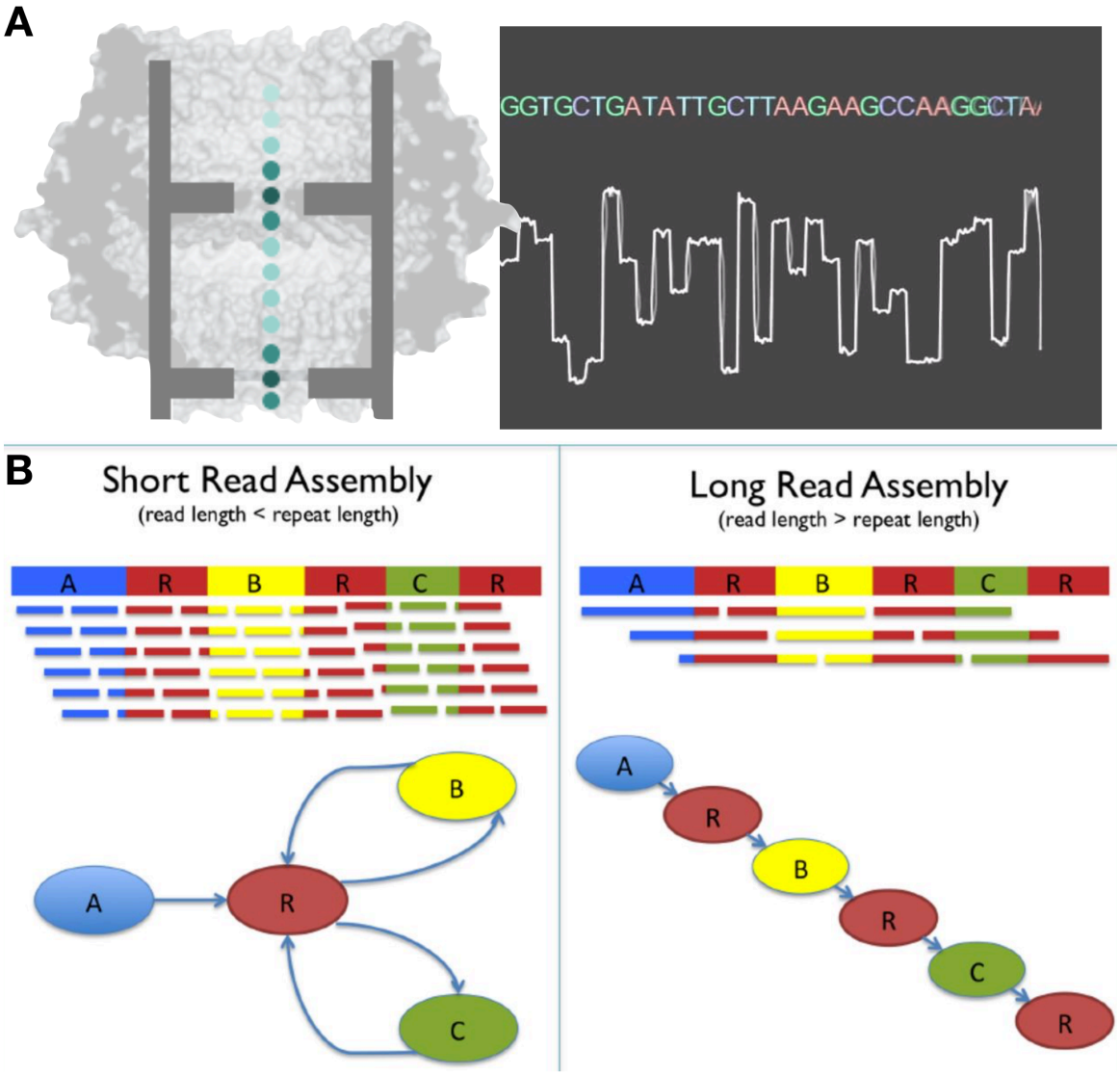


Figure 6 Advances in genome sequencing. (A) Visualization of a Nanopore used in the R10 chemistry of Oxford Nanopore Technologies [72]. **(B)** Comparison between short read and long read assembly. Short reads that do not cover a repeat cannot be fully assembled due to uncertainty. Long reads that span the repetitive region can be placed based on the variable sequence around it, thereby enabling complete assembly (adapted from [73]).

1.7 Wort-sugar metabolism and transport in brewing yeasts

Brewer's wort is a complex sugar mixture resulting from starch degradation during the malting and mashing steps. Over the previous century, this hydrolytic process was optimized to enrich for fermentable sugars such as glucose, fructose, maltose and maltotriose, which make up 8%, 1%, 47% and 14%, respectively, of the total sugar content of wort [74]. In addition to these sugars, wort contains dextrins, which are glucose polymers with α,1-4 or α,1-6 links. Dextrins represent 27% of the total sugar content of wort [74]. Glucose polymers that are highly branched and contain many α,1-6 linkages are not further digested and remain as fibers. Linear α,1-4 linked glucose polymers with more than 3 residues are generally not fermented by regular brewing yeast, but can be digested by humans. Glucose polymers are too large to be transported over the yeast cell membrane but contribute to the body and mouthfeel of the beer. However, some yeasts possess an extracellular amylase, which provides them with diastatic power, the ability to further hydrolyze polymers such maltotetraose and maltopentaose into glucose and maltose [75]. In some *Saccharomyces* species, glucose polymers up to 3 residues (maltotriose) can be transported over the cell membrane via proton symporters [76]. After uptake of malto(trio)se and a proton, the proton must be exported from the cytosol by the Pma1 proton ATPase which requires 1 ATP per proton. Research showed that transport is the rate limiting step in malto(trio)se utilization and therefore an important process to study [41, 77, 78].

In *S. cerevisiae*, wort-sugar consumption follows a sequential pattern, in which glucose is first transported over the membrane via hexose transporters [79, 80] (encoded by *HXT* genes) and then enters the Embden Meyerhof pathway, also called glycolysis [81], which converts glucose into pyruvate. In the absence of oxygen, brewer's yeast converts pyruvate into ethanol and CO_2. In the presence of oxygen, pyruvate can be completely respired via the TCA cycle into CO_2. However, even under certain aerobic conditions, *Saccharomyces* yeasts still produce ethanol, a phenomenon called the Crabtree effect [82]. Glucose represses the induction of maltose-related genes via the Mig1 protein and, in addition, triggers catabolite inactivation of maltose transporters via glucose-induced ubiquitination [80]. This mechanism ensures that metabolism is completely dedicated to glucose conversion as long as this monosaccharide is present in the medium [80, 83].

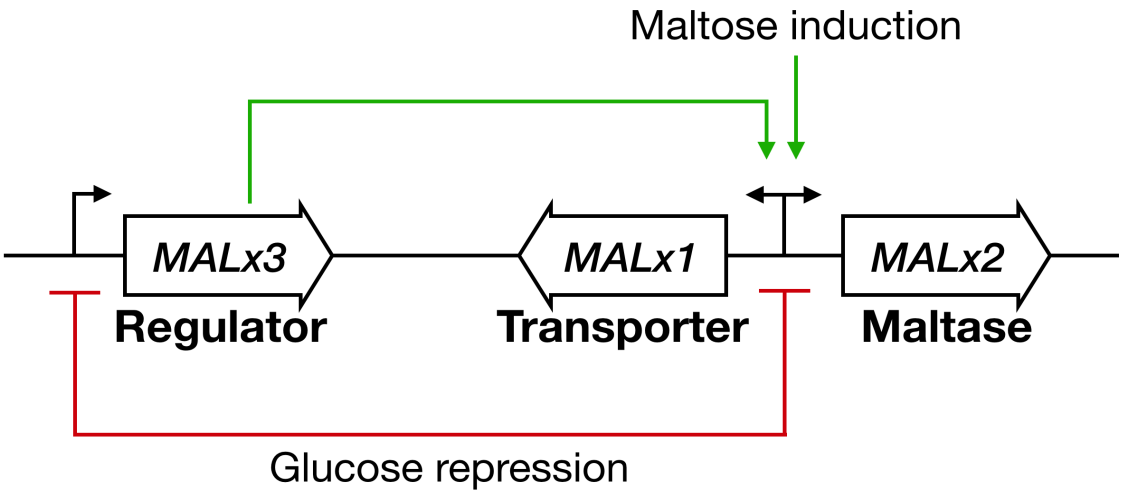


Figure 7 Schematic representation of a canonical *MAL* locus. Genes are depicted by large white arrows induced by their promoters (black arrows). *MAL* genes are repressed in the presence of glucose (red) and induced by intracellular maltose (green).

After depletion of glucose, repression of maltose metabolism is alleviated and the *MAL* loci can be actively transcribed. In *S. cerevisiae*, a *MAL* locus consists of three genes: *MALx1*, which encodes a maltose proton symporter, *MALx2*, encoding an intracellular α-glucosidase that hydrolyses maltose into two glucose molecules, and *MALx3*, encoding a transcriptional activator that acts on the bi-directional promoter of *MALx1* and *MALx2* (Figure 7). The "x" varies depending on where the locus is present. *MAL* loci have been identified on different chromosomes indicated in Table 1.

Table 1. *MAL* loci identified in *S. cerevisiae* and corresponding *MAL* genes.

Chromosome	Locus	Transporter gene	Hydrolase gene	Regulator gene
VII	*MAL1*	*MAL11*	*MAL12*	*MAL13*
III	*MAL2*	*MAL21*	*MAL22*	*MAL23*
II	*MAL3*	*MAL31*	*MAL32*	*MAL33*
XI	*MAL4*	*MAL41*	*MAL42*	*MAL43*
VIII	*MAL6*	*MAL61*	*MAL62*	*MAL63*

In *S. cerevisiae*, the *MAL* loci have the same gene organization and are generally located in subtelomeric regions that are prone to duplication events [49, 84]. The *MALx3* genes are the mostly diverse, whereas the *MALx2* genes are highly similar (>99% sequence similarity), just like the *MALx1* genes. *MAL11* represents an exception, as it shares only 67% sequence identity with *MAL31* in *S. cerevisiae* S288C. Since *MAL11* encodes a transporter that has different characteristics, it is also named *AGT1* [85]. The Malx1 proteins (Mal11/Agt1 excluded) are high- affinity, high-specificity maltose transporters (K_m 2-4 mM) that cannot transport maltotriose [80, 86]. In contrast, *AGT1* encodes a transporter that facilitates transport with different affinities for a wide variety of substrates, including maltose and maltotriose (K_m 20-35 mM) [78, 87]. The high K_m for maltotriose may reflect steric hindrance during transport. A different maltotriose transporter, encoded by a gene named *MTY1* or *MTT1,* was identified in some ale yeasts [88]. *MTT1* had a 92 % similarity to *ScMal31* [89, 90]. Interestingly, maltotriose affinity was higher than for maltose (K_m of 16–27 mM and 61–88 mM, respectively) [91, 92]. Finally, two *MPH2/MPH3* genes were identified [18, 91, 93]. However, these genes were poorly expressed in brewing yeast while grown on maltose [93] and are only able to transport turanose [94].

In comparison with maltose metabolism in *S. cerevisiae,* which has been intensively studied, maltose metabolism in *S. eubayanus*, is still poorly characterized. In Chapter 2 of this thesis, the *MAL* genes of the type strain *S. eubayanus* CBS 12357^T were investigated. *S. eubayanus* was re-sequenced to obtain all *MAL* genes present in the sub telomeric regions. *MAL* genes in *S. eubayanus* were designated as *SeMALT* (transporter)*, SeMALS* (maltase) and *SeMALR* (regulator). Sequencing revealed four complete maltose transporter genes to be present in CBS 12357^T on chromosome II (*SeMALT1*), V (*SeMALT2*), XIII (*SeMALT3*) and XVI (*SeMALT4*). All *SeMALT* genes showed 78%-82% identity to *ScMAL31*. All *SeMALT* genes encoded functional maltose transporters but were unable to transport maltotriose. Not all transporters were expressed in *S. eubayanus* when grown in maltose media. Only *SeMALT2* and *SeMALT4*, which are >99% identical, encode the same amino-acid sequence and were part of an intact *MAL* locus, were expressed [90].

In Chapter 4, a different *S. eubayanus* strain (CDFM21L.1), isolated in Tibet and belonging to the Patagonia B Holarctic group, was also sequenced and characterized. A different organization of *MAL* genes was found in this strain. Only *SeMALT1-3* were identified but

SeMALT2 was translocated to chromosome XII. Besides the *SeMALT* genes, three additional and identical transporter genes found on chromosomes VII, XIV and XV were most closely related to *ScAGT1* (80% sequence similarity) and therefore named *SeAGT1*. A 95% similar transporter was also identified in other strains that belong to the Patagonia B Holarctic group [34]. Transporter characterization showed that only *SeMALT1* and *SeAGT1* encoded functional maltose transporters, with *SeAGT1* also able to transport maltotriose. The native *S. eubayanus* strain was not able to consume maltose or maltotriose due to a mutated regulator gene (*SeMALR1*). Integration of a functional *ScMAL13* in CDFM21L.1 restored growth on maltose and maltotriose [35]. RNA sequencing revealed that *SeMALT1* was poorly expressed and that *Se*Agt1 was solely responsible for maltose and maltotriose transport. These studies gave more insight in the possible variation among wild *S. eubayanus* isolates and their malt sugar metabolism, and highlighted the importance of DNA sequencing in molecular biology.

Lager brewing yeast *S. pastorianus* has been known and studied prior to the discovery of *S. eubayanus*. Properties inherited from both parents make *S. pastorianus* an outstanding fermenter at low temperature and under harsh conditions such as high sugar and ethanol concentrations. Whole-genome sequencing of *S. pastorianus* strains WS34/70 and CBS1483 revealed that *S. pastorianus* inherited maltose transporter genes from both parents (Table 2) [18, 51]. Transporter genes related to wort sugar uptake that were identified in *S. pastorianus* were *MALx1*, *AGT1*, *MTT1*, and *MALT* [88]. However, not all these genes encompassed a full ORF. For example, *ScAGT1* located on the *S. cerevisiae* sub-genome was truncated in *S. pastorianus* because of an early stop codon that abolished the functionality of *Sc*Agt1 [95]. The *S. eubayanus* sub-genome allele *SeAGT1* however, does encode a functional transporter in *S. pastorianus* [96].

Table 2. Overview of maltose transporter genes in the yeasts *S. cerevisiae* (red), *S. eubayanus* (blue), and *S. pastorianus* (purple).

MAL genes	Maltotriose transport	Remarks	Reference
MAL11	Yes	Divergent from all other *MALx1* genes	[93]
MAL21	No	Expression is strain dependent	[93]
MAL31	No	Functional and expressed transporter	[93]
MAL41	No	Expression is strain dependent	[93]
MAL61	No	Expression is strain dependent	[93, 97]
SeMALT1	No	Poor expression	Chapter 2
SeMALT2	No	High expression	Chapter 2
SeMALT3	No	Poor expression	Chapter 2
SeMALT4	No	High expression	Chapter 2
*SeMALT413**	Yes	Laboratory evolved and chimeric	Chapter 3
SeAGT1	Yes	Functional but not expressed	Chapter 4
ScAGT1 (MAL11)	Yes	Truncated in *S. pastorianus*	[77, 85, 89, 91]
ScMALx1 (21,31,41,61)	No	Some are truncated, strain dependent	[18, 51]
ScMTT1 / ScMTY1	Yes	Common to *S. pastorianus*	[77, 85, 89, 91]
SeMALT1	No	Complete ORF	[63]
SeMALT2 / SeMALT4	No	Sometimes truncated, strain dependent	[63]
SeMALT3	No	Sometimes truncated, strain dependent	[63]
SeAGT1	Yes	Functional and expressed	[96]

Although different maltose transporters have been identified in lager yeast, not every *S. pastorianus* has the same maltose-transporter gene content [51, 63, 88, 98]. For example, *MTT/MTY1* which is present in Weihenstephan 34/70 and other *S. pastorianus* strains [88], is absent from the *S. pastorianus* strain CBS 1483, in which *ScMALx1*, *ScAGT1, SeAGT1* and *SeMALT* are present [51, 63]. Even though most *MAL* genes are present in lager brewing strains, they frequently carry mutations that introduce a premature stop codon [18, 95]. For example, *ScAGT1*, *SeMALT2* and *SeMALT3* are truncated in *S. pastorianus* CBS 1483 [25, 63].
The origin of the different *MAL* genes in *S. pastorianus* is becoming clearer with the accumulation in whole-genome sequence information of lager brewing yeasts. Near-complete chromosome assemblies of *S. eubayanus* provided new information of their *MAL* gene content and showed highly similarity with sequences on the *S. eubayanus* subgenome of *S. pastorianus*, as in the case of *SeAGT1*. All maltose-transporter genes identified in *S. pastorianus* and their similarity to parental transporter genes are visualized in Figure 8.

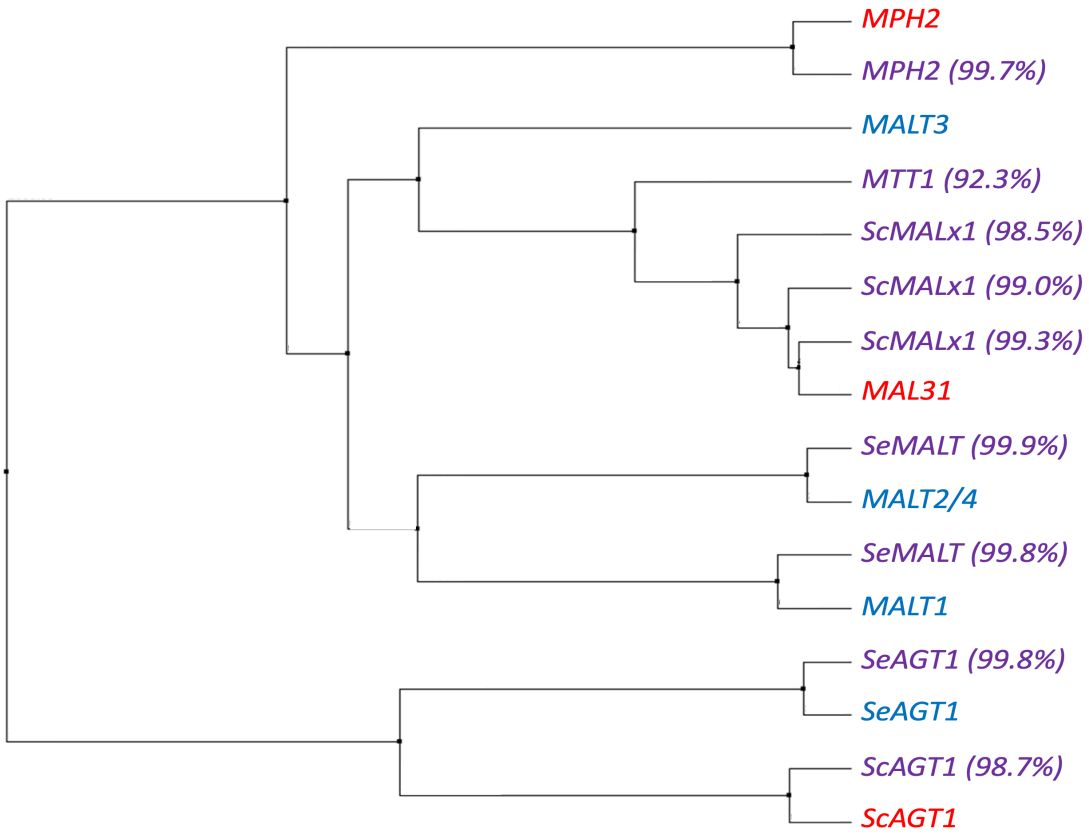


Figure 8 Schematic representation of maltose transporter genes in *S. cerevisiae* S288C (red), *S. eubayanus* CDFM21L.1 (blue) and *S. pastorianus* CBS1483 (purple). MTT1 sequence was obtained from Dietvorst *et al*. 2010. Average distances between transporters were calculated based on DNA sequences with Clustal 2.1 and visualized by Jalview 2.10.5. Sequence identities are based on the most closely related parental gene.

How these maltotriose transporters evolved was, until recently, subject to discussion and not witnessed in the laboratory. In Chapter 3, *S. eubayanus* was mutated and evolved to obtain a novel maltotriose transporter named *SeMALT413*. Gene neofunctionalization was hypothesized to occur when gene duplicates are present so that one of the two copies can

mutate freely without consequences [99]. The evolved *S. eubayanus* strain newly acquired the ability to consume maltotriose. DNA-sequence analysis showed that reciprocal recombination of three *MALT* transporter genes led to a chimeric transporter gene, encoding a functional maltotriose transporter. Generation of the chimeric *Se*MalT413 transporter was the first gene neofunctionalization to be observed in real time. Similar results were obtained in a different study [34], strengthening our findings. Sequence comparison of *MTT1* and other transporters indicates that *MTT1* is closely related to *ScMAL31* but has a few domains with low similarity, possibly originating from *S. paradoxus* [100]. It is hypothesized that *MTT1* might have originated in a similar manner as *SeMALT413* and could be the result of recombination between *ScMAL31* and a different *MAL* gene (Chapter 3) [100].

1.8 Strain improvement strategies in the alcoholic beverage industry

Use of genetically modified (GM) organisms in the food industry is not fully accepted by consumers [101]. Therefore, it is important for the brewing industry to exclusively use yeast strain improvement strategies that are legally 'non-GM'. To innovate brewing yeasts with non-GM fashion, strategies such random mutagenesis, breeding/hybridization and laboratory evolution can be applied (Figure 9).

Random mutagenesis, which is considered a non-GM technique, causes mutations all over the genome. While many of these mutations are lethal, cells that do survive may have obtained beneficial mutations. Improved growth performance is one of the criteria to select for mutants and is easy to measure. Complex traits can be also screened for but can require specific assays making it harder to achieve the required level of throughput.

Most alcoholic beverage companies already have a favorite strain for their processes but often they possess multiple strains with a natural variation in brewing-related properties. Although some strains might have very interesting properties, such as production of high concentrations of the flavor compound ethyl hexanoate, they are not necessarily also optimal performers in the industrial process. Hybridization is an effective method for obtaining new brewing strains and can be done sexually (mating) or asexually (protoplast fusion) [102]. Hybridization can be used to combine desired properties of individual strains, such as increased production of flavor components and performance under industrial conditions. One of the advantages of hybridization is that heterosis, the ability of the hybrid to outcompete its parental strains, sometimes occurs [103].

For further optimization of brewing strains, laboratory evolution can be applied. In laboratory evolution, prolonged cultivation under a set of carefully chosen conditions is used to select for micro-organisms with a specific trait. This technique can be used to optimize the fermentative behavior of *S. pastorianus* or its parents [100, 104]. Laboratory evolution is based on a "survival of the fittest" approach. Over time, mutations accumulate and the best mutant is selected based on higher specific growth rate, lower death rate and/or increased retention in the culture [105]. Several laboratory evolution setups exist, each designed for selection of different properties.

The simplest strategy is serial transfer in flasks. In this approach a mid-exponential culture is transferred to a new flask, thereby maintaining selective pressure for fast exponential growth. This process can be automated and controlled in sequential batch reactors (SBRs). By selecting for faster growth under specific conditions, these methods can, for example, be used to improve substrate-uptake rates or higher stress tolerance. With every cycle of batch cultivation, stress levels can be increased. This method has yielded strains with increased tolerance to a wide variety of stresses, including toxic metabolites, inhibitors, salinity, osmolarity, acidity and temperature [104, 106-108]. At

the end of a SBR cycle, the culture liquid is almost completely removed, while using the remainder of the culture as inoculum for the next batch. A risk of this process is that fast sedimenting cells may be selected [105, 109]. However, this feature can be also be used as a strategy as well to select for rapidly sedimenting cells that can, for example, be used for brewing.

In an industrial batch process, low substrate affinity may result in prolonged fermentation times. This problem occurs in beer brewing processes when maltotriose is very slowly consumed at the end of the fermentation process. For affinity improvement by laboratory evolution, nutrient-limited chemostat cultivation can be applied [110]. This method strongly selects for mutants with an improved affinity for the limiting nutrient. This strategy was successfully applied to maltotriose consumption in *S. pastorianus* and yielded evolved strains with improved maltotriose fermentation kinetics in wort [47].

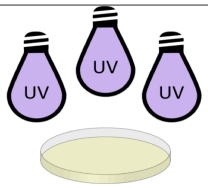
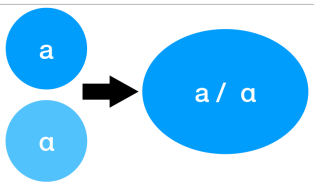
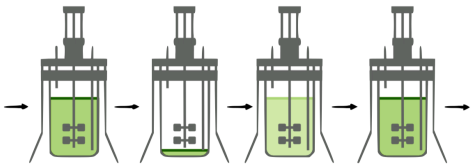
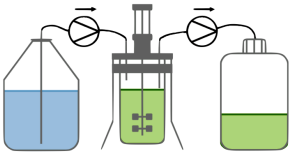
Graphical representation	non-GMO improvement	Application
	UV mutagenesis	Random mutation genome wide
	Sexual Hybridization (Rare/Direct/Mass Mating) Asexual Hybridization (protoplast fusion)	Chromosomal content of 2 or more parents
	Sequential Batch Reactor	Selection of high specific growth rates
	Chemostat	Selection for better substrate affinities

Figure 9 Non-GMO strategies for strain improvement. Methods such as UV mutagenesis, hybridization, sequential batch reactors or chemostats are used to improve or generate new strains with enhanced properties. Strategies are chosen based on the improvements needed and are often combined, such as UV mutagenesis followed by laboratory evolution. Partly adapted from [104]

1.9 Outline of this study

The lager yeast *S. pastorianus* is responsible for the production of lager beer, which is the most produced alcoholic beverage in the world and has a multi-billion-Euro market. Understanding the evolutionary history and properties of *S. pastorianus* can help improving the production process and also inspire strategies for developing new varieties of this yeast. The research described in this thesis focuses on understanding the genome composition and malt-sugar metabolism of *S. eubayanus*, one of the ancestor species of modern lager yeasts. Although *S. eubayanus* was discovered in 2011 in Patagonia, its brewing characteristics are barely understood and information about its wort sugar metabolism is incomplete.

In **Chapter 2** the genome of the *S. eubayanus* type strain CBS 12357^T was sequenced with long- read single-molecule sequencing. Combining the resulting sequence data with short-read sequencing data enable near-complete genome assembly and resolved the organization of all four *S. eubayanus MAL* loci. Using the *MAL* gene DNA sequences, we individually overexpressed all putative *S. eubayanus* MALT transporters in a maltose-negative *S. cerevisiae* strain and demonstrated their functionality as maltose transporters. In parallel, the first use of CRISPR-Cas9 technology in *S. eubayanus* enabled systematic knockouts of the Se*MALT* genes. The double deletion of *MALT2* and *MALT4,* whose translation result in an identical protein, abolished growth on maltose leading to the conclusion that these transporters play a dominant role in maltose uptake in *S. eubayanus*. Transcriptome analysis validated this conclusion, as *MALT2* and *MALT4* were the only Mal transporter genes that were strongly expressed under the tested conditions. The research provided a wealth of information for understanding malt sugar metabolism in *S. eubayanus* and can be used for strain selection and improvement for industrial applications.

The inability of *S. eubayanus* CBS 12357^T to consume maltotriose makes it challenging to brew a commercial product with this strain. **Chapter 3** describes the use of UV mutagenesis, combined with laboratory evolution, to generate a *S. eubayanus* strain that is able to consume not only glucose and maltose but also maltotriose in brewing wort. DNA sequencing revealed the mutations that had occurred and led to the discovery of a chimeric *SeMALT413* maltotriose-transporter gene. The chimeric gene has originated from in-frame recombinations between three different *SeMALT* transporters from three different loci. This is the first time that gene neofunctionalization was observed in real-time. Reverse engineering of the chimeric transporter gene demonstrated that *SeMALT413* indeed encodes a functional maltotriose transporter. Characterization in a 7 L industrial pilot fermentation showed the evolved strains were suitable for beer brewing.

Since its first discovery in Patagonia, *S. eubayanus* has been isolated from multiple locations in the world, including North-America, Tibet, Chili and New Zealand. Genetic analysis showed that a strain from Tibet, CDFM21L.1, is the closest related to the *S. eubayanus* sub-genome of *S. pastorianus*. Also for this strain, physiology and genome structure were poorly understood. Previous research showed that this strain harbors DNA sequences related to *SeAGT1* in *S. pastorianus*, a gene from which the origins still needed to be elucidated. **Chapter 4** focuses on resolving these knowledge gaps by sequencing strain CDFM21L.1 with short-read -and long read -technologies. The near-complete genome assembly revealed all *MAL* genes, including three copies of *SeAGT1*. Systematic physiological analysis including transporter overexpression and knockout studies demonstrated that two Asian *S. eubayanus* strains were unable to consume maltose or maltotriose. Overexpression of CDFM21L.1 *MAL* genes showed that *SeMALT1* and *SeAGT1* encode functional transporters and that the *SeMALS* genes encode functional

maltases. Integration of a functional *ScMAL13* regulator gene in CDFM21L.1 restored induction of the *MAL* genes and thereby enabled maltose and maltotriose consumption. Triple deletion of *SeAGT1* in CDFM21L.1 abolished growth on maltose and maltotriose. Hybridization of maltotriose-negative strains of *S. cerevisiae* and *S. eubayanus* resulted in an artificial *S. pastorianus* strain in which crosstalk between *MAL* genes of its two subgenomes enabled consumption of maltotriose. These results indicate that, in contrast to previous hypotheses, at least some *S. pastorianus* strains inherited the ability to consume maltotriose from a *S. eubayanus* ancestor.

Chapter 2

Structural, physiological and regulatory analysis of maltose transporter genes in *Saccharomyces eubayanus* CBS 12357^{T}

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Abstract

Saccharomyces pastorianus lager brewing yeasts are domesticated hybrids of *Saccharomyces cerevisiae* and cold-tolerant *Saccharomyces eubayanus*. To understand the contribution of both parental genomes to maltose metabolism in brewing wort, this study focuses on maltose transport in the *S. eubayanus* type strain CBS 12357^T/FM1318. To obtain complete sequences of the *MAL* loci of this strain, a near-complete genome assembly was generated using the Oxford Nanopore Technology MinION sequencing platform. Except for CHRXII, all sixteen chromosomes were assembled as single contigs. Four loci harboring putative maltose transporter genes (*SeMALT1-4*), located in subtelomeric regions of CHRII, CHRV, CHRXIII and CHRXVI, were completely resolved. The near-identical loci on CHRV and CHRXVI strongly resembled canonical *S. cerevisiae MAL* loci, while those on CHRII and CHRXIII showed different structures suggestive of gene loss. Overexpression of *SeMALT1-4* in a maltose-transport-deficient *S. cerevisiae* strain restored growth on maltose, but not on maltotriose, indicating maltose-specific transport functionality of all four transporters. Simultaneous CRISPR-Cas9-assisted deletion of only *SeMALT2* and *SeMALT4*, which shared 99.7 % sequence identity, eliminated growth of *S. eubayanus* CBS 12357^T on maltose. Transcriptome analysis of *S. eubayanus* CBS 12357^T established that *SeMALT1* and *SeMALT3*, are poorly expressed in maltose-grown cultures, while *SeMALT2* and *SeMALT4* were expressed at much higher levels than *SeMALT1* and *SeMALT3*, indicating that only *SeMALT2/4* are responsible for maltose consumption in CBS 12357^T. These results represent a first genomic and physiological characterization of maltose transport in *S. eubayanus* CBS 12357^T and provides a valuable resource for further industrial exploitation of this yeast.

Introduction

Saccharomyces eubayanus was first isolated from Nothofagus trees and stromata of *Cyttaria harioti* in North-Western Patagonia [19]. Strains of *S. eubayanus* have subsequently been also isolated from locations in North America [59], Asia [58] and Oceania [60]. Initial physiological characterization of the Patagonian *S. eubayanus* strain CBS 12357^T revealed that it grows faster than *S. cerevisiae* at temperatures below 10 ^oC [111], shows poor flocculation [112] and consumes maltose but not maltotriose [111, 113].

Isolation and characterization of *S. eubayanus* provided a strong impetus for research on *S. pastorianus* lager brewing yeasts. The hybrid nature of lager yeast genomes was already shown by Southern hybridization [114, 115]; RFLP genotyping, Sanger sequencing [54, 56] and comparative proteomics [116, 117]. However, release of the first *S. eubayanus* genome sequence [19] unequivocally established that this cold-tolerant *Saccharomyces* species contributed the non-*cerevisiae* part of *S. pastorianus* genomes [18, 51, 65, 118]. Access to this genome sequence and its updates [62, 111] proved invaluable for resolving the complex structure of aneuploid *S. pastorianus* genomes. Moreover, access to *S. eubayanus* strains stimulated vigorous research into *de novo* generation of hybrids between *S. cerevisiae* and *S. eubayanus* in the laboratory [111, 112, 119, 120]. This approach has the potential to increase our understanding of the domestication process of lager brewing strains and, moreover, to strongly increase the genetic and phenotypic variety of lager yeast strains available to the brewing industry. *De novo* constructed *S. cerevisiae* x *S. eubayanus* hybrids have been demonstrated to combine advantageous brewing-related properties of both parents (cryo-tolerance, maltotriose utilization and strong flocculation) and even exhibited best parent heterosis also referred to as hybrid vigour [111, 120-123]. However, generation of new hybrids is, by itself, not sufficient to understand the genetic basis for the exceptional performance of *S. pastorianus* under brewing conditions.

Lager brewing strains of *S. pastorianus* have, over several centuries, been selected for rapid, near-complete fermentation of all-malt brewer's wort fermentable sugars, which typically comprise 60 % maltose, 25 % maltotriose and 15 % glucose, with trace amounts of fructose [78]. Lager brewing therefore critically depends on the capacity of *S. pastorianus* strains to efficiently take up and ferment the wort α-glucosides maltose and maltotriose. The required maltose fermentation characteristics of *S. pastorianus* strains are conferred by genes originating from each of the parents and from a set that likely arose during its domestication history (e.g. *MTT1*) [85, 88, 89, 91-93, 96, 124].

In *S. cerevisiae*, maltose metabolism and the responsible *MAL* genes are well characterized in term of sequence, genetics, regulation and biochemistry. *S. cerevisiae MAL* loci harbor the three key genes essential for maltose utilization, encoding a transcriptional activator (*MALx3*), a maltose permease (*MALx1*) and a maltase (*MAx2*) [125]. Numbers and identities of *MAL* loci are highly strain dependent, with up to five *MAL* loci (*MAL1*, *2*, *3*, *4* and *6*) occurring in haploid *S. cerevisiae* genomes. *MAL* loci are typically located in subtelomeric regions, with the structurally identical *MAL1*, *2*, *3*, *4* and *6* located near telomeres of CHRVII, III, II, XI and VIII, respectively [87, 124-129].

Maltose is transported across the *S. cerevisiae* plasma membrane by maltose-proton symport, mediated by Malx1 transporters [130, 131] and, to a lesser extent, by facilitated diffusion [132]. All *MALx1* genes are highly similar, with the exception of *MAL11* and its allele *AGT1*, whose DNA sequence shows only 57% identity to the other four *MALx1* transporter genes [87, 95]. This sequence difference is accompanied by a difference in

substrate range, with Agt1 also being able to transport other α-glucosides, such as trehalose [133], sucrose [134, 135] and, importantly for brewing applications, maltotriose [85, 95]. The *S. cerevisiae* genome harbors two additional maltose permease genes, *MPH2* and *MPH3*, which are located subtelomerically on CHRIV and X, respectively. Although the transport mechanisms of Mph2 and Mph3 have not been experimentally established, both carriers were shown to transport of a range of substrates including glucose, maltose, maltotriose, α-methylglucoside and turanose [132].

In contrast to the wealth of information on *S. cerevisiae*, knowledge on maltose transport in *S. eubayanus* is limited. The type strain *S. eubayanus* CBS 12357^T grows on maltose, but not on maltotriose [111]. Annotation of its genome sequence revealed four open reading frames sharing similarity with *S. cerevisiae MAL31* (*SeMALT1*; *SeMALT2*, *SeMALT3*, and *SeMALT4*) [62]. The hybrid *S. pastorianus* genome harbors two additional maltose transporter gene variants that were not found in either of the reference parental genomes. The first of these, *MTT1/MTY1*, shares 90 % and 54 % DNA sequence identity with *S. cerevisiae MAL31* and *MAL11*, respectively [91, 92]. The second *S. pastorianus*-specific maltose-transporter gene, *SeAGT1*, shared significant identity with *S. cerevisiae AGT1* (85% *ScAGT1*) [96]. *SeAGT1* was unexpectedly found to be located on the *S. eubayanus*-derived CHRVIII-XV, suggesting a *S. eubayanus* origin, despite the absence of similar genes in currently available *S. eubayanus* genome sequences [18, 51]. However, genome assembly of an Asian *S. eubayanus* strain (CDFM21L.1 [58]) revealed short (<200bp) sequences reminiscent of a putative *SeAGT1* gene [111]. Both *MTT1/MTY1* and *SeAGT1* were shown to confer low- temperature dependent transport of both maltose and maltotriose [136]. To illustrate the complexity of α-glucoside transport in *S. pastorianus*, the model strain Weihenstephan 34/70 contains all *S. cerevisiae MAL* loci except for *MAL2*, a single *MPH* gene (*MPH2*), as well as all four *S. eubayanus* genes (*MALT1* to *4*) [18, 51] and the two *S. pastorianus*-specific genes *MTT1* and *SeAGT1* [88]. In this *S. pastorianus* background, the *S. cerevisiae* allele of *AGT1* (*MAL11*) carries a nonsense mutation [95].

Hitherto, no study has systematically investigated the functionality of the individual α-glucoside transporters in *S. pastorianus*. In addition to the complexity of maltose metabolism in *S. pastorianus* strains, genetic analysis is complicated by the limited genetic accessibility of industrial lager brewing yeasts [137]. However, availability of the *S. eubayanus* type strain and of its genome sequence offers an alternative approach to fill existing knowledge gaps on transport of wort sugars. The aim of this study was therefore to investigate the contribution of individual putative maltose-transporter (*SeMALT*) genes in *S. eubayanus* CBS 12357^T. To this end, a new near-complete genome sequence of the strain CBS 12357^T was assembled using Oxford Nanopore Technology's MinION long-read sequencing platform. Subsequently, CRISPR-Cas9 gene editing was used to systematically delete the *MALT* genes in *S. eubayanus*. In a complementary approach, all four *S. eubayanus MALT* open reading frames were cloned and constitutively expressed alongside the *S. cerevisiae MAL12* maltase gene in a *S. cerevisiae* strain lacking all maltose utilization genes (*MALx1*, *MALx2* and *MALx3*), *MPH1/2*, *SUC2* and *IMA1-5* genes; [138]. Subsequently, growth of the genetically modified yeast strains was analysed on different carbon sources. Finally, RNA sequencing was performed on glucose- and maltose-grown cultures to study differential expression of the *S. eubayanus MALT* genes.

Materials and Methods

Strains and maintenance

S. eubayanus strain CBS 12357^T (alias FM1318 [19] was obtained from the Westerdijk Fungal Biodiversity Institute (Utrecht, the Netherlands,). The *S. cerevisiae* strain IMZ616 [138] was derived from the CEN.PK lineage [70, 139]. All strains used in this study are listed in Table 1. Stock cultures of *S. eubayanus* and *S. cerevisiae* strains were grown in YPD (10 g L^{-1} yeast extract, 20 g L^{-1} peptone and 20 g L^{-1} glucose) until late exponential phase, complemented with sterile glycerol to a final concentration of 30 % (v/v) and stored at -80 ^{0}C as 1.5 ml aliquots until further use.

Table 1: Strains used in this study

Strain	Genotype	Sp	Reference
CBS 12357^T/ FM1318	*MATa/MATα SeMALT1/SeMALT1 SeMALT2/SeMALT2 SeMALT3/SeMALT3 SeMALT4/SeMALT4*	Se[a]	[19]
IMK816	*MATa/MATα SemalT1Δ/SemalT1Δ SeMALT2/SeMALT2 SeMALT3/SeMALT3 SeMALT4/SeMALT4*	Se	This study
IMK817	*MATa/MATα SeMALT1/SeMALT1 SemalT2Δ/SemalT2Δ SeMALT3/SeMALT3 SemalT4Δ/SemalT4Δ*	Se	This study
IMK818	*MATa/MATα SeMALT1/SeMALT1 SeMALT2/SeMALT2 SemalT3Δ/SemalT3Δ SeMALT4/SeMALT4*	Se	This study
IMZ616	*MATa ura3-52 HIS3 LEU2 TRP1 mal1Δ::loxP mal2Δ::loxP mal3Δ::loxP mph2Δ::loxP mph3Δ::loxP suc2Δ::loxP-KanMX-loxP ima1Δ ima2Δ ima3Δ ima4Δ ima5Δ* pUDC156 (*Spcas9::URA3 CEN6*)	Sc[b]	[138]
IMX1253	*MATa ura3-52 HIS3 LEU2 TRP1 mal1Δ::loxP mal2Δ::loxP mal3Δ::loxP mph2Δ::loxP mph3Δ::loxP suc2Δ::loxP-KanMX-loxP ima1Δ ima2Δ ima3Δ ima4Δ ima5Δ sga1Δ::ScTEF1$_{pr}$-SeMALT1-ScCYC1$_{ter}$::ScTDH3$_{pr}$-ScMAL12- ScADH1$_{ter}$* pUDC156 (*Spcas9::URA3 CEN6*)	Sc	This study
IMX1254	*MATa ura3-52 HIS3 LEU2 TRP1 mal1Δ::loxP mal2Δ::loxP mal3Δ::loxP mph2Δ::loxP mph3Δ::loxP suc2Δ::loxP-KanMX-loxP ima1Δ ima2Δ ima3Δ ima4Δ ima5Δ sga1Δ:: ScTEF1$_{pr}$-SeMALT2-ScCYC1$_{ter::Sc}$TDH3pr-ScMAL12- ScADH1$_{ter}$* pUDC156 (*Spcas9::URA3 CEN6*)	Sc	This study
IMX1255	*MATa ura3-52 HIS3 LEU2 TRP1 mal1Δ::loxP mal2Δ::loxP mal3Δ::loxP mph2Δ::loxP mph3Δ::loxP suc2Δ::loxP-KanMX-loxP ima1Δ ima2Δ ima3Δ ima4Δ ima5Δ sga1Δ:: ScTEF1$_{pr}$-SeMALT3-ScCYC1$_{ter}$::ScTDH3$_{pr}$-ScMAL12- ScADH1$_{ter}$* pUDC156 (*Spcas9::URA3 ARS4 CEN6*)	Sc	This study
IMX1365	*MATa ura3-52 HIS3 LEU2 TRP1 mal1Δ::loxP mal2Δ::loxP mal3Δ::loxP mph2Δ::loxP mph3Δ::loxP suc2Δ::loxP-KanMX-loxP ima1Δ ima2Δ ima3Δ ima4Δ ima5Δ sga1Δ:: ScTEF1$_{pr}$-ScAGT1-ScCYC1$_{ter}$::ScTDH3$_{pr}$-ScMAL12- ScADH1$_{ter}$* pUDC156 (*Spcas9::URA3 ARS4 CEN6*)	Sc	This study

[a] *S. eubayanus*
[b] *S. cerevisiae*

Media and cultivation

S. eubayanus batch cultures were grown on synthetic medium (SM) containing 3.0 g L^{-1} KH_2PO_4, 5.0 g L^{-1} $(NH_4)_2SO_4$, 0.5 g L^{-1} $MgSO_4$, 7 H_2O, 1 mL L^{-1} trace element solution, and 1 mL L^{-1} vitamin solution [140]. The pH was set to 6 with 2 M KOH prior to autoclaving at 120 °C for 20 min. Vitamin solutions [140] were sterilized by filtration and added to the sterile medium. Concentrated sugar solutions were autoclaved at 110 °C for 20 min and added to the sterile flasks to give a final concentration of 20 g L^{-1} carbon source (glucose (SMG), maltose (SMM) or maltotriose (SMMt)). *S. cerevisiae* batch cultures were grown on SM supplemented with 150 mg L^{-1} uracil to compensate for loss of plasmid pUDC156 that carried the *cas9* endonuclease gene, and supplemented with 20 g L^{-1} carbon source (glucose (SM_uG), maltose (SM_uM) or maltotriose (SM_uMt)). All batch cultures were grown in 500 mL shake flasks with a working volume of 100 mL. The cultures were inoculated at an initial OD_{660nm} of 0.1 and incubated under an air atmosphere and shaken at 200 rpm and at 20 ^oC in a New Brunswick™ Innova44 incubator (Eppendorf Nederland B.V, Nijmegen, The Netherlands).

Selection of the *S. eubayanus* strains transformed with plasmids pUDP062 ($gRNA_{SeMALT1}$), pUDP063 ($gRNA_{SeMALT2}$) and pUDP064 ($gRNA_{SeMALT3}$) was carried out on a modified SMG medium, in which $(NH_4)_2SO_4$ was replaced by 5 g.L^{-1} K_2SO_4 and 10 mM acetamide ($SM_{Ace}G$) [141]. SM- based solid medium contained 2 % Bacto Agar (BD, Franklin Lakes, NJ). Selection of *S. cerevisiae* integration strains was carried out on $SM_{Ace}G$. For plasmid propagation, *E. coli* XL1-Blue-derived strains (Agilent Technologies, Santa Clara, CA) were grown in Lysogeny Broth medium (LB, 10 g L^{-1} tryptone, 5 g L^{-1} yeast extract, 5 g L^{-1} NaCl) supplied with 100 mg L^{-1} ampicillin.

Plasmid and strain construction

Plasmid construction Guide-RNA (gRNA) sequences for deletion of *SeMALT1*, *Se*MALT2/4 and *SeMALT3* were designed following the guiding principles recommended in [142]. The DNA sequences encoding these gRNAs were synthesised at GeneArt (Thermo Fisher Scientific, Waltham, MA) and were delivered in pUD631, pUD632 and pUD633 respectively (Table 2). The gRNA spacer sequences (Se*MALT1* 5'ATTCCAAACGACAATAAAGA3', Se*MALT2/T4* 5'-TACAGGAGAATGGGAGATTT-3' and Se*MALT3* 5'- GTTTTCAAAGCTTGCAGAAG-3') and the structural gRNA sequence were flanked at their 5' ends by the Hammerhead ribozyme (HH) and at their 3' ends by the Hepatitis Delta Virus ribozyme (HDV) [143]. The HH-gRNA-HDV fragment was flanked on both ends with a BsaI site for further cloning [142, 144]. In the next step, the gRNAs were transferred into the pUDP004 plasmid [142], which enables combined expression of the gRNA cassette of $Spcas9^{D147Y\ P411T}$ [145]. The plasmid pUDP062, expressing $gRNA_{SeMALT1}$ was constructed in a one-pot reaction by digesting pUDP004 and pUD631 using BsaI and ligating with T4 ligase. Similarly, pUDP063, expressing $gRNA_{SeMAT2/T4}$ and $Spcas9^{D147Y\ P411T}$ was assembled from pUDP004 and pUD632. The plasmid pUDP064 expressing $gRNA_{SeMALT3}$ and $Spcas9^{D147Y\ P411T}$ was assembled from pUDP004 and pUD633. Correct assembly of pUDP062-064 was verified by restriction analysis with SspI and PdmI.

Table 2: Plasmids used in this study

Name	Relevant characteristics	Origin
p426-TEF-amdS	ori (ColE1) *bla* 2µ amdSYM *TEF1$_{pr}$-CYC1$_{ter}$*	[141]
pUDP004	ori (ColE1) *bla* panARSopt amdSYM Sc*TDH3$_{pr}$*-BsaI-BsaI-Sc*CYC1$_{ter}$* *AaTEF1$_{pr}$-Spcas9*$^{D147Y\ P411T}$*-ScPHO5$_{ter}$*	[142]
pUDP052	ori (ColE1) *bla* panARSopt amdSYM Sc*TDH3$_{pr}$-gRNA$_{SGA1}$*-Sc*CYC1$_{ter}$* *AaTEF1$_{pr}$-Spcas9*$^{D147Y\ P411T}$*-ScPHO5$_{ter}$*	This study
pUDP062	ori (ColE1) *bla* panARSopt amdSYM Sc*TDH3$_{pr}$-gRNA$_{SeMALT1}$*-Sc*CYC1$_{ter}$* *AaTEF1$_{pr}$-Spcas9*$^{D147Y\ P411T}$*-ScPHO5$_{ter}$*	This study
pUDP063	ori (ColE1) *bla* panARSopt amdSYM Sc*TDH3$_{pr}$-gRNA$_{SeMALT2/T4}$*-Sc*CYC1$_{ter}$* *AaTEF1$_{pr}$-Spcas9*$^{D147Y\ P411T}$*-ScPHO5$_{ter}$*	This study
pUDP064	ori (ColE1) *bla* panARSopt amdSYM Sc*TDH3$_{pr}$-gRNA$_{SeMALT3}$*-Sc*CYC1$_{ter}$* *AaTEF1$_{pr}$-Spcas9*$^{D147Y\ P411T}$*-ScPHO5$_{ter}$*	This study
pUDE044	ori (ColE1) *bla* 2µ *ScTDH3$_{pr}$-ScMAL12-ScADH1$_{ter}$ URA3*	[134]
pUD479	ori (ColE1) *bla* 2µ amdSYM *ScTEF1$_{pr}$-SeMALT1-ScCYC1$_{ter}$*	This study
pUD480	ori (ColE1) *bla* 2µ amdSYM *ScTEF1$_{pr}$-SeMALT2/4-ScCYC1$_{ter}$*	This study
pUD481	ori (ColE1) *bla* 2µ amdSYM *ScTEF1pr-SeMALT3-ScCYC1ter*	This study
pUD445	ori (ColE1) *bla* 2µ amdSYM *ScTEF1pr-ScAGT1-ScCYC1ter*	This study
pUDR119	ori (ColE1) *bla* 2µ AmdSYM *SNR52*$_{pr}$-gRNA$_{SGA1}$-*SUP4$_{ter}$*	[146]

The coding regions of *SeMALT1*, *SeMALT2* and *SeMALT3* were amplified from CBS 12357^{T} genomic DNA with Phusion High-Fidelity DNA polymerase (ThermoFisher Scientific), according to the supplier's instructions with primers pairs 10491/10492, 10632/10633 and 10671/10672 (Table S5), respectively. The coding sequence of *ScAGT1* was amplified from CEN.PK113-7D genomic DNA with Phusion High-Fidelity DNA polymerase (ThermoFisher Scientific), according to the supplier's instructions with primers pairs 9940/9941. Each primer carried a 40 bp extension complementary to the plasmid backbone of p426-TEF-amdS [138, 141], which was PCR amplified using Phusion High-Fidelity DNA polymerase (ThermoFisher Scientific) and primers 7812 and 5921 (Table S5). p426-TEF-amdS is an expression plasmid that harbors the promoter of the translational elongation factor EF-1 alpha (*TEF1*) of *S. cerevisiae*. Each *SeMALT* fragment was assembled with the p426-TEF-amdS backbone fragment using NEBuilder HiFi DNA Assembly (New England Biolabs, Ipswich, MA), resulting in plasmids pUD479 (*SeMALT1*), pUD480 (*SeMALT2/T4*), pUD481 (*SeMALT3*) and pUD445 (*ScAGT1*) (Table 2).

Strains construction *S. eubayanus* IMK816 (Se*malT1Δ*) was constructed by transforming CBS 12357^T by electroporation [142] with 200 ng of pUDP062 and 1 µg of 120 bp repair fragment obtained by mixing an equimolar amount of primers 11850 and 11851 (Table S5) [147] (Figure 1). As control, the same transformation was performed without including the repair DNA fragment. Transformants were selected on $SM_{Ace}G$ plates. Strain IMK817 (*SemalT2Δ SemalT4Δ*) and IMK818 (*SemalT3Δ*) were constructed in the same way. The *SeMALT2/T4* deletion was constructed by co-transforming pUDP063 and a repair DNA fragment formed by primers 11328 and 11329, while the *SeMALT3* deletion involved pUDP064 and a repair DNA formed by primers 11330 and 11331 (Table S5). Deletion of *SeMALT1*, *SeMALT2/T4* and *SeMALT3* was verified by diagnostic PCR, using primers pairs 11671/11672, 11673/11674 and 11675/11676 (Table S5), respectively (Figure 1C). Prior to storing at -80 ^oC, transformants were successively streaked on $SM_{Ace}G$ and YPD plates. The genotype was verified after each plating round with the primers pairs mentioned above.

S. cerevisiae IMZ616 (*mal1Δ mal2Δ mal3Δ mph2Δ mph3Δ suc2Δ ima1Δ ima2Δ ima3Δ ima4Δ ima5Δ* pUDC156 (*Spcas9 URA3 ARS4 CEN6*)), which cannot grow on α-glucosides [138] was used as a host to test the functionality of individual *S. eubayanus* (putative) maltose transporter genes. *S. cerevisiae* IMX1253 was constructed by integrating the *S. cerevisiae* maltase gene *ScMAL12* and the *SeMALT1* transporter gene at the *ScSGA1* locus of strain IMZ616 (Figure 2). The *ScSGA1* gene encodes an intracellular sporulation-specific glucoamylase [148] that is not expressed during vegetative growth [149]. This integration site was shown suitable for expression of single or multiple genes as previously demonstrated in [147, 150-152]. The fragment containing *ScMAL12* was PCR amplified using Phusion High-Fidelity DNA polymerase (Thermo FisherScientific) from pUDE044 [134] with primers 9596 and 9355, which included a 5' extension homologous to the upstream region of the *S. cerevisiae SGA1* locus and an extension homologous to the co-transformed transporter fragment, respectively. The DNA fragment carrying the *S. eubayanus SeMALT1* maltose symporter was PCR amplified from pUD479 using primers 9036 and 9039, which included a 5' extension homologous to the co-transformed transporter fragment and an extension homologous to the downstream region of the *S. cerevisiae SGA1* locus, respectively. To facilitate integration in strain IMZ616, the two PCR fragments were co-transformed with plasmid pUDR119, which expressed a gRNA targeting Sc*SGA1* (spacer sequence: 5'-ATTGACCACTGGAATTCTTC-3') [146] (Figure 2A). The plasmid and repair fragments were transformed using the LiAc protocol [153] and transformed cells were plated on $SM_{Ace}G$. Correct integration was verified by diagnostic PCR with primers pairs 4226/5043 and 942/4224 (Figure 2 and Table S5). Strains *S. cerevisiae* IMX1254, IMX1255 and IMX1365 were constructed following the same principle, but instead of using pUD479 to generate the transporter fragment, pUD480 pUD481 and pUD445 were used to PCR amplify Se*MALT2/T4*, Se*MALT3* and *ScAGT1* respectively. Correct integration was verified by diagnostic PCR with primers pairs 4226/5043 and 942/4224 (Figure 2 and Table S5). All PCR-amplified gene sequences were Sanger sequenced (Baseclear, Leiden, The Netherlands).

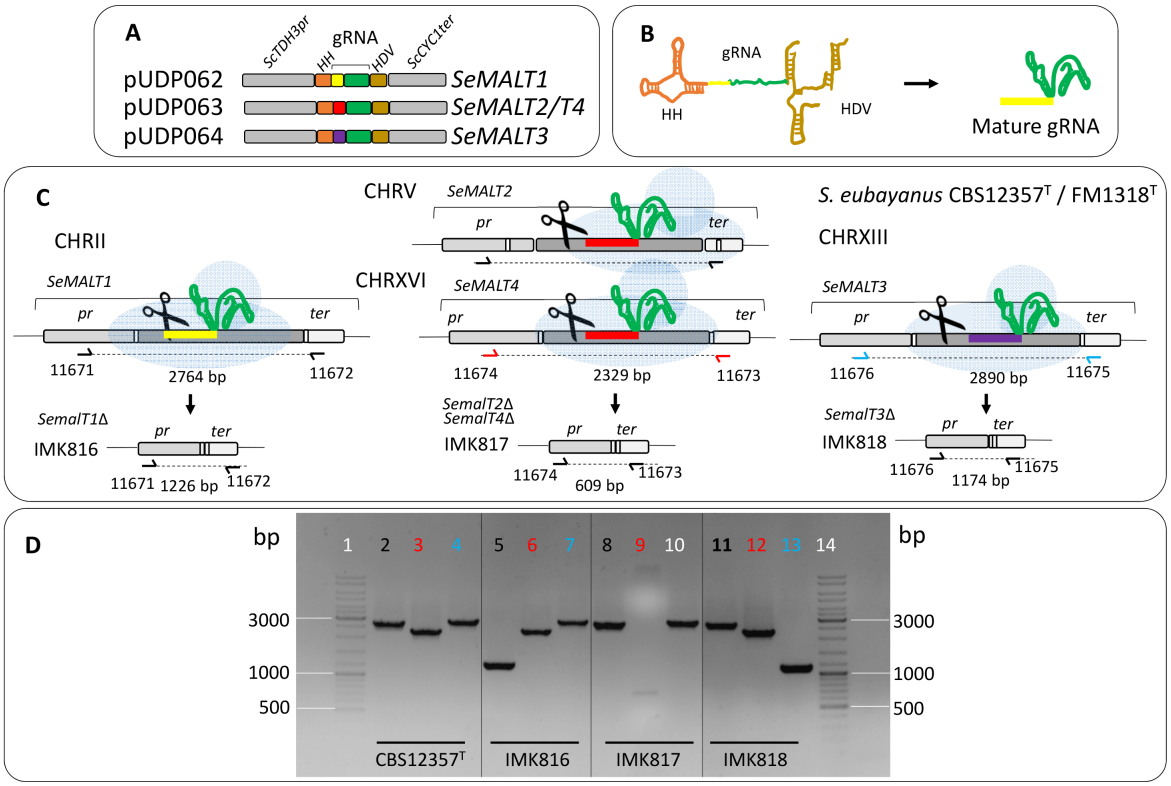

Figure 1: Deletion of *SeMALT* genes using CRISPR-Cas9-assisted genome editing in *S. eubayanus* CBS 12357^T. (A) Representation of the gRNA expression cassette in pUDP062, pUDP063 and pUDP064. gRNAs targeting either *SeMALT1*, *SeMALT2/T4* or *SeMALT3* were flanked by a 5' hammerhead ribozyme (HH, orange) and a 3' hepatitis-δ virus ribozyme (HD, bronze). These constructs were expressed from the RNA polymerase II *ScTDH3* promoter and the *ScCYC1* terminator. **(B)** Upon ribozyme self-cleavage, a mature gRNA comprising the Se*MALT* guiding spacer (yellow) and the constant structural gRNA fragment (green) is released. **(C)** Schematic representation of Se*MALT* gene editing upon transformation of pUDP062 or pUDP063 or pUDP064 into *S. eubayanus* CBS 12357^T. Primers used for verification of transformants from transformation are indicated together with the size of the expected PCR products. **(D)** Validation of transformants derived from transformations of *S. eubayanus* CBS 12357^T with either pUDP062, pUDP063 or pUDP064 in presence of the corresponding 120 bp repair DNA fragments. Lanes 1 and 14 GeneRuler DNA Ladder Mix (Thermo Fischer Scientific). Lanes 2, 5, 8 and 11 fragments amplified with primers 11671 and 11672 black label). Lanes 3, 6, 9, 12 fragments amplified with primers 11674 and 11673 (red label). Lanes 4, 7, 10 and 13 fragments amplified with primers 11676 and 11675 (blue label) from genomic DNA from CBS 12357^T (Lanes 2, 3 and 4), from IMK816 (*SemalT1Δ*) (Lanes 5, 6 and 7), from IMK817 (*SemalT2Δ/ SemalT4Δ*) (Lanes 8, 9 and 10) and from IMK 818 (*SemalT3Δ*) (Lanes 11, 12 and 13).

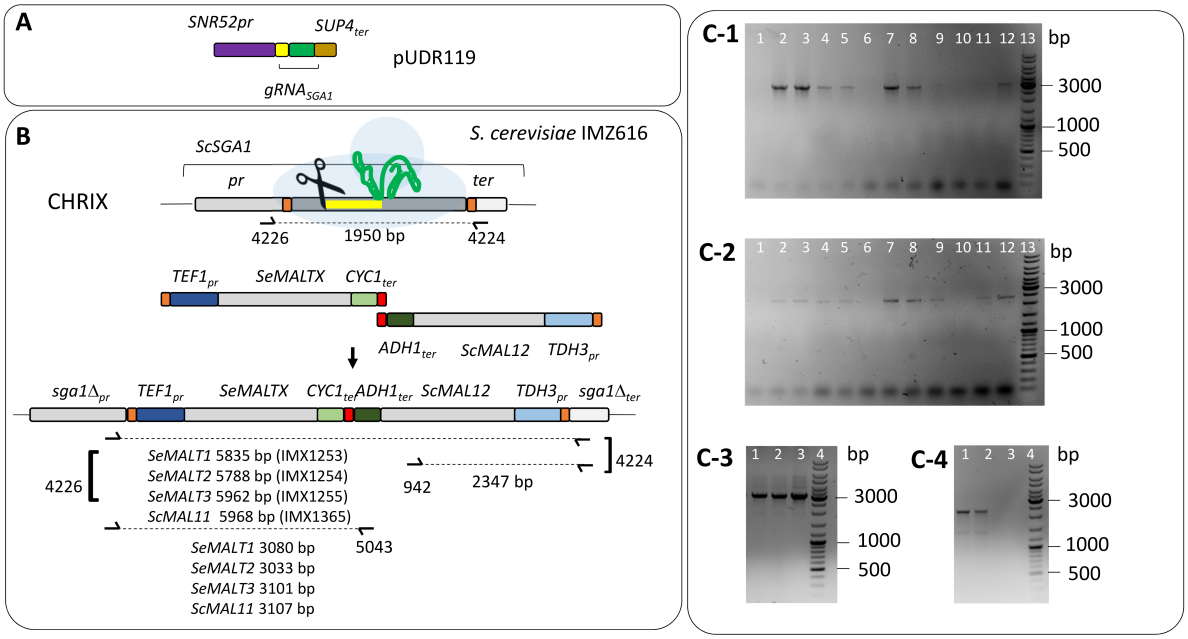

Figure 2: Integration of *S. eubayanus* CBS 12357^T maltose transporter genes at the *ScSGA1* locus of *S. cerevisiae* IMZ616 (*mal1Δ mal2Δ mal3Δ mph2Δ mph3Δ::suc2Δ ima1Δ ima2Δ ima3Δ ima4Δ ima5Δ Spcas9*) [138]. (A) Integration at the Sc*SGA1* locus by Cas9-assisted genome editing. The Cas9-targeting gRNA was expressed from pUDR119 [146]. **(B)** Schematic representation of the integration of *SeMALT* expression cassettes at the *ScSGA1* locus. Upon cleavage, the Cas9-induced double strand break was repaired by the two co-transformed fragments harboring a transporter gene expression cassette and the *S. cerevisiae* maltase gene *MAL12*, respectively. Primers used for verification of transformants from transformation are indicated together with the size of the expected PCR products. Integration of *SeMALT1*, *SeMALT2*, *SeMALT3* or *ScMAL11* resulted in *S. cerevisiae* strains IMX1253, IMX1254, IMX1255 and IMX1365 respectively. **(C)** Validation of the *S. cerevisiae* IMX1253, IMX1254, IMX1255 and IMX1365. **(C1)** Lane 13 GeneRuler DNA Ladder Mix (Thermo Fischer Scientific). Lanes 1 to 12 fragments amplified with primers 4226 and 5043. Lanes 1, 2, 3 and 4 fragments amplified from clones transformed with *SeMALT1*. The strain corresponding to lane 3 was renamed IMX1253. Lanes 5, 6, 7 and 8 fragments amplified from clones transformed with *SeMALT2*. The strain corresponding to lane 7 was renamed IMX1254. Lanes 9, 10, 11 and 12 fragments amplified from clones transformed with *SeMALT3*. The strain corresponding to lane 12 was renamed IMX1255. **(C2)** Lane 13 GeneRuler DNA Ladder Mix (Thermo Fischer Scientific). Lanes 1 to 12 fragments amplified with primers 942 and 4224. Lanes 1, 2, 3 and 4 fragments amplified from clones transformed with *SeMALT1*. Lane 3 corresponds to IMX1253. Lanes 5, 6, 7 and 8 fragments amplified from clones transformed with *SeMALT2*. Lane 7 corresponds to IMX1254. Lanes 9, 10, 11 and 12 fragments amplified from clones transformed with *SeMALT3*. Lane 12 corresponds to IMX1255. **(C3)** Lanes 1 to 3 fragments amplified with primers 4226 and 5043. Lanes 1, 2, 3 and 4 fragments amplified from clones transformed with *ScMAL11*. The strain corresponding to lane 3 was renamed IMX1365. Lane 4 GeneRuler DNA Ladder Mix (Thermo Fischer Scientific). **(C4)** Lanes 1 to 4 fragments amplified with primers 942 and 4224. Lanes 1, 2, and 3 fragments amplified from clones transformed with *ScMAL11*. Lane 3 corresponds to IMX1365. Lane 4 GeneRuler DNA Ladder Mix (Thermo Fischer Scientific).

Illumina sequencing
Genomic DNA from *S. eubayanus* CBS 12357^T was isolated as previously described in [51]. Paired-end sequencing (2-fold 150 bp) was performed on a 350 bp PCR-free insert library using Illumina HiSeq2500 (San Diego, CA) by Novogene (HK) Company Ltd (Hong Kong, China) with a sample size of 3.2 Gbase. Sequence data are available at NCBI under Bioproject accession number PRJNA450912.

MinION sequencing
For Nanopore sequencing, a 1D sequencing library (SQK-LSK108) was prepared according to the manufacturer's recommendation and loaded onto a FLO-MIN106 (R9.4) flow cell, connected to a MinION Mk1B unit (Oxford Nanopore Technology, Oxford, United Kingdom). MinKNOW software (version 1.5.12; Oxford Nanopore Technology) was used for quality control of active pores and for sequencing. Raw files generated by MinKNOW were base called using Albacore (version 1.1.0; Oxford Nanopore Technology). Reads, in fastq format, with a minimum length of 1000 bp were extracted, yielding 3.26 Gb of sequence with an average read length of 8.07 kb. Sequencing data are available at NCBI under Bioproject accession number PRJNA450912.

De novo assembly
De novo assembly of the Oxford Nanopore MinION dataset was performed using Canu (v1.4, setting: genomesize=12m) [154]. Assembly correctness was assessed using Pilon [155] and further correction "polishing" of sequencing/assembly errors was performed by aligning Illumina reads with BWA [156] using correction of only SNPs and short indels (–fix bases parameter). Genome assembly gene annotation was performed with the MAKER2 annotation pipeline (version 2.31.9) [157] using SNAP (version 2013–11-29) [158] and Augustus (version 3.2.3) [159] as *ab initio* gene predictors. *S. cerevisiae* S288C EST and protein sequences were obtained from SGD (Saccharomyces Genome Database, http://www.yeastgenome.org/) and were aligned using BLASTX on the obtained polished sequence assembly (BLAST version 2.2.28+) [160]. Predicted translated protein sequences of the final gene model were aligned to the *S. cerevisiae* S288C protein Swiss-Prot database using BLASTP (http://www.uniprot.org/). Custom-made Perl scripts were used to map systematic names to the annotated gene names. Error rates in the nanopore-sequencing data were estimated from the q score (Phred scaled) per read, as calculated by the base caller Albacore (version 1.1.0) (Oxford Nanopore Technology). Average q score was used to calculate the error $P= 10^{q/10}$.

RNA isolation
S. eubayanus CBS 12357^T was grown in either SMG or SMM until mid-exponential phase (OD_{660nm} of 12.5). Culture samples corresponding to ca. 240 mg of biomass wet weight were directly quenched in liquid nitrogen. The resulting frozen pellet was gently thawed on ice and spun down at 4700 x g for 5 min at 0 ^oC. Pellets were then resuspended in 1.2 mL of ice-cold AE buffer (50 mM sodium acetate and 10 mM EDTA, pH 5.0), followed by addition of 1.2 mL of acid phenol/chloroform/isoamyl alcohol mix and 0.12 mL 10 % sodium dodecyl sulfate. The resulting mix was vortexed for 30 s and incubated for 5 min at 65 °C. After homogenizing for 30 sec by vortexing, 800 µL aliquots were distributed in RNase-free screw-cap tubes [110]. After centrifugation (15 min at 10,000 x g), the aqueous phase was transferred to a new tube containing 0.4 mL of acid phenol/chloroform. The mix was vortexed for 30 seconds, centrifuged (15 min at 10,000 x g) and the aqueous phase was again transferred to a new tube. RNA was then ethanol

precipitated and re-suspended in RNAse-free water. Prior to cDNA synthesis, purity, concentration and integrity of the RNA in the samples was assessed with the Nanodrop (Thermo Fisher Scientific), Qubit (Thermo Fisher Scientific) and Tapestation 220 with RNA Screen Tape (Agilent Technologies), respectively, according the manufacturers' recommendations. cDNA libraries were prepared using the TruSeq RNA V2 kit (Illumina) and sequenced on HISeq 2500 (Illumina) at Novogene (HK) Company Ltd (Hong Kong, China).

Transcriptome analysis

Libraries with 300 bp insert size were paired end sequenced (150 bp). Duplicate biological samples were processed, generating an average sequence quantity of 23.7M reads per sample. Reads were aligned to the Oxford Nanopore CBS 12357^T reference assembly using a two-pass STAR [161] procedure. In the first pass, splice junctions were assembled and used to inform the second round of alignments. Introns between 15 and 4000 bp were allowed, and soft clipping was disabled to prevent low-quality reads from being spuriously aligned. Ambiguously mapped reads were removed from the dataset. Expression level for each transcript were quantified using htseq-count [162] in union mode. Fragments per kilo-base of feature (gene) per million reads mapped (FPKM) values were calculated by "Applying the rpkm method" from the edgeR package [163, 164]. Differential expression analysis was performed using DESeq [165]. Transcript data can be retrieved at the Genome Omnibus Database (GEO: https://www.ncbi.nlm.nih.gov/geo/) under accession number:

Analytical methods

Optical densities of yeast cultures were measured with a Libra S11 spectrophotometer (Biochrom, Cambridge, UK) at a wavelength of 660 nm. Biomass dry weight was measured by filtering 10-mL culture samples over pre-weighed nitrocellulose filters with a pore size of 0.45 µm. Filters were washed with 10 mL water, dried in a microwave oven (20 min at 350 W) and reweighed. Each measurement was performed in duplicate. For glucose, maltose, maltotriose and ethanol analysis, culture samples were centrifuged five minutes at 10,000 g and supernatants were analyzed by high-performance liquid chromatography (HPLC) analysis on an Agilent 1260 HPLC equipped with a Bio-Rad HPX 87 H column (Bio-Rad, Hercules, CA). Elution was performed at 65 °C with 5mM H_2SO_4 at a flow rate of 0.8 mL min^{-1}. Detection was by means of an Agilent refractive-index detector and an Agilent 1260 VWD detector.

Viability measurements using fluorescence-assisted cell sorting

Cultures were analysed on a BD FACSAria™ II SORP Cell Sorter (BD Biosciences, Franklin Lakes, NJ) equipped with 355 nm, 445 nm, 488 nm, 561 nm and 640 nm lasers and a 70 µm nozzle, and operated with filtered FACSFlow™ (BD Biosciences). Correct cytometer performance was evaluated prior to each experiment by running a CST cycle with corresponding CS&T Beads (BD Biosciences). Drop delay for sorting was determined by running an Auto Drop Delay cycle with Accudrop Beads (BD Biosciences). Morphology of the cells was analysed by plotting forward scatter (FSC) against side scatter (SSC). 96 single cells were sorted onto 96-well format Nunc omnitray (Thermo Scientific) plates containing YPD agar using a "single cell" sorting mask, corresponding to a yield mask of 0, a purity mask of 32 and a phase mask of 16. Viability was measured as the average percentage of sorted cells able to form a colony after 48h incubation at 30˚C on three triplicate plates.

Results

A high-quality *S. eubayanus* genome assembly with 330 kb of previously unexplored sequence including four *MAL* loci.

Owing to advances in genome sequencing technology, the quality of genome sequence data of *S. eubayanus* CBS 12357^T/FM1318 has gradually improved [19, 62, 111, 166]. The currently available reference sequence is based on second generation sequencing technology (Illumina generated data), obtained from libraries with different insert sizes that were co-assembled into a 11.66 Mb genome, comprising 144 contigs forming 22 scaffolds. While representing an important resource for research on *S. eubayanus* and *S. pastorianus*, this most advanced draft genome sequence is incomplete [62]. In particular multiple repeated regions, such as subtelomeric regions, are not yet fully resolved due to limitations of short-read sequencing technology. In total, approximately 122 kb of the scaffolded genome remain undefined.

To generate a near-complete, chromosome-level *de novo* assembly of *S. eubayanus* CBS 12357^T, we used long-read sequencing with third-generation single-molecule technology (Oxford Nanopore Technology MinION platform). A single flow cell was used to generate 3.3 Gb of sequence reads, with an average read length of 8 kb and an estimated average error rate of 9.6 %. These data represented a genome coverage of 135 fold of the estimated diploid genome size (24 Mb). An assembly exclusively based on the MinION reads was generated with the Canu program [154]. This assembly yielded 19 contigs, which is 8-fold fewer than obtained in the short-read-only assembly of the latest CBS 12357^T draft genome [62]. In the MinION-based assembly, the mitochondrial genome and all chromosomes except for CHRXII were assembled as single contigs. CHRXII was manually reconstructed by joining three contigs, with a 1000 N residues gaps introduced between the contigs. The sequence discontinuity was caused by the inability of the assembly software to handle the highly repetitive DNA organization of the rDNA locus (Figure 3). This approach yielded a nearly complete 11.9 Mb genome assembly.

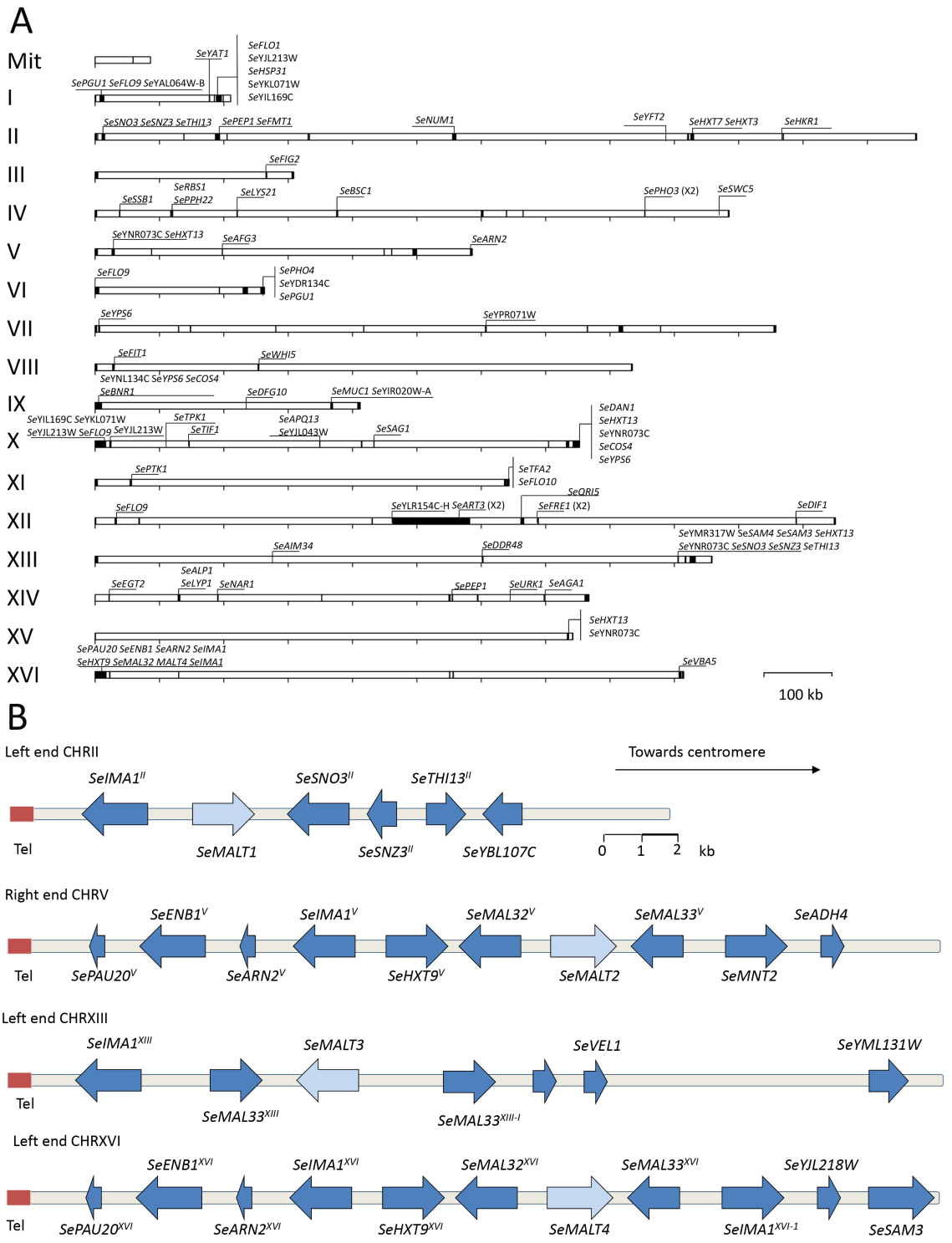

Figure 3: High-quality *S. eubayanus* genome assembly with 330 kb of unexplored sequence including four MAL loci. (A) Representation of the assembled *S. eubayanus* chromosomes, the black boxes denote newly added sequences. New annotated open reading frames and gene entries modified relative to the earlier draft genome [62] often leading to a redefinition of start and stop codons. **(B).** Organization of subtelomeric regions including maltose metabolism genes. Arrows denote the direction of transcription. The label "Tel" indicates the position of the telomere. The *SeMALT* genes are indicated in light blue. The gene and interval sizes are approximately to scale.

Prior to annotation, the assembly based on MinION sequencing data was "polished" with additional Illumina sequencing data using Pilon [155]. *In fine* this polished genome assembly included 330 kb of sequence which were not assembled in the previous genome assembly. With the exception of a region on chromosome XII that corresponded to a partially reconstructed rDNA locus, additional sequences were mainly located in the subtelomeric regions (Figure 3A). A total of 5444 ORFs were annotated, including 41 previously unassembled ORFs (Baker *et al*. 2015). Additionally, 60 ORFs were modified relative to the earlier draft genome, often leading to a redefinition of start and stop codons (Figure 3A and Table S6). For 65 of the 101 new genes, a paralog or an identical copy of the gene had already been assembled at a different location in the previous assembly. Gene ontology analysis using Fischer's Exact Test revealed an overrepresentation among the new genes of three GO categories related to sugar transport and cell wall (Table 3). This is not that surprising since, subtelomeres are acknowledged to be the major chromosomal regions involved functional evolution as they are sites for large rearrangement in term of structure, gene content and copy number (not only gain but also loss-of-function variants) [94, 167].

Table 3: Overrepresented GO functional categories among 101 newly identified genes in a MinION-sequencing based *S. eubayanus* CBS 12357 genome assembly annotation. Enrichment of functional categories was assessed by Fisher's exact test using Bonferroni correction.

GO		Genes	P-value*
GO:0031225	anchored component of membrane (15)	*SeFLO1*, *SeFLO9* (X4), Se*FLO10*, *SeFIG2*, *SeYPS6* (X3), Se*SAG1*, *SeDAN1*, *SeEGT2*, *SeAGA1*, *Se*YIL169C	1.78E-10
GO:0005618	cell wall (15)		7.04E-09
GO:0008645	hexose transport (8)	*SeHXT7*, *SeHXT3*, *Se HXT13* (X4), Se*MAL31/SeMALT4*, *SeHXT9*	4.77E-4

Four subtelomeric regions harbored complete sequences of putative maltose transporters. Two of these, which contained *SeMALT1* and *SeMALT3*, showed structural features that differed from those of canonical *S. cerevisiae MAL* loci. The CHRII locus only contained a transporter gene (Se*MALT1*) while the CHRXIII '*SeMAL* locus' consisted of a transporter gene (Se*MALT3*) flanked by two non-identical genes that strongly resembled the *S. cerevisiae* regulator genes *MAL33* and *MAL63* (Figure 3B). In contrast, the *S. eubayanus MAL* loci on CHRV and CHRXVI showed the same organization as the well described *S. cerevisiae MAL* loci. Starting from their telomeric ends, they contained a maltase gene (*SeMAL32*), followed by the transporter gene (*SeMALT2* on CHRV and *SeMALT4* on CHRXVI), which shared a bi-directional promoter with the maltase gene, and a *MAL* regulator gene (Figure 3B and Figure S8). Similarity between the right-arm CHRV and left-arm CHRXVI subtelomeric regions extended beyond the *SeMAL* genes, with a sequence identity of 94 % and shared gene synteny over a 20 kb region (Figure 3B). The fully assembled *SeMALT4* gene shared 99.7 % identity with *SeMALT2*, from which it differed by only five nucleotides. None of these five nucleotide variations affected the predicted amino acid sequence of the encoded transporters.

Systematic deletion of *MALT* genes revealed that Se*MALT2/SeMALT4* are essential for growth maltose.

To explore the contribution of the four *S. eubayanus MALT* genes to maltose consumption, deletion strains were constructed. Because the high sequence similarity of *SeMALT2* and *SeMALT4* complicated individual deletion of these genes, three strains were constructed with either a single deletion of *SeMALT1* or *SeMALT3* or a double deletion of *SeMALT2* and *SeMALT4* (Figure 1). The option offered by CRISPR-Cas9 to simultaneously delete of multiple gene copies in a single transformation step [147] is especially helpful in diploid strains such as *S. eubayanus* CBS 12357^T. To explore the use of this methodology in *S. eubayanus*, we used a broad-host-range yeast plasmid for co-expression of *Spcas9* and a cassette encoding a ribozyme-flanked gRNA, which was successfully used in the Saccharomycotina yeasts *S. pastorianus* [142], *Kluyveromyces* sp. and *Ogataea* sp. [144]. Cloning of specific gRNA cassettes targeting *SeMALT1*, *SeMALT2/4* and *SeMALT3* in pUDP002 resulted in pUDP062, pUDP063 and pUDP064, respectively. These plasmids were then transformed into *S. eubayanus* CBS 12357^T, either alone or in combination with a 120-bp double stranded repair DNA fragment for the targeted Se*MALT* gene (Figure 1). In the absence of a repair fragment, transformation with a gRNA-expressing construct was expected to be fatal if both gene copies were cut, unless both breaks were repaired by non-homologous end joining (NHEJ) of the induced double strand breaks. However, transformation of *S. eubayanus* CBS 12357^T with pUDP062, pUDP063 or pUDP064 alone yielded 1100, 128 and nine transformants, respectively. These numbers were not substantially different from those observed upon co-transformation of the corresponding repair fragments (3000, 114 and 13 colonies, respectively). Based on a set of 30 transformants, genome editing with the gRNA$_{SeMALT1}$ yielded the lowest frequency of transformants in which both gene copies were deleted (3 %). The *SeMALT3* gRNA performed better with a 7 % frequency out of 13 transformants tested, while the gRNA targeting Se*MALT2/T4* showed an efficiency of 40 % of accurate deletion of both copies of the two genes out of a set of eight transformants.

The resulting *S. eubayanus* deletion strains IMK816 (*SemalT1*Δ), IMK817 (*SemalT2*Δ *SemalT4*Δ) and IMK818 (*SemalT3*Δ), as well as the wild-type strain CBS 12357^T, were grown in SMG and SMM media. While specific growth rates of all four strains in SMG were the same (0.22 h^{-1} at 20 ^{o}C, Table 4), strain IMK187 (*SemalT2Δ SemalT4Δ*) did not grow on maltose (Figure 4). Conversely, strains IMK816 and IMK818 exhibited the same specific growth rate on maltose as the reference strain (0.17 h^{-1} at 20 ^{o}C, Table 4). These data suggested that only *SeMALT2* and/or *SeMALT4* only contributed to growth on maltose of wild- type *S. eubayanus* CBS 12357^T.

Table 4: Specific growth rates (h^{-1}) of *S. eubayanus* CBS 12357^T [19], *S. eubayanus* (*Se*) maltose transporter deletion mutants, *S. cerevisiae* (*Sc*) strains overexpressing individual *S. eubayanus* maltose transporter genes and the maltose-consumption-deficient host strain *S. cerevisiae* IMZ616 [135]. *S. eubayanus* strains were grown on SMG and SMM media at 20 °C. *S. cerevisiae* strains were grown on SM_UG and SM_UM at 20 °C. Data represent average and standard deviation of three biological replicates. "*mal*Δ" denotes the following genotype *mal1Δ mal2Δ mal3Δ mph2Δ mph3Δ suc2Δ ima1Δ ima2Δ ima3Δ ima4Δ ima5Δ.*

strain		Relevant genotype or phenotype			Specific growth rate (h^{-1}) Glucose	Maltose
CBS 12357^T	*Se*	*SeMALT1* *SeMALT4*	*SeMALT2*	*SeMALT3*	0.24 ± 0.003	0.17 ± 0.001
IMK816	*Se*	*SemalT1Δ* *SeMALT4*	Se*MALT2*	*SeMALT3*	0.22 ± 0.001	0.17 ± 0.000
IMK817	*Se*	*SeMALT1* *SemalT4Δ*	Se*malT2Δ*	Se*MALT3*	0.22 ± 0.002	0.002 ± 0.000
IMK818	*Se*	*SeMALT1* *SeMALT4*	*SeMALT2*	*SemalT3Δ*	0.22 ± 0.001	0.17 ± 0.002
IMZ616	Sc	*"malΔ"*			0.19 ± 0.002	0.00 ± 0.000
IMX1253	*Sc*	*"malΔ" ScTEF1$_{pr}$-SeMALT1-ScCYC1$_{ter}$ - ScMAL12*			0.20 ± 0.003	0.13 ± 0.002
IMX1254	*Sc*	*"malΔ" ScTEF1$_{pr}$-SeMALT2-ScCYC1$_{ter}$ ScMAL12*			0.18 ±0.002	0.13 ± 0.001
IMX1255	*Sc*	*"malΔ" ScTEF1$_{pr}$-SeMALT3-ScCYC1$_{ter}$ ScMAL12*			0.19 ±0.002	0.13 ± 0.004
IMX1365	*Sc*	*"malΔ" ScTEF1$_{pr}$-ScMAL11-ScCYC1$_{ter}$ ScMAL12*			0.19 ±0.001	0.10

*Se*MalT1, *Se*MalT2/4, and *Se*MalT3 all support maltose uptake.

For further functional analysis, *SeMALT1*, *SeMALT2* and *SeMALT3* were expressed in a maltose-transporter negative *S. cerevisiae* strain background. *SeMALT4* was not included in this comparison, as it encodes a protein with the same amino acid sequence as *SeMALT2.* The maltose-negative *S. cerevisiae* strain IMZ616 originates from *S. cerevisiae* CEN.PK102-3A, which carries three *MAL loci* (*MAL1*, *MAL2* and *MAL3*) [134]. To eliminate growth on α-glucosides, these three *MAL* loci, *MPH2* and *MPH3* as well as the α-glucoside hydrolase-encoding genes *SUC2* and *IMA1-5* were deleted, yielding *S. cerevisiae* IMK291 [135]. Introduction of *cas9* into this strain yielded IMZ616 [138].

Restoration of growth on maltose of *S. cerevisiae* IMZ616 requires simultaneous expression of a maltose transporter and a maltase. Therefore, the three *S. eubayanus* transporter genes were cloned behind the constitutive *ScTEF1* promoter and, together with an expression cassette for the *ScMAL12* maltase gene, integrated at the *SGA1* locus of *S. cerevisiae* IMZ616 (Figure 2). The resulting *S. cerevisiae* strains IMX1253, IMX1254, IMX1255, which expressed *SeMALT1*, *SeMALT2* and *SeMALT3*, respectively, were grown on SM_UM. As expected, the host strain IMZ616 did not show any growth on maltose, while the three *SeMALT*-expressing strains showed different growth profiles on this disaccharide. Strain IMX1255 (*SeMALT3*) resumed growth after a lag phase of ~10 h and consumed half of the maltose supplied (Figure 5). Strains IMX1253 (*SeMALT1*) and IMX1254 (*SeMALT2*) showed lag phases of 100 and 250 h, respectively. However, after these lag phases, maltose was consumed. Strain IMX1254 (*SeMALT2*) consumed 75 % of the supplied maltose in 150 h. In the same conditions the control strain IMX1365 co expressing *ScAGT1* and *ScMAL12* showed a short lag phase of 10h and that was

immediately followed by exponential growth, IMX1365 reached stationary phase and full maltose consumption in less than 100 h a performance comparable to the IMX1255 (*SeMALT3*) (Figure 5).

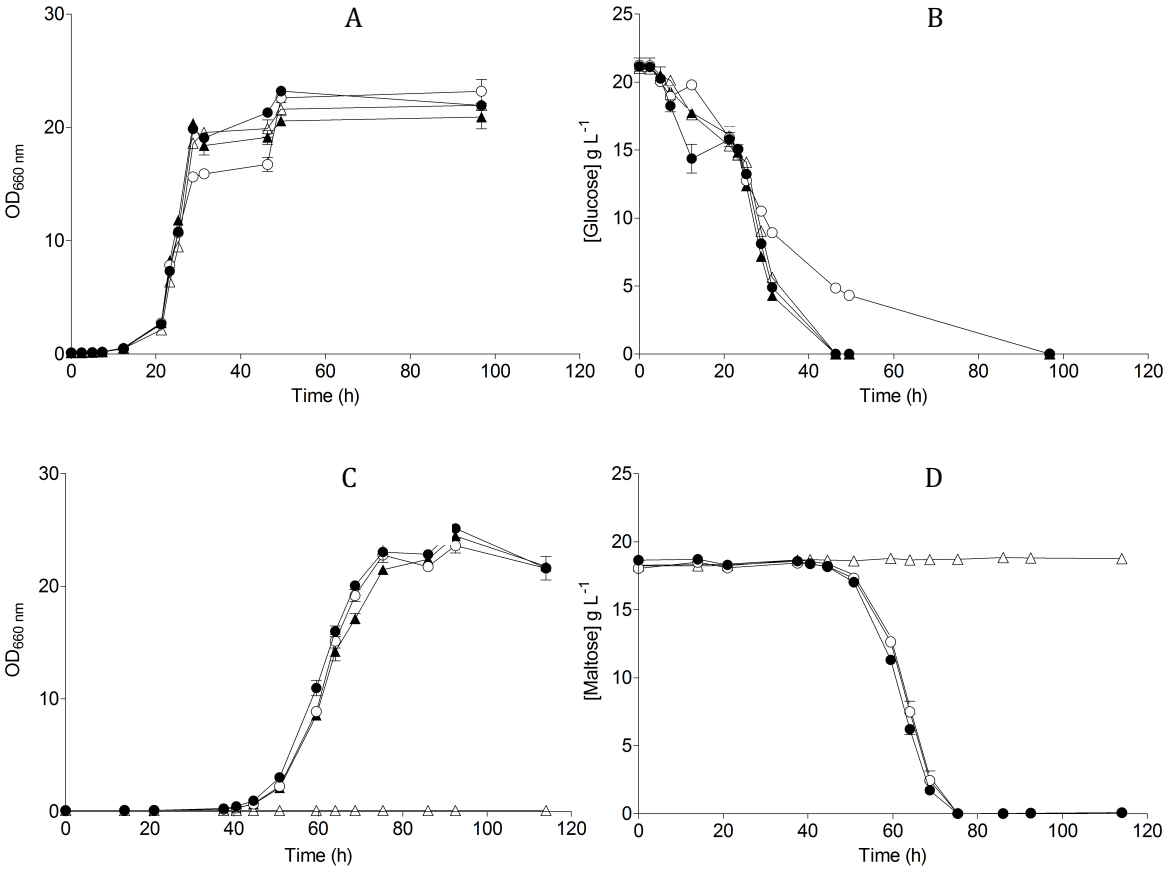

Figure 4: Characterization of the *S. eubayanus* strains (●) CBS 12357^T, (▲) IMK816 (*SemalT1*Δ), (△) IMK817 (*SemalT2Δ SemalT4Δ*) and (○) IMK818 (*SemalT3*Δ) during growth on glucose and maltose as sole carbon source. *S. eubayanus* strains were grown on SMG or SMM at 20 °C. Growth on glucose **(A)** and on maltose **(C)** was monitored based on optical density measurement at 660 nm (OD_{660nm}). Concentrations of glucose **(B)** and maltose **(D)** in culture supernatants were measured by HPLC. Data are presented as average and standard deviation of three biological replicates.

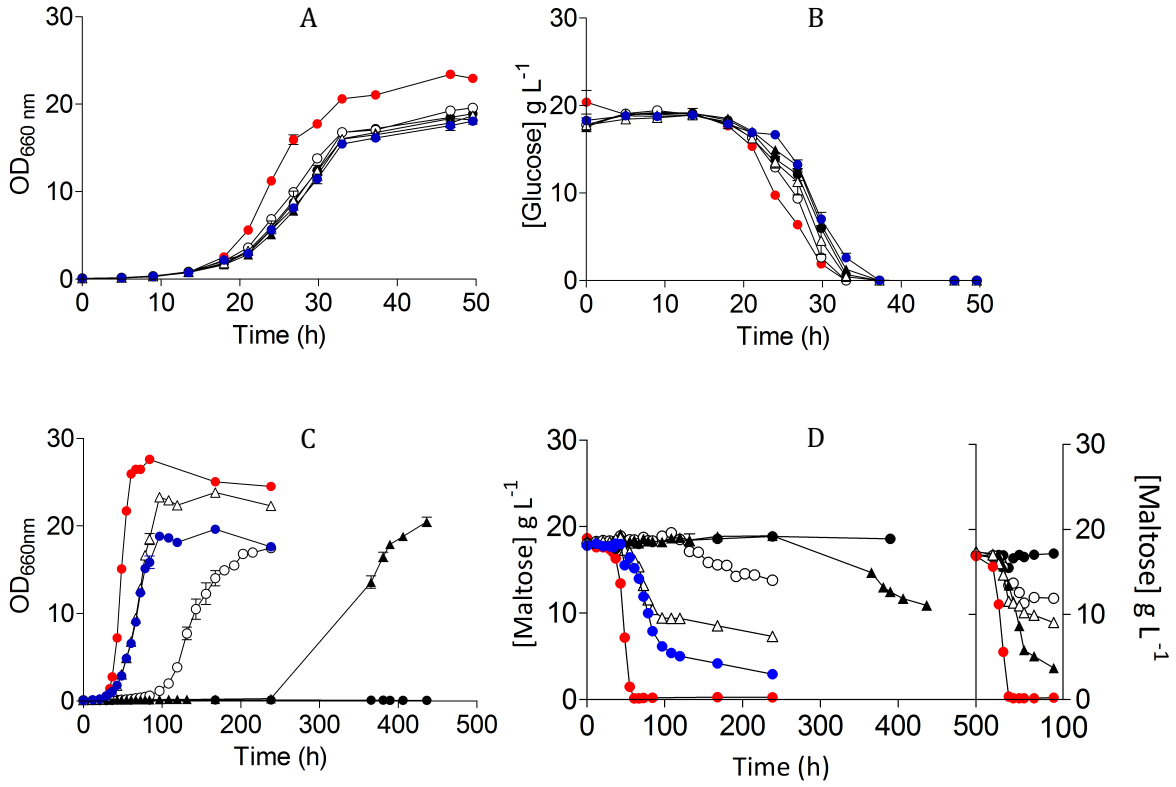

Figure 5: Characterization of the *S. cerevisiae* strains (●) IMZ616 [138], (○) IMX1253 (Sc*TEF1*$_{pr}$-*SeMALT1-ScCYC1*$_{ter}$), (▲) IMX1254 (Sc*TEF1*$_{pr}$-*SeMALT2-ScCYC1*$_{ter}$), (△) IMX1255 (Sc*TEF1*$_{pr}$-*SeMALT3-ScCYC1*$_{ter}$), (●) IMX1365 (*ScTEF1*$_{pr}$-*ScMAL11-SeCYC1*$_{ter}$) and (●) *S. eubayanus* CBS 12357^{T}. The strains were grown in SM$_U$G and SM$_U$M at 20 °C. Growth on glucose **(A)** and on maltose **(C)** was monitored based on optical density measurement at 660 nm (OD_{660nm}). Concentrations of glucose **(B)** and maltose **(D)** in culture supernatants were measured by HPLC. Panel D shows the data of two consecutive batches. Data are presented as average and standard deviation of three biological replicates.

These delayed growth phenotypes of the Se*MALT*-expressing strains resemble those observed during growth on lactose of S. *cerevisiae* strains expressing the β-galactosidase (*LAC4*) and the lactose permease (*LAC12*) genes of *Kluyveromyces marxianus* and *Kluyveromyces lactis* [168, 169]. In those studies, lactose utilization first had to be improved by laboratory evolution which resulted in lowering copy number of the plasmid harboring the permease and hydrolase genes as well as a short internal deletion located in the bi-directional promoter driving expression of the two genes [168]. The same way, an adaptation step have been included in the workflow for complementation of the hexose transporter null (*hxt^{0}*) strain EBYWV4000 with human glucose transporter (*GLUT*) [170] suggesting that swapping transporter or implementing new assimilatory pathway remains non trivial.

When, at the end of a first round of batch cultivation on maltose, cells of the *SeMALT*-expressing strains were transferred to fresh maltose medium, they all showed instantaneous growth at a specific growth rate of 0.13 h^{-1} (Table 4). Under the same conditions, *S. eubayanus* CBS 12357^{T} grew on maltose at a specific growth rate of 0.17 h^{-1} (Figure 5 and Table 4). Even after transfer to fresh maltose medium, none of the heterologously expressed *SeMALT* transporters enabled full maltose consumption in these cultures. Similary to the first cycle of batch cultivation on maltose, strain IMX1254 (*MALT2*) consumed 75 % of the maltose supplied, while strains IMX1255 (Se*MALT3*) and

IMX1253 (Se*MALT1*) consumed ca. 50 % and 35 %. Even after prolonged incubation, none of the *S. cerevisiae* strains expressing an *SeMALT* gene nor *S. eubayanus* CBS 12357^T showed growth on maltotriose, while the positive control IMX1365 (*ScAGT1*) showed growth at a rate of 0.08 ± 0.007 h^{-1} (Figure S9).
In contrast to the deletion study, which suggested that only *Se*Malt2 and/or *Se*Malt4 were able to transport maltose, heterologous expression in the Mal$^-$ *S. cerevisiae* strain IMZ616 showed that all four transporter genes encode transporters that, in combination with a maltase, allow growth on maltose.

Slow growth of Se*MALT*-expressing *S. cerevisiae* strains is not caused by maltose accelerated death.
Since maltose is imported by proton symporters energized by the plasma-membrane proton-motive force [171, 172], an unrestricted influx of maltose can lead to a fast influx of protons [173]. Unless the resulting rate of proton influx can be countered by the proton-pumping plasma-membrane ATPase (Pma1, [171], dissipation of the proton motive force and cytosolic acidification can cause maltose-induced cell death. Indeed, pronounced maltose-accelerated death has been observed in *S. cerevisiae* evolved an increased maltose-transport capacity [173]. To test whether this phenomenon was responsible for the observed delayed growth of *S. cerevisiae* strains expressing *S. eubayanus* maltose transporters, the corresponding *S. cerevisiae* strains (IMX1243, IMX1254 and IMX1255) were first grown on SMG. Upon reaching late exponential phase, cells after washing were transferred to SM medium (without C-source) to give an OD_{660} of 1.0. The resulting cell suspension was then sampled before and 30, 120 and 270 min after addition of 20 $g.L^{-1}$maltose. From each sample, 96 cells were sorted using gated Forward scatter signal and side scatter signal intensities on SMG medium and the viability was estimated based on the number of growing colonies. Neither the three *SeMALT*-expressing *S. cerevisiae* strains, nor *S. eubayanus* CBS 12357^T or the Mal$^-$ *S. cerevisiae* IMZ616 showed a decreased viability over a period of 270 min exposure to maltose (Figure 6). This result indicated that delayed growth of the *SeMALT*-expressing *S. cerevisiae* strains was not due to maltose accelerated death.

In contrast to *SeMALT2/T4*, the transporter genes Se*MALT3* and Se*MALT1* were not efficiently transcribed in maltose grown cells.
The inability of the double deletion mutant *S. eubayanus* IMK817 (Se*malT2Δ*-Se*malT4Δ*) to grow on maltose, despite the demonstration that *SeMALT1* and *SeMALT3* have the potential to encode functional maltose transporters, might indicate that *SeMALT1* and *SeMALT3* are not expressed in maltose-grown cultures. To investigate the impact of carbon sources on genome-wide transcript profiles and, more specifically, on transcriptional regulation of maltose metabolism genes, duplicate cultures of the *S. eubayanus* wild-type strain CBS 12357^T were grown on SMG and SMM and sampled in mid-exponential phase (OD_{660nm} = 12.5 ± 1.0). After mRNA isolation and processing, cDNA libraries were sequenced with Illumina sequencing technology. cDNA sequencing reads were mapped onto the newly annotated *S. eubayanus* CBS 12357^T genome assembly and used to calculate FPKM (fragments per kilobase of feature (gene) per million reads mapped) expression values. FPKM results represent normalized expression values that take into account gene length and sequencing depth. Statistical analysis showed that 125 genes were differentially expressed in the glucose- and maltose-grown cultures with a fold difference > 4 (Table S7). All four *S. eubayanus MALT* transporters were significantly upregulated during growth on maltose and three (*SeMALT2, SeMALT4* and *SeMALT1*)

were found among the ten most upregulated genes (Table 5). *SeMALT2* and *SeMALT4* exhibited 262- and 244-fold higher transcript levels during growth on maltose than during growth on glucose. *SeMALT1* and *SeMALT3* represented a substantially lower fold-difference between maltose- and glucose-grown cultures (67- and 6.8- fold, respectively; Figure 7 and Table 5). The most pronounced difference between Se*MALT2/SeMALT4* and Se*MALT1/SeMALT3* concerned their expression level. The FPKM value of Se*MALT1* in maltose-grown cultures was 48-fold lower than that of *SeMALT2* and *SeMALT4* value ($FPKM_{SeMALT1}$ = 30; $FPKM_{SeMALT2}$ = 1683, $FPKM_{SeMALT4}$ = 1451). Similarly, *SeMALT3* exhibited a FPKM value of only 200. In the same analysis, Se*ACT1* and the glycolytic gene Se*TDH3*, genes commonly used as internal standard in transcript analysis exhibited substrate-independent FPKM values of 1600 and 6000, respectively. The maltase genes that were physically associated to *SeMALT2* and *SeMALT4* (Figure 3) were also strongly overexpressed in maltose-grown cultures, representing the highest upregulation fold difference in expression in glucose- and maltose-grown cultures of *S. eubayanus* (Table 5). This result confirms the functionality of the bidirectional promoters controlling the maltase and transporter genes in the *S. eubayanus MAL* loci harbouring *SeMALT2* and *SeMALT4*.

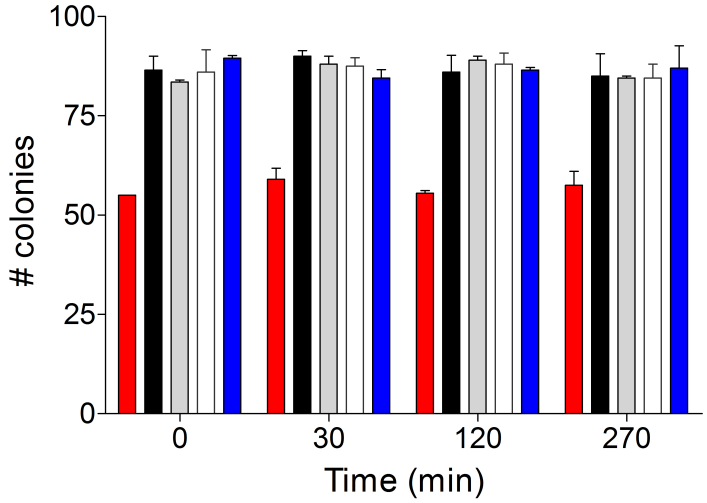

Figure 6: Cell viability after exposure to maltose of glucose-pregrown cultures of *S. eubayanus* CBS 12357^T (■), *S. cerevisiae* IMZ616 (■), IMX1253 (*SeMALT1*) (■), IMX1254 (*SeMALT2*) (□) and IMX1255 (*SeMALT3*) (■). Cells from glucose-grown batch cultures were resuspended in SM. Prior addition of 20 g L^{-1} of maltose, the initial viability was measured by sorting 96 cells per strain on SM_UG plates. The SM_UG cultures were sampled after 30, 120 and 270 minutes. The viability data are represented as averages ± mean deviations of three independent experiments for each strain.

Table 5: Transcript level of the 15 most strongly upregulated genes in maltose-grown *S. eubayanus* CBS 12357^T. FPKM gene expression values of *S. eubayanus* CBS 12357^T grown on SMG (glucose) and SMM (Maltose) were calculated from duplicate RNA seq experiments (2 x 150bp; 7.1 Gb) using the "Applying rpkm" method from the EdgeR package [163]. *p*-values were calculated using DESeq and adjusted for multi-testing. The descriptions were based on similarity with *S. cerevisiae* orthologs.

CHR	Coordinates	Gene name	Description	FPKM		Fold-change	adjusted p-value
				Glucose	Maltose		
CHRXVI	13815-15572	*SeMAL32^{XVI}*	Maltase (alpha-D-glucosidase)	9.9 ± 0.2	5218.4 ± 365	525.6	0
CHRV	569448-571205	*SeMAL32^{V}*	Maltase (alpha-D-glucosidase)	9.6 ± 0.0	4620.4 ±3 84	481.3	0
CHRV	566765-568606	*SeMALT2*	Maltose permease-member of the 12 TMB domain family of sugar transporters	5.5 ± 0.4	1451 ± 39	262.7	0
CHRXVI	16413-18254	*SeMALT4*	Maltose permease-member of the 12 TMB domain family of sugar transporters	6.9 ± 0.4	1683.8 ± 30	244.5	0
CHRIV	971754-973529	*SeDAK2*	Dihydroxyacetone kinase required for detoxification of dihydroxyacetone	19.2 ± 0.6	3342.3 ± 171	174.1	0
CHRII	8497-10338	*SeMALT1*	Maltose permease-member of the 12 TMB domain family of sugar transporters	0.5 ± 0.0	30.8 ± 1	67.7	3.2E-82
CHRXVI	25036-25626	*SeYJL218W*	Putative acetyltransferase	8.3 ± 2.3	528.3 ± 4	63.9	2.1E-246
CHRIV	968740-970695	*SeYFL054C*	Putative channel-like protein; similar to Fps1p	10.7 ±2.2	529.6 ± 22	49.3	0
CHRII	5766-7535	*SeIMA1^{II}*	Isomaltase, α -1,6-glucosidase; required for isomaltose utilization	1.6 ± 0.1	66.6 ± 5	41.7	2.7E-150
CHRIV	974654-975250	*SeREE1*	Cytoplasmic protein involved in the regulation of enolase	27.0 ± 1.1	1079.8 ± 76	40.0	2.3E-293
CHRV	564762-565970	*SeMAL33^{V}*	MAL-activator protein	10.0 ± 1.2	196.3 ± 17	19.6	4.4E-171
CHRIV	512918-513952	*SeYRO2*	Protein with a putative role in response to acid stress	128.1 ±6.9	2348.5 ± 110	18.3	2.5E-238
CHRXVI	732395-734113	*SeHXT13^{XVI}*	Putative transmembrane polyol transporter	14.2 ± 3.9	202.2 ± 19	14.3	3.4E-113
CHRIV	552424-553068	*SeHSP26*	Small heat shock protein (sHSP) with chaperone activity	137.5 ± 19.5	1520.3 ±33	11.1	1.2E-147
CHRVII	934199-934486	*SeSPG1*	Protein required for high temperature survival during stationary phase	6.3 ± 1.6	65.3 ± 5	10.3	2.3E-31

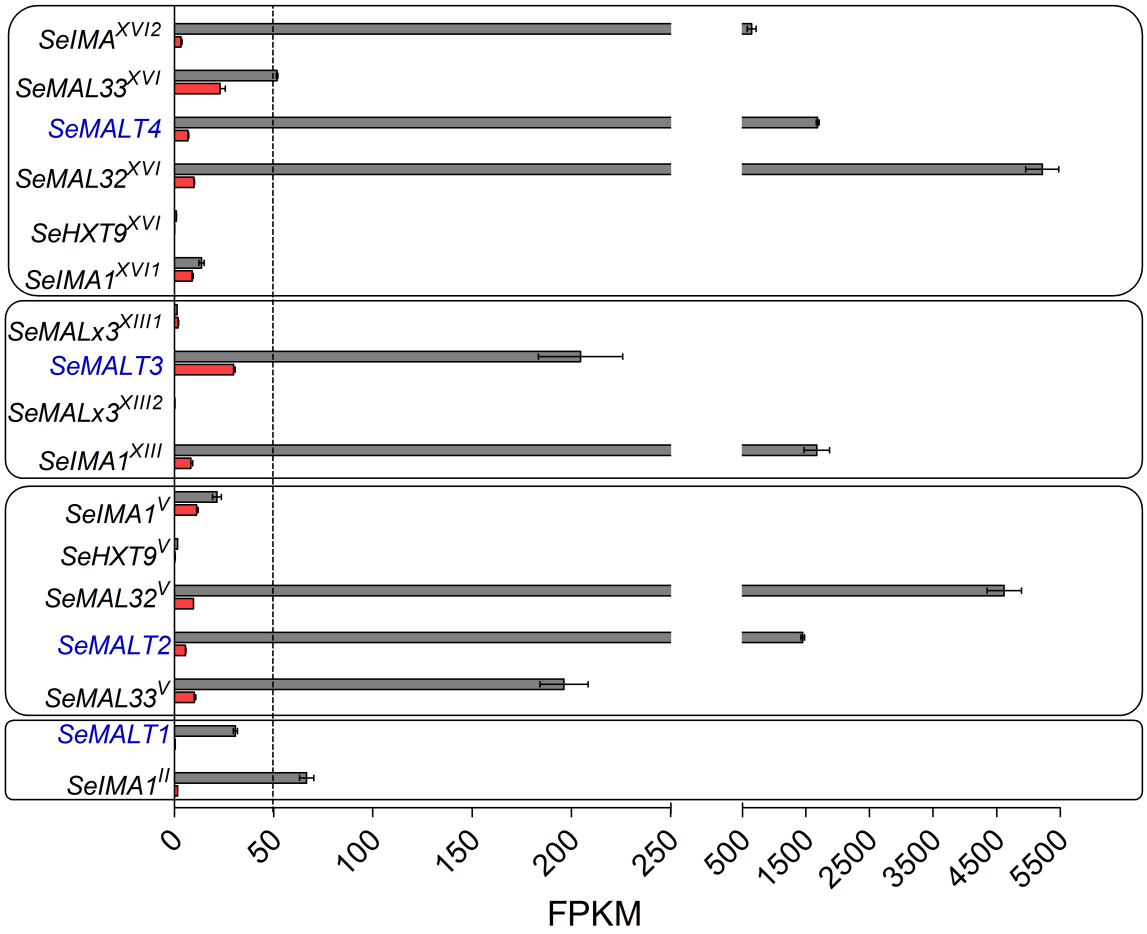

Figure 7: Expression of the maltose metabolism associated genes in *S. eubayanus* CBS 12357^T.
Transcript levels of maltose metabolism genes located at subtelomeric regions of CHRII, V, XII and XVI. FPKM gene expression values of *S. eubayanus* CBS 12357^T grown on SMG (glucose, (■)) and SMM (maltose, (■)) at 20 ^oC were calculated from duplicate RNA seq experiments (2 x 150bp; 7.1 Gb) using the "Applying rpkm" method in the EdgeR package [163]. Data represent average and mean deviation of two biological replicates.

Discussion

S. eubayanus is not only a key contributor to the hybrid genomes of current *S. pastorianus* lager brewing strain and a basis for developing new brewing yeast hybrids [113] but is also directly used for brewing specialty lager beer (https://www.cnbc.com/2017/09/26/heineken-unveils-h41-a-beer-that-puts-yeast-into-focus.html) [174]. This study provided an upgraded, near-complete genome sequence of the *S. eubayanus* type strain CBS 12357^{T}and the first characterization of its four maltose transporter genes.

Complete and accurate *de novo* assembly of eukaryotic genomes has long been a major challenge. While short-read, high-coverage technologies such as the popular Illumina platforms enable accurate sequencing and assembly of unique genomic sequences, they cannot resolve repetitive sequences. As previously demonstrated for *S. cerevisiae* CEN.PK113-7D [70, 175, 176], use of the Oxford Nanopore MinION platform generated long-reads that enabled a near-complete assembly of 15 of the 16 chromosomes of *S. eubayanus* CBS 12357^{T} (Figure 3A). Correct assembly of subtelomeric regions, which are known for their high sequence redundancy, was especially important in the context of this study in view of the subtelomeric location of the four *MAL* loci in this strain (Figure 3B). Despite the massive sequencing coverage yielded by a single flow cell, which was invaluable for genome scaffold construction, the intrinsic higher error rate of MinION sequencing [177] required polishing with additional Illumina sequencing data. Although highly effective, as illustrated by the complete assembly of the four *MAL* loci and the use of resulting high-quality genome sequence information for transcriptome analysis, this approach could not correct all errors. In particular, INDELs causing omissions of single nucleotides in homopolymer regions were left in the final assembly, which is a known pitfall of the single molecule nanopore sequencing (Oxford Nanopore Technology MinIOn). Manual curation and, in particular, validation of relevant sequences by Sanger sequencing will be required to further refine the current genome sequence of *S. eubayanus* CBS 12357^{T}.

Also, the first application of CRISPR-Cas9-assisted genome editing in *S. eubayanus* reported in this study was greatly facilitated by the availability of an accurate genome sequence. Resolution of the sequences of the four *SeMALT* genes enabled optimization of the gRNA spacer selection, thereby minimizing the risk of undesirable off-target events. Although targeting efficiencies of the employed ribozyme-flanked-gRNA expression system [142], which ranged from 3 to 40 % were not ideal, Cas9-assisted gene deletion offered clear advantages over traditional methods that rely on a double crossover event that inserts a DNA fragment containing a selection marker in the recipient strain's genome [178, 179]. Cas9-assisted genome editing in S*. eubayanus* did not make use of a marker cassette and, most importantly, enabled simultaneous marker-free editing both alleles of the *SeMALT1* or the *SeMALT3* gene. In the case of *SeMALT2* and *SeMALT4*, a single transformation was even sufficient to delete both alleles of two genes [147, 180]. Achieving these objectives with conventional techniques would have been extremely time consuming as multiple rounds of transformation and marker recovery would be required. Depending on consumer acceptance and regulations in place [181, 182], marker-free, Cas9-assisted genome editing of *S. eubayanus* may be combined with the generation of new *Saccharomyces* hybrids to accelerate development to novel brewing strains.

Cas9-mediated gene disruption and transcriptome analysis in *S. eubayanus* CBS 12357 showed that, in maltose-grown cultures, *SeMALT2* and *SeMALT4* were predominantly responsible for maltose uptake. These transporters are located within two nearly

identical (97% identity) *SeMAL* loci that strongly resemble canonical *S. cerevisiae MAL* loci [93, 124, 125]. Although two other genes, Se*MALT3* and Se*MALT1*, could restore growth upon their expression in the Mal$^-$ *S. cerevisiae* strain IMZ616, their low expression levels in *S. eubayanus* CBS 12357^T were apparently not sufficient to support growth on maltose when *SeMALT2* and *SeMALT4* were both deleted (Figures 3 and 7). This study did not provide new insights into the origin of *MTT1/MTY1* [91, 92] and *SeAGT1* [18, 96, 111] in industrial *S. pastorianus* strains as, consistent with earlier observations [62], no genes with strong sequence similarity to these maltose transporter genes were identified in the improved genome sequence of *S. eubayanus* CBS 12357^T.

The low expression levels of *SeMALT1* and *SeMALT3* may be related to their genomic context, as they were located in atypical Se*MAL* loci on CHRII and CHRXIII, respectively (Figure 3B). Assuming that these two atypical Se*MAL* loci evolved from a complete *MAL* locus, loss of the maltase gene may have disrupted the bi-directional promoter that, in *S. cerevisiae*, controls expression of both the maltose and the maltose transporter genes [183]. In *S. cerevisiae*, the maltose regulator Mal63 binds two regulatory sites (5'MGSN$_9$MGS3') located between positions -465 and -579 in the region separating the two divergent genes [184]. While two of these elements were also found in the promoter regions of *SeMALT2* and *SeMALT4* (Figure S8), the *SeMALT1* and *SeMALT3* promoters each harbored only a single element (Figure S8). Alternatively, low expression of *SeMALT1* and *SeMALT3* in maltose-grown cultures may reflect a sub-functionalization that, during evolution, led to a different regulation and/or catalytic properties of the encoded transporters [185, 186]. Indeed, such a sub-functionalization has been experimentally reconstructed for yeast α-glucoside hydrolases [99].

None of the four Se*MALT* genes identified in *S. eubayanus* CBS 12357^T were found to encode a functional maltotriose transporter. Although, the *S. eubayanus* Patagonian lineage is unlikely to have contributed the *S. eubayanus* subgenome of *S. pastorianus* lager brewing strains [18], this observation would be consistent with the notion that *S. cerevisiae* has contributed the vital ability to ferment this trisaccharide, which is abundantly present in wort as in contrast multiple *S. cerevisiae* ale strains mainly issued from the beer 1 and 2 groups have been shown to use this sugar [187]. However, CBS 12357 is a representative of sole the Patagonia B group, one of five groups defined based of the phylogenetic distribution of *S. eubayanus* strains isolated so far [61]. Therefore, we cannot at all exclude the possibility that this brewing relevant phenotypic trait of lager yeast would originate from the *S. eubayanus* parent. The only elements so far that could tilt towards this hypothesis are very fragmented sequencing data of an isolate from the Holarctic group, that suggested occurrence of *S. eubayanus* ortholog of the *S. cerevisiae AGT1/MAL11* gene [111]. Therefore, the resource and methodology used in this study paved the way for further exploration of the diversity of *S. eubayanus* population and elucidation of *S. eubayanus* parental lineage of *S. pastorianus*.

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Authors' contributions

JD, JP, AB, NB designed experiments, AB, NB, JSGM performed experiments, MvdB, NB and JD performed bioinformatics work, JD, JP, AB, NB, JSGM analyzed result data. JD and JP wrote the manuscript. All authors read and approved the final manuscript.

Conflict of interest

The authors declare no conflicts of interest

Chapter 3

In vivo recombination of *Saccharomyces eubayanus* maltose-transporter genes yields a chimeric transporter that enables maltotriose fermentation

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Abstract

Saccharomyces eubayanus is the non-S. *cerevisiae* parent of the lager-brewing hybrid *S. pastorianus*. In contrast to most *S. cerevisiae* and Frohberg-type *S. pastorianus* strains, *S. eubayanus* cannot utilize the α-tri-glucoside maltotriose, a major carbohydrate in brewer's wort. In *Saccharomyces* yeasts, utilization of maltotriose is encoded by the subtelomeric *MAL* gene family, and requires transporters for maltotriose uptake. While *S. eubayanus* strain CBS 12357^{T} harbors four *SeMALT* genes which enable uptake of the α-di-glucoside maltose, it lacks maltotriose transporter genes. In *S. cerevisiae*, sequence identity indicates that maltotriose and maltose transporters likely evolved from a shared ancestral gene. To study the evolvability of maltotriose utilization in *S. eubayanus* CBS 12357^{T}, maltotriose-assimilating mutants obtained after UV mutagenesis were subjected to laboratory evolution in carbon-limited chemostat cultures on maltotriose-enriched wort. An evolved strain showed improved maltose and maltotriose fermentation in 7-L fermenter experiments on industrial wort. Whole-genome sequencing revealed a novel mosaic *SeMALT413* gene, resulting from repeated gene introgressions by non-reciprocal translocation of at least three *SeMALT* genes. The predicted tertiary structure of *Se*MalT413 was comparable to the original *Se*MalT transporters, but overexpression of *SeMALT413* sufficed to enable growth on maltotriose, indicating gene neofunctionalization had occurred. The mosaic structure of *SeMALT413* resembles the structure of *S. pastorianus* maltotriose-transporter gene *SpMTY1*, which has high sequences identity to alternatingly *S. cerevisiae MALx1, S. paradoxus MALx1* and *S. eubayanus SeMALT3.* Evolution of the maltotriose transporter landscape in hybrid *S. pastorianus* lager-brewing strains is therefore likely to have involved mechanisms similar to those observed in the present study.

Author Summary

Fermentation of the wort sugar maltotriose is critical for the flavor profile obtained during beer brewing. The recently discovered yeast *Saccharomyces eubayanus* is gaining popularity as an alternative to *S. pastorianus* and *S. cerevisiae* for brewing, however it is unable to utilize maltotriose. Here, a combination of non-GMO mutagenesis and laboratory evolution of the *S. eubayanus* type strain CBS 12357^{T} was used to enable maltotriose fermentation and improve brewing performance. The improved strain expressed a novel transporter gene, *SeMALT413,* which was formed by recombination between three different *SeMALT* maltose-transporter genes*.* Overexpression of *SeMALT413* in CBS 12357^{T} confirmed its neofunctionalization as a maltotriose transporter. As the *S. pastorianus* maltotriose transporter *Sp*Mty1 has a mosaic structure similar to *Se*MalT413, maltotriose utilization likely involved similar recombination events during the domestication of current lager brewing strains. Based on a posteriori sequence analysis, the emergence of gene functions has been attributed to gene neofunctionalization in a broad range of organisms. The real-time observation of neofunctionalization during laboratory evolution constitutes an important validation of the relevance and importance of this mechanism for Darwinian evolution.

Introduction

Saccharomyces eubayanus was discovered in Patagonia and identified as the non-*S. cerevisiae* parental species of hybrid *S. pastorianus* lager-type beer brewing yeasts [19, 188]. While *S. cerevisiae* is strongly associated with biotechnological processes, including dough leavening, beer brewing and wine fermentation [189], *S. eubayanus* has only been isolated from the wild [58-60]. Beer brewing is performed with wort, a complex substrate containing a fermentable sugar mixture of 15% of the monosaccharide glucose, 60% of the α-di-glucoside maltose and 25% of the α-tri-glucoside maltotriose [78]. While many *S. cerevisiae* and *S. pastorianus* strains utilize all three sugars, *S. eubayanus* isolates do not utilize maltotriose [90, 111, 187]. In *Saccharomyces*, the ability to utilize maltotriose requires its uptake into the cell and subsequent hydrolysis into glucose [85, 86]. Maltose and maltotriose utilization are encoded by genes clustered in the *MAL* loci, which can be present on up to five different chromosomes [190]. *MAL* loci typically harbor genes from up to three gene families (Fig 1): a *MALT* polysaccharide proton-symporter gene, a *MALS* α-glucosidase gene which hydrolyses α-oligo-glucosides into glucose, and a *MALR* regulator gene that induces the transcription of *MALT* and *MALS* genes in the presence of maltose [125]. While *MALS* genes enable hydrolysis of both maltose and maltotriose, the *MALT* gene family comprises transporters with diverse substrate specificities [85, 99]. In *S. cerevisiae*, most *MAL* loci harbor an *ScMalx1* transporter (Fig 1), which transports maltose and other disaccharides, such as turanose and sucrose [86, 135], but cannot import maltotriose [85]. In contrast, the *MAL1* locus located on chromosome VII of *S. cerevisiae* contains *ScAGT1*, a transporter gene with only 57% nucleotide identity with *ScMALx1* transporter genes. *ScAGT1* encodes a broad-substrate-specificity sugar-proton symporter that enables maltotriose uptake [77, 85, 87].

The *S. eubayanus* type strain CBS 12357^T is able to utilize maltose, but cannot utilize maltotriose, suggesting that it expresses a functional maltase and a functional maltose transporter, but no maltotriose transporter [90]. Indeed, the four *MAL* loci in CBS 12357^T harbor a total of two *MALS* genes and four *MALT* genes with high homology to *ScMALx1: SeMALT1*, *SeMALT2*, *SeMALT3* and *SeMALT4* (Fig 1) [62]. Deletion of these genes in *S. eubayanus* type strain CBS 12357^T indicated that its growth on maltose relies on expression of *SeMALT2* and *SeMALT4* [90]. *SeMALT1* and *SeMALT3* were found to be poorly expressed in the presence of maltose in this strain, supposedly due to incompleteness of the *MAL* loci which harbor them. However, no homolog of *ScAGT1* was found in the genome of CBS 12357^T, and neither CBS 12357^T nor its derivatives overexpressing *SeMALT* genes were able to utilize maltotriose.

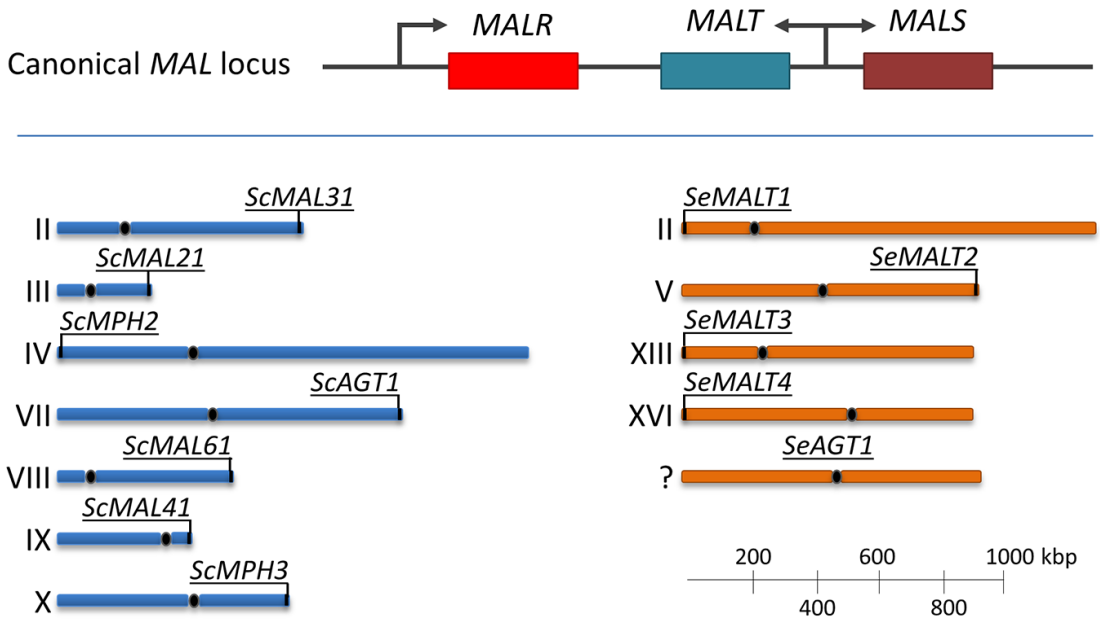

Fig 1: Organization of maltose and maltotriose transporter genes in *S. cerevisiae* and *S. eubayanus*. In *Saccharomyces* species, maltose and maltotriose utilization is encoded in the *MAL* genes, which are located in subtelomeric regions and comprise three types of genes: a *MALT* α-oligo-glucoside proton-symporter gene, a *MALS* α-glucosidase gene which hydrolyses α-(di or tri)-glucosides into glucose, and a *MALR* regulator gene that induces the transcription of *MALT* and *MALS* genes in the presence of maltose. In canonical MAL loci, the *MALT* and *MALS* are expressed from a bi-directional *MALR*-dependent promoter sequence. The chromosomal location of known maltose and maltotriose transporter genes in *S. cerevisiae* and *S. eubayanus* is shown, although the presence of these genes varies among isolates. *ScMPH2* and *ScMPH3* encode α-glucoside permeases which do not enable efficient maltotriose uptake [85]. *ScMAL31*, *ScMAL21*, *ScMAL61* and *ScMAL41* encode maltose transporters of the *Sc*Malx1 family. *ScAGT1* encodes a maltotriose transporter. *SeMALT1*, *SeMALT2*, *SeMALT3* and *SeMALT4* encode maltose transporters with high sequence identity to the *Sc*Malx1 family. *SeAGT1* is an maltotriose transporter which has recently been discovered in the north American *S. eubayanus* isolate yHRVM108 [34].

The *MALT* transporter genes in *Saccharomyces* yeasts are localized to the subtelomeric regions (Fig 1) [62, 85, 86, 90-92], which are gene-poor and repeat-rich sequences adjacent to the telomeres [191-193]. The presence of repeated sequences makes subtelomeric regions genetically unstable by promoting recombination [194, 195]. As a result, subtelomeric gene families are hotspots of genetic diversity [94, 167, 196]. In *S. cerevisiae*, subtelomeric gene families contain more genes than non-subtelomeric gene families, reflecting a higher incidence of gene duplications [94]. As previously shown in *Candida albicans* submitted to long term laboratory evolution, the gene repertoire of the subtelomeric *TLO* family can be extensively altered due to ectopic recombinations between subtelomeric regions of different chromosomes, resulting in copy number expansion, in gene disappearance and in formation of new chimeric genes [197]. Despite their common origin, genes within one family can have different functions, due to the accumulation of mutations [198, 199]. *In silico* analysis of the sequences and functions of genes from the *MALT*, *MALS* and *MALR* gene families indicated functional diversification through gene duplication and mutation [94]. Indeed, the presence of multiple gene copies can facilitate the emergence of advantageous mutations mainly by one of three

mechanisms: (i) neofunctionalization, corresponding to the emergence of a novel function which was previously absent in the gene family [200], (ii) subfunctionalization, corresponding to the specialization of gene copies for part of the function of the parental gene [201] and (iii) altered expression due to gene dosage effects resulting from the increased copy number [202]. While the different functions of *MALS* genes were assigned to subfunctionalization of the ancestral *MALS* gene [99], the maltotriose transporter gene *ScAGT1* was proposed to result from neofunctionalization within the *MALT* family [94]. In general, the emergence of a large array of gene functions was attributed to subfunctionalization and neofunctionalization [94, 99, 197, 203-206]. However, current evidence for neofunctionalization within subtelomeric gene families is based on *a posteriori* analysis and rationalization of existing sequence diversity. While in some cases the genetic process leading to neofunctionalization could be reconstructed at the molecular level [206-208], the emergence of a completely new function within a subtelomeric gene family was never observed within the timespan of an experiment to the best of our knowledge. However, the genetic diversity within *Saccharomyces MALT* transporters suggests that evolution of *Se*MalT transporters could lead to the emergence of a maltotriose transporter by neofunctionalization [94]. Therefore, laboratory evolution may be sufficient to obtain maltotriose utilization in *S. eubayanus* strain CBS 12357^T.

Laboratory evolution is a commonly-used method for obtaining desired properties by prolonged growth and selection under conditions favoring cells which develop the desired phenotype [104, 209]. Similarly as in Darwinian natural evolution, the conditions under which laboratory evolution is conducted shape the phenotypes acquired by evolved progeny following the process of survival of the fittest [210]. In *Saccharomyces* yeasts, selectable properties include complex and diverse phenotypes such as high temperature tolerance, efficient nutrient utilization and inhibitor tolerance [211-214]. Laboratory evolution was successfully applied to improve sugar utilization for arabinose, galactose, glucose and xylose [212, 215-217]. In *S. pastorianus*, improved maltotriose uptake was successfully selected for in a prolonged chemostat cultivation on medium enriched with maltotriose [47]. Theoretically, laboratory evolution under similar conditions could select *S. eubayanus* mutants which develop the ability to utilize maltotriose.

In this study, we submitted *S. eubayanus* strain CBS 12357^T to UV-mutagenesis and laboratory evolution in order to obtain maltotriose utilization under beer brewing conditions. While obtaining a non-GMO maltotriose-consuming *S. eubayanus* strain was a goal in itself for industrial beer brewing, we were particularly interested in the possible genetic mechanisms leading to the emergence of maltotriose utilization. Indeed, we hypothesized that the genetic plasticity and functional redundancy of the four subtelomeric *SeMALT* genes of CBS 12357^T could facilitate the emergence of maltotriose transport by neofunctionalization. The evolution process leading to maltotriose utilization in a strain with only maltose transporters, such as CBS 12357^T, may provide insight in the emergence of maltotriose utilization in general.

Results

Mutagenesis and evolution enable *S. eubayanus* to utilize maltotriose

To obtain maltotriose-consuming mutants, CBS 12357^T was grown on synthetic medium containing 20 g L^{-1} glucose (SMG) until stationary phase, and approximately 10^8 cells were used to inoculate synthetic medium containing 20 g L^{-1} maltotriose (SMMt) as sole carbon source. After incubation at 20 ˚C for three months, neither growth nor maltotriose utilization was observed.

Exposure to UV radiation can cause DNA damage, resulting in the emergence of cells with diverse mutations due to error-prone repair. Therefore, UV-mutagenesis was applied to increase the likelihood of obtaining a mutation enabling maltotriose utilization. To this end, CBS 12357^T was grown in SMG medium, sporulated, submitted to mild UV-mutagenesis (46% survival rate) and approximately 10^8 cells of the mutagenized population were used to inoculate SMMt containing 20 g L^{-1} maltotriose. After two weeks at 20 ˚C, growth was observed and, after 3 weeks, the maltotriose concentration had decreased to 10.5 g L^{-1}. After two subsequent transfers in fresh SMMt, 96 single cells were sorted into a 96 well plate containing YPD medium by fluorescence-activated cell sorting (FACS). The resulting single-cell cultures were transferred to a 96 well plate containing SMMt, in which growth was monitored by OD_{660} measurements. The seven single-cell isolates with the highest final OD_{660} were selected and named IMS0637-IMS0643. To characterize growth on maltotriose, the strain CBS 12357^T, the single-cell isolates IMS0637-IMS0643 and the maltotriose-consuming *S. pastorianus* strain CBS 1483 were grown in shake flasks on SMMt (Fig 2A and Supplementary Fig S1). After 187 h, *S. eubayanus* CBS 12357^T did not show any maltotriose consumption. Conversely, isolates IMS0637-IMS0643, all showed over 50% maltotriose consumption after 91 h (as compared to 43 h for CBS 1483). Upon reaching stationary phase, isolates IMS0637-IMS0643 had consumed 93 ± 2% of the initial maltotriose concentration, which was similar to the 92 % conversion reached by *S. pastorianus* CBS 1483. While these results indicated that the single cell isolates IMS0637-IMS0643 utilized maltotriose in synthetic medium, they did not consume maltotriose in presence of glucose and maltose after 145 h of incubation (Fig 2B). Under the same conditions, *S. pastorianus* CBS 1483 consumed 50% of the maltotriose after 145 h (Fig 2B).

Nutrient-limited growth confers a selective advantage to spontaneous mutants with a higher nutrient affinity [47, 104]. Therefore, to improve maltotriose utilization under industrially relevant conditions, the pooled isolates IMS0637-IMS0643 were subjected to laboratory evolution in a chemostat culture on modified brewer's wort. To avoid the presence of residual maltose, which would prevent selection for maltotriose utilization, brewer's wort was diluted 6-fold. To strengthen the selective advantage for maltotriose-consuming cells, the diluted wort was complemented with 10 g L^{-1} maltotriose, yielding concentrations of 2 g L^{-1} glucose, 15 g L^{-1} maltose and 15 g L^{-1} maltotriose in the medium feed. To prevent growth limitation due to the availability of limited oxygen or nitrogen, the medium was supplemented with 10 mg L^{-1} ergosterol, 420 mg L^{-1} Tween 80 and 5 g L^{-1} ammonium sulfate [140].

During the batch cultivation phase that preceded continuous chemostat cultivation, glucose and maltose were completely consumed, leaving maltotriose as the only carbon source. After initiation of continuous cultivation at a dilution rate of 0.03 h^{-1}, the medium outflow initially contained 13.2 g L^{-1} of maltotriose.

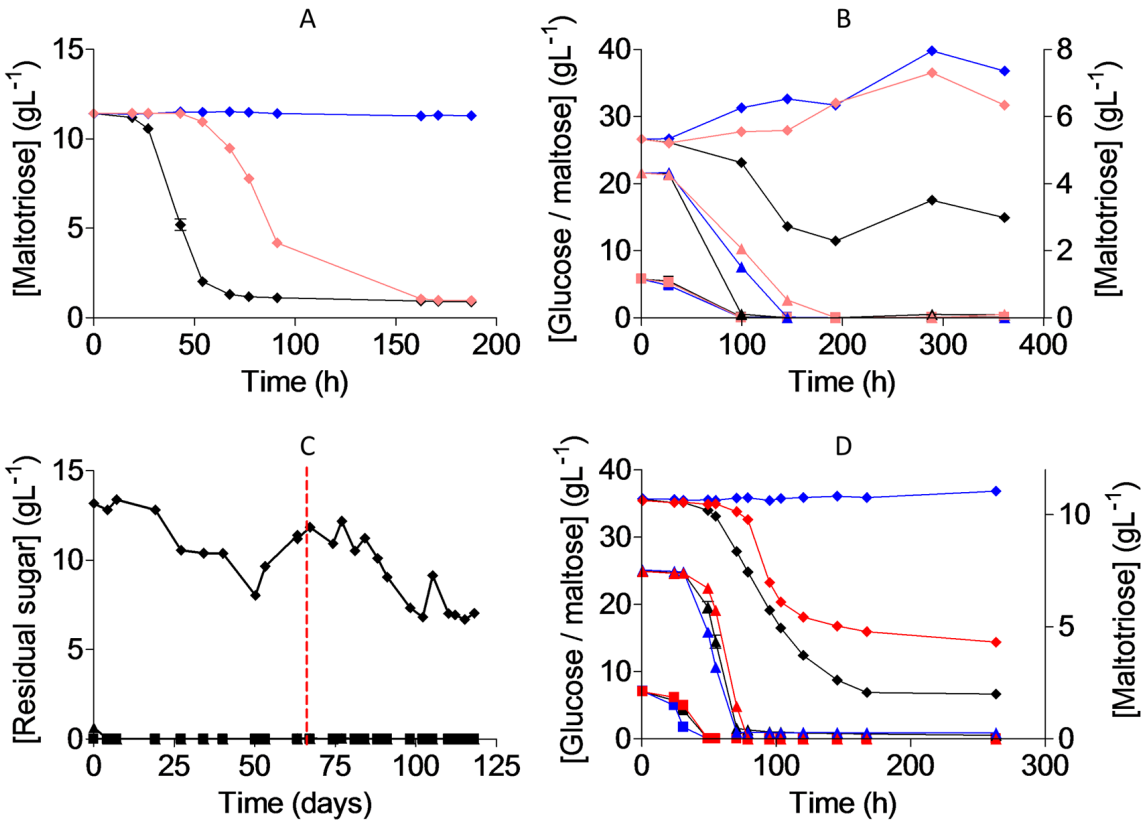

Fig 2: Mutagenesis and evolution to obtain maltotriose consuming *S. eubayanus*. (A) Characterization of *S. pastorianus* CBS 1483(black), *S. eubayanus* CBS 12357^T(blue) and IMS0637 (light red) on SMMt at 20 °C. The data for IMS0637 is representative for the other mutants IMS0638-IMS0643 (Supplementary Fig S1). The average concentration of maltotriose (◆) and average deviation were determined from two replicates (Supplementary data file 2). **(B)** Characterization of *S. pastorianus* CBS 1483 (black), *S. eubayanus* CBS 12357^T (blue) and IMS0637 (light red) on wort at 20 °C. The concentrations of (■) glucose, (▲) maltose and (◆) maltotriose were measured from single biological measurements (Supplementary data file 3). **(C)** Residual maltotriose concentration in the outflow during laboratory evolution of strains IMS0637-IMS0643 in an anaerobic chemostat at 20 °C on maltotriose enriched wort. The concentrations of (■) glucose, (▲) maltose and (◆) maltotriose were measured by HPLC. The chemostat was restarted after a technical failure (red dotted line, Supplementary data file 4). **(D)** Characterization of *S. pastorianus* CBS 1483 (black), *S. eubayanus* CBS 12357^T (blue) and IMS0750 (red) on wort at 12 °C in 250 mL micro-aerobic Neubor infusion bottles. The average concentration and standard deviation of (■) glucose, (▲) maltose and (◆) maltotriose were determined from three biological replicates. The data for IMS0751 and IMS0752 are shown in Supplementary data file 5 and Fig S2.

After 121 days of chemostat cultivation, the maltotriose concentration had progressively decreased to 7.0 g L^{-1} (Fig 2C). At that point, which corresponded to *ca.* 125 generations, 10 single colonies were isolated from the culture on SMMt agar plates and incubated at 20 °C. Three single-cell lines were named IMS0750, IMS0751 and IMS0752 were selected for further characterization in micro-aerobic cultures, grown at 12 °C on 3-fold diluted wort, along with *S. eubayanus* CBS 12357^T and *S. pastorianus* CBS 1483 (Fig 2D). In these cultures, strains CBS 12357^T and IMS0751 only consumed glucose and maltose, while *S. pastorianus* CBS 1483, as well as the evolved isolates IMS0750 and IMS0752, also consumed maltotriose (Supplementary Fig S2). After 263 h, maltotriose concentrations in cultures of strains IMS0750 and IMS0752 had decreased from 20 to 4.3 g L^{-1} maltotriose as compared to 2.0 g L^{-1} in cultures of strain CBS 1483.

Whole genome sequencing reveals a new recombined chimeric *SeMALT* gene

We sequenced the genomes of the *S. eubayanus* strain CBS 12357^T, of the UV-mutagenized isolates IMS0637-IMS0643 and of the strains isolated after subsequent chemostat evolution IMS0750-IMS0752 using paired-end Illumina sequencing. Sequencing data were mapped to a chromosome-level assembly of strain CBS 12357^T [90] to identify SNPs, INDELs and copy number changes. The genomes of the UV-mutants IMS0637, IMS0640, IMS0641 and IMS0642 shared a set of 116 SNPs, 5 INDELs and 1 copy number variation (Supplementary data file 1). In addition to these shared mutations, isolates IMS0638, IMS0639 and IMS0643 carried three identical SNPs. Overall, 97% of SNPs and INDELs of IMS0637-IMS0643 were heterozygous, indicating that the haploid spores of CBS 12357^T diploidized by mating after mutagenesis (Supplementary data file 1). Of the mutations present in all isolates, 34 SNPs and all 5 INDELs affected intergenic regions, 30 SNPs were synonymous, 48 SNPs resulted in amino acid substitutions and 4 SNPs resulted in a premature stop codon (Supplementary data file 1). None of the 52 non-synonymous SNPs affected genes previously linked to maltotriose utilization. The only copy number variation concerned a duplication of the right subtelomeric region of CHRVIII. Read mate-pairing indicated that the duplicated region was attached to the left arm of CHRII, causing the replacement of left subtelomeric region of CHRII by a non-reciprocal translocation. The recombination resulted in loss of one of the *SeMALT1* allele, which is not expressed in CBS 12357^T [90].

Since the ability to utilize maltotriose in wort emerged only after laboratory evolution during chemostat cultivation, mutations present in the chemostat-evolved strains IMS0750 and IMS0752 were studied in more detail. With the exception of one synonymous SNP, IMS0750 and IMS0752 were identical and shared 100 SNPs, 3 INDELs and 5 copy number changes (Supplementary data file 1). The non-maltotriose utilizing strain IMS0751 shared only 63 SNPs and 3 INDELs with IMS0750 and IMS0752, of which 98% were homozygous, indicating a recent loss of heterozygosity event affected its entire genome. Of the mutations in maltotriose-utilizing strains IMS0750 and IMS0752, only 5 SNPs and 4 copy number changes were absent in IMS0637-IMS0643, and could therefore explain the ability to utilize maltotriose in wort (Fig 3A). The 5 SNPs consisted of two intergenic SNPs and three non-synonymous SNPs in genes with no link to maltotriose. However, the changes in copy number affected several regions harboring *SeMALT* genes: a duplication of 550 bp of CHRII including Se*MALT1* (coordinates 8,950 to 9,500), a duplication of the left arm of CHRXIII including Se*MALT3* (coordinates 1-10,275), loss of the left arm of CHRXVI (coordinates 1-15,350), and loss of 5.5 kb of CHRXVI including *SeMALT4* (coordinates 16,850-22,300). Analysis of read mate pairing indicated that the

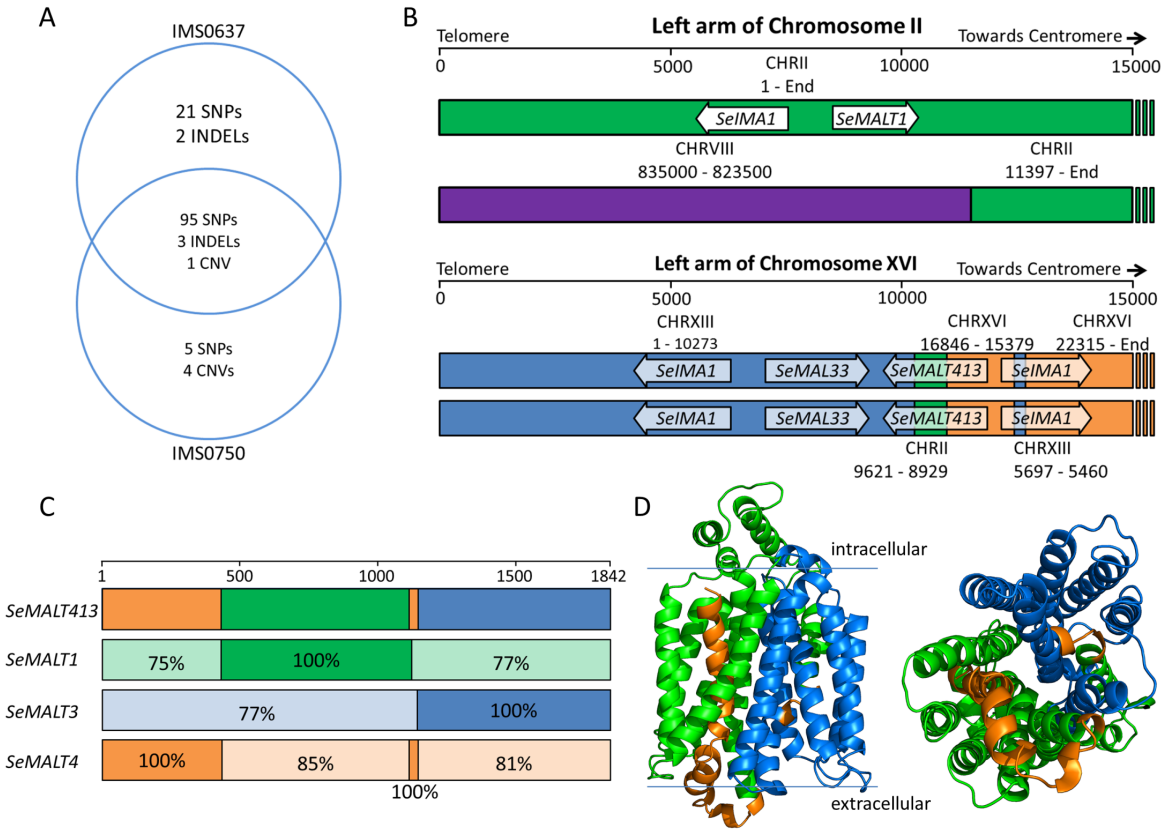

Fig 3: Identification of mutations in the mutagenized strain IMS0637 and the evolved strain IMS0750. (**A**) Venn diagram of the mutations found in UV-mutagenized IMS0637 and evolved IMS0750 relative to wildtype CBS 12357^T. Single nucleotide polymorphisms (SNPs), small insertions and deletions (INDELs) and copy number variation (CNV) are indicated as detected by Pilon. (**B**) Recombined chromosome structures in IMS0637 and IMS0750 as detected by whole genome sequencing using MinION nanopore technology and *de novo* genome assembly. The first 15,000 nucleotides of the left arm of CHRII and CHRXVI are represented schematically. The origin of the sequence is indicated in green for CHRII, purple for CHRVIII, blue for CHRXIII and orange for CHRXVI. The ORFs of *SeMALT* transporter genes, *SeIMA* isomaltase genes and the MALR-type regulator *SeMAL33* which were affected by the recombinations are indicated by arrows. While the recombination of CHRII and CHRVIII was present in IMS0637 and IMS0750, the recombination of both copies of CHRXVI was found only in IMS0750 but not in IMS0637. The recombination on CHRXVI created the chimeric *SeMALT413* transporter gene. (**C**) Overview of the sequence identity of the 1,842 nucleotides of *SeMALT413* relative to *SeMALT1*, *SeMALT3* and *SeMALT4*. The open reading frames of the genes were aligned (Supplementary Fig S3) and regions with 100% sequence identity were identified. For regions in which the sequence identity was lower than 100%, the actual sequence identity is indicated for each *SeMALT* gene. The origin of the sequence is indicated in green for CHRII, red for CHRVIII, blue for CHRXIII and orange for CHRXVI. (**D**) Prediction of the protein structure of SeMalt413 with on the left side a transmembrane view and on the right a transport channel view. Domains originated from *S. eubayanus* SeMalt transporters are indicated by the colors orange (*Se*Malt4 chromosome XVI), green (*Se*Malt1 chromosome II) and blue (SeMalt3 chromosome XIII).

copy number variation resulted from a complex set of recombinations between chromosomes II, XIII and XVI.
The high degree of sequence identity of the affected *MAL* loci and their localization in the subtelomeric regions made exact reconstruction of the mutations difficult. Therefore, IMS0637 and IMS0750 were sequenced using long-read sequencing on ONT's MinION platform, and a *de novo* genome assembly was made for each strain. Comparison of the resulting assemblies to the chromosome-level assembly of CBS 12357^T indicated that two recombinations had occurred. Both in IMS0637 and IMS0750, an additional copy of the terminal 11.5 kb of the right arm of chromosome VIII had replaced the terminal 11.4 kb of one of the two copies of the left arm of chromosome II (Fig 3B). This recombination was consistent with the copy number changes of the affected regions in IMS0637-IMS0643, IMS0750 and IMS0752 and resulted in the loss of one copy of the *MAL* locus harboring Se*MALT1*. In addition, the genome assembly of IMS0750 indicated the replacement of both copies of the first 22.3 kb of CHRXVI by complexly rearranged sequences from CHRII, CHRXVIII and CHRXVI. The recombined region comprised the terminal 10,273 nucleotides of the left arm of CHRIII, followed by 693 nucleotides from CHRII, 1,468 nucleotides from CHRXVI and 237 nucleotides from CHRXIII (Fig 3B). The recombinations were non reciprocal, as the regions present on the recombined chromosome showed increased sequencing coverage while surrounding regions were unaltered. This recombination resulted in the loss of the canonical *MAL* locus harboring Se*MALT4* on chromosome XVI. However, the recombined sequence contained a chimeric open reading frame consisting of the 5' part of Se*MALT4* from CHRXVI, the middle of Se*MALT1* from CHRII and the 3' part of Se*MALT3* from CHRXIII (Fig 3C, Supplementary Fig S3). To verify this recombination, the ORF was PCR amplified using primers binding on the promotor of *SeMALT4* and the terminator of *SeMALT3,* yielding a fragment for strain IMS0750, but not for CBS 12357^T and IMS0637. Sanger sequencing of the fragment amplified from strain IMS0750 confirmed the chimeric organization of the ORF, which we named Se*MALT413*. The sequence of Se*MALT413* showed 100% identity to Se*MALT4* for nucleotides 1-434 and 1113-1145, 100% identity to Se*MALT1* for nucleotides 430-1122 and 100% identity to Se*MALT3* for nucleotides 1141-1842 (Fig 3C). Nucleotides 1123-1140, which showed only 72% identity with *SeMALT1* and 61% identity with *SeMALT3*, were found to represent an additional introgression (Fig 3B). While the first 434 nucleotides can be unequivocally attributed to *SeMALT4* due to a nucleotide difference with *SeMALT2*, the nucleotides 1123-1140 are identical in *SeMALT2* and Se*MALT4*. Therefore, this part of the sequence of Se*MALT413* might have come from Se*MALT2* on CHRV or from *SeMALT4* on CHRXVI. Overall, Se*MALT413* showed a sequence identity of only 85 to 87% with the original Se*MALT* genes, with the corresponding protein sequence exhibiting between 52 and 88% similarity. We therefore hypothesized that the recombined *Se*MalT413 transporter might have an altered substrate specificity and thereby enable maltotriose utilization.
The tertiary protein structure of the chimeric *SeMALT413* gene was predicted with SWISS-MODEL (https://swissmodel.expasy.org/), based on structural homology with the *Escherichia coli* xylose-proton symporter XylE [218], which has previously been used as a reference to model the structure of the maltotriose transporter *Sc*Agt1 [219]. Similarly to the maltose transporters in *Saccharomyces*, XylE is a proton symporter belonging to the major facilitator superfamily with a transmembrane domain composed of 12 α-helixes (Supplementary Fig S4). The same structure was predicted for *Se*MalT413, with 1 α-helix formed exclusively by residues from *Se*MalT4, 4 α-helixes formed exclusively by residues from *Se*MalT1 and 5 α-helixes formed exclusively by residues from *Se*MalT3 (Fig 3D). The

remaining two α-helixes were composed of residues from more than one transporter. Since the first 100 amino acids were excluded from the model due to absence of similar residues in the xylose symporter reference model, the structure prediction underestimated the contribution of *Se*MalT4. The three-dimensional arrangement of the α-helixes of *Se*MalT413 was almost identical to *Se*MalT1, *Se*MalT3 and *Se*MalT4, indicating that it retained the general structure of a functional maltose transporter (Supplementary Fig S5).

Introduction of the *SeMALT413* gene in wildtype CBS 12357^T enables maltotriose utilization

The small structural differences identified between *Se*MalT413 and the wild-type *S. eubayanus Se*MalT transporters could not be used to predict the ability of *Se*MalT413 to transport maltotriose [219]. Therefore, to investigate its role in maltotriose transport, *SeMALT413* and, as a control, Se*MALT2* were overexpressed in the wild-type strain *S. eubayanus* CBS 12357^T (Fig 4A and Supplementary Fig S6). Consistently with previous gene editing in CBS 12357^T [90], the expression cassettes were inserted at the Se*SGA1* locus, encoding an intracellular sporulation-specific glucoamylase which is not expressed during vegetative growth [148, 149]. Growth of the resulting strains *S. eubayanus* IMX1941 (*SeSGA1*Δ::*ScTEF1*$_{pr}$-*SeMALT2-ScCYC1*$_{ter}$) and IMX1942 (*SeSGA1*Δ::*ScTEF1*$_{pr}$-*SeMALT413-ScCYC1*$_{ter}$), as well as the wild-type strain CBS 12357^T and the evolved isolate IMS0750 was tested on SM supplemented with different carbon sources (Supplementary Fig S7). On glucose, strains IMX1941 and IMX1942 exhibited the same specific growth rate of 0.25 ± 0.01 h^{-1} as CBS 12357^T, while IMS0750 grew faster with a growth rate of 0.28 ± 0.01 h^{-1}. Glucose was completely consumed after 33 h (Fig 4B). On maltose, the specific growth rates of CBS 12357^T, IMX1941, IMX1942 and IMS0750 ranged between 0.17 and 0.19 h^{-1} and did not differ significantly. Maltose was completely consumed after 43 h (Fig 4C). On maltotriose, only the evolved mutant IMS0750 and reverse engineered strain IMX1942 (*ScTEF1*$_{pr}$-*SeMALT413-ScCYC1*$_{ter}$) showed growth. IMS0750 grew with a specific growth rate of 0.19 ± 0.01 h^{-1} and consumed 55% of maltotriose within 172 h. Over the same period, IMX1942 grew at 0.03 ± 0.00 h^{-1} and consumed 45% of the maltotriose after 172 h (Fig 4D), demonstrating the capacity of *SeMALT413* to transport maltotriose.

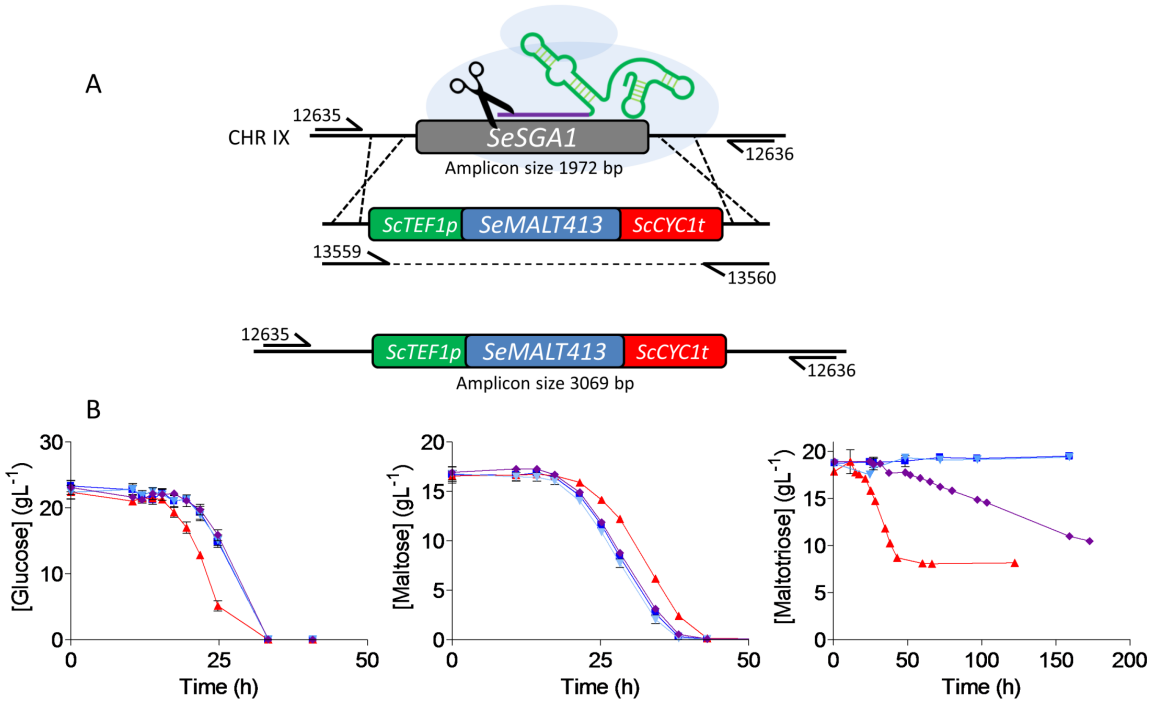

Fig 4: Reverse engineering of *SeMALT413* in CBS 12357^T and characterization of transporter functionality in SM. (**A**) Representation of the CRISPR-Cas9 gRNA complex (after self-cleavage of the 5' hammerhead ribozyme and a 3' hepatitis-δ virus ribozyme from the expressed gRNA) bound to the *SeSGA1* locus in CBS 12357^T. Repair fragment with transporter cassette *ScTEF1p-SeMALT413-ScCYC1t* was amplified from pUD814(*SeMALT413*) with primers 13559/13560 and contains overhangs with the *SeSGA1* locus for recombination. *SeSGA1* was replaced by the *ScTEF1p-SeMALT413-ScCYC1t* cassette. Correct transformants were checked using primers 12635/12636 upstream and downstream of the *SeSGA1* locus (Supplementary Fig S6). Strains were validated using Sanger sequencing. (**B**) Characterization of (■) CBS 12357, (▲) IMS0750, (▼) IMX1941, (◆) IMX1942 on SM glucose, maltose and maltotriose. Strains were cultivated at 20 °C and culture supernatant was measured by HPLC. Data represent average and standard deviation of three biological replicates (Supplementary data file 6).

The *SpMTY1* maltotriose transporter gene displays a similar chimeric structure as *SeMALT413*

The emergence of the maltotriose transporter *Se*MalT413 by recombination between different *MALT* genes during laboratory evolution demonstrates that *MALT* gene neofunctionalization can contribute to the emergence of maltotriose utilization. To investigate if such neofunctionalization could have played a role in the emergence of maltotriose transporter genes in *Saccharomyces* yeasts, we analyzed the sequences of existing maltotriose transporter genes in *S. cerevisiae* and *S. pastorianus* genomes. In *S. cerevisiae* strains, maltotriose transport is encoded by the *ScAGT1* gene [77, 85, 87]. However, *ScAGT1* is truncated and non-functional in *S. pastorianus* [95]. Instead, maltotriose utilization has been attributed to two *S. pastorianus*-specific genes: *LgAGT1* and *SpMTY1.* The maltotriose transporter gene *LgAGT1* was identified on *S. eubayanus* chromosome XV of *S. pastorianus* and shares 85% sequence identity with *ScAGT1* [18, 96]. Although it is absent in CBS 12357^T [62], *LgAGT1* was found to enable maltotriose transport in the north-American *S. eubayanus* isolate yHRVM108 [34, 62]. The *SpMTY1* gene, also referred to as *MTT1*, was found in the *MAL1* locus of *S. pastorianus.* In addition to *S. cerevisiae* chromosome VII, *SpMTY1* was also found on *S. eubayanus* chromosome VII

of *S. pastorianus*, of which the right arm originates from S. cerevisiae due to a recombination [18]. *SpMTY1* shows 90% sequence identity with *ScMALx1* genes [91, 92], but also displays segmental sequence identity with *SeMALT* genes [89, 220].
The relatively low homology of *ScAGT1* and *LgAGT1* genes indicates that they are less related to maltose transporter genes such as *ScMALx1* and *SeMALT* than *SpMTY1*. Moreover, their sequence identity to maltose transporters from the *MALT* family such as *ScMAL31* is roughly homogenous over their coding region. Therefore, there is no evidence that they resulted from recombinations between other *MALT* genes. In contrast, the identity of some segments of *SpMTY1* relative to *ScMAL31* deviates strongly from the average identity of 89% [92]. Indeed, sequence identity with *ScMAL31* of *S. cerevisiae* S288C [221] is above 98% for nucleotides 1-439, 627-776, 796-845, 860-968 and 1,640-1,844, while it is only 79% for nucleotides 440-626, 65% for nucleotides 777-795, 50% for nucleotides 846-859 and 82% for nucleotides 969-1,639 (Supplementary Fig S8). Alignment of the sequences of *S. eubayanus* CBS 12357^{T} *SeMALT* genes [90] to *SpMTY1* showed high sequence identity with *SeMALT3* across several regions that showed significant divergence from the corresponding *ScMAL31* sequences: 91% identity for nucleotides 478-533, 94% identity for nucleotides 577-626 and 94% identity for nucleotides 778-794 (Supplementary Fig S8). These observations would indicate that the evolution of *SpMTY1* might have involved introgression events similar to those responsible for the *SeMALT413* neofunctionalization described in the present study. However, introgressions from *SeMALT* genes cannot explain the entire *SpMTY1* gene structure. Its evolution may therefore have involved multiple introgressions, similarly as for *SeMALT413*. While most regions with low identity to *ScMAL31* and *SeMALT3* were too short to identify their provenance, the sequence corresponding to the 969th to 1,639th nucleotide of *SpMTY1* could be blasted on NCBI. In the S288C genome, *ScMAL31* was the closest hit with 82% identity. However, when blasting the sequence against the full repository excluding *S. pastorianus* genomes, the closest hit was the orthologue of *ScMAL31* on chromosome VII of *S. paradoxus* strain YPS138. In addition to an 89% identity to nucleotides 969-1,639 of *SpMTY1*, *SparMAL31* had an identity of 94% for nucleotides 544-575 and of 93% for nucleotides 846-859 (Supplementary Fig S8). Therefore, the sequence of *SpMTY1* may have resulted from recombination between different *MALT* genes, involving *ScMALx1* and other *MALT* genes such as *SeMALT3* and *SparMAL31*. While the chimeric *SeMALT413* ORF can be fully explained by recombination between *SeMALT* genes, *SpMTY1* probably accumulated additional mutations during its evolution/domestication.

Applicability of a maltotriose-consuming *S. eubayanus* strain for lager beer brewing
S. eubayanus strains are currently used for industrial lager beer brewing [90]. To test the evolved strain IMS0750 under laboratory-scale brewing conditions, its performance was compared with that of its parental strain CBS 12357^{T} in 7-L cultures grown on high-gravity (16.6 ° Plato) wort (Fig 5). After 333 h, IMS0750 had completely consumed all glucose and maltose, and the concentration of maltotriose had dropped from 19.3 to 4.7 g L^{-1} (Fig 5). In contrast, CBS 12357^{T} did not utilize any maltotriose. In addition to its improved maltotriose utilization, IMS0750 also showed improved maltose consumption: maltose was completely consumed within 200 h, while complete maltose consumption by strain CBS 12357^{T} took *ca.* 330 h (Fig 5). Consistent with its improved sugar utilization, the final ethanol concentration in cultures of strain IMS0750 was 18.5% higher than in corresponding cultures of strain CBS 12357^{T} (Fig 5). Brewing-related characteristics of IMS0750 were further explored by analyzing production of aroma-defining esters, higher

alcohols and diacetyl. Final concentrations of esters and higher alcohols were not significantly different in cultures of the two strains, with the exception of isoamylacetate, which showed a 240 % higher concentration in strain IMS0750 (Table 1). In addition, while the concentration of the off-flavour diacetyl remained above its taste threshold of 25 µg L^{-1} after 333h for CBS 12357^T, it dropped below 10 µg L^{-1} for IMS0750 (Table 1).

Table 1: Concentrations of alcohols, esters and diacetyl after fermentation of wort with a gravity of 16.6 °P by *S. eubayanus* strains CBS 12357^T and IMS0750. The data correspond to the last time point (330 h) of the fermentations shown in Fig 5. The average and average deviation of duplicate fermentations are shown for each strain.

Compound	Unit	CBS 12357^T	IMS0750
Methanol	mg L^{-1}	3.3 ± 0.3	3.7 ± 0.3
Propanol	mg L^{-1}	23.7 ± 2.1	24.1 ± 0.9
Isobutanol	mg L^{-1}	48.5 ± 2.4	42.9 ± 7.2
Amyl alcohol	mg L^{-1}	138.5 ± 9.0	155.9 ± 6.4
Diacetyl	µg L^{-1}	43.8 ± 22.9	7.5 ± 0.2
Ethylacetate	mg L^{-1}	24.5 ± 5.5	26.1 ± 0.8
Isoamylacetate	mg L^{-1}	1.4 ± 0.6	3.1 ± 0.3

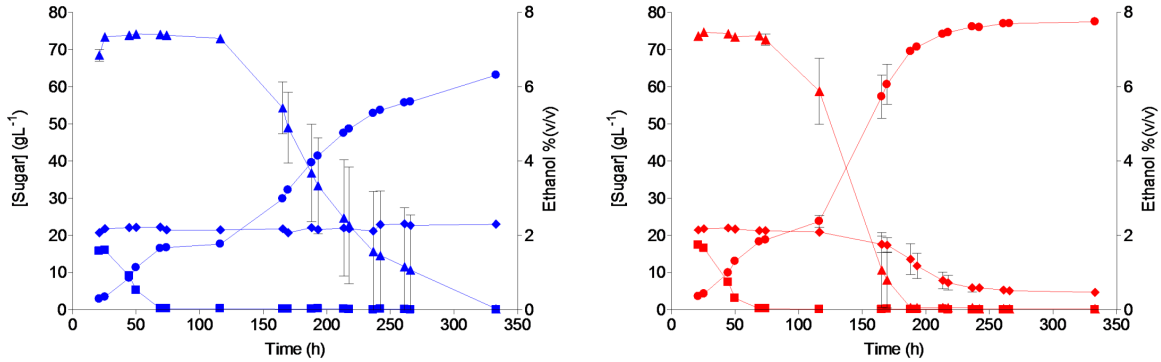

Fig 5: Extracellular metabolite profiles of *S. eubayanus* strains CBS 12357^T and IMS0750 in high-gravity wort at 7-L pilot scale. Fermentations were performed on wort with a gravity of 16.6 °Plato. The average concentrations of glucose (■), maltose (▲), maltotriose (◆) and ethanol (●) are shown for duplicate fermentations of CBS 12357^T (blue) and IMS0750 (red). The average deviations are indicated (Supplementary data file 7).

Discussion

UV mutagenesis and subsequent laboratory evolution yielded mutants which were able to utilize maltotriose in synthetic medium and in brewer's wort. In the resulting isolates IMS0750 and IMS0752, several recombinations affecting subtelomeric regions were identified. All four maltose transporter genes in *S. eubayanus* CBS 12357^T are localized in subtelomeric *MAL* loci: *SeMALT1* on chromosome II, *SeMALT2* on chromosome V, *SeMALT3* on chromosome XIII and *SeMALT4* on chromosome XVI [62, 90]. In the evolved strain IMS0750, a complex recombination between the subtelomeric regions of chromosomes II, XIII and XVI involved at least three of these *MAL* loci. Long-read nanopore sequencing enabled complete reconstruction of the recombined left arm of chromosome XVI, revealing recombinations between the ORFs of at least *SeMALT1*, *SeMALT3* and *SeMALT4*. These recombinations occurred within the open reading frame of *SeMALT4* and the newly-formed chimeric ORF *SeMALT413* encoded a full-length protein with a structure comparable to that of *Se*MalT transporters. In contrast to the original *SeMALT* genes, overexpression of *SeMALT413* enabled growth on maltotriose, indicating that *Se*MalT413 acquired the ability to import maltotriose.

The predicted structure of *Se*MalT413 was highly comparable to the structure of other transporters from the major facilitator superfamily [222] and to the structure of *Se*MalT1, *Se*MalT3 and *Se*MalT4. While nothing is known about the amino acid residues responsible for substrate specificity in *Se*MalT transporters, the threonine and serine residues at the 505th and 557th position respectively of *Sc*Agt1 were identified as critical for maltotriose transport [223]. In *Se*MalT413, the corresponding amino acids originate from *SeMALT3*. However, since *SeMALT3* itself is unable to utilize maltotriose, the ability of *Se*MalT413 to transport maltotriose likely depends on the interaction of residues from the different parental transporters, rather than from the residues of one of the transporters. Interestingly, CBS 12357^T was recently evolved for maltotriose utilization in another study, resulting in a chimeric *SeMALT434* transporter which enabled maltotriose uptake [34]. In this study, a 230-bp introgression of *SeMALT3* into the ORF of *SeMALT4* was found, including the 505th and 557th residues. While the shorter α-helixes of *SeMALT434* could lead to broader substrate-specificity by increasing structural flexibility, the length of these helixes was not affected in *SeMALT413*. As a result, we hypothesize that the acquired maltotriose utilization does not depend solely on specific residues, but rather on the interaction of the residues from the different parental transporter, either by increasing structural flexibility, or by the properties of several critical residues from different α-helixes. The 230-bp from *SeMALT3* which were present in *SeMALT413* and in *SeMALT434* may be of particular importance. However, the specific combination of sequences from *SeMALT4*, *SeMALT1* and *SeMALT3* in *SeMALT413* may further contribute to the maltotriose specificity.

Recombinations are an important driver of evolution, as illustrated by the emergence of aerobic growth on citrate during laboratory evolution of *Escherichia coli* [224]. Indeed, a tandem repeat of the citrate/succinate antiporter *citT* placed under the constitutive *RNA* promoter enabled aerobic growth on citrate. Moreover, the emergence of a new ORF by recombination has been observed previously between the *TLO* genes of *C. albicans*, although it was not associated with a new gene function [197]. In contrast, the emergence of *SeMALT413* is an example of gene neofunctionalization, which occurred by recombination within genes of the subtelomeric *MALT* family. Neofunctionalization by *in vivo* formation of chimeric sequences is reminiscent of the mechanism used by the pathogen *Trypanosoma brucei* to evade its host's immune system [225]. *T. brucei*

expresses a single variant surface glycoprotein (VSG) gene from a subtelomeric location and its genome contains many VSG pseudogenes [226]. Due to 70 bp repetitive elements, the actively expressed VSG gene can be altered by gene conversion from pseudogenes, resulting in a chimeric VSG gene [227-229]. While antigen switching may not qualify as neofunctionalization, it demonstrates the ability of recombinations to diversify gene functions by creating chimeric ORFs. This ability has also been exploited for *in vitro* protein engineering, a strategy known as gene shuffling or gene fusion [230, 231]. Gene shuffling involves randomized assembly of diverse DNA sequences into chimeric genes, followed by screening for novel or improved functions. Analogously to *in vitro* gene shuffling, the complex protein remodeling caused by *in vivo* formation of chimeric sequences may be particularly potent for protein neofunctionalization [232]. The demonstration of neofunctionalization of a sugar transporter in *S. eubayanus* by *in vivo* gene shuffling supports the notion that gene fusion is an essential driver of evolution by accelerating the emergence of new enzymatic functions [233]. Moreover, analysis of the *SpMTY1* maltotriose transporter gene revealed a chimeric structure similar to that of *SeMALT413*, albeit with alternating sequence identity with *ScMAL31*, *SeMALT* and *SparMAL31*. While sequences from *S. cerevisiae* and *S. eubayanus* were already present in the genome of *S. pastorianus*, the presence of sequences from *S. paradoxus* is plausible as introgressions from *S. paradoxus* were commonly found in a wide array of *S. cerevisiae* strains [234]. Therefore, the sequence of *SpMTY1* could have resulted from *in vivo* gene shuffling between genes from the *MALT* family, followed by accumulation of mutations. The emergence of *SeMALT413* could therefore be representative of the emergence of maltotriose utilization during the evolution of *S. pastorianus*. Moreover, the emergence of a maltotriose transporter after laboratory evolution of CBS 12357^T, which was discovered at the same time as *SeMALT413* provides further credibility to the evolutionary importance of *in vivo* gene shuffling for gene neofunctionalization [34].

No evidence of reciprocal translocations between *SeMALT1*, *SeMALT3* and *SeMALT4* was found in the genome of IMS0750, indicating genetic introgression via non-conservative recombinations. Such introgressions can occur during repair of double strand breaks by strand invasion of a homologous sequence provided by another chromosome and resection [235], leading to localized gene conversion and loss of heterozygosity. This model, which was proposed to explain local loss of heterozygosity of two orthologous genes in an *S. cerevisiae* x *S. uvarum* hybrid [235], provides a plausible explanation of the emergence of *SeMALT413* through non-reciprocal recombination between paralogous *SeMALT* genes in *S. eubayanus*. The mosaic sequence composition of the resulting transporter gene suggests that neofunctionalization required multiple successive introgression events. As a result of these genetic introgressions, the *SeMALT4* gene was lost. The fact that IMS0750 harbored two copies of *SeMALT413* and no copy of *SeMALT4* indicates a duplication of the newly-formed ORF at the expense of *SeMALT4* via loss of heterozygosity. As functional-redundancy enables the accumulation of mutation without losing original functions [94, 197, 198, 236], the loss of *SeMALT4* was likely facilitated by the presence of the functionally-redundant maltose transporter *SeMALT2* [90]. The observation that introgressions were only found at *SeMALT4* may be due to the low number of tested mutants. However, it should be noted that introgressions in the *SeMALT1* and *SeMALT3* ORF's would have been unlikely to be beneficial, since these genes are not expressed in CBS 12357^T [90].

This study illustrates the role of the rapid evolution of subtelomeric genes in adaptation to environmental changes. In *Saccharomyces* yeasts, subtelomeric regions contain a large number of gene families encoding functions critical to the interaction of a cell with its

environment, such as nutrient uptake, sugar utilization, inhibitor tolerance and flocculation [94, 237-242]. The high number of genes within subtelomeric families results in functional redundancy and therefore in mutational freedom [94, 197, 198, 236]. *In Saccharomyces* species, many industrially-relevant brewing traits are encoded by subtelomeric gene families, such as the *MAL* genes encoding maltose utilization and the *FLO* genes encoding flocculation [243]. While subtelomeric regions are difficult to reconstruct due to their repetitive nature, they encode much of the genetic diversity between genomes [70, 244]. *A posteriori* sequence analysis of existing gene families can elucidate their evolutionary history. For example, the α-glucosidase genes from the *MALS* family emerged by expansion of an ancestral pre-duplication gene with maltose-hydrolase activity and trace isomaltose-hydrolase activity [99]. The evolution of *MALS* isomaltase genes from this ancestral gene is an example of subfunctionalization: the divergent evolution of two gene copies culminating in their specialization for distinct functions which were previously present to a lesser extent in the ancestral gene. The generation of functional redundancy by gene duplication is critical to this process as it enables mutations to occur which result in loss of the original gene function without engendering a selective disadvantage [94, 197, 198, 200, 201, 236]. In contrast to subfunctionalization, neofunctionalization consists of the emergence of a function which was completely absent in the ancestral gene [204]. While the emergence of many genes from a large array of organisms has been ascribed to subfunctionalization and to neofunctionalization, these conclusions were based on *a posteriori* analysis of processes which had already occurred, and not on their experimental observation [94, 99, 197, 203-206]. *Ex-vivo* engineering of the subtelomeric *FLO* genes demonstrated that recombinations within subtelomeric gene families can alter their function [203]. However, *in vivo* neofunctionalization within a subtelomeric gene family was never observed in real time. Here we present clear experimental evidence of neofunctionalization within a laboratory evolution experiment. The ability of *SeMALT413* to transport maltotriose proves that such *in vivo* gene shuffling is relevant for evolutionary biology. Given their high genetic redundancy of subtelomeric gene families, and the large body of evidence of gene sub- and neofunctionalization in their evolutionary history, it is likely that subtelomeric localization of genes facilitates the emergence of new functions. As a result, subtelomeric regions would not only be a hotspot of genetic diversity between different genomes, but also a preferred location for the birth of new genes and new gene functions.

While *SeMALT413* was shown to enable maltotriose utilization, it remained unclear how the UV-mutagenized cells acquired the ability to utilize maltotriose and why these mutations were insufficient to enable maltotriose utilization in wort. Since maltotriose-consuming mutants did not arise in the absence of UV-mutagenesis, the ability to utilize maltotriose likely emerged as a result of genetic evolution rather than due to epigenetic adaptation. Moreover, the introduction of *SeMALT413* in CBS 12357^{T} resulted in slower maltotriose utilization than IMS0750, suggesting that other mutations may contribute to the maltotriose-utilization phenotype of IMS0750. While whole genome sequencing of IMS0637-IMS0643 revealed a wide array of mutations, none affected genes which were previously linked to maltotriose utilization. The fact that *SeMALT1*, *SeMALT2*, *SeMALT3* and *SeMALT4* could be PCR amplified from the IMS0637 genome while *SeMALT413* could not, indicates that maltotriose utilization was not due to an undetected *SeMALT413* gene. In addition, alignment of short-read data to the reference genome and *de novo* genome assembly based on long-read data did not reveal any mutations affecting MAL genes, except a recombination between CHRII and CHRVII, which resulted in the loss of one of

the two copies of *SeMALT1*. Since deletion of *SeMALT1* does not enable maltotriose utilization in CBS 12357^T [90], this mutation is unlikely to be causal. While one of the 122 mutations affecting the UV-mutagenized strains or additional undetected mutations may have contributed to maltotriose utilization, their elucidation is beyond the scope of this study. Moreover, while overexpression of *SeMALT413* in CBS 12357^T resulted in lesser maltotriose utilization than the evolved strain IMS0750, the maltotriose transporter *Se*MalT413 is not necessarily suboptimal. Indeed, when overexpressing transporters suboptimal growth is commonly observed and has been attributed to imbalances between transporter activity and the subsequent metabolic steps [168, 170]. Overall, regardless of the presence of other mutations contributing to maltotriose utilization, the emergence of the maltotriose transporter gene *SeMAL413* from parental genes which do not enable maltotriose transport demonstrates that gene neofunctionalization occurred.

While the introduction of *SeMALT413* in CBS 12357^T via genetic engineering demonstrated its neofunctionalization, the use of GMO-strains is limited in the brewing industry by customer acceptance issues [245]. However, the non-GMO evolved *S. eubayanus* isolate IMS0750 could be tested on industrial brewing wort at 7 L scale. In addition to near-complete maltotriose conversion, the maltose consumption, isoamylacetate production and diacetyl degradation of IMS0750 were superior to CBS 12357^T. While the increased maltotriose consumption could be at least partially attributed to the emergence of the *SeMALT413*, it remained unclear if and what mutations could underlie the other changes. However, efficient maltose and maltotriose consumption, as well as the concomitantly increased ethanol production, are important factors determining the economic profitability of beer brewing processes [246]. In addition, low residual sugar concentration, low concentrations of diacetyl and high concentrations of Isoamylacetate are desirable for the flavor profile of beer [16, 247]. *S. eubayanus* strains typically generate high concentrations of 4-vinyl guaiacol, a clove-like off flavor [38, 248], but strategy to eliminate this production in *S. eubayanus* have recently been described [38]. Therefore, expansion of phenotypic landscape of *S. eubayanus* might be accelerated by combining these domesticated traits. In terms of application, the laboratory evolution approach for conferring maltotriose utilization into *S. eubayanus* presented in this paper is highly relevant in view of the recent introduction of this species in industrial-scale brewing processes [90, 174]. The ability to ferment maltotriose can be introduced into other natural isolates of *S. eubayanus*, either by laboratory evolution or by crossing with evolved strains such as *S. eubayanus* IMS0750. Besides their direct application for brewing, maltotriose-consuming *S. eubayanus* strains are of value for the generation of laboratory-made hybrid *Saccharomyces* strains for brewing and other industrial applications [111, 121, 123, 249].

Materials and methods

Strains and maintenance

All yeast strains used and generated in this study are listed in Table 2. *S. eubayanus* type strain CBS 12357^T [19] and *S. pastorianus* strain CBS 1483 [47, 51] were obtained from the Westerdijk Fungal Biodiversity Institute (Utrecht, the Netherlands). Stock cultures were grown in YPD, containing 10 g L^{-1} yeast extract, 20 g L^{-1} peptone and 20 g L^{-1} glucose, at 20 ˚C until late exponential phase, complemented with sterile glycerol to a final concentration of 30% (v/v) and stored at -80 ˚C until further use.

Table 2: *Saccharomyces* strains used during this study

Name	Species	Relevant genotype	Origin
CBS 12357	*S. eubayanus*	Wild-type diploid	[19]
IMS0637	*S. eubayanus*	Evolved strain derived from CBS 12357	This study
IMS0638	*S. eubayanus*	Evolved strain derived from CBS 12357	This study
IMS0639	*S. eubayanus*	Evolved strain derived from CBS 12357	This study
IMS0640	*S. eubayanus*	Evolved strain derived from CBS 12357	This study
IMS0641	*S. eubayanus*	Evolved strain derived from CBS 12357	This study
IMS0642	*S. eubayanus*	Evolved strain derived from CBS 12357	This study
IMS0643	*S. eubayanus*	Evolved strain derived from CBS 12357	This study
IMS0750	*S. eubayanus*	Evolved strain derived from CBS 12357	This study
IMS0751	*S. eubayanus*	Evolved strain derived from CBS 12357	This study
IMS0752	*S. eubayanus*	Evolved strain derived from CBS 12357	This study
IMX1941	*S. eubayanus*	*ΔSesga1::ScTEF1p-SeMALT2-ScCYC1t*	This study
IMX1942	*S. eubayanus*	*ΔSesga1::ScTEF1p-SeMALT413-ScCYC1t*	This study
CBS 1483	*S. pastorianus*	Group II brewer's yeast, Brewery Heineken, bottom yeast, July 1927	[51]

Media and cultivation

Plasmids were propagated overnight in *Escherichia coli* XL1-Blue cells in 10 mL LB medium containing 10 g L^{-1} peptone, 5 g L^{-1} Bacto Yeast extract, 5 g L^{-1} NaCl and 100 mg L^{-1} ampicillin at 37 °C. YPD medium was prepared using 10 g L^{-1} yeast extract, 20 g L^{-1} peptone and 20 g L^{-1} glucose. Synthetic medium (SM) contained 3.0 g L^{-1} KH_2PO_4, 5.0 g L^{-1} $(NH_4)_2SO_4$, 0.5 g L^{-1} $MgSO_4$, 7 H_2O, 1 mL L^{-1} trace element solution, and 1 mL L^{-1} vitamin solution [140], and was supplemented with 20 g L^{-1} glucose (SMG), maltose (SMM) or maltotriose (SMMt) by addition of autoclaved 50% w/v sugar solutions. Maltotriose (95.8% purity) was obtained from Glentham Life Sciences, Corsham, United Kingdom. Industrial wort was provided by HEINEKEN Supply Chain B.V., Zoeterwoude, the Netherlands. The wort was supplemented with 1.5 mg L^{-1} of Zn^{2+} by addition of $ZnSO_4 \cdot 7H_2O$, autoclaved for 30 min at 121°C and filtered using Nalgene 0.2 µm SFCA bottle top filters (Thermo Scientific, Waltham, MA) prior to use. Where indicated, filtered wort was diluted with sterile demineralized water. Solid media were supplemented with 20 g L^{-1} of Bacto agar (Becton Dickinson, Breda, The Netherlands). *S. eubayanus* strains transformed with plasmids pUDP052 ($gRNA_{SeSGA1}$) were selected on medium in which $(NH_4)_2SO_4$ was replaced by 5 g L^{-1} K_2SO_4 and 10 mM acetamide ($SM_{Ace}G$: SMG) [141].

Shake-flask cultivation
Shake-flask cultures were grown in 500 mL shake flasks containing 100 mL medium and inoculated from stationary-phase aerobic precultures to an initial OD_{660} of 0.1. Inocula for growth experiments on SMMt were grown on SMM. In other cases, media for growth experiments and inoculum preparation were the same. Shake flasks were incubated at 20 ˚C and 200 RPM in a New Brunswick Innova43/43R shaker (Eppendorf Nederland B.V., Nijmegen, The Netherlands). Samples were taken at regular intervals to determine OD_{660} and extracellular metabolite concentrations. OD_{660} measurements were taken with a Jenway 7200 spectrometer (Cole-Parmer, Staffordshire, UK) unless indicated otherwise.

Microaerobic growth experiments
Microaerobic cultivation was performed in 250 mL airlock-capped Neubor infusion bottles (38 mm neck, Dijkstra, Lelystad, Netherlands) containing 200 mL 3-fold diluted wort supplemented with 0.4 mL L^{-1} Pluronic antifoam (Sigma-Aldrich). Bottle caps were equipped with a 0.5 mm x 16 mm Microlance needle (BD Biosciences) sealed with cotton to prevent pressure build-up. Sampling was performed aseptically with 3.5 mL syringes using a 0.8 mm x 50 mm Microlance needle (BD Biosciences). Microaerobic cultures were inoculated at an OD_{660} of 0.1 from stationary-phase precultures in 50 mL Bio-One Cellstar Cellreactor tubes (Sigma-Aldrich) containing 30 mL of the same medium, grown for 4 days at 12 °C. Bottles were incubated at 12 °C and shaken at 200 RPM in a New Brunswick Innova43/43R shaker. At regular intervals, 3.5 mL samples were collected in 24 deep-well plates (EnzyScreen BV, Heemstede, Netherlands) using a LiHa liquid handler (Tecan, Männedorf, Switzerland) to measure OD_{660} and external metabolites. 30 µL of each sample was diluted 5 fold in demineralized water in a 96 well plate and OD_{660} was measured with a Magellan Infinite 200 PRO spectrophotometer (Tecan, Männedorf, Switzerland). From the remaining sample, 150 µL was vacuum filter sterilized using 0.2 µm Multiscreen filter plates (Merck, Darmstadt, Germany) for HPLC measurements.

7-L wort fermentation cultivations
Batch cultivations under industrial conditions were performed in 10 L stirred stainless-steel fermenters containing 7 L of 16.6 °Plato wort. Fermentations were inoculated to a density of 5 x 10^6 cells mL^{-1} at 8 °C. The temperature was raised during 48 hours to 11 °C and increased to 14 °C as soon as the gravity was reduced to 6.5 °Plato. Samples were taken daily during weekdays and the specific gravity and alcohol content were measured using an Anton Paar density meter (Anton Paar GmbH, Graz, Austria).

Adaptive Laboratory Evolution - UV mutagenesis and selection
First, we attempted to obtain maltotriose-consuming mutants without UV-mutagenesis. To this end, *S. eubayanus* CBS 12357^T was grown in a 500 mL shake flask containing 100 mL SMG at 20 ˚C until stationary phase. Cells were washed twice with demineralized water and used to inoculate a 50 mL shake flask containing 10 mL SMMt to an OD_{660} of 2, corresponding to an initial inoculum of approximately 10^8 cells. The SMMt culture was incubated at 20 °C and 200 RPM during three months. During this period, no growth was observed and HPLC measurements did not show any maltotriose consumption after three months.
In parallel, we mutagenized spores of *S. eubayanus* CBS 12357^T to increase the likelihood of beneficial mutations. To this end, *S. eubayanus* CBS 12357^T was grown in a 500 mL shake flask containing 100 mL SMG at 20 ˚C until stationary phase. The resulting cells were washed twice with demineralized water and transferred to a 500 mL shake flask

containing 100 mL of 20 g/L potassium acetate at pH 7.0 to sporulated. After three days, the presence of ascospores was verified by optic microscopy and diluted to an OD_{660} of 1. Of the resulting suspension, 50 mL was spun down at 4816 g for 5 min and washed twice with demineralized water. 25 mL of washed cells was poured into a 100 mm x 15 mm petri dish (Sigma-Aldrich) without lid and irradiated with a UV lamp (TUV 30 W T8, Philips, Eindhoven, The Netherlands) at a radiation peak of 253.7 nm. 25 mL of non-mutagenized and 5 mL of mutagenized cells were kept to determine survival rate. From both samples, a 100-fold dilution was made, from which successive 10-fold dilutions were made down to a 100,000-fold dilution. Then, 100 µL of each dilution was plated on YPD agar and the number of colonies were counted after incubation during 48h at room temperatures. After 10,000-fold dilution, 182 colonies formed from the non-mutagenized cells against 84 colonies for the mutagenized cells, indicating a survival rate of 46%. The remaining 20 mL of mutagenized cells, corresponding to about 10^8 cells, was spun down at 4816 g for 5 min and resuspended in 1 mL demineralized water. These mutagenized cells were added to a 50 mL shake flask containing 9 mL SMMt and incubated for 21 days at 20 °C and 200 RPM. Maltotriose concentrations were analyzed at day 0, 19 and 21. After 21 days, two 100 µL samples were transferred to fresh shake flasks containing SMMt and incubated until stationary phase. At the end of the second transfer, single cell isolates were obtained using the BD FACSAria™ II SORP Cell Sorter (BD Biosciences, Franklin Lakes, NJ) equipped with a 488 nm laser and a 70 µm nozzle, and operated with filtered FACSFlow™ (BD Biosciences). Cytometer performance was evaluated by running a CST cycle with CS&T Beads (BD Biosciences). Drop delay for sorting was determined by running an Auto Drop Delay cycle with Accudrop Beads (BD Biosciences). Cell morphology was analysed by plotting forward scatter (FSC) against side scatter (SSC). Gated single cells were sorted into a 96 well microtiter plates containing SMMt using a "single cell" sorting mask, corresponding to a yield mask of 0, a purity mask of 32 and a phase mask of 16. The 96 well plates were incubated for 96 h at room temperature in a GENIos Pro micro plate spectrophotometer (Tecan, Männedorf, Switzerland), during which period growth was monitored as OD_{660}. After 96 h, biomass in each well was resuspended using a sterile pin replicator and the final OD_{660} was measured. The 7 isolates with the highest final OD_{660} were picked, restreaked and stocked as isolates IMS0637-643. PCR amplification of the *S. eubayanus*-specific *SeFSY1* gene and ITS sequencing confirmed that all 7 isolates were *S. eubayanus*.

Laboratory evolution in chemostats

Chemostat cultivation was performed in Multifors 2 Mini Fermenters (INFORS HT, Velp, The Netherlands) equipped with a level sensor to maintain a constant working volume of 100 mL. The culture temperature was controlled at 20 °C and the dilution rate was set at 0.03 h^{-1} by controlling the medium inflow rate. Cultures were grown on 6-fold diluted wort supplemented with 10 g L^{-1} additional maltotriose (Glentham Life Sciences), 0.2 mL L^{-1} anti-foam emulsion C (Sigma-Aldrich, Zwijndrecht, the Netherlands), 10 mg L^{-1} ergosterol, 420 mg L^{-1} Tween 80 and 5 g L^{-1} ammonium sulfate. Tween 80 and ergosterol were added as a solution as described previously [140]. IMS0637-IMS0643 were grown overnight at 20 ˚C and 200 RPM in separate shake flasks on 3-fold diluted wort. The OD_{660} of each strain was measured and the equivalent of 7 mL at an OD_{660} of 20 from each strain was pooled in a total volume of 50 mL. The reactor was inoculated by adding 20 mL of the pooled culture. After overnight growth, the medium inflow pumps were turned on and the fermenter was sparged with 20 mL min^{-1} of nitrogen gas and stirred at 500 RPM. The pH was not adjusted. Samples were taken weekly. Due to a technical failure on the 63rd

day, the chemostat was autoclaved, cleaned and restarted using a sample taken on the same day. After a total of 122 days, the chemostat was stopped and 10 single colony isolates were sorted onto SMMt agar using FACS, as for IMS0637-IMS0643. PCR amplification of the *S. eubayanus* specific *SeFSY1* gene and ITS sequencing confirmed that all ten single-cell isolates were *S. eubayanus*. Three colonies were randomly picked, restreaked and stocked as IMS0750-752.

Genomic isolation and whole genome sequencing

Yeast cultures were incubated in 50 mL Bio-One Cellstar Cellreactor tubes (Sigma-Aldrich) containing liquid YPD medium at 20°C on an orbital shaker set at 200 RPM until the strains reached stationary phase with an OD_{660} between 12 and 20. Genomic DNA for whole genome sequencing was isolated using the Qiagen 100/G kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions and quantified using a Qubit® Fluorometer 2.0 (Thermo Scientific).

Genomic DNA of the strains CBS 12357^T and IMS0637-IMS0643 was sequenced by Novogene Bioinformatics Technology Co., Ltd (Yuen Long, Hong Kong) on a HiSeq2500 sequencer (Illumina, San Diego, CA) with 150 bp paired-end reads using PCR-free library preparation. Genomic DNA of the strains IMS0750 and IMS0752 was sequenced in house on a MiSeq sequencer (Illumina) with 300 bp paired-end reads using PCR-free library preparation. All reads are available at NCBI (https://www.ncbi.nlm.nih.gov/) under the bioproject accession number PRJNA492251.

Genomic DNA of strains IMS0637 and IMS0750 was sequenced on a Nanopore MinION (Oxford Nanopore Technologies, Oxford, United Kingdom). Libraries were prepared using 1D-ligation (SQK-LSK108) as described previously [70] and analysed on FLO-MIN106 (R9.4) flow cell connected to a MinION Mk1B unit (Oxford Nanopore Technology). MinKNOW software (version 1.5.12; Oxford Nanopore Technology) was used for quality control of active pores and for sequencing. Raw files generated by MinKNOW were base called using Albacore (version 1.1.0; Oxford Nanopore Technology). Reads with a minimum length of 1000 bp were extracted in fastq format. All reads are available at NCBI (https://www.ncbi.nlm.nih.gov/) under the bioproject accession number PRJNA492251.

Genome analysis

For the strains CBS 12357^T, IMS0637-IMS0643, IMS0750 and IMS0752, the raw Illumina reads were aligned against a chromosome-level reference genome of CBS 12357^T (NCBI accession number PRJNA450912, https://www.ncbi.nlm.nih.gov/) [90] using the Burrows–Wheeler Alignment tool (BWA), and further processed using SAMtools and Pilon for variant calling [155, 156, 250]. Heterozgous SNPs and INDELs which were heterozygous in CBS 12357^T were disregarded. Chromosomal translocations were detected using Breakdancer [251]. Only translocations which were supported by at least 10% of the reads aligned at that locus were considered. Chromosomal copy number variation was estimated using Magnolya [252] with the gamma setting set to "none" and using the assembler ABySS (v 1.3.7) with a k-mer size of 29 [253]. All SNPs, INDELs, recombinations and copy number changes were manually confirmed by visualising the generated .bam files in the Integrative Genomics Viewer (IGV) software [254]. The complete list of identified mutations can be found in Supplementary Data File 1.

For strains IMS0637 and IMS0750, the nanopore sequencing reads were assembled de novo using Canu (version 1.3) [154] with –genomesize set to 12 Mbp. Assembly correctness was assessed using Pilon [155], and sequencing/assembly errors were polished by aligning Illumina reads with BWA [156] using correction of only SNPs and

short indels (–fix bases parameter). Long sequencing reads of IMS0637 and IMS0750 were aligned to the obtained reference genomes and to the reference genome of CBS 12357^{T} using minimap2 [255]. The genome assemblies for IMS0637 and IMS0750 are available at NCBI (https://www.ncbi.nlm.nih.gov/) under the bioproject accession number PRJNA492251.

Molecular biology methods

For colony PCR and Sanger sequencing, a suspension containing genomic DNA was prepared by boiling biomass from a colony in 10 µL 0.02 M NaOH for 5 min, and spinning cell debris down at 13,000 g. To verify isolates belonged to the *S. eubayanus* species, the presence of *S. eubayanus*-specific gene *SeFSY1* and the absence of *S. cerevisiae*-specific gene *ScMEX67* was tested by DreamTaq PCR (Thermo Scientific) amplification using primer pair 8572/8573 [256], and primer pair 8570/8571 [257], respectively. Samples were loaded on a 1% agarose gel containing SYBR Green DNA stain (Thermo Scientific). GeneRuler DNA Ladder Mix (Thermo Scientific) was used as ladder and gel was run at a constant 100V for 20 min. DNA bands were visualized using UV light. For additional confirmation of the *S. eubayanus* identity, ITS regions were amplified using Phusion High-Fidelity DNA polymerase (Thermo Scientific) and primer pair 10199/10202. The purified (GenElute PCR Cleanup Kit, Sigma-Aldrich) amplified fragments were Sanger sequenced (BaseClear, Leiden, Netherlands) [258]. Resulting sequences were compared using BLAST to available ITS sequences of *Saccharomyces* species and classified as the species to which the amplified region had the highest sequence identity. The presence of the *SeMALT* genes was verified by using Phusion High-Fidelity DNA polymerase and gene specific primers: 10491/10492 for *SeMALT1*, 10632/10633 for *SeMALT2* and *SeMALT4/2*, 10671/10672 for *SeMALT3*, 10491/10671 for *SeMALT13*, and 10633/10671 for *SeMALT413*. The amplified fragments were purified using the GenElute PCR Cleanup Kit (Sigma-Aldrich) and Sanger sequenced (BaseClear) using the same primers used for amplification.

Plasmid construction

All plasmids and primers used in this study are listed in Table 3 and Supplementary Table S1, respectively. DNA amplification for plasmid and strain construction was performed using Phusion High-Fidelity DNA polymerase (Thermo Scientific) according to the supplier's instructions. The coding region of S*eMALT413* was amplified from genomic DNA of IMS0750 with primer pair 10633/10671. Each primer carried a 40 bp extension complementary to the plasmid backbone of p426-TEF-amds [135], which was PCR amplified using primer pair 7812/5921. The transporter fragment and the p426-TEF-amdS backbone fragment were assembled [259] using NEBuilder HiFi DNA Assembly (New England Biolabs, Ipswich, MA), resulting in plasmid pUD814. The resulting pUD814 plasmid was verified by Sanger sequencing, which confirmed that its *SeMALT413* ORF was identical to the recombined ORF found in the nanopore assembly of IMS0750 (Fig 3C).

Table 3: Plasmids used during this study

Name	Relevant genotype	Source
pUDP052	*ori* (ColE1) *bla* panARSopt *amdSYM ScTDH3*$_{pr}$-gRNA*$_{SeSGA1}$*-*ScCYC1*$_{ter}$ *AaTEF1*$_{pr}$-*Spcas9*$^{D147Y\ P411T}$-*ScPHO5*$_{ter}$	[90]
pUDE044	*ori* (ColE1) *bla* 2µ *ScTDH3*$_{pr}$-*ScMAL12-ScADH1*$_{ter}$ *URA3*	[260]
p426-TEF-amdS	*ori* (ColE1) *bla* 2µ *amdSYM ScTEF1$_{pr}$-ScCYC1$_{ter}$*	[135]
pUD479	*ori* (ColE1) *bla* 2µ *amdSYM ScTEF1*$_{pr}$-*SeMALT1-ScCYC1*$_{ter}$	[90]
pUD480	*ori* (ColE1) *bla* 2µ *amdSYM ScTEF1*$_{pr}$-*SeMALT2-ScCYC1*$_{ter}$	[90]
pUD814	*ori* (ColE1) *bla* 2µ *amdSYM ScTEF1*$_{pr}$-*SeMALT413-ScCYC1*$_{ter}$	This study

Strain construction
To integrate and overexpress Se*MALT2* and Se*MALT413* ORFs in *S. eubayanus* CBS 12357^T, Se*MALT2* and Se*MALT413* were amplified from pUD480 and pUD814 respectively with primers 13559/13560 that carried a 40 bp region homologous to each flank of the *SeSGA1* gene located on *S. eubayanus* chromosome IX. To facilitate integration, the PCR fragments were co-transformed with the plasmid pUDP052 that expressed *Spcas9*$^{D147Y\ P411T}$ [142, 145] and a gRNA targeting *SeSGA1* [90]. The strain IMX1941 was constructed by transforming CBS 12357^T with 1 µg of the amplified Se*MALT2* expression cassette and 500 ng of plasmid pUDP052 by electroporation as described previously [142]. Transformants were selected on $SM_{Ace}G$ plates. Similarly, IMX1942 was constructed by transforming CBS 12357^T with 1 µg of the amplified *SeMALT413* expression cassette for *SeMALT413* instead of *SeMALT2*. Correct integration was verified by diagnostic PCR with primer pair 12635/12636 (Supplementary Fig S9). All PCR-amplified gene sequences were Sanger sequenced (BaseClear).

Protein structure prediction
Homology modeling of the *Se*MalT413 transporter was performed using the SWISS-MODEL server (https://swissmodel.expasy.org/) [261]. The translated amino acid sequence of *SeMALT413* was used as input (Supplementary Fig S4). The model of the xylose proton symporter XylE (PDB: 4GBY) was chosen as template [218]. Models were built based on the target-template alignment using ProMod3. Coordinates which are conserved between the target and the template are copied from the template to the model. Insertions and deletions are remodeled using a fragment library. Side chains are then rebuilt. Finally, the geometry of the resulting model is regularized by using a force field. In case loop modelling with ProMod3 fails, an alternative model is built with PROMOD-II [262]. 3D model was assessed and colored using Pymol (The PyMOL Molecular Graphics System, Version 2.1.1 Schrödinger, LLC.).

Sequence analysis of *SpMTY1*
The sequence of *SpMTY1* was analyzed by aligning *ScMAL31*, *ScAGT1*, *ScMPH2* and *ScMPH3* from *S. cerevisiae* strain S288C (63) and *SeMALT1*, *SeMALT2*, *SeMALT3*, *SeMALT4* from *S. eubayanus* strain CBS 12357^T [90] to the sequence of *SpMTY1* from *S. pastorianus* strain Weihenstephan 34/70 [92] using the Clone manager software (version 9.51, Sci-Ed Software, Denver, Colorado). The origin of nucleotides 969 to 1,639 of *SpMTY1* was further investigated using the blastn function of NCBI (https://www.ncbi.nlm.nih.gov/). The sequence was aligned against *S. cerevisiae* S288C (taxid:559292) to identify closely related homologues. In addition, *SpMTY1* was aligned against the complete nucleotide collection. To avoid matches with genomes harboring an *MTY1* gene, sequences from *S. pastorianus* (taxid:27292), *S. cerevisiae* (taxid:4932), *S. eubayanus* (taxid:1080349), *S. cerevisiae* x *eubayanus* (taxid:1684324) and *S. bayanus (taxid:4931)* were excluded. The most significant alignment was with nucleotides 1,043,930 to 1,044,600 of chromosome VII of *S. paradoxus* strain YPS138 (GenBank: CP020282.1). As the most significant alignment of these nucleotides to *S. cerevisiae* S288C (taxid:559292) was *ScMAL31*, the gene was further referred to as *SparMAL31*.

Analytics
The concentrations of ethanol and of the sugars glucose, maltose and maltotriose were measured using a high pressure liquid chromatography (HPLC) Agilent Infinity 1260 series (Agilent Technologies, Santa Clara, CA) using a Bio-Rad Aminex HPX-87H column

at 65 °C and a mobile phase of 5 mM sulfuric acid with a flow rate of 0.8 mL per minute. Compounds were measured using a RID at 35 °C. Samples were spun down (13,000 g for 5 min) to collect supernatant or 0.2 µm filter-sterilized before analysis. The concentrations of ethylacetate and isoamylacetate, methanol, propanol, isobutanol, isoamyl alcohol and diacetyl were determined as described previously [47].

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Chapter 4

Maltotriose consumption by hybrid *Saccharomyces pastorianus* is heterotic and results from regulatory cross-talk between parental sub-genomes

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Abstract

S. pastorianus strains are hybrids of *S. cerevisiae* and *S. eubayanus* that have been domesticated for several centuries in lager-beer brewing environments. As sequences and structures of *S. pastorianus* genomes are being resolved, molecular mechanisms and evolutionary origin of several industrially relevant phenotypes remain unknown. This study investigates how maltotriose metabolism, a key feature in brewing, may have arisen in early *S. eubayanus* x *S. cerevisiae* hybrids. To address this question, we generated a near-complete genome assembly of Himalayan *S. eubayanus* strains of the Holarctic subclade. This group of strains have been proposed to be the origin of the *S. eubayanus* subgenome of current *S. pastorianus* strains. The Himalayan *S. eubayanus* genomes harbored several copies of an *SeAGT1* *α*-oligoglucoside transporter gene with high sequence identity to genes encountered in *S. pastorianus*. Although Himalayan *S. eubayanus* strains are unable to grown on maltose and maltotriose, their maltose-hydrolase and *SeMALT1* and *SeAGT1* maltose-transporter genes complemented the corresponding null mutants of *S. cerevisiae*. Expression, in a Himalayan *S. eubayanus* strain, of a functional *S. cerevisiae* maltose-metabolism regulator gene (*MALx3*) enabled growth on oligoglucosides. The hypothesis that the maltotriose-positive phenotype in *S. pastorianus* is a result of heterosis was experimentally tested by constructing a *S. cerevisiae* x *S. eubayanus* laboratory hybrid with a complement of maltose-metabolism genes that resembles that of current *S. pastorianus* strains. The ability of this hybrid to consume maltotriose in brewer's wort demonstrated regulatory cross talk between sub-genomes and thereby validated this hypothesis. These results provide experimental evidence of the evolutionary origin of an essential phenotype of lager-brewing strains and valuable knowledge for industrial exploitation of laboratory-made *S. pastorianus*-like hybrids.

Introduction

Saccharomyces pastorianus is an interspecific hybrid of *S. cerevisiae* and *S. eubayanus* [18, 19, 51, 65]. *S. pastorianus* strains are widely used for production of lager beer, which is currently the most produced alcoholic beverage worldwide. Lager brewing requires alcoholic fermentation at relatively low temperatures. *S. pastorianus* was hypothesized to have emerged by spontaneous hybridization and to have colonized early lager-brewing processes due to a combination of cold-tolerance inherited from *S. eubayanus* and superior fermentation kinetics inherited from *S. cerevisiae* [111, 112, 121]. Lager beer is brewed from barley wort, whose sugar composition consists, by weight, of approximately 15% glucose, 60% maltose and 25% maltotriose [78]. During wort fermentation, maltotriose is generally only utilized after glucose and maltose are depleted, while its consumption is also relatively slow and often incomplete [47, 85, 95].

Complete sugar utilization is desirable for lager beer fermentation to optimize concentrations of ethanol and flavor compounds and to avoid residual sweetness [246]. While *S. pastorianus* and *S. cerevisiae* strains are capable of consuming maltotriose, none of the wild isolates of *S. eubayanus* characterized thus far have been shown to possess this trait [34, 90, 111]. These observations led to the hypothesis that the ability of *S. pastorianus* to ferment maltotriose was inherited from *S. cerevisiae* [90, 111, 112, 121, 263].

The genetic information for maltose utilization is well conserved in *Saccharomyces* species and depends on three gene families. *MALT* genes encode plasma-membrane proton symporters with varying substrate specificities and affinities [25, 264], *MALS* genes encode α-glucosidases that hydrolyze *α*-oligoglucosides into glucose, while *MALR* genes encode a regulator required for transcriptional induction of *MALT* and *MALS* genes by maltose [125, 190]. In *Saccharomyces* species, maltose-utilization genes are generally organized in *MAL* loci. These loci contain a *MALT* gene (called *ScMALx1* and *SeMALTx* in *S. cerevisiae* and *S. eubayanus*, respectively), a *MALS* gene referred to as *ScMALx2* or *SeMALSx* and a *MALR* gene referred to as *ScMALx3* or *SeMALRx* [90, 265]. In the absence of glucose and presence of maltose, the MalR regulator binds a bi-directional promoter, thereby simultaneously activating expression of *MALT* and *MALS* genes [266].

The Sc*MAL1*-Sc*MAL4* and Sc*MAL6* loci of *S. cerevisiae* as well as the *SeMAL1*-*SeMAL4* loci of *S. eubayanus* are located in subtelomeric regions [62, 70, 90, 267]. While all *S. cerevisiae* *Sc*Malx1 transporters transport maltose, only *Sc*Mal11 is able to also transport maltotriose [85]. *ScMAL11* (also known as *ScAGT1*) shares only 57% nucleotide identity with other *ScMALx1* genes [87]. The four *SeMALT* (*SeMALT1-4*) genes identified in the genome of the Patagonian type strain CBS12357 of *S. eubayanus* were shown to encode functional maltose transporters, but none of these genes enabled maltotriose transport [90]. While no clear *ScAGT1* ortholog was found in *S. eubayanus* CBS12357^{T}, such an ortholog was recently found in the genomes of two North American isolates assigned to the Holarctic subclade of *S. eubayanus* [34].

S. pastorianus inherited *MAL* genes from both *S. cerevisiae* and *S. eubayanus* [18, 51, 268]. However, the *S. cerevisiae*-derived maltotriose- transporter gene *ScAGT1* is truncated and, therefore, non-functional in *S. pastorianus* [95]. Instead, maltotriose consumption by *S. pastorianus* strains was attributed to *SeAGT1* and *SpMTY1*/*SpMTT1* genes [89, 91, 92, 220]. In *S. pastorianus*, *SeAGT1* is located on *S. eubayanus* CHRXV and was therefore, already before the identification of an *AGT1* ortholog in Holarctic *S. eubayanus* strains [34], assumed to originate from *S. eubayanus* [18]. *SpMTY1,* also referred to as *SpMTT1,* is located on *S. cerevisiae* CHRVII and has less than 92% sequence identity with other

Saccharomyces maltose transporters [91]. However, *SpMTY1* contains sequence patches with high similarity to maltose transporters from *S. eubayanus* and *S. paradoxus* [100]. Recently, two independent laboratory evolution studies with *S. eubayanus* demonstrated that recombination of different *SeMALT* genes yielded chimeric, neo-functionalized genes that encoded maltotriose transporters [34, 100]. *SpMTY1* may have resulted from successive introgressions of maltose-transporter genes from *S. cerevisiae*, *S. eubayanus* and *S. paradoxus*.

Recently made *S. cerevisiae* x *S. eubayanus* laboratory hybrids showed similar lager-brewing performance as *S. pastorianus* strains, also with respect to maltotriose utilization [111, 112, 248, 263]. In these hybrids, maltotriose consumption depended on the presence of a functional *Sc*Agt1 transporter encoded by the *S. cerevisiae* subgenome [29]. However, in view of the non-functionality of *ScAGT1* in current *S. pastorianus* strains, these laboratory hybrids did not fully recapitulate the genetic landscape of *S. pastorianus* with respect to maltotriose fermentation [18, 111, 248].

Studies on laboratory hybrids based on *S. eubayanus* strains whose genomes are more closely related to the *S. eubayanus* subgenome of *S. pastorianus* strains than that of the Patagonian type strain CBS12357 might generate new insights into the evolution of maltotriose utilization in *S. pastorianus*. To date, Himalayan *S. eubayanus* isolates show the highest sequence identity with the *S. eubayanus* sub-genome of *S. pastorianus*, with up to 99.82% identity, as opposed to 99.56% for *S. eubayanus* CBS12357^{T} [58].

Here, we investigated if and how the genomes of Himalayan *S. eubayanus* strains could have contributed to maltotriose utilization in the earliest hybrid ancestors of current *S. pastorianus* strains. To this end, we generated chromosome-level genome assemblies of these strains by long-read DNA sequencing. Since the Himalayan strains were unable to utilize maltotriose, we functionally characterized the assembled *MAL* genes and identified genetic determinants that prevented maltotriose utilization. Subsequently, a laboratory hybrid of a representative Himalayan *S. eubayanus* strain and a maltotriose-deficient ale strain of *S. cerevisiae* was generated to investigate the genetics of maltotriose utilization in a hybrid context. We discuss the implications of the experimental results for the proposed role and origin of *SeAGT1* in *S. pastorianus* and for the potential of hybridization to enable maltotriose consumption in novel *Saccharomyces* hybrids.

Materials and methods

Strains and maintenance

All strains used in this study are listed in Table 1. Stock cultures of *S. eubayanus* and *S. cerevisiae* strains were grown in YPD (10 g L^{-1} yeast extract, 20 g L^{-1} peptone and 20 g L^{-1} glucose) until late exponential phase, complemented with sterile glycerol to a final concentration of 30 % (v/v) and stored at -80 °C as 1 mL aliquots until further use.

Table 1: *Saccharomyces* strains used in this study. The abbreviation *malΔ* indicates *mal11-mal12*::loxP *mal21-mal22*::loxP *mal31-32*::loxP. Mal and Mtt denote the maltose and maltotriose phenotype respectively.

Name	Species	Relevant genotype	Origin
CDFM21L.1	*S. eubayanus*	Wildtype Mal^- Mtt^-	[58]
ABFM5L.1	*S. eubayanus*	Wildtype Mal^- Mtt^-	[58]
CBS 12357	*S. eubayanus*	Wildtype Mal^+ Mtt^-	[19] Westerdijk institute[a]
CBS 1483	*S. pastorianus*	Wildtype Mal^+ Mtt^+	Westerdijk institute[a]
CEN.PK113-7D	*S. cerevisiae*	*MATa MAL1x MAL2x MAL3x MAL4x MAL2-8C SUC2 LEU2 URA3*	[139]
IMZ616	*S. cerevisiae*	*MATa ura3-52 LEU2 MAL2-8C malΔ mp2/3hΔ suc2Δ ima1Δ* ima2Δ *ima3Δ ima4Δ ima5Δ* pUDC156 (*Spcas9 URA3 ARS4 CEN6*)	[138]
IMX1365	*S. cerevisiae*	*MATa ura3-52 LEU2 MAL2-8C malΔ mph2/3Δ suc2Δ ima1Δ ima2Δ ima3Δ ima4Δ ima5Δ* pUDC156 (*URA3 cas9*) *sga1Δ:: $ScTDH3_p$-ScMAL12-ScADH1$_t$ $ScTEF1_p$-ScAGT1-ScCYC1$_t$*	[90]
IMX1702	*S. cerevisiae*	*MATa ura3-52 LEU2 MAL2-8C malΔ mph2/3Δ suc2Δ ima1Δ ima2Δ ima3Δ ima4Δ ima5Δ* pUDC156 (*URA3 cas9*) *sga1Δ:: $ScTDH3_p$-ScMAL12-ScADH1$_t$ $ScTEF1_p$-SeMALT1-ScCYC1$_t$*	This study
IMX1704	*S. cerevisiae*	*MATa ura3-52 LEU2 MAL2-8C malΔ mph2/3Δ suc2Δ ima1Δ ima2Δ ima3Δ ima4Δ ima5Δ* pUDC156 (*URA3 cas9*) *sga1Δ:: $ScTDH3_p$-ScMAL12-ScADH1$_t$ $ScTEF1_p$-SeMALT2-ScCYC1$_t$*	This study
IMX1706	*S. cerevisiae*	*MATa ura3-52 LEU2 MAL2-8C malΔ mph2/3Δ suc2Δ ima1Δ ima2Δ ima3Δ ima4Δ ima5Δ* pUDC156 (*URA3 cas9*) *sga1Δ:: $ScTDH3_p$-ScMAL12-ScADH1$_t$ $ScTEF1_p$-SeMALT3-ScCYC1$_t$*	This study
IMX 1708	*S. cerevisiae*	*MATa ura3-52 LEU2 MAL2-8C malΔ mph2/3Δ suc2Δ ima1Δ ima2Δ ima3Δ ima4Δ ima5Δ* pUDC156 (*URA3 cas9*) *sga1Δ:: $ScTDH3_p$-ScMAL12-ScADH1$_t$ $ScTEF1_p$-SeAGT1-ScCYC1$_t$*	This study
IMX1313	*S. cerevisiae*	*MATa ura3-52 LEU2 MAL2-8C malΔ mph2/3Δ suc2Δ ima1Δ ima2Δ ima3Δ ima4Δ ima5Δ* sga1*Δ*::*$ScTEF1_p$-ScMAL31-ScCYC1$_t$* pUDC156 (*URA3 cas9*)	This study
IMX1313Δ	*S. cerevisiae*	*MATa ura3-52 LEU2 MAL2-8C malΔ mph2/3Δ suc2Δ ima1Δ ima2Δ ima3Δ ima4Δ ima5Δ* sga1*Δ*::*$ScTEF1_p$-ScMAL31-ScCYC1$_t$*	This study

IMZ752	*S. cerevisiae*	*MATa ura3-52 LEU2 MAL2-8C malΔ mph2/3Δ suc2Δ ima1Δ ima2Δ ima3Δ ima4Δ ima5Δ* sga1*Δ*::*ScTEF1*$_p$-*ScMAL31-ScCYC1*$_t$ pUDE843 (*ori* (*ColE1*) *bla* 2µ *ScTDH3*$_p$-*SeMALS1-ScADH1*$_t$ *URA3*)	This study
IMZ753	*S. cerevisiae*	*MATa ura3-52 LEU2 MAL2-8C malΔ mph2/3Δ suc2Δ ima1Δ ima2Δ ima3Δ ima4Δ ima5Δ* sga1*Δ*::*ScTEF1*$_p$-*ScMAL31-ScCYC1*$_t$ pUDE844 (*ori* (*ColE1*) *bla* 2µ *ScTDH3*$_p$-*SeMALS2-ScADH1*$_t$ *URA3*)	This study
IMK820	*S. eubayanus*	*MATa/MATα Semalt1Δ/Semalt1Δ*	This study
IMK823	*S. eubayanus*	*MATa/MATα Seagt1Δ/Seagt1Δ* (X3)	This study
IMX1939	*S. eubayanus*	*MATa/MATα Semalt1Δ/Semalt1Δ Sesga1Δ*::*ScMAL13/Sesga1Δ*::*ScMAL13*	This study
IMX1940	*S. eubayanus*	*MATa/MATα Seagt1Δ/Seagt1Δ Sesga1Δ*::*ScMAL13/Sesga1Δ*::Sc*MAL13*	This study
IMX1762	*S. eubayanus*	*MATa/MATα Sesga1Δ*::*ScMAL12/Sesga1Δ*::Sc*MAL12*	This study
IMX1765	*S. eubayanus*	*MATa/MATα Sesga1Δ*::*ScMAL13/Sesga1Δ*::Sc*MAL13*	This study
CBC-1	*S. cerevisiae*	*MATa/MATα* Mal$^+$ Mtt$^-$	Lallemand
HTSH020	*S. cerevisiae X S. eubayanus*	*MATa/MATα* Mal$^+$ Mtt$^+$	This study

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Media and cultivation

S. eubayanus batch cultures were grown on synthetic medium (SM) containing 3.0 g L^{-1} KH_2PO_4, 5.0 g L^{-1} $(NH_4)_2SO_4$, 0.5 g L^{-1} $MgSO_4.7H_2O$, 1 mL L^{-1} trace element solution, and 1 mL L^{-1} vitamin solution [140]. The pH was set to 6.0 with 2 M KOH prior to autoclaving at 120 °C for 20 min. Vitamin solutions were sterilized by filtration and added to the sterile medium. Concentrated sugar solutions were autoclaved at 110 °C for 20 min or filter sterilized and added to the sterile flasks to give a final concentration of 20 g L^{-1} glucose (SMG), maltose (SMM) or maltotriose (SMMt). With the exception of IMZ752 and IMZ753, *S. cerevisiae* batch cultures were grown on SM supplemented with 150 mg L^{-1} uracil [269] to compensate for loss of plasmid pUDC156 that carried the *Spcas9* endonuclease gene, and supplemented with 20 g L^{-1} glucose (SM_uG), maltose (SM_uM) or maltotriose (SM_uMt). All batch cultures were grown in 250 mL shake flasks with a working volume of 50 mL. The cultures were inoculated at an initial OD_{660nm} of 0.1 and incubated under an air atmosphere and shaken at 200 rpm and at 20 °C in a New Brunswick™ Innova44 incubator (Eppendorf Nederland B.V, Nijmegen, The Netherlands).

S. eubayanus strains transformed with plasmids pUDP052 ($gRNA_{SeSGA1}$), pUDP091 ($gRNA_{SeMALT1}$) and pUDP090 ($gRNA_{SeAGT1}$) were selected on modified SMG medium ($SM_{Ace}G$) in which $(NH_4)_2SO_4$ was replaced by 6.6 g L^{-1} K_2SO_4 and 10 mM acetamide [141]. SM-based solid media contained 2 % Bacto Agar (BD Biosciences, Franklin Lakes, NJ). *S. cerevisiae* strains expressing either *SeMALT, SeMALS* or *ScMALR* were selected on $SM_{Ace}G$. For plasmid propagation, *E. coli* XL1-Blue-derived strains (Agilent Technologies, Santa Clara, CA) were grown in Lysogeny Broth medium (LB, 10 g L^{-1} tryptone, 5 g L^{-1} yeast extract, 5 g L^{-1} NaCl) supplied with 100 mg L^{-1} ampicillin. Synthetic wort medium (SWM) for growth studies contained 14.4 g·L^{-1} glucose, 2.3 g·L^{-1} fructose, 85.9 g·L^{-1} maltose, 26.8 g·L^{-1} maltotriose, 5 g·L^{-1} $(NH_4)_2SO_4$, 3 g·L^{-1} KH_2PO_4, 0.5 g·L^{-1} $MgSO_4.7H_2O$, 1 mL·L^{-1} trace element solution, 1 mL·L^{-1} vitamin solution, supplemented with the anaerobic growth factors ergosterol and Tween 80 (0.01 g·L^{-1} and 0.42 g·L^{-1} respectively), as previously described [140].

Industrial wort (containing 14.4 g L^{-1} glucose, 85.9 g L^{-1} maltose, 26.8 g L^{-1} maltotriose, 2.3 g L^{-1} fructose and 269 mg L^{-1} FAN) was provided by Heineken Supply Chain B.V. (Zoeterwoude, the Netherlands). The wort was supplemented with 1.5 mg L^{-1} of Zn^{2+} by addition of $ZnSO_4.7H_2O$, autoclaved for 30 min at 121 ºC, filtered using Nalgene 0.2 µm SFCA bottle top filters (Thermo Scientific) and diluted with sterile demineralized water. Sporulation medium consisted of 2 % (w/v) potassium acetate in MilliQ water set to pH 7.0 with KOH, autoclaved at 121 °C for 20 min.

Microaerobic growth experiments

Microaerobic cultures were grown in 250-mL airlock-capped Neubor infusion bottles (38 mm neck, Dijkstra, Lelystad, Netherlands) containing 200 mL three-fold diluted industrial wort supplemented with 0.4 mL L^{-1} Pluronic antifoam (Sigma-Aldrich, St. Louis, MO). Bottle caps were equipped with a 0.5 mm x 16 mm Microlance needle (BD Biosciences) sealed with cotton to prevent pressure build-up. Sampling was performed aseptically with 3.5 mL syringes using a 0.8 mm x 50 mm Microlance needle (BD Biosciences). Microaerobic cultures were inoculated at an OD_{660nm} of 0.1 from stationary-phase precultures in 50 mL Bio-One Cellstar Cellreactor tubes (Sigma-Aldrich) containing 30 mL of the same medium, grown for 4 days at 12 °C. Bottles were incubated at 12 °C and shaken at 200 rpm in a New Brunswick Innova43/43R shaker (Eppendorf Nederland B.V.). At regular intervals, 3.5 mL samples were collected in 24 deep-well plates (EnzyScreen BV, Heemstede, Netherlands) using a LiHa liquid handler (Tecan, Männedorf, Switzerland) to measure OD_{660nm} and external metabolites. 30 µL of each sample was diluted 5 fold in demineralized water in a 96 well plate and OD_{660nm} was measured with a Magellan Infinite 200 PRO spectrophotometer (Tecan). From the remaining sample, 150 µL was vacuum filter sterilized using 0.2 µm Multiscreen filter plates (Merck, Darmstadt, Germany) for HPLC measurements.

Analytical methods

Optical densities of yeast cultures were measured with a Libra S11 spectrophotometer (Biochrom, Cambridge, United Kingdom) at a wavelength of 660 nm. Biomass dry weight was measured by filtering 10-mL culture samples over pre-weighed nitrocellulose filters with a pore size of 0.45 µm. Filters were washed with 10 mL water, dried in a microwave oven (20 min at 350 W) and reweighed. Sugars were measured using a high pressure liquid chromatography Agilent Infinity 1260 series (Agilent Technologies) using a Bio-Rad Aminex HPX-87H column at 65 °C with 5 mM sulfuric acid at a flow rate of 0.8 mL min^{-1}. Compounds were measured using a RID at 35 °C. Samples were centrifuged at 13,000 g for 5 min to collect supernatant or 0.2 µm filter-sterilized before analysis.

Plasmid construction

Plasmids used and constructed in this study are listed in Table 2, oligonucleotide primers used in this study are listed in Supplementary Table 1. Coding regions of *SeMALT1*, *SeMALT2*, *SeMALT3* and *SeAGT1* were amplified from CDFM21L.1 genomic DNA with Phusion High-Fidelity DNA polymerase (Thermo Scientific), according to the supplier's instructions with primers pairs 12355/12356, 12357/12358, 12359/12360 and 12361/12362, respectively. The coding sequence of *ScMAL31* was amplified from CEN.PK113-7D genomic DNA with Phusion High-Fidelity DNA polymerase (Thermo Scientific), according to the supplier's instructions with primer pairs 9942/9943. Each primer carried a 40 bp extension complementary to the plasmid backbone of p426-TEF-amdS [135], which was PCR amplified using Phusion High-Fidelity DNA polymerase

(Thermo Scientific) and primers 7812/5921. Each transporter fragment was assembled with the p426-TEF-amdS backbone fragment using NEBuilder HiFi DNA Assembly (New England Biolabs, Ipswich, MA), resulting in plasmids pUD444 (*ScMAL31*), pUD794 (*SeMALT1*), pUD795 (*SeMALT2*), pUD796 (*SeMALT3*) and pUD797 (*SeAGT1*). All plasmids were verified for correct assembly by Sanger sequencing (Baseclear, Leiden, The Netherlands).

Table 2 Plasmids used in this study

Plasmid	Relevant genotype	Origin
p426-TEF-amdS	*ori* (*ColE1*) *bla* 2µ amdSYM *TEF1*$_{pr}$-*CYC1*$_{ter}$	[135]
pUD444	*ori* (*ColE1*) *bla* 2µ amdSYM *ScTEF1*$_{pr}$-*ScMAL31-ScCYC1*$_{ter}$	This study
pUD794	*ori* (*ColE1*) *bla* 2µ amdSYM *ScTEF1*$_{pr}$-*SeMALT1-ScCYC1*$_{ter}$	This study
pUD795	*ori* (*ColE1*) *bla* 2µ amdSYM *ScTEF1*$_{pr}$-*SeMALT2-ScCYC1*$_{ter}$	This study
pUD796	*ori* (*ColE1*) *bla* 2µ amdSYM *ScTEF1*$_{pr}$-*SeMALT3-ScCYC1*$_{ter}$	This study
pUD797	*ori* (*ColE1*) *bla* 2µ amdSYM *ScTEF1*$_{pr}$-*SeAGT1-ScCYC1*$_{ter}$	This study
pUDE044	*ori* (*ColE1*) *bla* 2µ *ScTDH3*$_{pr}$-*ScMAL12-ScADH1*$_{ter}$ *URA3*	[260]
pUDE843	*ori* (*ColE1*) *bla* 2µ *ScTDH3*$_{pr}$-*SeMALS1-ScADH1*$_{ter}$ *URA3*	This study
pUDE844	*ori* (*ColE1*) *bla* 2µ *ScTDH3*$_{pr}$-*SeMALS2-ScADH1*$_{ter}$ *URA3*	This study
pUDE780	*ori* (*ColE1*) *bla* 2µ *ScPGK1*$_{pr}$-*ScMAL13-ScTEF2*$_{ter}$ *URA3*	This study
pUDP002	*ori* (*ColE1*) *bla* panARSopt *Hyg ScTDH3*$_{pr}$-BsaI-BsaI-*ScCYC1*$_{ter}$ *AaTEF1*$_{pr}$-*Spcas9*$^{D147Y\ P411T}$-*ScPHO5*$_{ter}$	[144]
pUDP004	ori (ColE1) *bla* panARSopt amdSYM *ScTDH3*$_{pr}$-BsaI-BsaI-Sc*CYC1*$_{ter}$ *AaTEF1*$_{pr}$-*Spcas9*$^{D147Y\ P411T}$-*ScPHO5*$_{ter}$	[142]
pUDP052	ori (ColE1) *bla* panARSopt amdSYM *ScTDH3*$_{pr}$- gRNA$_{SGA1}$-*ScCYC1*$_{ter}$ *AaTEF1*$_{pr}$-*Sp*cas9$^{D147Y\ P411T}$-*ScPHO5*$_{ter}$	[90]
pUDP091	ori (ColE1) *bla* panARSopt amdSYM *ScTDH3*$_{pr}$- gRNA$_{SeMALT1}$-*ScCYC1*$_{ter}$ *AaTEF1*$_{pr}$-*Sp*cas9$^{D147Y\ P411T}$-*ScPHO5*$_{ter}$	This study
pUDP090	ori (ColE1) *bla* panARSopt amdSYM *ScTDH3*$_{pr}$- gRNA$_{SeAGT1}$-*ScCYC1*$_{ter}$ *AaTEF1*$_{pr}$-*Sp*cas9$^{D147Y\ P411T}$-*ScPHO5*$_{ter}$	This study
pUDR119	ori (ColE1) *bla* 2µ amdSYM *SNR52*$_{pr}$-gRNA$_{ScSGA1}$-*SUP4*$_{ter}$	[146]
pYTK074	ori (ColE1) *cat URA3*	[270]
pYTK082	*cat* 2µ	[270]
pYTK083	ori (ColE1) *bla*	[270]
pUD631	ori (ColE1) *bla* gRNA$_{SeMALT1}$	[90]
pUD634	ori (ColE1) *bla* gRNA$_{SeAGT1}$	This study
pUDC156	ori (ColE1) *bla ARS4 CEN6 URA3 Spcas9*	[138]

SeMALS1 and *SeMALS2* were amplified from CDFM21L.1 genomic DNA with Phusion High-Fidelity DNA polymerase (Thermo Scientific), with primers pairs 14451/14453 and 14452/14453, respectively. Each primer pair carried a 30 bp extension complimentary to the plasmid backbone of pUDE044 [260] which was PCR amplified using Phusion High-Fidelity DNA polymerase (Thermo Scientific) and primers 14449/14450. Resulting amplicons were assembled using NEBuilder HiFi DNA Assembly (New England Biolabs), resulting in plasmids pUDE843 (*SeMALS1*) and pUDE844 (*SeMALS2*) that were verified by Sanger sequencing (Baseclear).
S. cerevisiae ScMAL13, the *ScPGK1* promoter and the *ScTEF2* terminator were amplified from CEN.PK113-7D genomic DNA with Phusion High-Fidelity DNA polymerase (Thermo Scientific), with primer pairs 12915/12916, 9421/9422 and 10884/10885, respectively. Fragments were gel purified and used with pYTK074, pYTK082 and pYTK083 in Golden Gate assembly according to the yeast toolkit protocol [270] resulting in pUDE780, which was verified by Sanger sequencing (Baseclear).

Guide-RNA (gRNA) sequences for deletion of *SeMALT1* and *SeAGT1* in CDFM21L.1 were designed as described previously [142]. The DNA sequences encoding these gRNAs were synthesised at GeneArt (Thermo Scientific) and were delivered in pUD631 and pUD634, respectively. The gRNA spacer sequences (Se*MALT1* 5' CCCCGATATTCTTTACACTA 3', Se*AGT1* 5'- AGCTTTGCGAAAATATCCAA -3') and the structural gRNA sequence were flanked at their 5' ends by the Hammerhead ribozyme (HH) and at their 3' ends by the Hepatitis Delta Virus ribozyme (HDV) [143]. The HH-gRNA-HDV fragment was flanked on both ends with a BsaI site for further cloning [142, 144]. Plasmids pUDP091 ($gRNA_{SeMALT1}$) and pUDP090 ($gRNA_{SeAGT1}$) were constructed by Golden Gate cloning by digesting pUDP004 and the gRNA-carrying plasmid (pUD631 and pUD634, respectively) using BsaI and ligating with T4 ligase [271]. Correct assembly was verified by restriction analysis with PdmI (Thermo Scientific) and Sanger sequencing (Baseclear).

Strain construction

S. cerevisiae IMZ616, which cannot grow on α-glucosides [138], was used as a host to test functionality of individual *S. eubayanus* (putative) maltose transporter genes [90]. *S. cerevisiae* IMX1702 was constructed by integrating $ScTDH3_p$-*ScMAL12*-$ScADH1_t$ and $ScTEF1_p$-*SeMALT1*-$ScCYC1_t$ at the *ScSGA1* locus of strain IMZ616. A fragment containing the $ScTDH3_p$-*ScMAL12*-$ScADH1_t$ transcriptional unit was PCR amplified using Phusion High-Fidelity DNA polymerase (Thermo Scientific) from pUDE044 with primers 9596/9355, which included a 5' extension homologous to the upstream region of the *ScSGA1* locus and an extension homologous to the co-transformed transporter fragment, respectively. The DNA fragment carrying the *S. eubayanus SeMALT1* maltose symporter ($ScTEF1_p$-*SeMALT1*-$ScCYC1_t$) was PCR amplified from pUD794 using primers 9036/9039, which included a 5' extension homologous to the co-transformed transporter fragment and an extension homologous to the downstream region of the *ScSGA1* locus, respectively. To facilitate integration in strain IMZ616, the two PCR fragments were co-transformed with plasmid pUDR119 (*amdS*), which expressed a gRNA targeting *ScSGA1* (spacer sequence: 5'-ATTGACCACTGGAATTCTTC-3'). The plasmid and repair fragments were transformed using the LiAc yeast transformation protocol [146, 153] and transformed cells were plated on $SM_{Ace}G$. Correct integration was verified by diagnostic PCR with primers pairs 4226/4224. Strains *S. cerevisiae* IMX1704, IMX1706 and IMX1708 were constructed following the same principle, but instead of using pUD794 to generate the transporter fragment, pUD795, pUD796 and pUD797 were used to PCR amplify $ScTEF1_p$-*SeMALT2*-$ScCYC1_t$, $ScTEF1_p$-*SeMALT3*-$ScCYC1_t$ and $ScTEF1_p$-*SeAGT1*-$ScCYC1_t$ respectively. IMX1313 was constructed in a similar way using only $ScTEF1_p$-*ScMAL31*-$ScCYC1_t$ amplified with primer pair 9036/11018 which contain 5'and 3' extensions homologous to the upstream and downstream region of the *ScSGA1* locus. Correct integration was verified by diagnostic PCR with primer pair 4226/4224 (Supplementary Figure 1). All PCR-amplified gene sequences were Sanger sequenced (BaseClear). IMX1313 was grown on YPD to lose pUDR119 (*URA3*) and pUDC156 (amdSYM). An isolate unable to grown on SMG without uracil and with acetamide was selected and named IMX1313Δ. This strain was able to grow on SMG supplemented with 150 mg L^{-1} uracil.

To assess functionality of CDFM21L.1 *SeMALS1*, IMX1313Δ was transformed with 100 ng pUDE843 ($ScTDH3_p$-*SeMALS1*-$ScADH1_t$) by electroporation [142], resulting in strain IMZ752. Transformants were selected on SMG plates after 5 days of incubation at 20 °C and validated by PCR (DreamTaq polymerase, Thermo Scientific) using primer pair 14454/14455 (Supplementary Figure 1). Similarly, functionality of the *SeMALS2* maltase

gene of CDFM21L.1 was assessed by transforming IMX1313Δ with pUDE844 (*ScTDH3*$_{pr}$-*SeMALS2-ScADH1*$_{ter}$), resulting in strain IMZ753.
S. eubayanus IMK820 (*SemalT1Δ*) was constructed by transforming CDFM21L.1 with 200 ng of pUDP091 and 1 µg of 120 bp repair fragment obtained by mixing an equimolar amount of primers 12442/12443, as previously described [142]. As a control, the same transformation was performed without including the repair DNA fragment. Transformants were selected on SM$_{Ace}$G plates. *S. eubayanus* IMK823 (*Seagt1Δ*) was constructed similarly, using pUDP090 and primer pair 11320/11321. Deletion of *SemalT1* was verified by PCR with primer pair 11671/11672 and Sanger sequencing. The *Seagt1* deletion was verified by PCR using primer pair 12273/12274, and by Illumina whole-genome sequencing and read alignment to the reference genome of CDFM21L.1 (Bioproject accession number PRJNA528469).
Strains IMX1765, IMX1939 and IMX1940 were constructed by inserting *ScPGK1*$_p$-*ScMAL13-ScTEF2*$_t$ at the *SeSGA1* locus of CDFM21L.1, IMK820 and IMK823, respectively. A repair fragment containing *ScPGK1*$_p$-*ScMAL13-ScTEF2*$_t$ was amplified from pUDE780 with primer pair 12917/12918. Strains CDFML21L.1, IMK820 and IMK823 were transformed by electroporation by addition of 350 ng of repair fragment and 560 ng pUDP052 (*amdSYM*) into the cells as previously described [142]. Transformants were plated on SM$_{Ace}$G and incubated at 20 °C. IMX1762 was constructed similarly using a repair fragment with *ScTDH3*$_p$-*ScMAL12-ScADH1*$_t$ amplified from pUDE044 with primer pair 12319/12320. Strains were verified by PCR using primer pair 12635/12636 and Sanger sequencing.

Hybrid Construction
The *S. cerevisiae* X *S. eubayanus* hybrid HTSH020 was constructed by spore-to spore-mating. The *S. eubayanus* strain CDFM21L.1 and the *S. cerevisiae* strain CBC-1 were grown in 20 mL YPD at 20 °C until late exponential phase. Cells were centrifuged for 5 min at 1000 g and washed twice in demineralized water. Cells were re-suspended in 20 mL sporulation medium and incubated for 64 h at 20 °C. Presence of spores was verified by microscopy. Spores were harvested by centrifugation for 5 min at 1000 g, and washed with demineralized water, resuspended in 100 µL demineralized water containing 100 U/mL of Zymolyase (MP Bio, Santa Ana, CA) and incubated for 10 min at 30 °C. Spores were washed and plated on the edge of a YPD agar plate. Spores from the two strains were brought in contact with each other with an MSM System 400 micromanipulator (Singer Instruments, Watchet, United Kingdom). Zygote formation was observed after 6-8 h. Emerging colonies were re-streaked twice on SM 2% maltose at 12 °C. Successful hybridization was verified by multiplex PCR using DreamTaq DNA polymerase (Thermo Scientific), by amplifying the *S. cerevisiae* specific *MEX67* gene with primer pairs 8570/8571 and by amplifying the *S. eubayanus* specific gene *SeFSY1* with primers 8572/8573 (Supplementary Figure 2), as previously described [256].

Illumina sequencing
Genomic DNA of *S. eubayanus* strains CDFM21L.1 and ABFM5L.1, *S. cerevisiae* strain CBC-1 and *S. cerevisiae* x *S. eubayanus* strain HTSH020 was isolated as previously described [51]. Paired-end sequencing (2 X 150 bp) was performed on a 350 bp PCR-free insert library using Illumina HiSeq2500 (San Diego, CA) by Novogene (HK) Company Ltd (Hong Kong, China). Genomic DNA of the strains CBC-1 and HTSH020 was sequenced in house on a MiSeq sequencer (Illumina) with 300 bp paired-end reads using PCR-free library preparation. Sequence data are available at NCBI under Bioproject accession number PRJNA528469.

MinION sequencing
For long-read sequencing, a 1D sequencing library (SQK-LSK108) was prepared for CDFM21L.1 and CBC-1 and loaded onto a FLO-MIN106 (R9.4) flow cell, connected to a MinION Mk1B unit (Oxford Nanopore Technology, Oxford, United Kingdom), according to the manufacturer's instructions. MinKNOW software (version 1.5.12; Oxford Nanopore Technology) was used for quality control of active pores and for sequencing. Raw files generated by MinKNOW were base-called using Albacore (version 1.1.0; Oxford Nanopore Technology). Reads with a minimum length of 1000 bp were extracted in fastq format. For CDFM21L.1, 3.26 Gb of sequence with an average read length of 8.07 kb was obtained and for CBC-1 3.04 Gb sequence with an average read length of 7.27 kb. Sequencing data are available at NCBI under Bioproject accession number PRJNA528469.

De novo *assembly*
De novo assembly of the Oxford Nanopore MinION dataset was performed using Canu (v1.4, setting: genomesize=12m) [154]. Assembly correctness was assessed using Pilon [155] and further corrected by "polishing" of sequencing/assembly errors by aligning Illumina reads with BWA [156] using correction of only SNPs and short indels (–fix bases parameter). For HTSH020, an artificial reference genome was made by combining the assembly of CBC-1 and CDFM21L.1. The genome assemblies were annotated using the MAKER2 annotation pipeline (version 2.31.9) [157], using SNAP (version 2013–11-29) [158] and Augustus (version 3.2.3) [159] as *ab initio* gene predictors. *S. cerevisiae* S288C EST and protein sequences were obtained from SGD (*Saccharomyces* Genome Database, http://www.yeastgenome.org/) and were aligned using BLASTX on the obtained polished sequence assembly (BLAST version 2.2.28+) [160]. Predicted translated protein sequences of the final gene model were aligned to the *S. cerevisiae* S288C protein Swiss-Prot database using BLASTP (http://www.uniprot.org/). Custom-made Perl scripts were used to map systematic names to the annotated gene names. Error rates in nanopore-sequencing data were estimated from the q score (Phred scaled) per read, as calculated by the base caller Albacore (version 1.1.0) (Oxford Nanopore Technology). Average q score was used to calculate the error $P = 10^{q/10}$.

RNA isolation
CDFM21L.1, IMX1765, IMX1939 and HTS020 were grown in SMG, SMM and SMMt until mid-exponential phase (OD_{660nm} of 12 for SMG/SMM and of OD_{660nm} 15 for SMMt). Culture samples corresponding to ca. 200 mg of biomass dry weight were directly quenched in liquid nitrogen. The samples were processed and total RNA extracted as previously described [110]. Prior to cDNA synthesis, purity, concentration and integrity of the RNA in the samples was assessed with Nanodrop (Thermo Scientific), Qubit (Thermo Scientific) and Tapestation 220 with RNA Screen Tape (Agilent Technologies),

respectively, according the manufacturers' recommendations. cDNA libraries were prepared using the TruSeq RNA V2 kit (Illumina). Paired-end sequencing (2 X 150 bp) was performed on a 300 bp PCR-free insert library on a HISeq 2500 (Illumina) at Novogene (HK) Company Ltd (Hong Kong, China). Duplicate biological samples were processed, generating an average sequence quantity of 23.7M reads per sample. Reads were aligned to the CDFM21L.1 reference assembly (GEO accession number) using a two-pass STAR [161] procedure. In the first pass, splice junctions were assembled and used to inform the second round of alignments. Introns between 15 and 4000 bp were allowed, and soft clipping was disabled to prevent low-quality reads from being spuriously aligned. Ambiguously-mapped reads were removed from the dataset. Expression level for each transcript were quantified using htseq-count [162] in union mode. Fragments per kilo-base of feature (gene) per million reads mapped (FPKM) values were calculated by "Applying the fpkm method" from the edgeR package [163, 164]. Differential expression analysis was performed using DESeq [165].

Results

Sequencing of Himalayan *S. eubayanus* strains revealed variations of sub-telomeric regions and presence of novel putative maltose transporter genes.

It has been proposed that the *S. eubayanus* genetic pool of *S. pastorianus* was inherited from an ancestor of the Asian *S. eubayanus* lineage [58]. With 99.82% identity, the Himalayan *S. eubayanus* strains CDFM21L.1 and ABFM5L.1 that belong to the Holarctic lineage [61], present the closest characterized relatives of the *S. eubayanus* ancestor of lager brewing yeasts. However, this distance was based on a limited sequencing space [58] and the analysis did not investigate the presence of specific *S. eubayanus* genetic markers found in *S. pastorianus* hybrids. Therefore, the genome of the Himalayan *S. eubayanus* strain CDFM21L.1 was sequenced with a combination of long-read and short-read techniques (Oxford Nanopore MinION and Illumina technologies, respectively) to generate a near-complete draft reference genome sequence. The resulting CDFM21L.1 genome assembly comprised 19 contigs, including its mitochondrial genome. All chromosomes were completely assembled from telomere to telomere, except for chromosome XII, which was fragmented into 3 contigs due to the repetitive rDNA region and manually assembled into a single scaffold. With a total size of 12,034,875 bp and a set of X ORFs, this assembly represents the first near-complete draft genome of a *S. eubayanus* strain of the Holarctic clade [61].

Chromosome-level assemblies were hitherto only available for the Patagonia B-clade strain CBS 12357^{T} [19, 90]. We identified three major structural differences in CDFM21L.1 relative to CBS12357^{T} using Mauve [272]: (i) a paracentric inversion in the sub-telomeric region of chromosome VII, involving approximately 8 kbp, (ii) a translocation of approximately 12 kbp from the left sub-telomeric region of chromosome VIII to the right sub-telomeric region of chromosome VI, and (iii) a reciprocal translocation between approximately 20 kbp from the right sub-telomeric region of chromosome V and approximately 60 kbp from the center of chromosome XII (Figure 1A). All structural variation involved sub-telomeric regions, in accordance with their known relative instability [94].

An alignment comparison of the CDFM21L.1 and CBS 12357^{T} genomes with MUMmer revealed that 557 kb were unique to CDFM21L.1 and reciprocally 428 kb were unique to CBS 12357^{T}. Sequences unique to CBS 12357^{T} (3.6% of its genome) and to CDFM21L.1 (4.6% of its genome) were located primarily in sub-telomeric regions and in repetitive regions, such as rDNA on chromosome XII (Figure 1B). Out of the 32 sub-telomeric regions, 23 exhibited absence of synteny. Conserved synteny was observed for sub-telomeric regions on CHRIII (left), CHRIV (left and right), CHRVI (left), CHRIX (right), CHRXI (right), CHRXII (right), CHRXIV (left) and CHRXV (right) (Supplementary File 1).

The 428 kb of sequence that were absent in the Himalayan *S. eubayanus* strain included 99 annotated ORFs (Supplementary File 1). Of the 99 ORFs that were (partly) affected, 11 were completely absent in CDFM21L.1, involving mostly genes implicated in iron transport facilitation (Supplementary File 1). The 557 kb of sequence that was not present in CBS 12357^{T} included 113 annotated ORFs (Supplementary File 1). Of these 113 ORFs, 15 were completely absent in CBS 12357^{T}. These 15 ORFs showed an overrepresentation of genes involved in transmembrane transport (Fishers exact test, P-value 4.8E^{-5}) (Figure 1C).

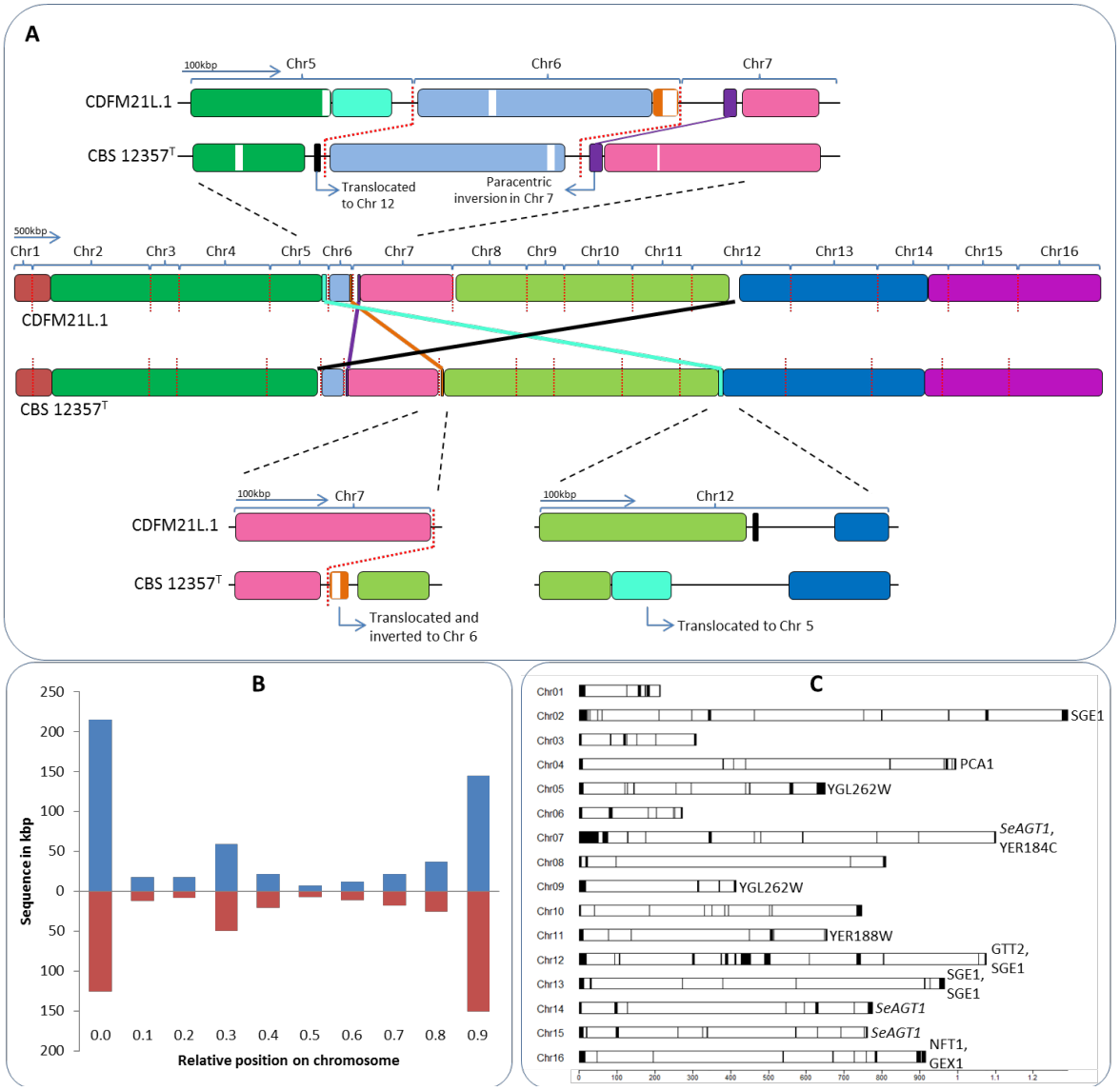

Figure 1. Genome comparison between CDFM21L.1 and CBS 12357T. (A) Translocations in CDFM21L.1 relative to CBS12357T, (i) a paracentric inversion in the sub-telomeric region of chromosome VII, involving approximately 8 kbp, (ii) a translocation of approximately 12 kbp from the left sub-telomeric region of chromosome VIII to the right sub-telomeric region of chromosome VI, and (iii) a reciprocal translocation between approximately 20 kbp from the right sub-telomeric region of chromosome V and approximately 60 kbp from the center of chromosome XII. Red lines depicted chromosomes separation. Genome syntheny is indicated with colored blocks. **(B)** Relative chromosome position of gene presence differences between CDFM21L.1 (blue) and CBS 12357T (red). **(C)** Representation of the assembled CDFM21L.1 *S. eubayanus* chromosomes, the black boxes denote newly added sequences. New annotated open reading frames and gene entries modified relative to the CBS 12357T draft genome [90].

Of the 15 ORFs unique to CDFM21L.1, three were identical orthologs of *S. cerevisiae MAL11/AGT1*. These three ORFs were found in the sub-telomeric regions of chromosomes VII, XIV and XV. Their sequence similarity with the *S. cerevisiae* CEN.PK113.7D and *S. pastorianus* CBS 1483 *MAL11/AGT1* genes was 82.7% and 99.89%, respectively. In addition to these *SeAGT1* genes, CDFM21L.1 genome sequence harbored three maltose transporters (*SeMALTx*), two maltases (*SeMALSx*) and three regulators (*SeMALRx*) encoding genes. In contrast to the situation in *S. eubayanus* CBS 12357^T, none of the *SeMAL* genes formed a canonical *MAL* locus in CDFM21L.1 (Figure 2). A systematic sequence inspection of these CDFM21L.1 *SeMAL* genes revealed mutations that prematurely interrupted the reading frames of *SeMALR1* (CHRV), *SeMALT2* (CHRXII) and *SeMALT*3 (CHRXII).

In addition to *S. eubayanus* CDFM21L.1 strain, a second Himalayan *S. eubayanus* isolate (ABFM5L.1) was sequenced. These two strains were 99.97% genetically identical at the nucleotide level, their *MAL* genes were syntenic and the premature stop codons in *SeMALR1* (CHRV), *SeMALT2* (CHRXII) and *SeMALT*3 (CHRXII) were conserved. Two additional mutations were identified in one of the three *SeAGT1* genes. A nucleotide variation on position 53 and 939 (T instead of an A and A instead of a G)) resulted in a glycine to valine and arginine to lysine change, respectively.

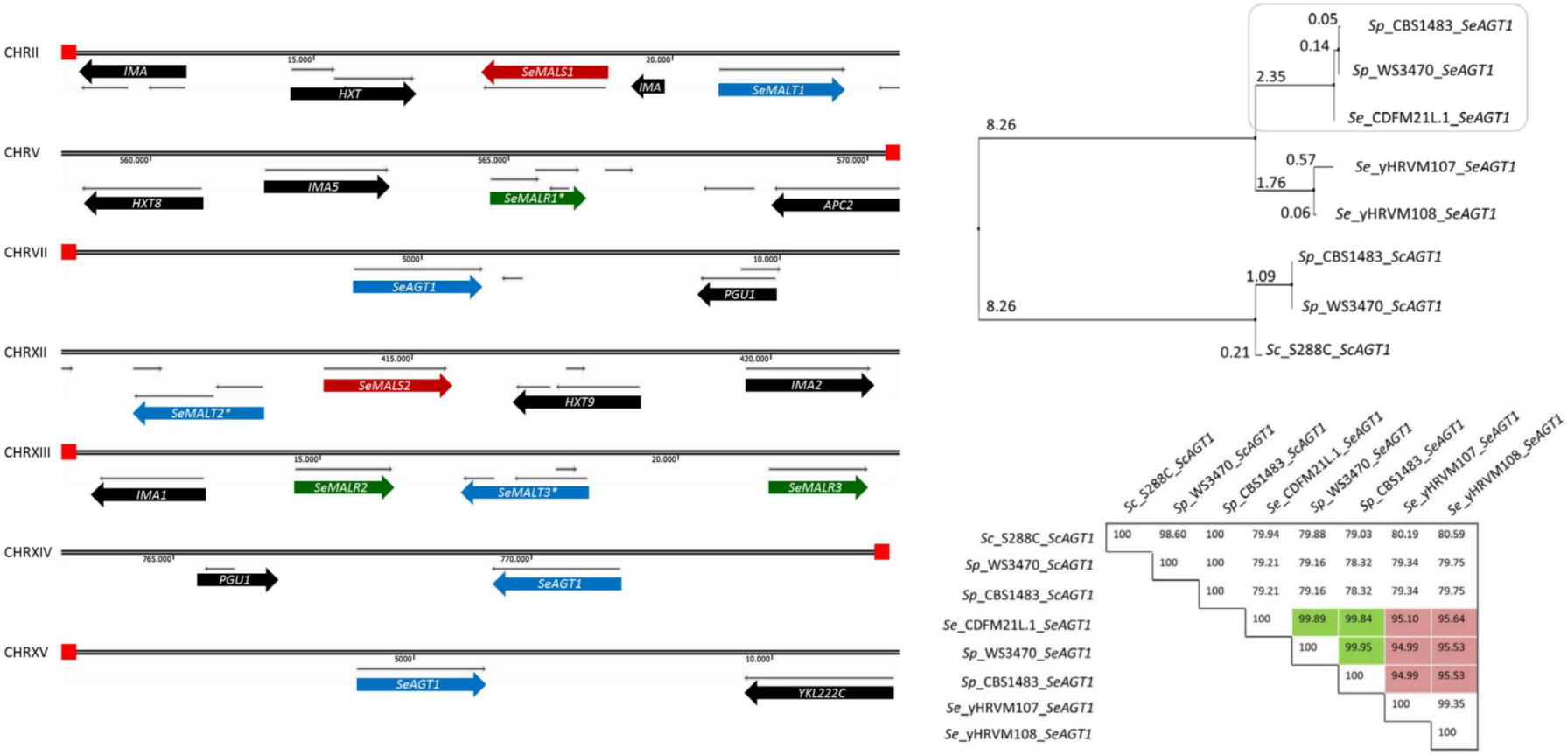

Figure 2. Organization of sub-telomeric regions involving *MAL* genes and *SeAGT1* in CDFM21L.1. (A) Chromosome sections are represented as lines and red boxes denote telomeres. CDFM21L.1 its genome harbors three *SeMALT* genes of which *SeMALT2* and *SeMALT3* have a mutation resulting in an early stop codon and truncated protein (denoted with *). Three copies of *SeAGT1* were found close to the telomeres on CHRVII, XIV and XV. Furthermore, there are two intact *SeMALS* genes on CHRII (1) and XII (2) and three *SeMALR* genes on CHRV (1) and XIII (2) whose copy on CHRV is also mutated (*SeMALR1**). The gene and interval sizes are approximately to scale. (**B**) Phylogeny of *Saccharomyces SeAGT1* genes described in *S. cerevisiae*, *S. eubayanus*, and lager-brewing hybrid *S. pastorianus*. (C) Nucleotide percentage identities between *AGT1* orthologs from *S. cerevisiae*, *S. eubayanus*, and lager-brewing hybrid *S. pastorianus*. Green color indicates highest similarity between *SeAGT1* and *SeAGT1* genes from *S. pastorianus* strains CBS 1483 and WS3470. Red color indicates similarity between *SeAGT1* from North American strains with *SeAGT1* genes from Asian *S. eubayanus* and *S. pastorianus* strains CBS 1483 and WS3470.

Paradoxically, Himalayan *S. eubayanus* strains do not utilize maltose and maltotriose.

Identification of *SeAGT1* in the two Himalayan *S. eubayanus* strains suggests an ability to not only grow on maltose but also on maltotriose. Strains from the Holarctic clade have previously been hypothesized to be the donor of the *S. eubayanus* sub-genome in *S. pastorianus* hybrids [58, 61]. However, no physiological data regarding their ability to grow on the sugars present in wort are available. To assess their growth characteristics, the *Asian S. eubayanus* strains CDFM21L.1, ABFM5L.1, the Patagonian *S. eubayanus* type strain CBS 12357^T and the *S. pastorianus* strain CBS 1483 were grown on diluted industrial brewer's wort at 12 ˚C. As reported previously, *S. pastorianus* strain CBS 1483 could utilize all three sugars but did not fully consume maltotriose (Figure 3) [47]. Also in accordance with previous observations [111], CBS 12357^T consumed glucose and maltose completely, but left maltotriose untouched. However, in marked contrast to *S. eubayanus* CBS 12357^T, neither CDFM21L.1 nor ABFM5L.1 consume maltose after growth on glucose. Moreover, like CBS 12357^T, maltotriose was not metabolized by these two *S. eubayanus* strains. While in CBS 12357^T an ability to grow on maltose and an inability to grow on maltotriose could be readily attributed to its *MAL* genes complement, CDFM21L.1 and ABFM5L.1 failed to grow on maltose even though they appeared to contain complete genes encoding maltose (*SeMALT1* and *SeAGT1*) and maltotriose (*SeAGT1*) transporters.

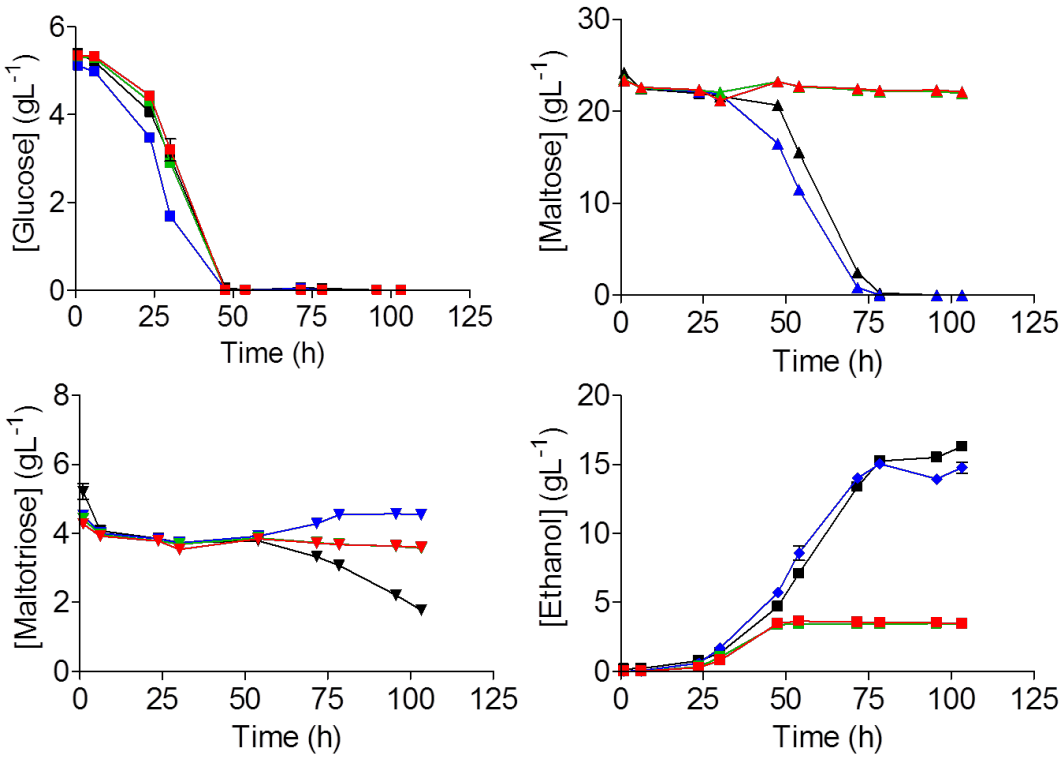

Figure 3. Characterization of sugar consumption of *S. pastorianus* CBS1483 (black) and *S. eubayanus* CBS 12357^T (blue), CDFM21L.1 (red), AS 2.4940 (green) on wort. For every sample, glucose (■), maltose (▲), maltotriose (▼) and ethanol (◆) were measured from the supernatant. Strains were grown at 12 °C for 110 hours in infusion Neubor flasks. Samples were filtered through a 0.22 µm filter and analyzed on HPLC. Data represents average and standard deviation of three biological replicates.

Growth defects on maltose and maltotriose are caused by deficiency of the regulatory *Se*MalR proteins in *S. eubayanus* CDFM21L.1.

The recent characterization of maltose metabolism in CBS 12357^T showed that the coding regions of transcriptionally silent maltose-transporter genes in *S. eubayanus* can potentially encode functional proteins [90]. The inability of the *S. eubayanus* Himalayan isolates to grow on α-oligosaccharides precluded direct testing of transporter-gene functionality by deletion studies. Instead, these genes were expressed in *S. cerevisiae* IMZ616, which is devoid of all native maltose metabolism genes [138]. The CDFM21L.1 transporter genes *SeMALT1*, *SeMALT2*, *SeMALT3* or *SeAGT1* were integrated at the *ScSGA1* locus in IMZ616 along with the *S. cerevisiae* maltase gene *ScMAL12* [90], yielding a series of strains overexpressing a single transporter (IMX1702 (*SeMALT1*), IMX1704 (*SeMALT2*), IMX1706 (*SeMALT3*) and IMX1708 (*SeAGT1*)). These strains, as well as the negative and positive control strains IMZ616 and IMX1365 (IMZ616 expressing *ScAGT1 ScMAL12*), were grown on SM media supplemented with either maltose or maltotriose. On maltose, not only the positive control strain IMX1365, but also IMX1702 (*SeMALT1*) and IMX1708 (*SeAGT1*) were able to grow on maltose, consuming 30 and 60%, respectively, of the initially present maltose after 100 h (Figure 4A). As anticipated, the *SeMALT2* and *SeMALT3* alleles with premature stop codons did not support growth on maltose. Of the two strains that grew on maltose, only IMX1708 (*SeAGT1*) also grew on maltotriose. These results demonstrate that *SeAGT1* from a Holarctic *S. eubayanus* encoded a functional maltotriose transporter and, consequently, that the inability of Holarctic strains to grow on maltose and maltotriose was not caused by transporter dysfunctionality.

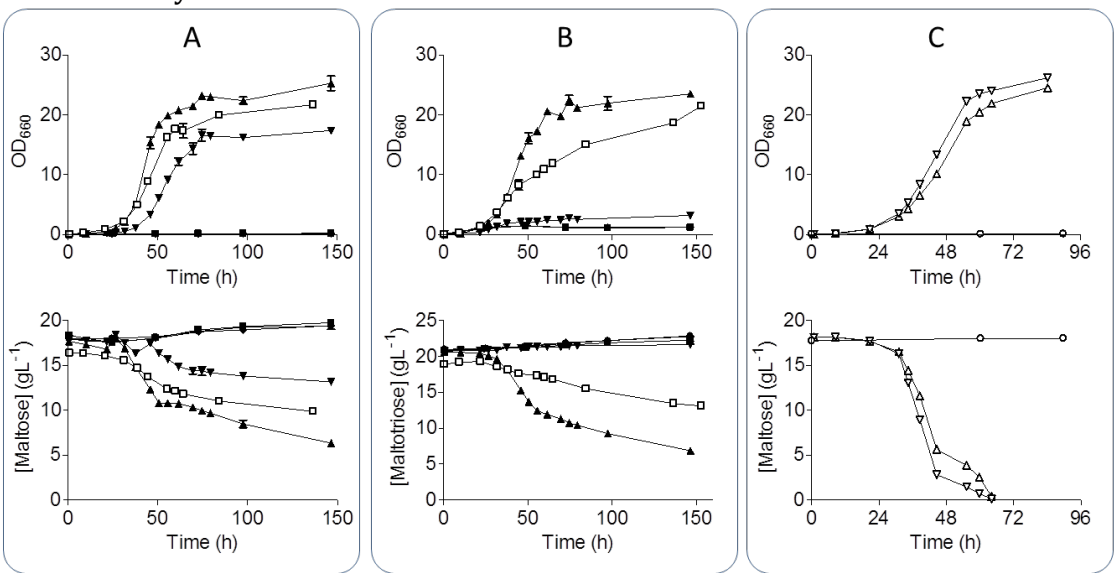

Figure 4. Overexpression of *SeMALT*, *SeAGT1* and *SeMALS* genes in a maltose negative background *S. cerevisiae* strain. Maltose negative background strain IMZ616 (■), IMX1365 overexpressing *ScMAL11* (▲), IMX1702 overexpressing *SeMALT1* (▼), IMX1704 overexpressing *SeMALT2* (◆) IMX1706 overexpressing *SeMALT3* (●) and IMX1708 overexpressing SeAGT1 (□) were grown on SM 2% maltose or maltotriose at 20 °C. Growth on maltose **(A)** and on maltotriose **(B)** was monitored based on optical density (OD_{660nm}) and concentrations of maltose and maltotriose in culture supernatants were measured by HPLC. Data are presented as average and standard deviation of two biological replicates. **(C)** IMX1313 overexpressing only *ScMAL31* (○), IMZ752 overexpressing *ScMAL31* and *SeMALS1* (△) and IMZ753 overexpressing *ScMAL31* and *SeMALS2* (▽) grown on SM maltose 2%. Growth was monitored based on optical density measurement at 660 nm (OD_{660nm}) and maltose in culture supernatants was measured by HPLC. Data represents average and standard deviation of two biological replicates.

In addition to transport, metabolism of α-oligoglucosides requires maltase activity. Functionality of the putative *SeMALS1* and *SeMALS2* maltase genes was tested by constitutive expression in strain IMZ616, together with a functional *Sc*MAL31 transporter genes, yielding strains IMZ752 and IMZ753, respectively. The maltase-negative strain IMX1313 was used as negative control. In SM medium with maltose, both IMZ752 (*SeMALS1*) and IMZ753 (*SeMALS2*) grew and completely consumed maltose within 65 h, demonstrating functionality of both hydrolase genes (Figure 4C).

In *S. cerevisiae* Transcriptional regulation of *MALx2* and *MALx1* genes is tightly controlled by a transcription factor encoded by *MALx3* genes. Malx3 binds an activating site located in the bidirectional promoters that control expression of *MALx2* and *MALx1* genes [183, 273]. To test whether absence of maltose consumption in Himalayan *S. eubayanus* strains was caused by a lack transcriptional upregulation of *SeMALT* and *SeMALS*, the *S. cerevisiae ScMAL13* gene was integrated at the *SeSGA1* locus in *S. eubayanus* CDFM21L.1, under the control of a constitutive *ScPGK1* promoter and *ScTEF2* terminator. *ScMAL13* expression in CDFM21L.1 enabled growth on maltose and maltotriose (Figure 5A), indicating that a lack of transcriptional upregulation was indeed the cause of the parental strain's inability to grow on these oligoglucosides. However, consumption of maltose and maltotriose was incomplete and consumed sugars were almost exclusively respired, as no ethanol was measured after 60 h of cultivation.

The possibility to grow an engineered variant of *S. eubayanus* CDFM21L.1 on α-oligoglucosides offered an opportunity to study transporter function in its native context. Complementary functional characterization by gene deletion of *SeMALT1* and *SeAGT1* was performed using CRISPR-Cas9 genome editing method [90, 142]. Deletion of *SeMALT1* and *SeAGT1* in CDFM21L.1 resulted in strains IMK820 and IMK823, respectively. Complete deletion of *SeAGT1* required disruption of six alleles. To confirm the complete removal of all copies, the genome of IMK823 was sequenced. Mapping reads onto the reference *S. eubayanus* CDFM21L.1 genome assembly confirmed that all six alleles were removed simultaneously. Subsequently, the regulator expression cassette (*ScPGK1*$_p$-*ScMAL13*-*ScTEF2*$_t$) was integrated in IMK820 and IMK823 at the *SeSGA1* locus yielding strains IMX1939 and IMX1940, respectively (Figure 5B). The four deletion strains IMK820 (*SemalT1*Δ), IMK823 (*Seagt1*Δ), IMX1939 (*SemalT1*Δ *Sesga1*Δ::*ScMAL13*) and IMX1940 (*Seagt1*Δ *Sesga1*Δ::*ScMAL13*) were characterized on SMG, SMM or SMMt. All four strains were able to grow on glucose (Supplementary Figure 3). While strains IMK820, IMK823 and IMX1940 were unable to grow on maltose or maltotriose (Figure 5C), strain IMX1939 (*SemalT1*Δ *Sesga1*Δ::*SeMAL13*), which harbored functional *SeAGT1* copies, grew on maltose as well as on maltotriose. However, after 64 h of growth, these sugars were only partially consumed. Only 1.2 g L^{-1} ethanol was produced from maltose and no ethanol formation was observed during growth on maltotriose. The low ethanol concentration and the relatively high OD_{660nm} suggest that, under the experimental conditions strain IMX1939 exhibited a Crabtree negative phenotype and exclusively respired maltotriose. *S. eubayanus* IMX1940 (*Seagt1*Δ *Sesga1*Δ::*SeMAL13*) did not consume maltotriose after 84 h of incubation. Moreover, despite the presence of *SeMALT1*, which encoded a functional maltose transporter upon expression in *S. cerevisiae* IMZ616, strain IMX1940 was also unable to consume maltose.

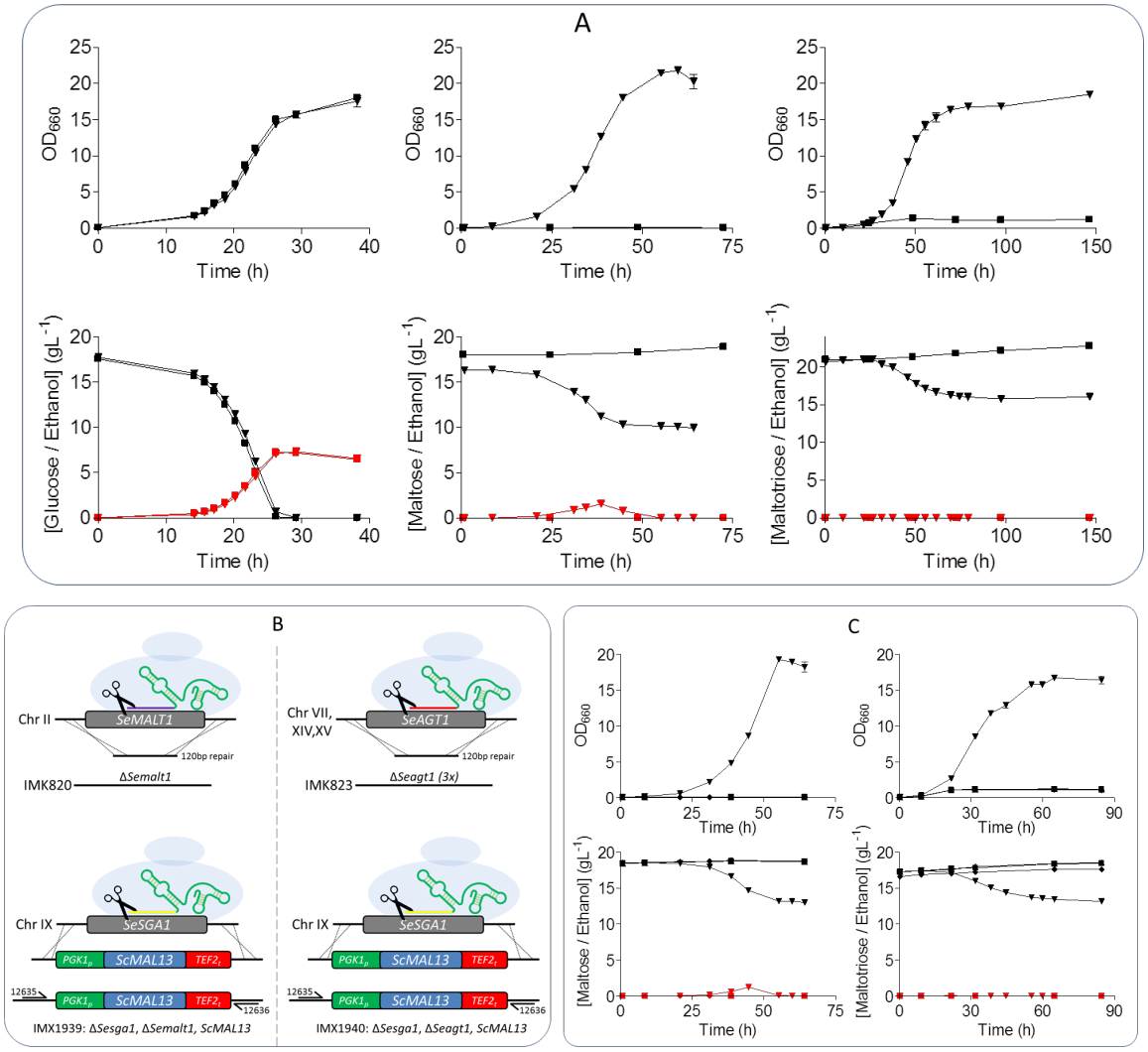

Figure 5. Integration of *ScMAL13* in CDFM21L.1 restores growth on maltose and maltotriose and enables native *SeMALT1* and *SeAGT1* characterization in knockout strains IMK820 and IMK823. (A) Characterization of *S. eubayanus* IMX1765 overexpressing *ScMAL13* (▼) and CDFM21L.1 (■) on SM with glucose, maltose or maltotriose at 20 °C. OD_{660nm} was measured (black) and sugar (black) and ethanol (red) concentrations were determined from the supernatant by HPLC. **(B)** Overview of constructed knockout strains. Knockouts of *SeMALT1* (IMK820) and *SeAGT1* (IMK823) were made with CRISPR-Cas9. Subsequently the *SeSGA1* locus was replaced by *ScPGK1*$_p$- *ScMAL13-ScTEF2*$_t$ using CRISPR-Cas9 in both strains resulting in IMX1939 and IMX1940, respectively. **(C)** *S. eubayanus* strains IMK820 (■), IMK823 (▲), IMX1939 (▼) and IMX1940 (◆) were characterized on SM with maltose or maltotriose at 20 °C. OD_{660nm} was measured (black) and sugar (black) and ethanol (red) concentrations were determined from the supernatant by HPLC. All data represents average and standard deviation of biological duplicates.

In addition to a functional Malx3 transcription factor, transcriptional activation of *MAL* genes also requires presence of a cis-regulatory motif in the promoter of regulated genes. Transcriptome analysis of *S. eubayanus* CBS 12357^T recently showed that absence of a canonical cis-regulatory motif in *SeMALT1* and *SeMALT3* of *S. eubayanus* CBS 12357^T caused a deficiency in their expression [90]. To further explore regulation of *SeMAL* and *SeAGT1* genes, we investigated the impact of carbon sources on genome-wide transcriptome and, specifically, on transcriptional activation of genes involved in maltose metabolism. Duplicate cultures of *S. eubayanus* strain IMX1765 (*ScPGK1*$_p$-*ScMAL13*-*ScTEF2*$_t$) were grown on SMG, SMM and SMMt at 20 °C and sampled in mid-exponential phase. After mRNA isolation and processing, cDNA libraries and reads were assembled onto the newly annotated *S. eubayanus* CDFM21L.1 genome to calculate FPKM (fragments per kilobase of feature (gene) per million reads mapped) expression values. The heterologous regulator *ScMAL13*, expressed from the constitutive *ScPGK1* promoter, displayed the same expression in glucose- and maltose-grown cultures. Although *ScMAL13* was efficiently expressed on glucose, none of the nine *S. eubayanus* maltose genes (the three identical *SeAGT1* copies being undistinguishable) were transcriptionally induced under these conditions (Figure 6), confirming that the hierarchical regulatory role of glucose catabolite repression [273, 274] also takes place in *S. eubayanus*. During growth on maltose, all nine genes were significantly upregulated relative to glucose-grown cultures but large variations in expression level were observed. The maltase genes *SeMALS1* and *SeMALS2* and the transporter gene *SeAGT1* showed the highest upregulation, with fold changes of 148, 161 and 2355 respectively. Although upregulated *SeMALT1* displayed a fold change of 13, its normalized expression in maltose-grown cultures was 886-fold lower than that of *SeAGT1*. This weaker upregulation might explain why, despite the ability of its coding region to support synthesis of a functional maltose transporter, *SeMALT1* alone could not restore growth on maltose.

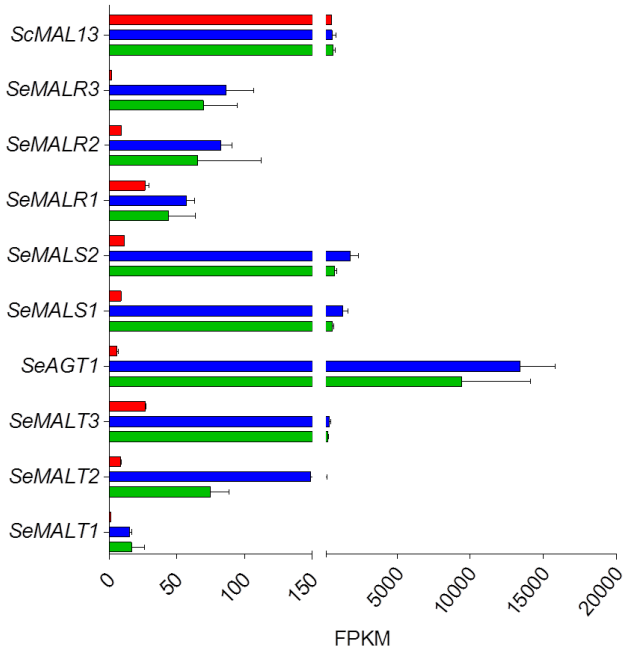

Figure 6. Expression levels of maltose metabolism genes in IMX1765. Normalized transcript levels of maltose metabolism genes from IMX1765mid-exponential phase grown on glucose (red), maltose (blue)and maltotriose (green) at 20 °C were calculated from duplicate RNA sequencing experiments (2x 150 bp) using the FPKM method. All data represents average and standard deviation of two biological duplicates.

Hybridization of two maltotriose-deficient *S. eubayanus* and *S. cerevisiae* lineages results in heterosis through regulatory crosstalk.

The genetic make-up of *S. pastorianus* lager-brewing yeasts strongly advocates that they originate from hybridization of *S. cerevisiae* and *S. eubayanus* parental lineages that were both unable to metabolize maltotriose [18]. This hypothesis is consistent with the recurrent mutation in the *S. cerevisiae AGT1* allele of *S. pastorianus* strains as well as with the inability of Himalayan strains of *S. eubayanus* to grow on these oligoglucosides.

Spores of the Himalayan *S. eubayanus* CDFM21L.1 were hybridized with *S. cerevisiae* CBC-1. This top-fermenting *S. cerevisiae* is recommended for cask and bottle conditioning and unable to consume maltotriose (Lallemand, Montreal, Canada). Analysis of the CBC-1 assembly, obtained by a combination of long and short read sequencing, linked its maltotriose-negative phenotype to a total absence of the *MAL11/AGT1* gene. The resulting laboratory interspecific hybrid HTSH020 was characterized at 12 °C on synthetic wort, a defined medium who composition resembles that of brewer's wort. While *S. eubayanus* CDFM21L.1 only consumed glucose and *S. cerevisiae* CBC-1 consumed glucose and maltose after 103 h (Figure 7A), the interspecific hybrid HTSH020 completely consumed glucose, maltose and partially consumed maltotriose after 105 h, thus resembling characteristics of *S. pastorianus* CBS1483 [47]. In addition to this gain of function, the hybrid HTSH020 outperformed both its parents on maltose consumption, since it depleted this sugar in 70 h instead of 95 h for strain CBC-1. Since *S. cerevisiae* grows generally slower at 12 °C, the experiments were also performed at 20 °C where HTSH020 consumed all maltose 16 h earlier than CBC-1 (Supplementary Figure 4).

Transcriptome analysis of the hybrid strain HTSH020 grown on SM with different carbon sources showed that *SeAGT1* expression was repressed during growth on glucose, with a normalized expression level of 7 FPKM (Figure 7B). When grown on SM maltose, *SeAGT1, SeMALS1* and *SeMALS2* were significantly induced, with fold increases of 816, 109 and 116, respectively (Figure 7B). Although *SeMALT1* and *SeMALT2* were induced, these transporters do not contribute to maltose metabolism due to truncation of their ORFs. These transcriptome data demonstrated that *SeAGT1* and *SeMALS* genes are induced by regulatory crosstalk between regulators encoded from the CBC-1 *S. cerevisiae* sub-genome and maltotriose transporter genes harbored by the *S. eubayanus* genome. This laboratory hybridization experiment may be the closest reproduction yet of how, centuries ago, maltotriose-fermentation capacity arose in the first hybrid ancestor of *S. pastorianus*.

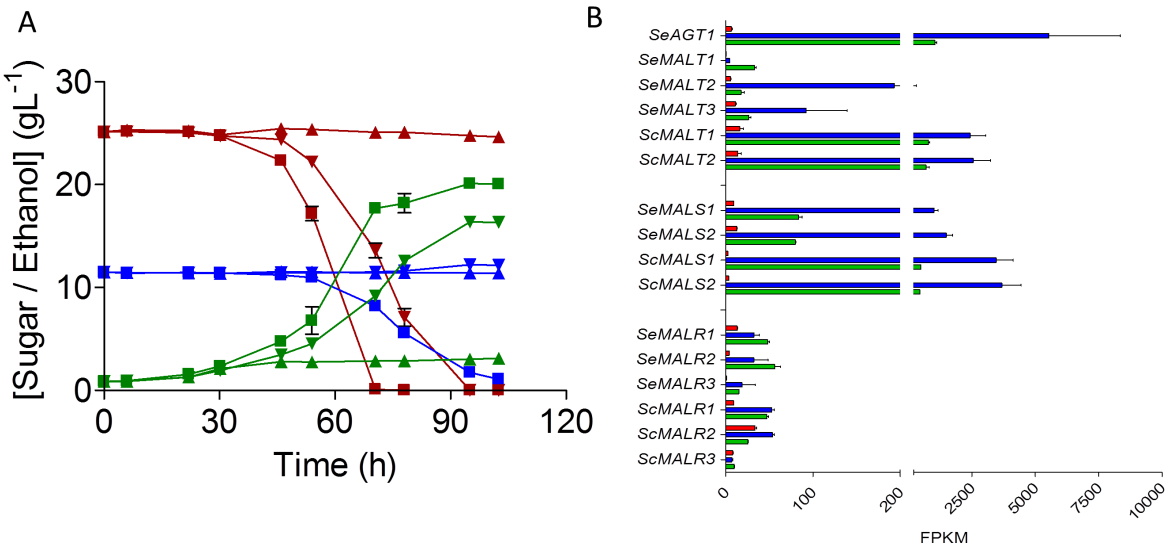

Figure 7. Hybridization of maltotriose deficient *S. cerevisiae* and *S. eubayanus* leading to crosstalk restoring maltotriose utilization, explains *S. pastorianus* phenotype. (A) Characterization of *S. cerevisiae* CBC-1 (▼), *S.* eubayanus CDFM21L.1 (▲) and hybrid HTS020 (■) on mock wort at 12 °C. Consumption of maltose (red), maltotriose (blue) and production of ethanol (green) was measured from supernatant by HPLC. Data represents average and standard deviation from biological triplicates. **(B)** Normalized transcript levels of maltose metabolism genes from HTS020 mid-exponential phase grown on glucose (red), maltose (blue)and maltotriose (green) at 20 °C were calculated from duplicate RNA sequencing experiments (2x 150 bp) using the FPKM method. All data represents average and standard deviation of two biological duplicates.

Discussion

The ability to consume maltose and maltotriose represents a key performance indicator of *S. pastorianus* lager-brewing strains [95]. This study demonstrates how mating of *S. cerevisiae* and *S. eubayanus* strains that cannot themselves ferment maltotriose, can yield maltotriose-fermenting hybrids. This laboratory study illustrates how, centuries ago, maltotriose-fermentation capacity may have arisen in the first hybrid ancestor of *S. pastorianus*.

While the origin of the *S. eubayanus* parent of *S. pastorianus* strains is still under debate [22, 50, 166], phylogenetic analysis suggested a far East Asian origin [58]. However, this interpretation was based on a limited sequencing space and was constrained by the quality of available sequence assemblies. Since an ortholog of *SeAGT1* had previously only been found in the *S. eubayanus* sub-genome of *S. pastorianus* strains, this finding revived the discussion on the geographical origin of the ancestral *S. eubayanus* parent [34]. The high-quality, annotated genome assemblies of the Himalayan *S. eubayanus* strains CDFM21L.1 and ABFM5L.1 presented in the present study revealed several copies of *SeAGT1*, whose very high sequence identity with *S. pastorianus SeAGT1* are consistent with the previously proposed Asian origin of the S*. eubayanus* sub-genome of *S. pastorianus* [34, 58, 61]. Next, genome-sequence comparison of the Patagonian B sub-clade *S. eubayanus* strain CBS12357^{T} and the Holarctic sub-clade strain CDFM21L.1 revealed homoplasy of *SeAGT1*, probably reflecting that these sub-clades evolved in different ecological niches.

The *S. eubayanus* wild stock whose genome sequence most closely corresponds to the *S. eubayanus* sub-genome of *S. pastorianus* originates from the Tibetan plateau of the Himalaya [58]. However, the first *S. cerevisiae x S. eubayanus* hybrid, from which current lager yeasts evolved by centuries of domestication, likely originates from a region between Bavaria and Bohemia in Central Europe. So far, European *S. eubayanus* isolates have not been reported. This may indicate that the original hybridization event occurred elsewhere or that the ancestral European lineage went extinct. The recent detection, in a metagenomics analysis of samples from the Italian Alps, of ITS1 sequences corresponding to *S. eubayanus* could indicate that a wild European lineage exists after all [275].

Functional characterization by heterologous complementation of an *S. cerevisiae* mutant strain established that the *Se*Agt1 transporters from the Himalayan *S. eubayanus* strains CDFM21L.1 and ABFM5L supported uptake of maltose and maltotriose. After showing that these strains also encoded a functional maltase gene, their inability to grow on maltose and maltotriose was attributed to an inability to transcriptionally upregulate maltose metabolism genes. In *S. cerevisiae* and, to some extent, ,in *S. eubayanus* strains of the Patagonian B sub-clade such as CBS12357^{T} [62, 90, 93], MAL loci exhibit a specific organization in which a transporter (*MALT*) and a hydrolase (*MALS*) gene are expressed from the same bidirectional promoter and are located adjacent to a regulator gene (*MALR*) [125]. In contrast, of the seven genomic regions harboring *MAL* genes in the two Asian *S. eubayanus* strains, none showed this canonical organization (Figure 2) and the subtelomeric regions carrying *SeAGT1* did not harbor sequences similar to hydrolase or regulator genes. Subtelomeric regions harboring the other *MAL* genes indicated intensive reorganization as a result of recombination. In particular, subtelomeric regions on CHRII, CHRV and CHRXII provide clear indications for recombination events that scattered genes from ancestral MAL1 and MAL2 loci over several chromosomes. A similar interpretation could explain the reorganization MAL3 on CHRXIII (Figure 2). Similar events may have contributed to loss of function of the MAL regulators (MalR), as exemplified by the

occurrence of a nonsense mutation in S*eMALR1*. These rearrangements did, however, not inactivate the *cis*-regulatory sequences of the *MAL* genes, since complementation with a functional *ScMAL13* allele caused induction of most *SeMAL* genes (Figure 6, Figure 7B) and, thereby, the heterotic maltotriose-positive phenotype of the hybrid strain HTSH020. Together with the high copy number of *SeAGT1*, this heterotic complementation may have been the main driver for colonization of low-temperature brewing processes by the early hybrid ancestors of current *S. pastorianus* strains.

Recent work on adaptation to brewing environments of laboratory *S. cerevisiae* × *S. eubayanus* hybrids showed loss maltotriose utilization during serial transfer in wort [29]. A similar loss of maltotriose utilization is frequently encountered in *S. cerevisiae* ale strains [187], as well as in some Saaz-type *S. pastorianus* strains [43]. This is thus in contrast with retention of a maltotriose assimilation phenotype by Frohberg-type *S. pastorianus* strains. This may have been facilitated by the occurrence of multiple copies of the *SeAGT1* gene in the *S. eubayanus* ancestor, which could act as a sequence buffer to counteracting adverse effects of gene copy loss. The recent release of the first long-read sequencing assembly of *S. pastorianus* enabled a precise chromosomal mapping of the maltose-metabolism genes [63] and showed that the Frohberg type *S. pastorianus* strains CBS 1483 harbored one copy of *SeAGT1* on the *S. eubayanus* CHR as in CDFM21L.1.

Differential retention and loss of maltotriose consumption in *S. pastorianus* lineages may reflect different brewing process conditions during domestication. In modern brewing processes based on high-gravity wort, cell division is largely constrained to the glucose and maltose phases, which occur before depletion of nitrogen sources [276]. It may be envisaged that, in early lager-brewing processes, unstandardized mashing processes generated wort with a higher maltotriose content, which would have allowed for continued yeast growth during the maltotriose consumption phase. During serial transfer on sugar mixtures, the selective advantage of consuming a specific sugar from a mixture correlates with the number of generations on that sugar during each cycle [215, 277]. Such conditions would therefore have conferred a significant selective advantage to a maltotriose-assimilating *S. cerevisiae* x *S. eubayanus* hybrid, especially if, similar to current ale yeasts, the *S. cerevisiae* parent was unable to ferment maltotriose.

The heterotic phenotype that was reconstructed in the interspecies *S. cerevisiae* x *S. eubayanus* hybrid HTS020 resulted from combination of dominant and recessive genetic variations from both parental genomes. *S. eubayanus* contributed the *SeAGT1* gene and its functional *cis*-regulatory sequences, but also harbored recessive mutations in *MALR* genes that allowed full expression of the heterotic phenotype. These mutations were complemented with a set of *S. cerevisiae* genes including a functional *MALR* and a non-functional *ScAGT1* gene to match the mutations found in *S. pastorianus* [18]. Although some *S. pastorianus* strains harbor an additional maltotriose transporter encoded by *SpMTT1 [91]*, this gene was recently proposed to have emerged after the original hybridization event as a result of repeated recombination between *MALT* genes from both sub-genomes [100].

Maltotriose fermentation is likely not the only heterotic phenotype of S*. pastorianus* strains. Flocculation or formation of complex aroma profiles [137, 268] are phenotypes that are not fully understood and difficult to reproduce, that also might result from heterosis [29].

Laboratory-made *S. cerevisiae* x *S. eubayanus* hybrids hold great potential for brewing process intensification and for increasing product diversity. In addition to increasing our understanding of the evolutionary history of lager yeast genomes evolutionary, this study has implications for the design of new hybrids. Hitherto, laboratory crosses of *S. cerevisiae*

x *S. eubayanus* strains were designed based on combination of dominant traits of the parental strains. Our results show that recessive traits can be just as important as contributors to the genetic diversity of such hybrids.

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Chapter 5

Outlook

The popularity of lager beer has resulted in a situation in which, for many decades, a single type of beer has dominated the global market. Fierce competition in the lager beer market makes it important to further reduce production costs. With respect to fermentation, brewers seek to make brewing more economical by process intensification, i.e., to more efficiently use fermentation tanks by increasing wort gravity. Obviously, increasing the concentration of fermentable sugars will only result in an increased volumetric productivity if its effects are not offset by negative effects on yeast performance.

The high concentrations of wort sugars that are inevitably associated with use of high-gravity wort can pose physiological challenges for yeast cells, for instance due to increased osmotic stress, higher final ethanol concentrations. Moreover, to achieve efficient conversion, wort sugars must be quickly transported at high concentrations (>150 g/L) as well as at low concentrations (<5 g/L). In general, brewing yeasts readily ferment glucose and maltose, while conversion of maltotriose of often slow and, in some cases, even incomplete. Efforts have been made to improve maltotriose consumption [47] but the molecular and physiological mechanisms that underlie suboptimal maltotriose uptake and fermentation under industrially relevant conditions are still not completely poorly understood.

With the availability of third-generation long-read sequencing techniques, the subtelomeric regions of brewing yeast genomes that harbor *MAL* genes can now be assembled with high accuracy, thereby providing a quick overview of the complement of potential maltose and maltotriose transporters in any brewing yeast strain. With the discovery of the *SeAGT1* gene in Chapter 4 [35] and of the evolved, chimeric *SeMALT413* gene in Chapter 3 [100], both of which encode functional maltotriose transporters, the genetics of maltotriose transport in brewing strains can now be better interpreted. In addition, identification of several genetically different maltotriose transporters, also including *Sc*Agt1 and Mtt1, is highly relevant for further studies on the genetics, physiology and genetic (in)stability of maltotriose transport in brewing strains and its interaction with maltose transport.

In addition to process intensification of 'conventional' lager brewing, product diversification plays an increasingly important role in the brewing industry. The clean taste of lager beer and the consistent use of *Saccharomyces pastorianus* resulted in a relatively small product diversity. The rapidly increasing number of craft breweries reflects a growing demand of consumers for more diverse brews. While variations of ingredients such as malts and hops can definitely also contribute to product diversity, the potential of yeast strain discovery and improvement is increasingly considered.

Recent genomic analysis suggests that phenotypic diversity in current hybrid *S. pastorianus* brewing strains is rather limited, as all current brewing strains are probably derived from a single hybridization event [63]. Generation of novel hybrids of 'wild' isolates opens up a plethora of possibilities to diversify brewing strains, possibly leading to entirely new products. In such strategies, use of phenotypic screening and genome sequencing for selecting and combining parental strains will increasingly facilitate prediction of hybrid performance and properties. The potential of this approach is demonstrated in Chapter 4, where knowledge-based design of a hybrid of two strains that were both unable to consume maltotriose yielded a maltotriose-fermenting hybrid. This

result also demonstrates that (ale) brewing strains can be used for hybridization when they have other interesting characteristics but cannot ferment maltotriose.
In addition to non-GMO methods such as hybridization and non-targeted mutagenesis, brewing yeasts are increasingly more accessible due to the implementation of CRISPR-Cas9 and improved genome-sequencing methods [90]. At present, concerns about consumer acceptance still preclude large-scale introduction of beverages and foods produced with GM microorganisms. However, gene editing will eventually also make their way into food and beverage fermentation when clear consumer benefits can be demonstrated [102]. In principle, the possibilities that could be opened up by application of gene editing are virtually endless. For example, gene editing could result in a next generation of brewing strains with a positive impact on various aspects of beer manufacturing, including energy costs and carbon footprint of the fermentation process. In addition, gene editing can be used to introduce new and specific flavors and organoleptic properties.

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Curriculum vitae

Nick Brouwers was born on 26 April, 1991 in Breda, Netherlands. After high school in 2009, doing Atheneum at the OLV in Breda, he moved to Wageningen to follow the BSc program Biotechnology at Wageningen University. During his bachelors, he joined the Wageningen iGEM 2012 team, where he contributed to the project "development of virus like particles for drug delivery" that was ranked within the top 16 worldwide. For his BSc thesis, Nick worked under supervision of Laura van der Straat and Leo de Graaff on the Funbrick™ system, a modular genetic integration system that was applied in *Aspergillus niger* for itaconic acid production.

Nick continued his studies at Wageningen University, doing the MSc Biotechnology program with a specialization in medical/molecular biotechnology. In 2013-2014 he did his internship at Harvard Medical School, Boston USA, where he worked with Brendon Dussel and Jeffrey Way on the development of chimeric activators, antibody based fusion protein for treatment of glioblastomas. The MSc program was finalized with a thesis where are protein scaffold system was designed for the degradation of xylan, again under the supervision of Laura van der Straat and Leo de Graaff. During his study, Nick was also strongly involved in starting the lacrosse association W.S.L.V. Wageningen Warriors, being part of the board for two years as vice president and president.

After graduation in 2014, Nick worked at the systems and synthetic biology department in Wageningen. Here a feasibility study was done on the use of phytochromes in metabolic networks. In 2015, Nick started his PhD in the Industrial Microbiology group at Delft University of Technology under the supervision of Jean-Marc Daran and Jack Pronk. During his PhD, Nick worked on the development of a screening platform to study lager brewing yeast brewing properties and genome composition. Furthermore, much research was done towards wort sugar metabolism in *S. eubayanus*, as described in this thesis. The PhD project was in collaboration with Niels Kuijpers, Ton Elink Schuurman, Jan-Maarten Geertman and Victor Boer from Heineken Supply chain, Global Innovation and Research. This work was performed within the BE-Basic R&D Program, which was granted a TKI subsidy from the Dutch Ministry of Economic Affairs, Agriculture and Innovation (EL&I).

During his PhD, Nick played with the Dutch National in-country lacrosse team and started Feet Factor-E, a family business focused on foot reflexology and employee well-being. Now he works at Amgen as a Sr. associate quality assurance outsourced by QbD as a pharmaceutical consultant.

List of publications

Structural, physiological and regulatory analysis of maltose transporter genes in *Saccharomyces eubayanus* CBS 12357^{T}
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