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 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, 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 SeMalT413 was comparable to the original SeMalT 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 12357T was used to enable maltotriose fermentation and improve brewing performance. The improved strain expressed a novel transporter gene, ScMALT413, which was formed by recombination between three different SeMALT maltose-transporter genes. Overexpression of ScMALT413 in CBS 12357T confirmed its neofunctionalization as a maltotriose transporter. As the S. pastorianus maltotriose transporter SpMty1 has a mosaic structure similar to SeMalt413, 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 [1,2]. While S. cerevisiae is strongly associated with biotechnological processes, including dough leavening, beer brewing and wine fermentation [3], S. eubayanus has only been isolated from the wild [4–6]. 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 [7]. While many S. cerevisiae and S. pastorianus strains utilize all three sugars, S. eubayanus isolates do not utilize maltotriose [8–10]. In Saccharomyces, the ability to utilize maltotriose requires its uptake into the cell and subsequent hydrolysis into glucose [11,12]. Maltose and maltotriose utilization are encoded by genes clustered in the MAL loci, which can be present on up to five different chromosomes [13]. 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 [14]. While MALS genes enable hydrolysis of both maltose and maltotriose, the MALT gene family comprises transporters with diverse substrate specificities [11,15]. In S. cerevisiae, most MAL loci harbor an ScMalx1 transporter (Fig 1), which transports maltose and other disaccharides, such as turanose and sucrose [12,16], but cannot import maltotriose [11]. 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 [11,17,18].

The S. eubayanus type strain CBS 12357T 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 [9]. Indeed, the four MAL loci in CBS 12357T harbor a total of two MALS genes and four MALT genes with high homology to ScMALx1: ScMALT1, ScMALT2, ScMALT3 and ScMALT4 (Fig 1) [20]. 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 [9]. 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 [9].

The MAL transporter genes in Saccharomyces yeasts are localized to the subtelomeric regions [9,11,12,20–22], which are gene-poor and repeat-rich sequences adjacent to the telomeres [23–25]. The presence of repeated sequences makes subtelomeric regions genetically unstable by promoting recombination [26,27]. As a result, subtelomeric gene families are hot-spots of genetic diversity [28–30]. In S. cerevisiae, subtelomeric gene families contain more genes than non-subtelomeric gene families, reflecting a higher incidence of gene duplications [28]. 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 [31]. Despite their common origin, genes within one family can have different functions, due to the accumulation of mutations [32,33]. In silico analysis of the sequences and functions of genes from the MAL, MALS and MALR gene families indicated functional diversification through gene duplication and mutation [28]. Gene duplication is a critical step for the evolution of new gene functions [34,35]. 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 [36], (ii) subfunctionalization, corresponding to the specialization of gene copies...
for part of the function of the parental gene [37] and (iii) altered expression due to gene dosage
effects resulting from the increased copy number [38]. While the different functions of MALS
genes were assigned to subfunctionalization of the ancestral MALS gene [15], it is not known
how the different functions of the maltotriose transporter gene ScAGT1 and of other maltose
transporter genes of the MALT family evolved from a common ancestor gene [28]. In general,
the emergence of a large array of gene functions was attributed to subfunctionalization and
neofunctionalization [15,28,31,39–42]. However, current evidence for neofunctionalization
within subtelomeric gene families is based on a posteriori analysis and rationalization of exist-
ing sequence diversity. While in some cases the genetic process leading to neofunctionalization
could be reconstructed at the molecular level [42–44], 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 ScMalT transporters could lead to the emergence
of a maltotriose transporter by neofunctionalization [28]. Therefore, laboratory evolution may
be sufficient to obtain maltotriose utilization in S. eubayanus strain CBS 12357T.

Laboratory evolution is a commonly-used method for obtaining desired properties by pro-
longed growth and selection under conditions favoring cells which develop the desired pheno-
type [45,46]. Similarly as in Darwinian natural evolution, the conditions under which
laboratory evolution is conducted shape the phenotypes acquired by evolved progeny follow-
ing the process of survival of the fittest [47]. In Saccharomyces yeasts, selectable properties
include complex and diverse phenotypes such as high temperature tolerance, efficient nutrient
utilization and inhibitor tolerance [48–51]. Laboratory evolution was successfully applied to
improve sugar utilization for arabinose, galactose, glucose and xylose [49,52–54]. In S. pasto-танус, improved maltotriose uptake was successfully selected for in a prolonged chemostat cul-
tivation on medium enriched with maltotriose [55]. 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 12357T to UV-mutagenesis and labora-
tory 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 12357T
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 12357T, may provide insight in the emergence of maltotriose utilization in general.

Results

Mutagenesis and evolution enables S. eubayanus to utilize maltotriose

To obtain maltotriose-consuming mutants, CBS 12357T was grown on synthetic medium con-
taining 20 g L⁻¹ glucose (SMG) until stationary phase, and approximately 10⁸ 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 12357T 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 S1 Fig). 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 [45,55]. 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 [56]. 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. 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 and selected for further characterization in micro-aerobic cultures, grown at 12˚C on 3-fold diluted wort, along with *S. eubayanus* CBS 12357ᵀ and *S. pastorianus* CBS 1483 (Fig 2D). In these cultures, strains CBS 12357ᵀ and IMS0751 only consumed glucose and maltose, while *S. pastorianus* CBS 1483, as well as the evolved isolates IMS0750 and IMS0752, also consumed maltotriose (S2 Fig). After 263 h, maltotriose concentrations in cultures of strains IMS0750 and IMS0752 had decreased from 10.7 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 recombinant chimeric *SeMALT* gene

We sequenced the genomes of the *S. eubayanus* strain CBS 12357ᵀ, 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 12357ᵀ [9] 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 (S1 Data File). 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 12357ᵀ diploidized by mating after mutagenesis (S1 Data File). 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 (S1 Data File). 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 12357T [9].

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 (S1 Data File). 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 SeMALT1 (coordinates 8,950 to 9,500), a duplication of the left arm of CHRXIII including SeMALT3 (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 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 12357T 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 SeMALT1. 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 SeMALT4 on chromosome XVI. However, the recombined sequence contained a chimeric open reading frame consisting of the 5’ part of SeMALT4 from CHRXVI, the middle of SeMALT1 from CHRII and the 3’ part of SeMALT3 from CHRXIII (Fig 3C, S3 Fig). 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 12357T and IMS0637. Sanger sequencing of the fragment amplified from strain IMS0750 confirmed the chimeric organization of the ORF, which we named SeMALT413. The sequence of SeMALT413 showed 100% identity to SeMALT4 for nucleotides 1–434 and 1113–1145, 100% identity to SeMALT1 for nucleotides 430–1122 and 100% identity to SeMALT3 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 SeMALT4. Therefore, this part of the sequence of SeMALT413 might have come from SeMALT2 on CHRV or from SeMALT4 on CHRXVI. Overall, SeMALT413 showed a sequence identity of only 85 to 87% with the original SeMALT genes, with the corresponding protein sequence exhibiting between 52 and 88% similarity. We therefore hypothesized that the recombined SeMalT413 transporter might have an altered substrate specificity and thereby enable maltotriose utilization.
The tertiary protein structure of the chimeric \textit{SeMALT413} gene was predicted with SWISS-MODEL (https://swissmodel.expasy.org/), based on structural homology with the \textit{Escherichia coli} xylose-proton symporter \textit{XylE} [57], which has previously been used as a reference to model the structure of the maltotriose transporter \textit{ScAGt1} [58]. Similarly to the maltose transporters in \textit{Saccharomyces}, \textit{XylE} is a proton symporter belonging to the major facilitator superfamily with a transmembrane domain composed of 12 $\alpha$-helixes (S4 Fig). The same structure was predicted for \textit{SeMalT413}, with 1 $\alpha$-helix formed exclusively by residues from \textit{SeMalT4}, 4 $\alpha$-helixes formed exclusively by residues from \textit{SeMalT1} and 5 $\alpha$-helixes formed exclusively by residues from \textit{SeMalT3} (Fig 3D). The remaining two $\alpha$-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 \textit{SeMalT4}. The three-dimensional arrangement of the $\alpha$-helixes of \textit{SeMalT413} was almost identical to \textit{SeMalT1}, \textit{SeMalT3} and \textit{SeMalT4}, indicating that it retained the general structure of a functional maltose transporter (S5 Fig).

**Introduction of the \textit{SeMALT413} gene in wildtype CBS 12357$^T$ enables maltotriose utilization**

The small structural differences identified between \textit{SeMalT413} and the wild-type \textit{S. eubayanus} \textit{SeMalT} transporters could not be used to predict the ability of \textit{SeMalT413} to transport maltotriose [58]. Therefore, to investigate its role in maltotriose transport, \textit{SeMALT413} and, as a control, \textit{SeMALT2} were overexpressed in the wild-type strain \textit{S. eubayanus} CBS 12357$^T$ (Fig 4A and S6 Fig). Consistently with previous gene editing in CBS 12357$^T$ [9], the expression cassettes were inserted at the \textit{SeSGA1} locus, encoding an intracellular sporulation-specific glucoamylase which is not expressed during vegetative growth [59,60]. Growth of the resulting strains \textit{S. eubayanus} IMX1941 (\textit{SeSGA1Δ::ScTEF1p-SeMALT2-ScCYCl}) and IMX1942 (\textit{SeSGA1Δ::ScTEF1p-SeMALT413-ScCYCl}), as well as the wild-type strain CBS 12357$^T$ and the evolved isolate IMS0750 was tested on SM supplemented with different carbon sources (S7 Fig). 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 (\textit{ScTEF1p-SeMALT413-ScCYCl}) 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 \textit{SeMALT413} to transport maltotriose.

**The \textit{SpMTY1} maltotriose transporter gene displays a similar chimeric structure as \textit{SeMALT413}**

The emergence of the maltotriose transporter \textit{SeMalT413} by recombination between different \textit{MALT} genes during laboratory evolution demonstrates that \textit{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 \textit{Saccharomyces} yeasts, we analyzed the sequences of existing maltotriose transporter genes in \textit{S. cerevisiae} and \textit{S. pastorianus} genomes. In \textit{S. cerevisiae} strains, maltotriose transport is encoded by the \textit{ScAGT1} gene [11,17,18]. However, \textit{SCAGT1} is truncated and non-functional in \textit{S. pastorianus} [61]. Instead, maltotriose utilization has been attributed to two \textit{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 [62,63]. Although it is absent in CBS 12357 [20], LgAGT1 was found to enable maltotriose transport in the north-American S. eubayanus isolate yHRVM108 [19,20]. The SpMTY1 gene, also referred to as MTT1, was found in the MAL1 locus of S. pastorianus [21,22]. 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 [62]. SpMTY1 shows 90% sequence identity with ScMALx1 genes [21,22], but also displays segmental sequence identity with ScMALT genes [64,65].

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% [21]. Indeed, sequence identity with ScMAL31 of S. cerevisiae S288C [66] is above 98% for

Fig 4. Reverse engineering of SeMALT413 in CBS 12357\textsuperscript{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\textsuperscript{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 (S6 Fig). Strains were validated using Sanger sequencing. (B) Characterization of CBS 12357 (blue squares), IMS0750 (red triangles), IMX1941 (cyan triangles), IMX1942 (purple diamonds) 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 (S6 Data File).

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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 (S8 Fig). Alignment of the sequences of S. eubayanus CBS 12357T SeMALT genes [9] to SpMYT1 showed high sequence identity with ScMAL3 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 (S8 Fig). These observations would indicate that the evolution of SpMYT1 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 SpMYT1 gene structure. Its evolution may therefore have involved multiple introgressions, similarly as for ScMAL31. While most regions with low identity to ScMAL31 and SeMAL3t were too short to identify their provenance, the sequence corresponding to the 969th to 1,639th nucleotide of SpMYT1 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 SpMYT1, SparMAL31 had an identity of 94% for nucleotides 544–575 and of 93% for nucleotides 846–859 (S8 Fig). Therefore, the sequence of SpMYT1 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, SpMYT1 probably accumulated additional mutations during its evolution.

Applicability of a maltotriose-consuming S. eubayanus strain for lager beer brewing

S. eubayanus strains are currently used for industrial lager beer brewing [9]. To test the evolved strain IMS0750 under laboratory-scale brewing conditions, its performance was compared with that of its parental strain CBS 12357T 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 12357T 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 12357T 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 12357T (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 333 h for CBS 12357T, it dropped below 10 μg L⁻¹ for IMS0750 (Table 1).

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 12357T are localized in subtelomeric MAL loci:
ScMALT1 on chromosome II, ScMALT2 on chromosome V, ScMALT3 on chromosome XIII and ScMALT4 on chromosome XVI [9,20]. 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 ScMALT1, ScMALT3 and ScMALT4. These recombinations occurred within the open reading frame of ScMALT4 and the newly-formed chimeric ORF ScMALT413 encoded a full length protein with a structure comparable to that of ScMalT transporters. In contrast to the original ScMALT genes, overexpression of ScMALT413 enabled growth on maltotriose, indicating that ScMalT413 acquired the ability to import maltotriose.

The predicted structure of ScMalT413 was highly comparable to the structure of other transporters from the major facilitator superfamily [67] and to the structure of ScMalT1, ScMalT3 and ScMalT4. While nothing is known about the amino acid residues responsible for substrate specificity in ScMalT transporters, the threonine and serine residues at the 505th and 557th position respectively of ScAgt1 were identified as critical for maltotriose transport [68]. In ScMalT413, the corresponding amino acids originate from ScMALT3. However, since ScMALT3 itself is unable to utilize maltotriose, the ability of ScMalT413 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 ScMALT434

### 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.

<table>
<thead>
<tr>
<th>Compound</th>
<th>Unit</th>
<th>CBS 12357 T</th>
<th>IMS0750</th>
</tr>
</thead>
<tbody>
<tr>
<td>Methanol</td>
<td>mg L⁻¹</td>
<td>3.3 ± 0.3</td>
<td>3.7 ± 0.3</td>
</tr>
<tr>
<td>Propanol</td>
<td>mg L⁻¹</td>
<td>23.7 ± 2.1</td>
<td>24.1 ± 0.9</td>
</tr>
<tr>
<td>Isobutanol</td>
<td>mg L⁻¹</td>
<td>48.5 ± 2.4</td>
<td>42.9 ± 7.2</td>
</tr>
<tr>
<td>Amyl alcohol</td>
<td>mg L⁻¹</td>
<td>138.5 ± 9.0</td>
<td>155.9 ± 6.4</td>
</tr>
<tr>
<td>Diacetyl</td>
<td>μg L⁻¹</td>
<td>43.8 ± 22.9</td>
<td>7.5 ± 0.2</td>
</tr>
<tr>
<td>Ethylacetate</td>
<td>mg L⁻¹</td>
<td>24.5 ± 5.5</td>
<td>26.1 ± 0.8</td>
</tr>
<tr>
<td>Isoamylacetate</td>
<td>mg L⁻¹</td>
<td>1.4 ± 0.6</td>
<td>3.1 ± 0.3</td>
</tr>
</tbody>
</table>

https://doi.org/10.1371/journal.pgen.1007853.t001
transporter which enabled maltotriose uptake [19]. 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 transporters, 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 [69]. 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 [31]. 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 [70]. T. brucei expresses a single variant surface glycoprotein (VSG) gene from a subtelomeric location and its genome contains many VSG pseudogenes [71]. 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 [72–74]. 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 [75,76]. Gene shuffling involves randomized assembly of diverse DNA sequences into chimeric genes, followed by screening for novel or improved functions. Analogous to in vitro gene shuffling, the complex protein remodeling caused by in vivo formation of chimeric sequences may be particularly potent for protein neofunctionalization [77]. 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 [78]. 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 SparMALT31. 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 [79]. 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 123577, which was discovered at the same time as SeMALT413 provides further credibility to the evolutionary importance of in vivo gene shuffling for gene neofunctionalization [19].

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 [80],
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 [80], 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 [28,31,32,81], the loss of SeMALT4 was likely facilitated by the presence of the functionally-redundant maltose transporter SeMALT2 [9]. 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 12357T [9].

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 [28,82–87]. The high number of genes within subtelomeric families results in functional redundancy and therefore in mutational freedom [28,31,32,81]. 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 [88]. While subtelomeric regions are difficult to reconstruct due to their repetitive nature, they encode much of the genetic diversity between genomes [89,90]. 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 [15]. 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 [28,31,32,36,37,81]. In contrast to subfunctionalization, neofunctionalization consists of the emergence of a function which was completely absent in the ancestral gene [40]. 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 [15,28,31,39–42]. Ex-vivo engineering of the subtelomeric FLO genes demonstrated that recombinations within subtelomeric gene families can alter their function [39]. 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\textsuperscript{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 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\textsuperscript{T} [9], 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\textsuperscript{T} resulted in lesser maltotriose utilization than the evolved strain IMS0750, the maltotriose transporter SeMalT413 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 [91,92]. 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\textsuperscript{T} via genetic engineering demonstrated its neofunctionalization, the use of GMO-strains is limited in the brewing industry by customer acceptance issues [93]. 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\textsuperscript{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 underlay 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 [94]. In addition, low residual sugar concentration, low concentrations of diacetyl and high concentrations of isoamylacetate are desirable for the flavor profile of beer [95,96]. S. eubayanus strains typically generate high concentrations of 4-vinyl guaiacol, a clove-like off-flavor [97,98], a strategy to eliminate this production in S. eubayanus have recently been described [97]. 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 [9,99]. The ability to ferment maltotriose can be introduced into other natural isolates of S. eubayanus, either by laboratory evolution or by crosses 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 [8,100–102].
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[1] and S. pastorianus strain CBS 1483 [55,103] 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.

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 KH₂PO₄, 5.0 g L⁻¹ (NH₄)₂SO₄, 0.5 g L⁻¹ MgSO₄·7 H₂O, 1 mL L⁻¹ trace element solution, and 1 mL L⁻¹ vitamin solution [56], 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 Zn²⁺ by addition of ZnSO₄·7H₂O, 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 (NH₄)₂SO₄ was replaced by 5 g L⁻¹ K₂SO₄ and 10 mM acetamide (SM_{Ace}G) [104].

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 OD₆₆₀ 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

Table 2. Saccharomyces strains used during this study.

<table>
<thead>
<tr>
<th>Name</th>
<th>Species</th>
<th>Relevant genotype</th>
<th>Origin</th>
</tr>
</thead>
<tbody>
<tr>
<td>CBS 12357</td>
<td>S. eubayanus</td>
<td>Wild-type diploid</td>
<td>[1]</td>
</tr>
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<td>S. eubayanus</td>
<td>Evolved strain derived from CBS 12357 This study</td>
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<tr>
<td>IMX1942</td>
<td>S. eubayanus</td>
<td>ΔSeSGA1::ScTEF1p-ScMALT413-ScCYC1</td>
<td>This study</td>
</tr>
<tr>
<td>CBS 1483</td>
<td>S. pastorianus</td>
<td>Group II brewer’s yeast, Brewery Heineken, bottom yeast, July 1927</td>
<td>[103]</td>
</tr>
</tbody>
</table>

https://doi.org/10.1371/journal.pgen.1007853.t002
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. Fermen-
tations 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\(^{18}\) 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 FACS \- Saria 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 (Glantham Life Sciences), 0.2 mL L\(^{-1}\) anti-foam emulsion C (Sigma-Aldrich), 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 [56]. 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 63\(^{rd}\) 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 Cellreector 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.
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 [90] 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/) [9] using the Burrows–Wheeler Alignment tool (BWA), and further processed using SAMtools and Pilon for variant calling [105–107]. Heterozygous SNPs and INDELs which were heterozygous in CBS 12357\(^T\) were disregarded. Chromosomal translocations were detected using Breakdancer [108]. 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 [109] with the gamma setting set to “none” and using the assembler ABySS (v 1.3.7) with a k-mer size of 29 [110]. All SNPs, INDELs, recombinations and copy number changes were manually confirmed by visualising the generated .bam files in the Integrative Genomics Viewer (IGV) software [111]. The complete list of identified mutations can be found in S1 Data File.

For strains IMS0637 and IMS0750, the nanopore sequencing reads were assembled de novo using Canu (version 1.3) [112] with--genomesize set to 12 Mbp. Assembly correctness was assessed using Pilon [107], and sequencing/assembly errors were polished by aligning Illumina reads with BWA [105] 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 [113]. 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 \(\mu\)L 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 *ScFSY1* and the absence of *S. cerevisiae*-specific gene *ScMEX67* was tested by DreamTaq PCR (Thermo Scientific) amplification using primer pair 8572/8573 [114], and primer pair 8570/8571 [115], 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) [116]. 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 S1 Table, 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 SeMALT413 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 [16], which was PCR amplified using primer pair 7812/5921. The transporter fragment and the p426-TEF-amdS backbone fragment were assembled [117] 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 recombinant ORF found in the nanopore assembly of IMS0750 (Fig 3C).

**Strain construction.** To integrate and overexpress SeMALT2 and SeMALT413 ORFs in S. eubayanus CBS 12357T, SeMALT2 and SeMALT413 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 Spcas9D147YP411T [119,120] and a gRNA targeting SeSGA1 [9]. The strain IMX1941 was constructed by transforming CBS 12357T with 1 μg of the amplified SeMALT2 expression cassette and 500 ng of plasmid pUDP052 by electroporation as described previously [120]. Transforms were selected on SM\_AceG plates. Similarly, IMX1942 was constructed by transforming CBS 12357T 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 (S9 Fig). All PCR-amplified gene sequences were Sanger sequenced (BaseClear).

**Protein structure prediction.** Homology modeling of the SeMalt413 transporter was performed using the SWISS-MODEL server (https://swissmodel.expasy.org/) [121]. The translated amino acid sequence of SeMALT413 was used as input (S4 Fig). The model of the xylose proton symporter XylE (PDB: 4GBY) was chosen as template [57]. 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

<table>
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<td>ori (ColEl1) bla panARSopt amdSYM ScTDH3p-gRNA\textsubscript{SeSGA1}-ScCYC1, AaTEF1\textsubscript{p}-Spcas9\textsubscript{D147YP411T}-ScPHO5</td>
<td>[9]</td>
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<tr>
<td>pUDE044</td>
<td>ori (ColEl1) bla 2μ ScTDH3p-ScMAL12-ScADH1, URA3</td>
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<tr>
<td>pUD814</td>
<td>ori (ColEl1) bla 2μ amdSYM ScTEF1\textsubscript{p}-SeMALT413-ScCYC1</td>
<td>This study</td>
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https://doi.org/10.1371/journal.pgen.1007853.t003
resulting model is regularized by using a force field. In case loop modelling with ProMod3 fails, an alternative model is built with PROMOD-II [122]. 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\(^T\) [9] to the sequence of SpMTY1 from *S. pastorianus* strain Weihenstephan 34/70 [21] 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: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 \(\mu\)m filter-sterilized before analysis. The concentrations of ethylacetate and isoamylacetate, methanol, propanol, isobutanol, isoamyl alcohol and diacetyl were determined as described previously [55].

**Supporting information**

S1 Table. Primers used in this study.

(S DOCX)

S1 Fig. Characterization of enriched mutants IMS0637-IMS0643 on SMMt. Characterization of *S. pastorianus* CBS 1483 (black), *S. eubayanus* CBS 12357\(^T\) (blue) and selected mutants IMS0637-IMS0643 (light red) on SMMt at 20°C. The average concentration of maltotriose (diamonds) and average deviation were determined from two replicates. IMS0637 was chosen as representative for all mutants (S8 Data File).

(TIF)

S2 Fig. Growth curves for the strains CBS 1483 (squares), CBS 12357\(^T\), (upward triangles), IMS0750 (downward triangles), IMS0751 (diamonds) and IMS0752 (circles) on 3x diluted wort at 12°C. (A) Consumption of glucose, (B) maltose, (C) maltotriose and (D) ethanol were measured by HPLC. Data represent average and standard deviation of three biological replicates (S9 Data File).

(TIF)

S3 Fig. Sequence alignment of the SeMALT transporter genes from CBS 12357\(^T\) to the new recombined SeMALT413 transporter gene from IMS0750. The alignment was performed using the Clone manager software (version 9.51, Sci-Ed Software). Identical nucleotides are shown by dots and nucleotides which differ from SpMTY1 are shown in orange. In addition, the sequences which match exactly are highlighted in yellow for SeMALT4, in green for
ScMALT1 and in blue for ScMALT3.

S4 Fig. Alignment of SeMalt413 to XylE using by ProMod3 for protein structure prediction. Transmembrane domain α-helices are indicated in red.

S5 Fig. Protein structure overlay of SeMalt1 (green), SeMalt4 (orange), SeMalt3 (blue) and SeMalt413 (magenta). ScMALT genes were translated into amino acid sequence and used for structural prediction using SWISS-MODEL with XylE as a structural template. Resulting SeMalt protein structures were overlayed using PyMOL.

S6 Fig. Amplification of the SeSGA1 locus to verify integration of SeMALT2 in IMX1941 and of ScMALT413 in IMX1942. The SeSGA1 locus was amplified from genomic DNA of IMX1941 (1), IMX1942 (2) and CBS 12357T (3) using primers 12635/12636 and Phusion polymerase (Thermo Fischer Scientific). At the SeSGA1 locus, IMX1941 should harbor ScTEF1p-ScMALT2-ScCYC1t and IMX1942 should harbor ScTEF1p-SeMALT413-ScCYC1t. As a negative control, a PCR was done with primers 12635/12636 without template DNA. L indicates the GeneRuler DNA Ladder Mix (Thermo Fischer Scientific).

S7 Fig. Characterization of CBS 12357T (blue squares), IMS0750 (red triangles), IMX1941 (cyan triangles), IMX1942 (purple diamonds) on SM (A) glucose, (B) maltose and (C) maltotriose. Strains were cultivated at 20˚C and optical densities were measured at 660 nm. Data represent average and standard deviation of three biological replicates (S10 Data File). Blue boxes represent the timeframe used to calculate growth rates.

S8 Fig. Alignment of ScMAL31, SeMALT3 and SparMAL31 to SpMY1 as obtained using Clone Manager. Identical nucleotides are shown by dots and nucleotides which differ from SpMY1 are shown in orange. In addition, the high-identity sequences described in the main text are highlighted in yellow for ScMAL31, in green for SeMALT3 and in blue for SparMAL31.

S9 Fig. PCR amplification of ScMALT genes in wild type CBS 12357T and evolved mutant IMS0750. The ScMALT genes were amplified from genomic DNA of CBS 12357T and IMS0750 using Phusion polymerase (Thermo Fischer Scientific). Lanes show PCR products for ScMALT1 (primers 10491/10492), ScMALT2 and SeMALT4 (primers 10633/10632), SeMALT3 (primers 10672/10671) and SeMALT413 (primers 10633/10671). As a negative control, a PCR was done with primers 10633/10632 without template DNA. L indicates the GeneRuler DNA Ladder Mix (Thermo Fischer Scientific).

S1 Data. Overview of all mutations identified in the mutagenized strains IMS0637-IMS0643 and in the evolved strains IMS0750-IMS0752. The mutations were obtained using Pilon by alignment against the chromosome level assembly of S. eubayanus strain CBS12357. The mutations are ordered per strain, and for each mutation, the strains in which the same mutation is found are indicated. SNP indicates single nucleotide polymorphisms, INDELs indicate small insertions and deletions and CNV indicates copy number variation. NON indicates a premature stop codon, NSY indicates a non-synonymous mutation and SYN a
A novel chimeric transporter enables maltotriose utilization.

S2 Data. Belonging to Fig 2A. Characterization of *S. pastorianus* CBS 1483, *S. eubayanus* CBS 12357 and IMS0637-0643 on SMMt at 20˚C. Maltotriose concentrations were measured for two biological replicates using HPLC.

S3 Data. Belonging to Fig 2B. Characterization of *S. pastorianus* CBS 1483, *S. eubayanus* CBS 12357 and IMS0637-0643 on wort at 20˚C. Concentrations of glucose, maltose and maltotriose were measured from single biological samples using HPLC.

S4 Data. Belonging to Fig 2C. 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 from a single chemostat by HPLC.

S5 Data. Belonging to Fig 2D. Characterization of *S. pastorianus* CBS 1483, *S. eubayanus* CBS 12357 and IMS0750 on wort at 12˚C. Glucose, maltose and maltotriose concentrations were determined from three biological replicates with HPLC.

S6 Data. Belonging to Fig 4B. Characterization of CBS 12357, IMS0750, IMX1941, IMX1942 on SM glucose, maltose and maltotriose. Strains were cultivated at 20˚C and culture supernatant was measured from three biological replicates using HPLC.

S7 Data. Belonging to Fig 5. Extracellular metabolite profiles of *S. eubayanus* strains CBS 12357 and IMS0750 in high-gravity wort at 7 L pilot scale. Fermentations were performed on wort with a gravity of 16.6˚ Plato. The concentrations of glucose, maltose, maltotriose and ethanol were measured from two biological replicates using HPLC.

S8 Data. Belonging to S1 Fig. Characterization of enriched mutants IMS0637-IMS0643 on SMMt at 20˚C. Concentrations of maltotriose were measured from two biological replicates using HPLC.

S9 Data. Belonging to S2 Fig. Growth curves for the strains CBS 1483 CBS 12357, IMS0750, IMS0751 and IMS0752 on 3x diluted wort at 12˚C. Concentrations of glucose, maltose, maltotriose and ethanol were measured from three biological replicates using HPLC.

S10 Data. Belonging to S7 Fig. Characterization of CBS 12357, IMS0750, IMX1941, IMX1942 on SM glucose, maltose and maltotriose. Strains were cultivated at 20˚C and optical densities were measured from three biological replicates at 660 nm.

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**References**


